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ORIGINAL RESEARCH

Association Between Plasma Trimethyllysine and Prognosis of Patients With Ischemic Stroke

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BACKGROUND: Trimethyllysine, a trimethylamine N-oxide precursor, has been identified as an independent cardiovascular risk factor in acute coronary syndrome. However, limited data are available to examine the role of trimethyllysine in the population with stroke. We aimed to examine the relationship between plasma trimethyllysine levels and stroke outcomes in patients presenting with ischemic stroke or transient ischemic attack.

METHODS AND RESULTS: Data of 10 027 patients with ischemic stroke/transient ischemic attack from the CNSR-III (Third China National Stroke Registry) and 1-year follow-up data for stroke outcomes were analyzed. Plasma levels of trimethyllysine were measured with mass spectrometry. The association between trimethyllysine and stroke outcomes was analyzed using Cox regression models. Mediation analysis was performed to examine the mediation effects of risk factors on the associations of trimethyllysine and stroke outcomes. Elevated trimethyllysine levels were associated with increased risk of cardiovascular death (quartile 4 versus quartile 1: adjusted hazard ratio [HR], 1.72; 95% CI, 1.03–2.86) and all-cause mortality (quartile 4 versus quartile 1: HR, 1.97; 95% CI, 1.40–2.78) in multivariate Cox regression model. However, no associations were found between trimethyllysine and nonfatal stroke recurrence or nonfatal myocardial infarction. Trimethyllysine was associated with cardiovascular death independent of trimethylamine N-oxide. Both estimated glomerular filtration rate and hs-CRP (high-sensitivity C-reactive protein) had significant mediation effects on the association of trimethyllysine with cardiovascular death, with a mediation effect of 37.8% and 13.4%, respectively.

CONCLUSIONS: Elevated trimethyllysine level is associated with cardiovascular death among patients with ischemic stroke/transient ischemic attack. Mediation analyses propose that trimethyllysine contributes to cardiovascular death through inflammation and renal function, suggesting a possible pathomechanistic link.

Key Words: gut microbiota ■ ischemic stroke ■ trimethylamine N-oxide ■ trimethyllysine

The gut microbiota has been acknowledged as a novel contributor affecting host metabolism.[1–4] Mounting evidence in mice and humans has shown that gut microbiota is capable of modifying the risk of developing cardiovascular disease.[5–7] The discovery of gut microbiota–derived metabolite, trimethylamine N-oxide (TMAO), has significantly contributed to the identification of the important role of gut microbiota in cardiovascular disease.[2,8–11] Studies also suggest great promise for TMAO as a strong cardiovascular diagnostic or prognostic marker.[8,10–12] However, except for TMAO, other microbiota-derived markers have emerged as predictive biomarkers.[13]

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CLINICAL PERSPECTIVE

What Is New?

- Elevated plasma trimethyllysine level is independently associated with cardiovascular death among patients with ischemic stroke and transient ischemic attack.
- Mediation analyses propose that trimethyllysine contributes to cardiovascular death through inflammation and renal function, suggesting a possible pathomechanistic link.

What Are the Clinical Implications?

- Plasma trimethyllysine level could provide important prognostic information for predicting the risk of cardiovascular death and all-cause mortality, and may represent a novel target for improving the prognosis of patients with ischemic stroke and transient ischemic attack.

Nonstandard Abbreviations and Acronyms

CNSR-III	Third China National Stroke Registry
FBG	fasting blood glucose
IS	ischemic stroke
MACE	major adverse cardiac event
Q1	quartile 1
Q2	quartile 2
Q3	quartile 3
Q4	quartile 4
SBP	systolic blood pressure
TMAO	trimethylamine N-oxide
TOAST	Trial of Org 10172 in Acute Stroke Treatment

Trimethyllysine is a relatively abundant posttranslational modification of protein in lysine residues in both plants and animals,[14–16] which serves as a nutrient precursor of TMAO.[17] Trimethyllysine was discovered and structurally identified as a plasma metabolite associated with incident cardiovascular risks in subjects with cardiovascular disease, independent of traditional cardiovascular risk factors and TMAO.[17] In patients with acute coronary syndrome, association was also found between elevated trimethyllysine levels and incident long-term (7-year) all-cause mortality and major adverse cardiac events (MACEs).[18] Other studies also suggested that elevated trimethyllysine levels were associated with progression of cardiac allograft vasculopathy,[19] coronary atherosclerosis,[20] and cardiovascular death.[21] However, limited data are available to examine the role of trimethyllysine in the population with stroke. Hence, in this study, we investigated whether the level of plasma trimethyllysine was associated with stroke outcomes in patients with ischemic stroke (IS) or transient ischemic attack (TIA) from the CNSR-III (Third China National Stroke Registry), and further examined which risk factors (including traditional risk factors and TMAO) mediated the association between trimethyllysine and stroke outcomes.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Study Populations

CNSR-III is a nationwide prospective registry for patients presented to hospitals with acute ischemic cerebrovascular events.[22] The registry recruited consecutive patients between August 2015 and March 2018 in China from 201 hospitals of 22 provinces and 4 municipalities, including 163 grade III (central hospitals for certain district or city, usually teaching hospitals) and 38 grade II (hospitals serving several communities) urban hospitals. There are a total of 15 166 patients with IS (n=14 146, 93.3%) or TIA (n=1020, 6.7%) within 7 days from the onset of symptoms to enrollment. Acute IS was diagnosed according to the World Health Organization criteria[23] and confirmed by magnetic resonance imaging or computed tomography. The details of imaging modality were described in the protocol of CNSR-III.[22] Among the total 201 sites in the CNSR-III, 171 participated in the prespecified gut microbial metabolites substudy. The study was approved by ethics committees of Beijing Tiantan Hospital and all other research centers, according to the principles expressed in the Declaration of Helsinki.[24] Written informed consent was obtained from all patients or legally authorized representatives before entering into the study.

Baseline Data Collection

The baseline data were collected prospectively using an electronic data capture system by face-to-face interviews, which included age, sex, ethnicity, region (North, Northeast, East, Central South, and West), hospital level (grade II and grade II), family income, education level, symptom to admission (<24 or ≥24 hours), intravenous thrombolysis and endovascular treatment, body mass index (calculated as weight in kilograms divided by height in meters squared, kg/m^2), current smoking, heavy drinking (≥2 standard alcohol consumption per day), medical history (stroke, coronary heart disease, hypertension, or diabetes), stroke type (TIA and IS), the causative subtypes of IS

classified according to the TOAST (Trial of Org 10172 in Acute Stroke Treatment) criteria,[25] severity of stroke on admission (National Institutes of Health Stroke Scale score),[26] systolic blood pressure (SBP) at admission, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride, fasting blood glucose (FBG) at admission, and discharge medications (antiplatelet, lipid-lowering,[27] and antihypertensive drugs).

Plasma Analysis

In this study, blood samples were collected from the 171 study sites that participated in the gut microbial metabolites substudy. Fasting blood samples were collected in serum-separation tubes and EDTA anticoagulation blood collection tube within 24 hours of admission. Blood samples were sent to the central laboratory to extract serum, plasma, and white blood cells. In this study, trimethyllysine, TMAO, homocysteine, hs-CRP (high-sensitivity C-reactive protein), and serum creatinine were based on centralized detection. Plasma total homocysteine was determined using the OLYMPUS AU2700 automatic biochemical analyzer (Homocysteine Assay Kit; Ausa Pharmed, Shenzhen, China) by the enzyme rate method. hs-CRP was measured on cobas c501 analyzer by using cardiac CRP (C-reactive protein) (latex) high sensitive assay (Roche, Basel, Switzerland).[28] Serum creatinine was measured using picric acid method (Roche cobas c501 analyzer with Roche Creatinine Jaffé Gen.2 assays).[29] Estimated glomerular filtration rate (eGFR) was calculated according to the Chronic Kidney Disease Epidemiology Collaboration creatinine.[30,31] Trimethyllysine and TMAO were measured with liquid chromatography–mass spectrometry, as previously reported.[18,32] The methods used for trimethyllysine and TMAO measurement were described in T appendix (Data S1 and Tables S1 and S2).

Patient Follow-Up and Outcome Evaluation

Patients were followed up for clinical outcomes at 3 months, 6 months, and 1 year after onset. Patients were interviewed face-to-face at 3 months and contacted over the telephone by trained research coordinators at 6 months and 1 year. Any death, stroke recurrence, or cardiovascular events during the follow-up periods were recorded. The stroke outcome includes MACEs (defined as nonfatal stroke recurrence, nonfatal myocardial infarction, or cardiovascular death), stroke recurrence (new IS or hemorrhagic stroke), cardiovascular death (cardiovascular death defined as IS, hemorrhagic stroke, sudden cardiac death, acute myocardial infarction, and death directly caused by heart failure or other cardiovascular death), and all-cause mortality. Confirmation of events was sought from the treating hospital, and suspected events without hospitalization were judged by independent end point judgment committee. Each case fatality was confirmed on either a death certificate from the attended hospital or the local citizen registry.

Statistical Analysis

Baseline characteristics were compared between groups categorized by trimethyllysine levels. Continuous variables were presented in medians (interquartile ranges) and compared between groups using the nonparametric Kruskal-Wallis test. Categorical variables were presented as percentages and tested by χ^2 test. The associations of trimethyllysine with outcomes (MACE, nonfatal stroke recurrence, nonfatal myocardial infarction, cardiovascular death, and all-cause mortality) were examined using Cox regression model.

