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ARTICLE

Relationship between elevated plasma trimethylamine N-oxide levels and increased stroke injury

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Abstract

Objective

To investigate whether elevated plasma trimethylamine N-oxide (TMAO) levels are associated with initial stroke severity and infarct volume.

Methods

This cross-sectional study included 377 patients with acute ischemic stroke and 50 healthy controls. Plasma TMAO levels were assessed at admission. Stroke infarct size and clinical stroke severity were measured with diffusion-weighted imaging and the NIH Stroke Scale (NIHSS). Mild stroke was defined as an NIHSS score <6.

Results

Plasma TMAO levels were higher in patients with ischemic stroke than in healthy controls (median 5.1 vs 3.0 μmol/L; $p < 0.001$). Every 1–μmol/L increase in TMAO was associated with a 1.13-point increase in NIHSS score (95% confidence interval [CI] 1.04–1.29; $p < 0.001$) and 1.69-mL increase in infarct volume (95% CI 1.41–2.03; $p < 0.001$) after adjustment for vascular risk factors. At admission, 159 patients (42.2%) had experienced a mild stroke, and their plasma TMAO levels were lower compared to those with moderate to severe stroke (median 3.6 vs 6.5 μmol/L; $p < 0.001$). The area under the receiver operating characteristics curve of plasma TMAO level in predicting moderate to severe stroke was 0.794 (95% CI 0.748–0.839; $p < 0.001$), and the optimal cutoff value was 4.95 μmol/L. The sensitivity and specificity of TMAO levels ≥4.95 μmol/L for moderate to severe stroke were 70.2% and 79.9%, respectively.

Conclusions

Patients with ischemic stroke had higher plasma TMAO levels compared to healthy controls. Higher plasma TMAO level at admission is an independent predictor of stroke severity and infarct volume in patients with acute ischemia.

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Glossary

CI = confidence interval; **DWI** = diffusion-weighted imaging; **eGFR** = estimated glomerular filtration rate; **IQR** = interquartile range; **LDL** = low-density lipoprotein; **NIHSS** = NIH Stroke Scale; **OR** = odds ratio; **ROC** = receiver operating characteristics; **TMAO** = trimethylamine N-oxide; **TOAST** = Trial of Org 10172 in Acute Stroke Treatment.

In recent years, there has been an increased focused on intestinal microbiota for their association with the pathogenesis of cardiovascular disease.[1–3] Some dietary nutrients such as carnitine, choline, and phosphatidylcholine are especially processed by intestinal microflora to produce trimethylamine, which is absorbed by the intestine and converted into trimethylamine N-oxide (TMAO) in the liver by heparin-containing monooxygenase.[4] Numerous studies have revealed an association between plasma TMAO levels and cardiovascular risk in different cohorts.[1–3,5–7] However, more evidence is needed to understand the effects of the relationship between TMAO and ischemic stroke.

We have found that elevated plasma TMAO levels before carotid artery stenting are independently associated with an increased risk of new ischemic lesions on diffusion-weighted imaging (DWI) after carotid artery stenting, which suggests that TMAO may also be associated with ischemic stroke.[8] This is particularly relevant because recent studies on human platelets and animal models have shown that TMAO promotes platelet hyperresponsiveness and increases thrombosis by altering the stimulation-dependent calcium signaling in platelets.[9] These are common physiopathologic processes in both cardiovascular and cerebrovascular diseases. Thus, we hypothesized that TMAO levels in peripheral blood would be elevated in patients with ischemic stroke and that TMAO level is a predictor for the severity of stroke.

The purpose of this study, therefore, was to investigate the association between systemic levels of TMAO and stroke severity as assessed by the NIH Stroke Scale (NIHSS) and infarct volume as measured by DWI in patients with acute ischemic stroke.

Methods

Study design and participants

This multicenter prospective cohort study was conducted between July 2016 and January 2018. Consecutive patients with first-ever ischemic stroke who presented within 12 hours of stroke onset were enrolled at 4 centers (First Affiliated Hospital of Zhengzhou University, Fifth Affiliated Hospital of Zhengzhou University, Second Hospital of Hebei Medical University, and Xuanwu Hospital Capital Medical University).

The inclusion criteria were as follows: age ≥18 years; first episode of ischemic stroke and presentation within 12 hours of onset of symptoms, which was defined per the "last known normal time" principle; and informed consent obtained from the patient or legal representative.