We created 2 multivariable adjusted models. Model 1 adjusted for confounders that were associated with both trimethyllysine and outcomes. Model 2 was performed to verify the robustness of the study findings and adjust for baseline variables that were associated with trimethyllysine or outcomes. We screened out 7 baseline variables independently associated with both trimethyllysine and outcome (Table S3), including age, region, intravenous thrombolysis and endovascular treatment, homocysteine, eGFR, hs-CRP, and TMAO. These variables plus sex were incorporated into model 1. Model 2 included the variables associated with trimethyllysine or outcomes: age, sex, region, body mass index, symptom to admission, intravenous thrombolysis and endovascular treatment, heavy drinking, medical history (stroke, coronary heart disease, or diabetes), stroke type, TOAST subtype, National Institutes of Health Stroke Scale score at admission, SBP at admission, HDL-C, LDL-C, FBG, homocysteine, eGFR, hs-CRP, TMAO, discharge with antiplatelet drugs, and discharge with lipid-lowering drugs (Table S3). The interactions between trimethyllysine levels and confounders for the risks of outcomes were also tested using the multivariable Cox regression. Kaplan-Meier product limit method was used to generate survival plots, and the significance of differences between groups was tested by the log-rank test. Data from patients who had no events during the follow-up were considered censored data. Hazard ratios (HRs) with 95% CIs were calculated to measure the strength of the associations. Two-sided $P<0.05$ was considered statistically significant. Above statistical analyses were conducted with SAS software version 9.4 (SAS Institute, Inc, Cary, NC).

Mediation models were used to examine whether the association of trimethyllysine and cardiovascular death was mediated by traditional risk factors (SBP, LDL-C, HDL-C, FBG, homocysteine, hs-CRP, or eGFR) or TMAO. If the variables were right skewed, log transformation was performed. Trimethyllysine was predictor variable (X); risk factors above mentioned were mediators (M); cardiovascular death was

outcome variables (Y), following a binomial distribution. In general, there are 4 steps for mediation analyses: (1) showing that the predictor variable determines the outcome (model Y=βTotX) (βTot=total effect); (2) showing that the predictor variable affects the mediator (model M=β1X) (β1=indirect effect 1); (3) showing that the mediator determines the outcome controlling for the predictor (model Y=β2M+βDirX) (β2=indirect effect 2, βDir=direct effect); (4) calculating the proportion of mediation: mediation effect (%)=(β1×β2/βTot)×100%. Mediation analysis was performed using R package lavaan. The mediation analysis of this study was divided into 2 steps: first, a simple mediation analysis of each risk factor (SBP, LDL-C, HDL-C, FBG, homocysteine, hs-CRP, eGFR, and TMAO) was done, and then the meaningful factors were incorporated into the multiple mediation analysis model.

RESULTS

Among a total of 15 166 patients with IS or TIA, 12 603 came from 171 sites that participated in the prespecified gut microbial metabolites substudy, among which, blood samples from 11 261 patients were collected and sent to the central laboratory. From the 11 261 patients with blood samples, we excluded 1234 either without trimethyllysine data or lost to follow-up, leaving 10 027 patients in the final analysis (Figure S1). The excluded population seemed likely to have lower income and education level, was more inclined to visit grade II hospital, was less likely to have a history of diabetes or dyslipidemia, and had a lower proportion with antiplatelet or lipid-lowering medication at discharge, compared with the study population. Other baseline characteristics were balanced between the study population and excluded population (Table S4).

Baseline Characteristics

The baseline characteristics of the cohort stratified by quartile of plasma trimethyllysine are shown in Table 1. The patients with elevated trimethyllysine levels seemed older and less likely to be women. The proportion of women in the quartile 4 group was only one third of the quartile 1 (Q1) group (17.1% versus 53.3%). Patients with elevated trimethyllysine levels were more likely to have higher TMAO, hs-CRP, and homocysteine, but lower eGFR. Notably, the proportion of large-artery atherosclerosis subtype did not differ among the 4 stratified groups of trimethyllysine.

Association Between the Levels of Trimethyllysine and Stroke Outcomes

Elevated levels of trimethyllysine were independently associated with cardiovascular death (quartile 4 [Q4] versus Q1: HR, 1.72; 95% CI, 1.03–2.86; quartile 3 [Q3] versus Q1: HR, 1.29; 95% CI, 0.77–2.16; quartile 2 [Q2] versus Q1: HR, 0.93; 95% CI, 0.54–1.60; *P* for trend=0.01) and all-cause mortality (Q4 versus Q1: HR, 1.97; 95% CI, 1.40–2.78; Q3 versus Q1: HR, 1.49; 95% CI, 1.06–2.11; Q2 versus Q1: HR, 1.01; 95% CI, 0.70–1.45; *P* for trend <0.0001) but not for MACE (Q4 versus Q1: HR, 1.18; 95% CI, 0.97–1.43; Q3 versus Q1: HR, 1.14; 95% CI, 0.95–1.37; Q2 versus Q1: HR, 1.12; 95% CI, 0.93–1.34; *P* for trend=0.16), after adjustment for age, sex, region, intravenous thrombolysis and endovascular treatment, homocysteine, eGFR, hs-CRP, and TMAO in model 1 (Table 2). Similar results were observed in model 2. Moreover, no associations were found between trimethyllysine levels and nonfatal stroke recurrence or nonfatal myocardial infarction, regardless in unadjusted or adjusted models (Table 2). Kaplan-Meier survival plots showed similar trends (Figure 1).

Concurrent Assessment of Trimethyllysine and TMAO on Stroke Outcomes

We also investigated the relationship between the levels of TMAO and stroke outcomes. Elevated TMAO levels were associated with significantly increased HRs for MACE (Q4 versus Q1: HR, 1.40; 95% CI, 1.17–1.67), but not with cardiovascular death (Q4 versus Q1: HR, 0.88; 95% CI, 0.56–1.39) in model 1. Multivariable analysis in model 2 showed similar results (Table S5).

Figure 2A showed a significant association between plasma trimethyllysine and TMAO levels using Spearman correlation analyses (r=0.29; P<0.001). Although both trimethyllysine and TMAO were included in the models, TMAO, but not trimethyllysine, was independently associated with MACE and nonfatal stroke recurrence (Table 2 and Table S5). Trimethyllysine predicted cardiovascular death, whereas TMAO did not (Table 2 and Table S5). Kaplan-Meier survival plots stratifying trimethyllysine and TMAO into low versus high levels (lower than median value versus median value or higher) showed similar trends (Figure 2B through 2D).

Subgroup Analysis for the Association Between Trimethyllysine and Cardiovascular Death

Table 3 shows the subgroup analysis for the association between trimethyllysine levels and cardiovascular death, which was consistent across various subgroups. There were no significant interactions in any of the predefined subgroups (P>0.10 for all comparisons). The subgroup analysis for the association between trimethyllysine levels and all-cause mortality is shown in Table S6.

Table 1. Baseline Clinical Characteristics

Characteristics	Trimethyllysine				*P* value
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	
	(n=2506)	(n=2504)	(n=2503)	(n=2514)	
Age, median (IQR), y	62 (54–69)	62 (54–70)	63 (54–70)	64 (55–72)	<0.001
Women, n (%)	1335 (53.3)	815 (32.6)	571 (22.8)	431 (17.1)	<0.001
Ethnicity (non-Han), n (%)	75 (3.0)	87 (3.5)	76 (3.0)	66 (2.6)	0.38
Region, n (%)					
North	830 (33.1)	947 (33.8)	784 (31.3)	629 (25.0)	<0.001
Northeast	207 (8.3)	245 (9.8)	268 (10.7)	274 (10.9)	
East	622 (24.8)	608 (24.3)	621 (24.8)	796 (31.7)	
Central South	519 (20.7)	539 (21.5)	603 (24.1)	618 (24.6)	
West	328 (13.1)	265 (10.6)	227 (9.1)	197 (7.8)	
Hospital level, n (%)					
Grade II	280 (11.2)	287 (11.5)	272 (10.9)	212 (9.4)	<0.01
Grade III	2226 (88.8)	2217 (88.5)	2231 (89.1)	2302 (91.6)	
Family income (RMB), n (%)					
<1500	527 (21.0)	504 (20.1)	451 (18.0)	382 (15.2)	<0.001
1501–2300	563 (22.5)	519 (20.7)	492 (19.7)	497 (19.8)	
>2300	858 (34.2)	842 (33.6)	908 (36.3)	960 (38.2)	
Unknown	558 (22.3)	639 (25.5)	652 (26.1)	675 (26.8)	
Education, n (%)					
Elementary or below	770 (30.7)	712 (28.4)	645 (25.8)	610 (24.3)	<0.001
Middle school	718 (28.6)	740 (29.6)	755 (30.2)	724 (28.8)	
High school or above	682 (27.2)	705 (28.2)	759 (30.3)	781 (31.1)	
Unknown	336 (13.4)	347 (13.9)	344 (13.7)	399 (15.9)	
Symptom to admission, n (%)					
<24 h	1535 (61.2)	1440 (57.5)	1488 (59.4)	1484 (59.0)	0.06
≥24 h	971 (38.8)	1064 (42.5)	1015 (40.6)	1030 (41.0)	
IT/ET, n (%)	343 (13.7)	249 (9.9)	270 (10.8)	217 (8.6)	<0.001
BMI, median (IQR), kg/m^2	24.4 (22.3–26.5)	24.5 (22.6–26.6)	24.5 (22.7–26.4)	24.4 (22.5–26.6)	0.30
Current smoking, n (%)	583 (23.3)	805 (32.2)	903 (36.1)	881 (35.0)	<0.001
Heavy drinking, n (%)	275 (11.0)	399 (15.9)	380 (15.2)	367 (14.6)	<0.001
Medical history, n (%)					
Stroke	504 (20.1)	562 (22.4)	585 (23.4)	604 (24.0)	<0.01
CHD	260 (10.4)	258 (10.3)	285 (11.4)	283 (11.3)	0.47
Hypertension	1525 (60.8)	1552 (62.0)	1558 (62.2)	1651 (65.7)	<0.01
Hyperlipidemia	203 (8.1)	234 (9.4)	215 (8.6)	221 (8.8)	0.47
Diabetes	649 (25.9)	583 (23.3)	561 (22.4)	602 (24.0)	0.03
Stroke type, n (%)					
TIA	176 (7.0)	169 (6.8)	164 (6.6)	174 (6.9)	0.92
Ischemic stroke	2330 (93.0)	2335 (93.2)	2339 (93.4)	2340 (93.1)	
TOAST subtype, n (%)					
LAA	568 (22.7)	571 (22.8)	561 (22.4)	579 (23.0)	0.31
SAO	478 (19.1)	487 (19.5)	493 (19.7)	457 (18.2)	
Cardioembolism	136 (5.4)	148 (5.9)	166 (6.6)	183 (7.3)	
Others	1324 (52.8)	1298 (51.8)	1283 (51.3)	1295 (51.5)	
NIHSS score at admission, n (%)					
0–3	1300 (51.9)	1385 (55.3)	1334 (53.3)	1374 (54.6)	0.07

(*Continued*)

Table 1. Continued

Characteristics	Trimethyllysine				*P* value
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	
	(n=2506)	(n=2504)	(n=2503)	(n=2514)	
≥4	1206 (48.1)	1119 (44.7)	1169 (46.7)	1140 (45.4)	
SBP at admission, median (IQR), mm Hg	148.0 (135.0–163.5)	147.5 (135.0–162.5)	148.0 (134.0–165.0)	149.0 (135.0–164.0)	0.53
HDL-C, median (IQR), mmol/L	1.2 (1.0–1.4)	1.1 (0.9–1.3)	1.1 (0.9–1.3)	1.1 (0.9–1.2)	<0.001
LDL-C, median (IQR), mmol/L	2.9 (2.3–3.4)	2.8 (2.2–3.4)	2.7 (2.2–3.4)	2.7 (2.2–3.3)	<0.001
Triglyceride, median (IQR), mmol/L	1.4 (1.0–1.9)	1.4 (1.0–2.0)	1.4 (1.0–2.0)	1.4 (1.0–2.0)	0.36
FBG, median (IQR), mmol/L	5.7 (5.0–7.3)	5.5 (4.9–6.9)	5.5 (4.8–6.6)	5.5 (4.8–6.7)	<0.001
Homocysteine, median (IQR), μmol/L	13.3 (10.9–17.2)	15.1 (12.2–20.3)	16.0 (12.7–21.8)	17.0 (13.2–23.2)	<0.001
eGFR, median (IQR), mL/min per 1.73 m^2	94.6 (85.1–102.7)	91.6 (81.0–100.1)	88.6 (76.5–97.9)	80.1 (64.2–93.6)	<0.001
hs-CRP, median (IQR), mg/L	1.8 (0.9–4.4)	1.7 (0.8–4.6)	1.8 (0.8–4.5)	2.0 (0.9–5.5)	<0.01
TMAO, median (IQR), μmol/L	1.4 (0.9–2.0)	1.6 (1.1–2.4)	1.8 (1.2–2.7)	2.3 (1.5–3.7)	<0.001
Discharge medications, n (%)					
Antiplatelet	2290 (91.8)	2302 (92.1)	2283 (91.3)	2279 (91.0)	0.51
Single	1503 (60.0)	1504 (60.1)	1513 (60.5)	1515 (60.3)	0.74
Dual	787 (31.4)	798 (37.9)	770 (30.8)	764 (30.4)	
Lipid lowering	2308 (92.5)	2319 (92.8)	2308 (92.3)	2329 (93.0)	0.79
Antihypertension	1184 (47.4)	1205 (48.2)	1213 (48.5)	1352 (54.0)	<0.001

Continuous data are presented as median (IQR), and categorical variables are presented as number (percentage). BMI indicates body mass index; CHD, coronary heart disease; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; IQR, interquartile range; IT/ET, intravenous thrombolysis and endovascular treatment; LAA, large-artery atherosclerosis; LDL-C, low-density lipoprotein cholesterol; NIHSS, National Institutes of Health Stroke Scale; RMB, chinese yuan; SAO, small-artery occlusion; SBP, systolic blood pressure; TIA, transient ischemic attack; TMAO, trimethylamine N-oxide; and TOAST, Trial of Org 10172 in Acute Stroke Treatment.

Notably, we did not find significant interaction between sex and trimethyllysine for cardiovascular death or all-cause mortality. Moreover, Kaplan-Meier survival plots showed that elevated trimethyllysine levels were associated with increased risk of MACE, cardiovascular death, and all-cause mortality in men and women alike (Figure S2).

However, in this cohort, we found that the trimethyllysine level was higher in men than women. Next, the associations between trimethyllysine levels and stroke outcomes in total population were analyzed on the basis of respective quartile of trimethyllysine of male and female patients (male: Q1: <0.91; Q2: 0.91–1.10; Q3: 1.10–1.36; Q4: ≥1.36; female: Q1: <0.73; Q2: 0.73–0.89; Q3: 0.89–1.10; Q4: ≥1.10). We found that trimethyllysine still remained a significant prognostic value for cardiovascular death and all-cause mortality in the total population (Table S7).

Mediation Analysis

We tested which risk factors (including traditional risk factors and TMAO) mediated the association between trimethyllysine and cardiovascular death. Except for hs-CRP and eGFR, other risk factors (SBP, LDL-C, HDL-C, FBG, homocysteine, and TMAO) did not have significant mediation effect on the association of trimethyllysine with cardiovascular death (Table S8). And then, the mediation effects of hs-CRP and eGFR on the trimethyllysine–cardiovascular death association were analyzed in a multivariate mediation analysis (Figure 3). Both hs-CRP and eGFR had significant mediation effects on the association of trimethyllysine with cardiovascular death. The percentage of the total effect mediated by hs-CRP was estimated at 13.4% (P<0.05), whereas the mediation effect by eGFR was 37.8% (P<0.05).

DISCUSSION

Previous studies have shown the association between trimethyllysine and cardiovascular event risk in patients with cardiovascular disease.[4,5] This is the first study, to our knowledge, investigating the role of trimethyllysine in patients with stroke. In this cohort study, we found that elevated levels of trimethyllysine were an independent risk factor for cardiovascular death and all-cause mortality but not for MACE. We did not find significant associations between trimethyllysine level and nonfatal stroke recurrence or nonfatal myocardial infarction. Mediation analyses suggested that eGFR and hs-CRP

Table 2. Association Between the Levels of Trimethyllysine and Outcomes

Variable	Hazard ratio (95% CI)			
	Event rate	Unadjusted	Adjusted*	Adjusted†
MACE				
Q1	9.30	Reference	Reference	Reference
Q2	10.50	1.14 (0.95–1.36)	1.12 (0.93–1.34)	1.14 (0.95–1.36)
Q3	10.99	1.19 (1.00–1.42)	1.14 (0.95–1.37)	1.14 (0.95–1.37)
Q4	11.69	1.27 (1.07–1.51)	1.18 (0.97–1.43)	1.17 (0.96–1.42)
P for trend		0.01	0.16	0.36
Nonfatal stroke recurrence				
Q1	8.06	Reference	Reference	Reference
Q2	9.23	1.15 (0.95–1.39)	1.13 (0.93–1.37)	1.13 (0.93–1.37)
Q3	8.95	1.12 (0.93–1.35)	1.06 (0.87–1.30)	1.06 (0.87–1.30)
Q4	8.83	1.11 (0.92–1.34)	1.02 (0.82–1.26)	1.01 (0.81–1.25)
P for trend		0.37	0.73	0.66
Nonfatal MI				
Q1	0.24	Reference	Reference	Reference
Q2	0.16	0.67 (0.19–2.39)	0.62 (0.17–2.25)	0.64 (0.18–2.34)
Q3	0.24	1.02 (0.33–3.15)	0.83 (0.26–2.76)	0.88 (0.26–2.92)
Q4	0.36	1.53 (0.55–4.30)	1.25 (0.39–4.05)	1.30 (0.04–4.24)
P for trend		0.31	0.50	0.34
Cardiovascular death				
Q1	1.12	Reference	Reference	Reference
Q2	1.08	0.96 (0.57–1.63)	0.93 (0.54–1.60)	1.07 (0.62–1.85)
Q3	1.52	1.36 (0.84–2.22)	1.29 (0.77–2.16)	1.27 (0.75–2.13)
Q4	2.15	1.95 (1.23–3.07)	1.72 (1.03–2.86)	1.78 (1.07–2.98)
P for trend		0.001	0.01	0.01
All-cause mortality				
Q1	2.35	Reference	Reference	Reference
Q2	2.48	1.05 (0.74–1.50)	1.01 (0.70–1.45)	1.05 (0.73–1.52)
Q3	3.60	1.53 (1.10–2.13)	1.49 (1.06–2.11)	1.29 (0.91–1.83)
Q4	5.17	2.23 (1.64–3.03)	1.97 (1.40–2.78)	1.87 (1.33–2.62)
P for trend		<0.0001	<0.0001	0.0003

MACE indicates major adverse cardiac event (defined as nonfatal stroke, nonfatal myocardial infarction, or cardiovascular death); MI, myocardial infarction; Q1, quartile 1; Q2, quartile 2; Q3, quartile 3; and Q4, quartile 4.