The exclusion criteria included baseline head CT or MRI diagnosed as cerebral hemorrhage, abscess, vascular malformation, tumor, or other nonischemic cerebrovascular disease; history of TIA or stroke; treatment with probiotics or antibiotics within 1 month before admission; contraindications to MRI (e.g., implanted metal devices); current or previous participation in any clinical study within 6 months before enrollment; inability to comprehend the study; renal insufficiency (creatinine clearance rate <30 mL/min); and hepatic dysfunction (serum alanine transaminase or serum aspartate transaminase 2 times higher than the normal value).

The enrolled patients received optimal medical management in the stroke centers. Patients eligible for alteplase or endovascular therapy received the appropriate treatment according to the Chinese guidelines. All patients received acute treatment and secondary prevention of stroke in accordance with Chinese guidelines during hospitalization and after discharge.

In addition, 50 age-matched healthy controls were recruited from relatives of outpatients or inpatients (mainly spouses). Whether there were cerebrovascular events was determined by neurologists through clinical interviews and neurologic examinations. The healthy controls also did not consume probiotics or antibiotics within 1 month before study enrollment.

Clinical assessment

In all patients, medical history, initial stroke severity, vascular risk factors, and diseases were recorded at baseline. To minimize bias, all patients and investigators involved in the clinical evaluation were blinded to the results of the biochemical tests.

Initial stroke severity was assessed with the NIHSS[10] at the time of admission by persons trained in it. The relationship between the plasma TMAO levels and NIHSS scores at presentation was analyzed by considering NIHSS score a continuous variable and by classifying stroke at admission as mild stroke or moderate to severe stroke. We defined a stroke with an NIHSS score <6 as a mild stroke and an NIHSS score of ≥6 as a moderate to severe stroke. Other characteristics and clinical variables were collected through clinical interviews and neurologic examinations by a board-certified neurologist within 1 day of admission. Diagnosis of hypertension was based on previous treatment history with antihypertensive drugs; diabetes mellitus was defined on the basis of treatment

history with hypoglycemic, glycosylated hemoglobin >6.4%, or serum random glucose level ≥11.1 mmol/L on admission; dyslipidemia was defined according to treatment history with antihyperlipidemia drugs or serum cholesterol level >5.17 mmol/L at admission; and current smoking was defined as any smoking within 28 days before admission. The estimated glomerular filtration rate (eGFR) was calculated by Chronic Kidney Disease Epidemiology Collaboration equation.

According to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification, the subtypes of stroke in the present study were classified as large artery atherosclerosis, small vessel occlusion, cardioembolic, undetermined etiology, or other determined etiology.[11]

Brain MRI

Imaging acquisition parameters were standardized across sites. All patients underwent MRI within 24 hours of admission with a 1.5T or 3.0T scanner. The sequence includes axial spin echo T1-weighted sequence, T2-weighted sequence, fluid-attenuated inversion recovery sequence, DWI sequence (b = 1,000 s/mm^2, 2-mm isotropic resolution, 30 diffusion directions), and 3D time-of-flight angiography. The slice thickness of MRI was 5 mm, and the slice gap was 1.5 mm. Acute cerebral infarction was defined as high signal intensity on DWI sequence. Infarct areas were manually traced on DWI sequences. Infarct volumes were calculated by slice thickness multiplied by infarct area in each slice. Patients without DWI lesions were excluded from further analyses.

All imaging measures were acquired with a common protocol and were processed and reviewed by 2 experienced stroke neurologists who were blinded to the other characteristics of the patients. Disagreements were resolved by consensus. A third reader helped resolve the disagreements if consensus between the 2 raters could not be reached. The interrater agreement number was 373 (98.9%).

TMAO measurement

A venous blood sample was collected as soon as possible after admission. Whole-blood samples were centrifuged immediately into plasma, separated into vials, and stored in a −80°C refrigerator until analysis. Another fasting blood sample was also collected for routine biochemical tests and TMAO levels on the second day of admission at 6 to 7 AM before breakfast. Overnight fasting blood samples also were collected from 50 healthy controls. All blood samples were handled with the same protocol at all the centers.