*Adjusted: adjust for age, sex, region, intravenous thrombolysis and endovascular treatment, homocysteine, estimated glomerular filtration rate, hs-CRP (high-sensitivity C-reactive protein), and trimethylamine N-oxide.

†Adjusted: adjust for age, sex, region, body mass index, symptom to admission, intravenous thrombolysis and endovascular treatment, heavy drinking, medical history (stroke, coronary heart disease, or diabetes), stroke type, TOAST (Trial of Org 10172 in Acute Stroke Treatment) subtype, National Institutes of Health Stroke Scale score at admission, systolic blood pressure at admission, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, fasting blood glucose, homocysteine, estimated glomerular filtration rate, hs-CRP (high-sensitivity C-reactive protein), trimethylamine N-oxide, discharge with antiplatelet drugs, and discharge with lipid-lowering drugs.

had significant mediation effects on the association of trimethyllysine with cardiovascular death, with a mediation effect of 37.8% and 13.4%, respectively.

In the study of Li et al,[18] because of the relatively small sample size, they did not show the subset outcome of MACE (nonfatal stroke, nonfatal myocardial infarction, need for revascularization, or all-cause mortality). In our study, trimethyllysine seems likely to be associated with MACE (nonfatal stroke, nonfatal myocardial infarction, and cardiovascular death) in an unadjusted model; however, the association does not exist after adjusting confounders. The results of this study indicated that the association between elevated trimethyllysine and MACE is mainly attributable to its association with death rather than vascular events. However, we found that TMAO was significantly associated with MACE and nonfatal stroke recurrence, but not with cardiovascular death. Our results indicated that trimethyllysine seems to be more related to cardiovascular death, whereas TMAO seems to be more related to vascular events, implying that trimethyllysine and TMAO may have different contributions

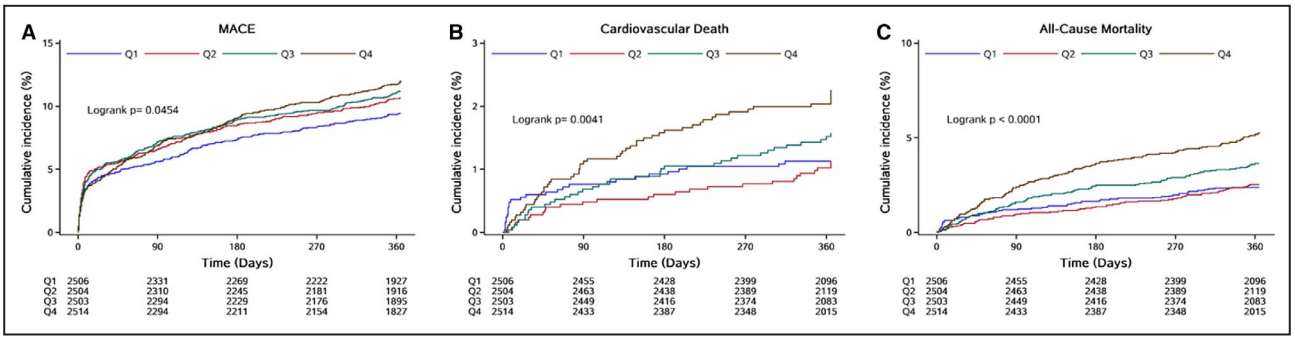

Figure 1. Cumulative incidence of 1-year outcomes, according to trimethyllysine (TML) quartiles.
A, TML and major adverse cardiac events (MACEs; defined as nonfatal stroke, nonfatal myocardial infarction, or cardiovascular death). **B**, TML and cardiovascular death. **C**, TML and all-cause mortality. Q1 indicates quartile 1; Q2, quartile 2; Q3, quartile 3; and Q4, quartile 4.

to cardiovascular risk. Moreover, we found men display a higher trimethyllysine level than women. In the articles published by Li et al, in Swiss ACS [Acute Coronary Syndrome] Cohort, the proportion of women in trimethyllysine Tertile3 was only 15.5%, and the proportion of women in trimethyllysine Tertile1 was 34.7%, which is similar to our results.[18] These results indicated men and women may have a different threshold to

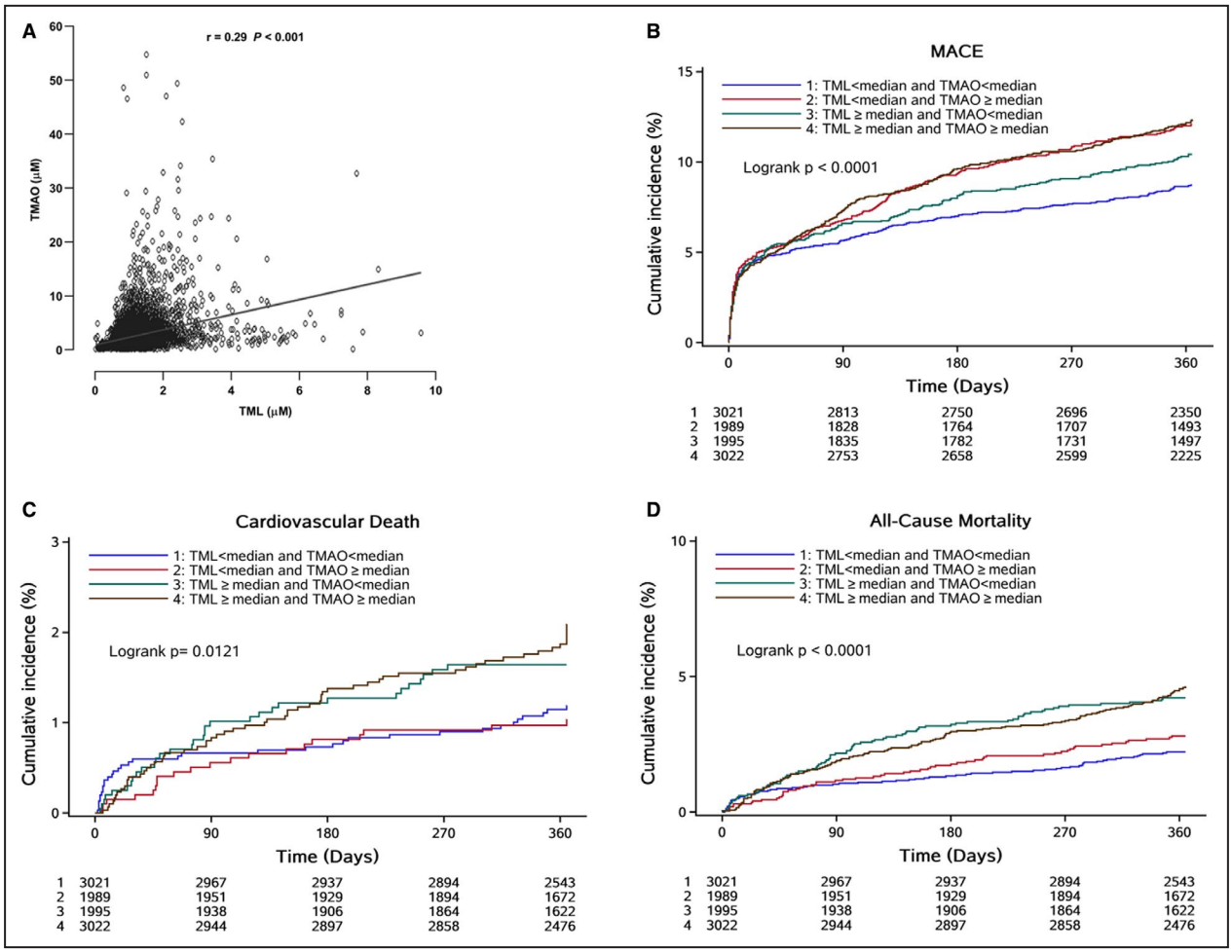

Figure 2. Concurrent assessment of trimethyllysine (TML) and trimethylamine N-oxide (TMAO) on stroke outcomes.
A, A significant association between plasma TML and TMAO levels using Spearman correlation analyses. **B** through **D**, Kaplan-Meier survival plots illustrating the association of TML and TMAO with major adverse cardiac events (MACEs; defined as nonfatal stroke, nonfatal myocardial infarction, or cardiovascular death) (**B**), cardiovascular death (**C**), and all-cause mortality (**D**), according to TML and TMAO levels, where each marker is categorized above vs below the median level; median plasma concentration of TML and TMAO within the cohort was used to stratify patients as "low" (lower than median) or "high" (median or higher) values.

Table 3. Subgroup Analysis for the Association Between Trimethyllysine Levels and Cardiovascular Death

Subgroup	Event rate		Hazard ratio (95% CI)		*P* for interaction (adjusted)
	Trimethyllysine < median	Trimethyllysine ≥ median	Unadjusted	Adjusted*	
Age, y					
<65	0.67	1.44	2.16 (1.26–3.65)	2.31 (1.23–4.36)	0.19
≥65	1.71	2.31	1.37 (0.89–2.10)	1.20 (0.75–1.93)	
Sex					
Men	1.05	1.72	1.65 (1.07–2.53)	1.43 (0.89–2.30)	0.63
Women	1.16	2.30	2.02 (1.14–3.55)	1.64 (0.86–3.11)	
TOAST subtype					
LAA	1.49	2.81	1.91 (1.06–3.43)	3.21 (1.33–7.75)	0.26
SAO	0.62	0.95	1.53 (0.55–4.30)	3.11 (0.71–13.66)	
Cardioembolism	2.46	2.87	1.19 (0.45–3.13)	0.24 (0.03–2.13)	
Others	0.95	1.59	1.68 (1.02–2.76)	1.81 (0.86–3.80)	
NIHSS score at admission					
0–3	0.71	1.07	1.52 (0.86–2.72)	0.98 (0.50–1.89)	0.47
≥4	1.55	2.73	1.78 (1.18–2.68)	1.73 (1.10–2.71)	
eGFR, mL/min per 1.73 m^2					
<90	1.66	2.28	1.38 (0.91–2.09)	1.32 (0.84–2.10)	0.59
≥90	0.72	1.13	1.59 (0.87–2.89)	2.10 (1.09–4.04)	
hs-CRP, mg/L					
<3	0.92	1.42	1.55 (0.95–2.52)	1.59 (0.91–2.81)	0.62
≥3	1.61	2.56	1.61 (0.99–2.62)	1.37 (0.80–2.36)	
TMAO, μmol/L					
Lower than median	1.16	1.60	1.40 (0.87–2.26)	1.53 (0.91–2.58)	0.41
Median or higher	1.01	1.99	1.99 (1.20–3.30)	1.55 (0.90–2.67)	
Homocysteine, μmol/L					
<15	1.05	1.77	1.70 (1.04–2.77)	1.92 (1.12–3.28)	0.68
≥15	1.15	1.83	1.61 (1.00–2.59)	1.17 (0.69–1.97)	
Antiplatelet therapy					
No	6.22	6.15	0.98 (0.57–1.67)	1.36 (0.73–2.54)	0.09
Single	0.70	1.62	2.36 (1.41–3.93)	2.24 (1.28–3.90)	
Dual	0.50	0.98	1.94 (0.82–4.58)	1.34 (0.51–3.49)	

eGFR indicates estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; LAA, large-artery atherosclerosis; NIHSS, National Institutes of Health Stroke Scale; SAO, small-artery occlusion; TMAO, trimethylamine N-oxide; and TOAST, Trial of Org 10172 in Acute Stroke Treatment.

*Adjusted: adjust for age, sex, region, body mass index, symptom to admission, intravenous thrombolysis and endovascular treatment, heavy drinking, medical history (stroke, coronary heart disease, or diabetes), stroke type, TOAST subtype, NIHSS score at admission, systolic blood pressure at admission, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, fasting blood glucose, homocysteine, eGFR, hs-CRP, TMAO, discharge with antiplatelet drugs, and discharge with lipid-lowering drugs.

stratify patient's risk. Although the level of trimethyllysine showed a difference between men and women, no sex-specific association of trimethyllysine levels with stroke outcomes were found. Our results showed elevated trimethyllysine levels were associated with increased risk of cardiovascular death and all-cause mortality in both men and women.

In the present study, we observed that the plasma levels of eGFR and hs-CRP were associated with both plasma trimethyllysine level and the risk of cardiovascular death. The prognostic value of trimethyllysine for mortality remained significant after adjustment for renal function, which is consistent with previous studies.[17,18] As a posttranslational modification of protein, trimethyllysine has been involved in the progress of histone modification during chromatin remodeling and regulation of gene expression.[33] Moreover, trimethyllysine has been also involved in the progress of methylation of protein lysine residues of both nuclear protein and cytosolic, secreted proteins.[34] Epigenetic modifications, such as DNA methylation and histone modification, have been demonstrated to play an important role in atherosclerosis and cardiovascular death.[35,36] However, whether any of these effects account for the significant association

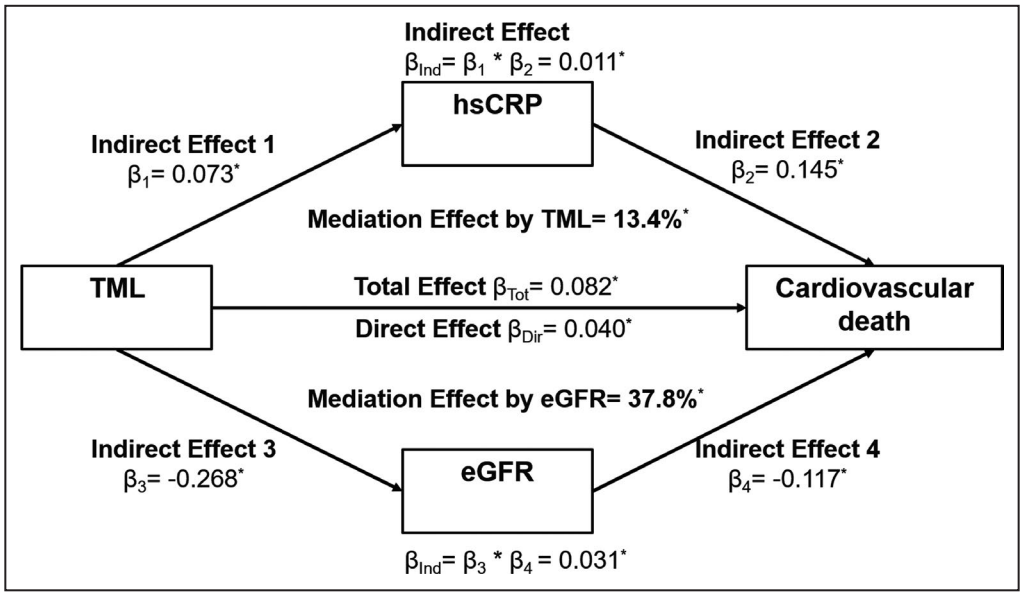

Figure 3. Mediation effect of trimethyllysine (TML)–hs-CRP (high-sensitivity C-reactive protein) and estimated glomerular filtration rate (eGFR)–cardiovascular death association.
Coefficients different from 0: *P<0.05.

observed between plasma trimethyllysine levels and cardiovascular death in populations with stroke remains unknown. Therefore, mediation analysis was used to explore the potential mechanisms. The results of mediation analysis revealed eGFR had a significant effect on trimethyllysine–cardiovascular death, which reached as high as 37.8%. We also found hs-CRP had a mediation effect on the trimethyllysine–cardiovascular death association. Considering that trimethyllysine is a precursor of TMAO, which has a role in renal function impairment[12,37–39] and proinflammatory effect,[40–42] we wondered whether the effect of trimethyllysine on cardiovascular death was mediated through TMAO. However, our study did not find that TMAO has a significant mediation effect on trimethyllysine-related cardiovascular death. Similar results were observed in stable patients with acute coronary syndromes, recently.[18] In our study, plasma trimethyllysine remained independently associated with cardiovascular death, even after adjustment for TMAO, suggesting that the mechanistic link between trimethyllysine and cardiovascular death is independent of TMAO pathway. We speculated that as a urinary excration metabolite, the accumulation of trimethyllysine may aggravate the function of kidney, as indicated by reduced eGFR. Moreover, as a methyl donor, trimethyllysine might regulate inflammation through epigenetic manner, such as methylation of key regulators of inflammation.[43] Given the fact that mediation analysis did not lead to causal relationship, these finding suggested that both renal function and inflammation might be 2 future directions for further studies to demonstrate the causal effect of trimethyllysine on cardiovascular death and its underlying mechanisms.

Strengths and Limitations

This study has several strengths and limitations. This study was a multicenter prospective registry with a fairly large sample size (>10 000 patients). To our knowledge, this was the first study investigating the role of trimethyllysine in patients with stroke, and for the first time in Asian population. In addition, the outcomes of this study not only included all-cause death, but also include cardiovascular death as the mortality end point. This study also has several limitations. This study did not collect patient-level diet information, so we could not evaluate the impact of diet on estimation of the association between trimethyllysine and stroke outcomes. For the different diet habits (such as red meat, eggs, and vegetables) in different regions of China, the region as an indirect indicator of diet was adjusted in the multivariate model. As an observational study, there may still be some unmeasured or unnoticed confounders that might lead to biased estimation. In addition, this study only monitored baseline trimethyllysine levels, but did not examine changes in trimethyllysine over time and at the time of the later events. This study was conducted exclusively in a Chinese patient population; our findings may not be generalizable to other populations.[44]

CONCLUSIONS

Among patients with IS/TIA, elevated plasma levels of trimethyllysine are associated with cardiovascular death independent of traditional risk factors and TMAO. Our mediation analyses imply that renal function and

inflammation might be 2 directions for future work to investigate the underlying mechanism between trimethyllysine and cardiovascular risk.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Material

Data S1
Tables S1–S8
Figures S1–S2

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Supplemental Material

Data S1

Supplemental Methods

TML and TMAO LC/MS/MS measurement

The plasma levels of TML (trimethyllysine) and TMAO (trimethylamine N-oxide) were measured as follows: First, 20 µl of plasma was mixed with 80 µl of a 5-µM internal standard comprised of d9-TML and d9-TMAO in methanol. Proteins were precipitated and the supernatant was recovered following centrifugation at 20,000 g at 4°C for 10 min. Supernatants (70 µl) were analysed by injection onto a silica column (2.0×150 mm, Luna 5u Silica 100 A; Cat. No. 00F-4274-B0, Phenomenex, Torrance, CA, USA) at a flow rate of 0.4 ml/min using an LC-20AD Shimadzu pump system, SIL-20AXR autosampler interfaced with an API 5500Q-TRAP mass spectrometer (AB SCIEX, Framingham, MA, USA). A discontinuous gradient was generated to resolve the analytes by mixing solvent A (0.1% formic acid and 10 mM ammonium formate in water) with solvent B (0.1% formic acid in acetonitrile). Analytes were monitored using electrospray ionization in positive-ion mode with multiple reaction monitoring (MRM) of precursor and characteristic product-ion transitions of TML at m/z 189.0→84.0, d9-TML at m/z 198.0→84.0, TMAO at m/z 76→58 and d9-TMAO at m/z 85→66, respectively. Standard curves were deemed acceptable if the coefficient of determination (R2) was 0.999 and accuracy (%) are displayed in Table S1. Quality control was measured every 20 samples. The calculated means and coefficient of variation (CV%) are listed in Table S2.

Table S1. Accuracy (%) of TML and TMAO determination by LC/MS/ MS.

Standard concentration (μM)	Accuracy of TML (%)	Accuracy of TMAO (%)
0.195	94.2	91
0.39	104	91.3
0.78	97	90
1.56	93.4	96.9
3.125	101	104
6.25	102	101
12.5	96.9	99.5

Table S2. Coefficient of variation (CV%) of TML and TMAO quality controls (QC).

		QC1	QC2	QC3
TML	Concentration (μM)	0.90	1.45	1.74
	CV (%)	2.74	6.77	3.15
TMAO	Concentration (μM)	0.64	2.26	9.92
	CV (%)	4.6	6.32	4.40

Table S3. Multivariable analysis of baseline variables for TML and stroke outcomes.

	TML	MACE	Cardiovascular death	All-cause mortality
Age, median (IQR), y	0.98(0.98-0.99)	-	-	1.05(1.03-1.06)
Female	0.29(0.25-0.33)	-	-	-
Region				
North	Ref	Ref	-	-
Northeast	1.39(1.13-1.70)	1.76(1.37-2.26)	-	-
East	1.33(1.16-1.54)	0.95(0.77-1.18)	-	-
Central South	1.31(1.13-1.53)	1.27(1.02-1.58)	-	-
West	0.66(0.54-0.82)	1.11(0.82-1.50)	-	-
Symptom to admission				
＜24h	-	Ref.	-	-
≥24h	-	0.85(0.72-1.00)	-	-
IT/ET	0.73(0.61-0.88)	-	-	0.53(0.32-0.89)

Heavy drinking	0.85(0.73-0.99)	-	-	
Medical history				
Stroke	-	1.65(1.39-1.95)	2.00(1.28-3.13)	1.60(1.17-2.18)
CHD	-	1.40(1.12-1.75)	1.98(1.16-3.38)	2.02(1.41-2.88)
Diabetes mellitus	0.79(0.69-0.90)	-	-	-
Stroke subtype				
Ischemic stroke	-	Ref.	-	-
TIA	-	0.42(0.27-0.65)	-	-
TOAST				
LAA	-	Ref.	-	-
CE	-	0.82(0.59-1.13)	-	-
SAO	-	0.61(0.47-0.78)	-	-
Other	-	0.78(0.65-0.94)	-	-
NIHSS at admission	-	1.02(1.00-1.04)	1.08(1.04-1.12)	1.11(1.09-1.14)
BMI (kg/m^2)	-	-	0.91(0.86-0.98)	0.94(0.90-0.98)

SBP (mmHg)	-	1.00(1.00-1.01)	-	-
HDL-C (mM)	0.65(0.54-0.79)	-	-	-
LDL-C (mM)	-	-	1.26(1.02-1.56)	1.30(1.12-1.52)
FBG (mM)	-	1.03(1.00-1.06)	-	-
HCY (μM)	1.01(1.01-1.02)	-	-	1.01(1.00-1.02)
eGFR (mL/min/1.73 m^2)	0.96(0.96-0.97)	-	0.99(0.98-1.00)	-
hsCRP (mg/L)	1.00(1.00-1.01)	-	1.01(1.00-1.02)	1.01(1.01-1.02)
TMAO (μM)	1.30(1.25-1.35)	1.02(1.01-1.04)	-	1.03(1.00-1.06)
Discharge with antiplatelet drugs	-	0.54(0.42-0.70)	0.29(0.18-0.48)	0.45(0.31-0.66)
Discharge with lipid-lowering drugs	-	0.74(0.56-0.98)	-	-

The stepwise multivariate logistic/cox regression model was used to investigate the association between all baseline variables in Table 1 and TML level/stroke outcomes.
For continuous variables, OR/HR represents change for per 1 unit increase.

BMI: body mass index; CHD: coronary heart diseases; eGFR, estimated glomerular filtration rate; FBG: fasting blood-glucose; HCY: homocysteine; hsCRP: high-sensitivity C-reactive protein; HDL-C: high density lipoprotein cholesterol; IT/ET: Intravenous thrombolysis and endovascular treatment; LDL-C: low density lipoprotein cholesterol; MACE: major adverse cardiac events (defined as non-fatal stroke, non-fatal myocardial infarction, or cardiovascular death); MI: myocardial infarction; NIHSS: National Institutes of Health Stroke Scale; SBP: systolic blood pressure; TMAO: trimethylamine-N-oxide; TML: trimethyllysine; OR: odds ratio; HR: hazard ratio.

Table S4. Baseline clinical characteristics between study population and excluded population.

Characteristics	Study population	Excluded population	*P*-value
N	10027	5139	
Age, median (IQR), y	63 (54-70)	62(54-70)	0.30
Female, n (%)	3152(31.4)	1650(32.1)	0.40
Ethnicity (non-Han), n (%)	304(3.0)	136(2.65)	0.18
Region, n (%)			
North	3090(30.82)	1580(30.8)	<0.001
Northeast	994(9.9)	732(14.2)	
East	2647(26.4)	1160(22.6)	
Central South	2279(22.73)	1328(25.8)	
West	1017(10.1)	339(6.6)	
Family income (RMB) , n (%)			
<1500	1864(18.6)	1088(21.2)	<0.001
1501~2300	2071(20.6)	1162(22.6)	
>2300	3568(35.6)	1659(32.3)	
UK	2524(25.2)	1230(23.9)	
Education, n (%)			
Elementary or below	2737(27.3)	1555(30.3)	<0.001
Middle school	2937(29.3)	1468(28.6)	
High school or above	2927(29.2)	1355(26.4)	
Unknown	1426(14.2)	761(14.8)	
Hospital level, n (%)			
Grade II	1051(10.5)	1144(22.3)	<0.001

Grade III	8976(89.5)	3995(77.7)	
Symptom to admission, n (%)			
＜24h	5947(59.3)	2999(58.4)	0.26
≥24h	4080(40.7)	2140(41.6)	
IT/ET, n (%)	1079(10.8)	516(10.0)	0.18
BMI (kg/m^2), median (IQR)	24.5 (22.5-26.6)	24.5 (22.8-26.6)	0.05
Current smoking, n (%)	3172 (31.6)	1580 (30.8)	0.26
Heavy drinking, n (%)	1421 (14.17)	705 (13.7)	0.45
Medical history, n (%)			
Stroke	2255 (22.5)	1100 (21.4)	0.13
CHD	1086 (10.8)	522 (10.2)	0.20
Hypertension	6286 (62.7)	3208 (62.4)	0.75
Hyperlipidemia	873 (8.7)	318 (6.2)	<0.001
Diabetes mellitus	2395 (23.9)	1115 (21.7)	<0.01
Stroke type, n (%)			0.55
TIA	9344 (93.2)	4802 (93.4)	
Ischemic stroke	683 (6.8)	337 (6.6)	
TOAST subtype, n (%)			0.16
LAA	2279 (22.7)	1235 (24.0)	
SAO	1915 (19.1)	981 (19.1)	
CE	633 (6.3)	291 (5.7)	
Others	5200 (51.9)	2632 (51.2)	
NIHSS at admission, n (%)			
NIHSS 0–3	5393 (53.8)	2867 (55.8)	0.02
NIHSS≥4	4634 (46.2)	2272 (44.2)	

SBP at admission (mmHg), median (IQR)	148.0 (135.0-164.0)	148.0 (135.0-162.5)	0.79
HDL-C (mM), median (IQR)	1.1 (0.9-1.3)	1.1 (0.9-1.3)	0.04
LDL-C (mM), median (IQR)	2.8 (2.2-3.4)	2.8 (2.3-3.5)	0.03
TG (mM), median (IQR)	1.4 (1.0-2.0)	1.4 (1.0-2.0)	0.14
FBG (mM), median (IQR)	5.5 (4.9-6.9)	5.5 (4.9-6.9)	0.97
Discharge medications, n (%)			
Antiplatelet	9154 (91.6)	4557 (88.8)	<0.001
Lipid-lowering	9264 (92.7)	4567 (89.0)	<0.001
Antihypertension	4954 (49.6)	2464 (48.0)	0.07

Continuous data are presented as median (interquartile range, IQR), and categorical variables are presented as %.

BMI: body mass index; CE: cardioembolism; CHD: coronary heart diseases; FBG: fasting blood-glucose; HDL-C: high density lipoprotein cholesterol; IT/ET: Intravenous thrombolysis or endovascular treatment; LAA: large-artery atherosclerosis; LDL-C: low density lipoprotein cholesterol; NIHSS: National Institutes of Health Stroke Scale; SAO: small-artery occlusion; SBP: systolic blood pressure; TIA: transient ischemic attack; TG: triglyceride.

Table S5. The association between the levels of TMAO and outcomes.

		Hazard Ratios (95% Confidence Interval)		
	Event rate	**Unadjusted**	**Adjusted[1]**	**Adjusted[2]**
MACE				
Q1	8.99	Reference	Reference	Reference
Q2	9.51	1.06(0.88-1.27)	1.02(0.85-1.23)	1.02(0.85-1.23)
Q3	10.38	1.16(0.97-1.38)	1.09(0.91-1.31)	1.13(0.94-1.36)
Q4	13.60	1.55(1.31-1.83)	1.40(1.17-1.67)	1.45(1.21-1.74)
Non-fatal stroke recurrence				
Q1	7.19	Reference	Reference	Reference
Q2	7.92	1.10(0.90-1.35)	1.10(0.90-1.35)	1.06(0.87-1.30)
Q3	8.79	1.22(1.00-1.49)	1.22(1.00-1.49)	1.20(0.98-1.47)
Q4	11.17	1.59(1.32-1.91)	1.57(1.29-1.91)	1.54(1.26-1.88)
Non-fatal MI				
Q1	0.24	Reference	Reference	Reference
Q2	0.16	0.67(0.19-2.36)	0.63(0.18-2.25)	0.56(0.16-2.01)
Q3	0.28	1.17(0.39-3.48)	1.08(0.36-3.26)	0.93(0.30-2.87)
Q4	0.32	1.39(0.48-3.99)	1.22(0.40-3.71)	0.98(0.31-3.11)
Cardiovascular death				
Q1	1.44	Reference	Reference	Reference
Q2	1.23	0.85(0.53-1.38)	0.77(0.47-1.24)	0.95(0.58-1.55)
Q3	1.32	0.91(0.57-1.46)	0.74(0.46-1.19)	1.11(0.67-1.84)
Q4	1.87	1.31(0.85-2.02)	0.88(0.56-1.39)	1.39(0.86-2.24)
All-cause mortality				
Q1	3.28	Reference	Reference	Reference

Q2	2.67	0.81(0.59-1.12)	0.71(0.51-0.98)	0.93(0.67-1.30)
Q3	3.00	0.91(0.66-1.24)	0.70(0.51-0.96)	1.12(0.80-1.56)
Q4	4.67	1.43(1.08-1.90)	0.85(0.63-1.13)	1.39(1.02-1.90)

Adjusted[1]: adjust for age, sex, region, IT/ET, HCY, eGFR, hsCRP, TML;

Adjusted[2]: adjust for age, sex, region, BMI, symptom to admission, IT/ET, heavy drinking, medical history (stroke, CHD, diabetes mellitus), stroke type, TOAST subtype, NIHSS at admission, SBP at admission, HDL-C, LDL-C, FBG, HCY, eGFR, hsCRP, TML, discharge with antiplatelet drugs, discharge with lipid-lowering drugs.

BMI: body mass index; CHD: coronary heart diseases; eGFR, estimated glomerular filtration rate; FBG: fasting blood-glucose; HCY: homocysteine; hsCRP: high-sensitivity C-reactive protein; HDL-C: high density lipoprotein cholesterol; IT/ET: Intravenous thrombolysis and endovascular treatment; LDL-C: low density lipoprotein cholesterol; MACE: major adverse cardiac events (defined as non-fatal stroke, non-fatal myocardial infarction, or cardiovascular death); MI: myocardial infarction; NIHSS: National Institutes of Health Stroke Scale; SBP: systolic blood pressure; TMAO: trimethylamine-N-oxide; TML: trimethyllysine.

Table S6. Subgroup analysis for the association between TML levels and all-cause mortality.

Subgroup	Event rate		Hazard Ratios (95%CI)		*P* for interaction (adjusted)
	TML<median	TML≥median	Unadjusted	Adjusted*	
Age					0.06
<65 yr	1.18	2.56	2.18 (1.46-3.27)	2.50 (1.57-4.00)	
≥65 yr	4.20	6.63	1.60 (1.22-2.08)	1.29 (0.97-1.72)	
Sex					0.68
Male	2.13	3.86	1.82 (1.35-2.45)	1.49 (1.08-2.05)	
Female	2.79	6.49	2.38 (1.67-3.38)	1.59 (1.07-2.36)	
TOAST subtype					0.66
LAA	3.16	5.96	1.92 (1.28-2.87)	1.53 (0.99-2.37)	
Non-LAA	2.20	3.92	1.80 (1.38-2.35)	1.63 (1.22-2.17)	
NIHSS at admission					0.99
0–3	1.30	2.44	1.88 (1.25-2.84)	1.30 (0.82-2.07)	
≥4	3.70	6.67	1.83 (1.40-2.38)	1.66 (1.25-2.21)	
eGFR(mL/min/1.73 m^2)					0.04
<90	4.14	5.51	1.34 (1.03-1.75)	1.24 (0.93-1.65)	
≥90	1.29	2.52	1.95 (1.28-2.98)	1.63 (1.17-2.27)	
hsCRP (mg/L)					0.58
<3	1.81	2.13	1.18 (0.80-1.75)	1.36 (0.90-2.07)	

≥3	3.67	7.93	2.20 (1.59-3.04)	1.58 (1.12-2.21)	
TMAO (μM)					0.83
<media	2.19	4.16	1.93 (1.40-2.66)	1.78 (1.25-2.52)	
≥media	2.77	4.53	1.65 (1.21-2.26)	1.52 (1.08-2.13)	
HCY (μM)					0.64
<15	2.36	3.74	1.60 (1.15-2.23)	1.53 (1.08-2.19)	
≥15	2.47	4.81	1.97 (1.44-2.69)	1.54 (1.10-2.16)	

Adjusted*: age, sex, region, BMI, symptom to admission, IT/ET, heavy drinking, medical history (stroke, CHD, diabetes mellitus), stroke type, TOAST subtype, NIHSS at admission, SBP at admission, HDL-C, LDL-C, FBG, HCY, eGFR, hsCRP,TMAO,discharge with antiplatelet drugs, discharge with lipid-lowering drugs

BMI: body mass index; CHD: coronary heart diseases; eGFR, estimated glomerular filtration rate; FBG: fasting blood-glucose; HCY: homocysteine; hsCRP: high-sensitivity C-reactive protein; HDL-C: high density lipoprotein cholesterol; IT/ET: Intravenous thrombolysis and endovascular treatment; LAA: large-artery atherosclerosis; LDL-C: low density lipoprotein cholesterol; MACE: major adverse cardiac events (defined as non-fatal stroke, non-fatal myocardial infarction, or cardiovascular death); MI: myocardial infarction; NIHSS: National Institutes of Health Stroke Scale; SBP: systolic blood pressure; TMAO: trimethylamine-N-oxide; TML: trimethyllysine.

Table S7. The association between the levels of TML and outcomes based on respective quartile of TML by sex.

		Hazard Ratios (95% Confidence Interval)		
	Event rate	Unadjusted	Adjusted[1]	Adjusted[2]
MACE				
Q1	9.21	Reference	Reference	Reference
Q2	10.02	1.10 (0.92-1.31)	1.05(0.88-1.26)	1.08 (0.90-1.30)
Q3	11.20	1.22 (1.03-1.46)	1.12(0.94-1.34)	1.14 (0.96-1.37)
Q4	12.07	1.34 (1.12-1.58)	1.16(0.96-1.39)	1.17 (0.97-1.40)
Cardiovascular death				
Q1	1.20	Reference	Reference	Reference
Q2	0.88	0.73 (0.42-1.27)	0.69(0.40-1.20)	0.85 (0.48-1.50)
Q3	1.56	1.30 (0.81-2.10)	1.16(0.71-1.89)	1.26 (0.76-2.08)
Q4	2.23	1.89 (1.22-2.95)	1.53(0.95-2.47)	1.68 (1.03-2.75)
All-cause mortality				
Q1	2.47	Reference	Reference	Reference
Q2	2.47	1.00 (0.70-1.42)	0.91(0.64-1.30)	1.04 (0.73-1.49)
Q3	3.20	1.29 (0.93-1.80)	1.10(0.78-1.55)	1.11 (0.78-1.57)
Q4	5.46	2.24 (1.66-3.03)	1.66(1.20-2.30)	1.68 (1.21-2.32)

Adjusted[1]: adjust for age, region, IT/ET, HCY, eGFR, hsCRP, TMAO;

Adjusted[2]: adjust for age, region, BMI, symptom to admission, IT/ET, heavy drinking, medical history (stroke, CHD, diabetes mellitus), stroke type, TOAST subtype, NIHSS at admission, SBP at admission, HDL-C, LDL-C, FBG, HCY, eGFR, hsCRP, TMAO, discharge with antiplatelet drugs, discharge with lipid-lowering drugs.

BMI: body mass index; CHD: coronary heart diseases; eGFR, estimated glomerular filtration rate; FBG: fasting blood-glucose; HCY: homocysteine; hsCRP: high-sensitivity C-reactive protein; HDL-C: high density lipoprotein cholesterol; IT/ET: Intravenous thrombolysis and endovascular treatment; LDL-C: low density lipoprotein cholesterol; MACE: major adverse cardiac events (defined as non-fatal stroke, non-fatal myocardial infarction, or cardiovascular death); MI: myocardial infarction; NIHSS: National Institutes of Health Stroke Scale; SBP: systolic blood pressure; TMAO: trimethylamine-N-oxide; TML: trimethyllysine.

Table S8. Mediation analysis of risk factors on TML- cardiovascular death.

Mediators	A (p-value)	B (p-value)	Med-eff (p-value)	Med-eff (%)	Direct effect (p-value)
SBP	0.034 (<0.01)	-0.031 (0.29)	-0.012 (0.33)	-1.20	0.087 (<0.01)
FBG	-0.019 (0.13)	0.007 (0.86)	0 (0.86)	0.00	0.075 (0.02)
LDL	0.011 (0.29)	-0.003 (0.93)	0 (0.94)	0.00	0.086 (<0.01)
HDL	-0.029 (0.08)	0.023 (0.05)	-0.011 (0.23)	-1.10	0.088 (<0.01)
HCY	0.081 (<0.001)	0.008 (0.80)	0.011 (0.80)	1.10	0.088 (<0.01)
eGFR	-0.29 (<0.001)	-0.079 (0.01)	0.307 (0.07)	30.70	0.052 (0.08)
hsCRP	0.075 (<0.001)	0.155 (<0.001)	0.133 (0.01)	13.30	0.078 (<0.01)
TMAO	0.309 (<0.001)	-0.014 (0.62)	-0.046 (0.63)	-4.60	0.091 (<0.01)

SBP: systolic blood pressure; FBG: fasting blood-glucose; LDL: low density lipoprotein cholesterol; HDL: high density lipoprotein cholesterol; HCY: homocysteine; eGFR, estimated glomerular filtration rate; hsCRP: high-sensitivity C-reactive protein; TMAO: trimethylamine-N-oxide; TML: trimethyllysine.

A=indirect effect of predictors on mediators; B=indirect effect of mediators on outcome; Med-eff=Mediation effect

Figure S1. Flow-chart of the Third China National Stroke Registry (CNSR-III).

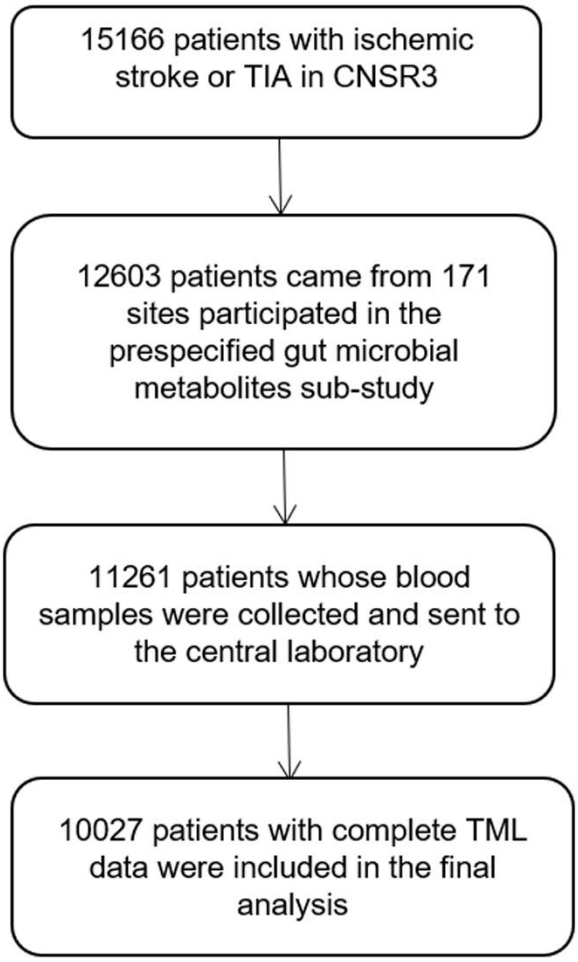
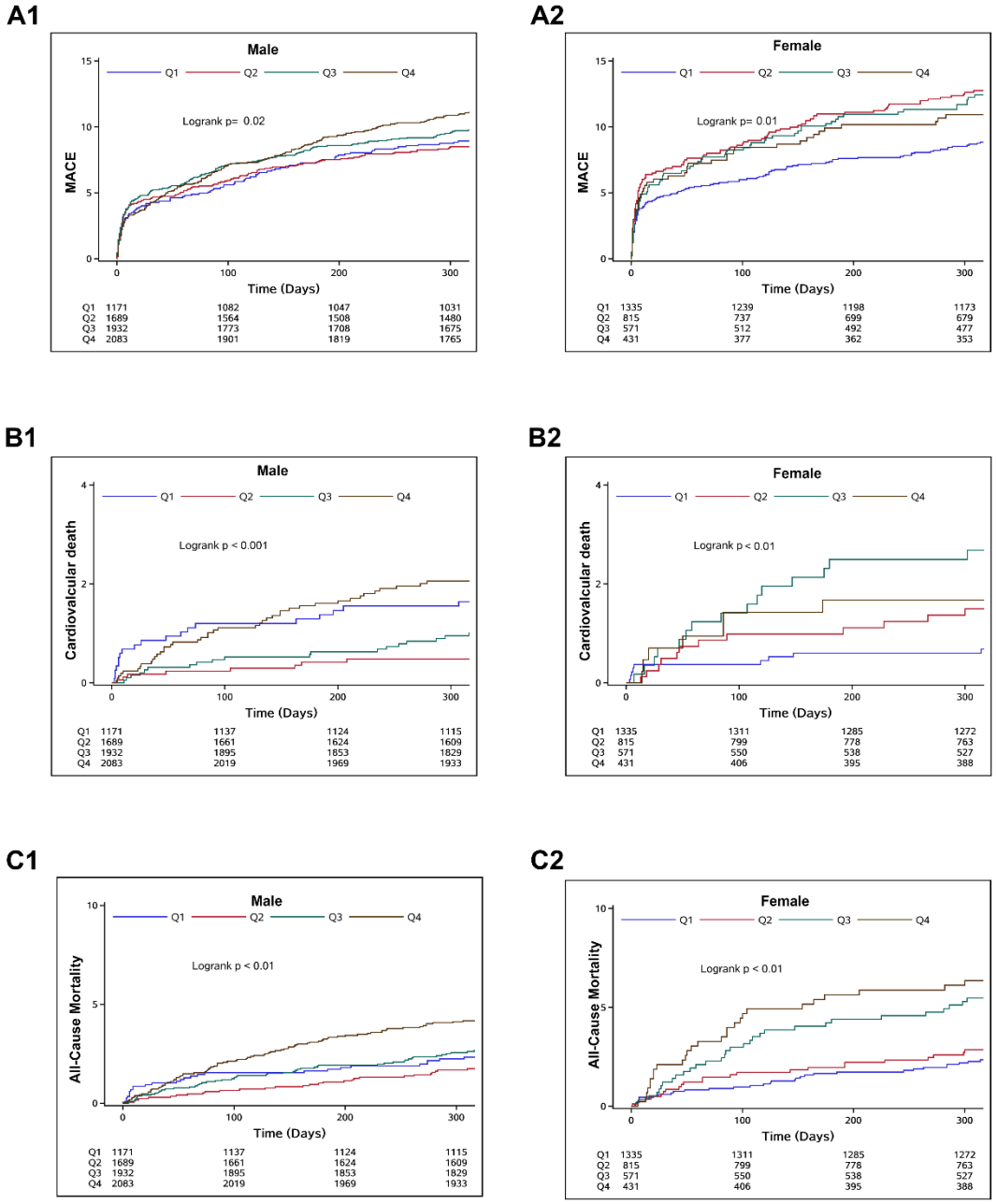


Figure S2. Cumulative incidence of 1-year outcomes (MACE, Cardiovascular death and All-cause Mortality) according to TML quartiles in male and female subgroups.



(A1, A2) TML and MACE; (B1, B2) TML and cardiovascular death; (C1, C2) TML and all-cause mortality; MACE, defined as defined as non-fatal stroke recurrence, non-fatal myocardial infarction and cardiovascular death.