As previously described, plasma levels of TMAO were detected with stable isotope dilution liquid chromatography–tandem mass spectrometry.[8,12] Briefly, 80 μL 10-μmol/L d9-TMAO was added to 20 μL plasma, and the sample was vortexed for 1 minute. The supernatant was then centrifuged for 25 minutes at 15,000*g* and transferred to a clean sampling bottle for testing. The supernatant of 10 mL was injected into SiO_2 column for analysis. The column temperature was 30°C; the flow rate was 0.8 mL/min, with mobile phase A with 0.1% formic acid aqueous solution and mobile phase B with 0.1% acetic acid in methanol. The concentration of TMAO and d9-TMAO was determined by positive multiple reaction monitoring mass spectrometry. We used various known TMAO concentrations to establish a standard curve for the determination of TMAO concentrations. The normal reference value (median, interquartile range [IQR]) of TMAO in healthy controls was 2.8 (IQR 1.9–4.8) μmol/L. The intra-assay coefficients of variation were 1.9% to 5.6%, and the interassay coefficients of variation were 2.9% to 8.4%. The analyses were performed with the same batch of reagents by board-certified laboratory technicians who had no access to the clinical data.

Statistical analyses

All the data were analyzed with SPSS 19.0 (SPSS Inc, Chicago, IL), GraphPad Prism 6 (GraphPad Software, La Jolla, CA), and SAS 9.4 (SAS Institute, Inc, Cary, NC). The data for categorical variables were described as proportions, and the data for continuous variables were described as medians (IQRs) or mean ± SD. The Kolmogorov-Smirnov test was used to assess the normality of data distribution. Continuous variables were analyzed by the Student *t* test or Mann-Whitney *U* test, and categorical variables were analyzed by the Pearson χ^2 test or Fisher exact test, depending on the nature of the underlying distributions. We used the Spearman rank correlation coefficient to assess the independent association between plasma TMAO levels and NIHSS scores, as well as infarct volumes. We adjusted any significant differences in the baseline clinical characteristics and other selected confounders in the subsequent multivariate analysis. The predictive value of TMAO for stroke and stroke severity (moderate to severe stroke) was assessed with the receiver operating characteristics (ROC) curve. We calculated the area under ROC, sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio. The best TMAO cutoff value was the threshold resulting in the maximum summation of sensitivity and specificity (i.e., the Youden index). Then, we assessed the association of the preestablished cutoff value of TMAO and stroke, as well as stroke severity, by logistic regression analysis. All tests were 2 sided. We set a value of $p < 0.05$ as statistically significant for all tests. Sample size calculation was based on the means and SD observed for TMAO levels of patients with stroke in a pilot study. An estimated 350 patients would be needed to yield a 90% power at $p = 0.05$ (2sided).

Standard protocol approvals, registrations, and patient consents

The study protocol was approved by medical ethics committee and the institutional research board of each participating center. The study was conducted according to the principles of the Declaration of Helsinki. Written informed consent was obtained from each patient or a legal representative before enrollment.

Data availability

Data related to the current article are available from the corresponding author on reasonable request.

Results

Patient characteristics and clinical variables

Between July 2016 and January 2018, 1,726 consecutive patients with acute ischemic stroke were screened. A total of 405 patients were enrolled in the study. Among those, 5 patients were excluded from final analysis because of missing NIHSS scores, 14 for missing MRI data within 24 hours of admission, and 10 for missing blood plasma results, resulting in a final sample size of 377 patients (figure 1). Mean ± SD age was 62.5 ± 10.7 years, and 162 (43.0%) of the patients were female. The median NIHSS score was 8 (IQR 4–13) at presentation, and the median infarct volume on DWI sequences was 16.6 (IQR 9.6–24.3) mL. The median time from stroke onset to blood collection was 8.4 (IQR 6.4–10.6) hours, and the median time from the stoke onset to MRI was 17.8 (IQR 12.2–23.8) hours. The median plasma TMAO levels did not vary significantly between blood samples taken at admission and after overnight fasting (5.1 vs 5.3 μmol/L; $p = 0.47$); therefore, the overnight fasting TMAO levels were excluded from further statistical analyses. The plasma TMAO levels did not vary significantly between different stroke subtype ($p = 0.36$) (data available from Dryad, table 1, doi.10.5061/dryad.kh304mq). The baseline demographic and clinical characteristics of the 377 patients with stroke are listed in table 1.

Differences in TMAO levels between patients with stroke and controls

The plasma TMAO levels at admission of patients with acute ischemic stroke were significantly higher than those of healthy controls (5.1 [IQR 3.3–7.6] vs 3.0 [IQR 2.2–4.5] μmol/L; $p < 0.001$) (figure 2). Further baseline characteristics for all participants are summarized in data available from Dryad (table 2, doi.10.5061/dryad.kh304mq).

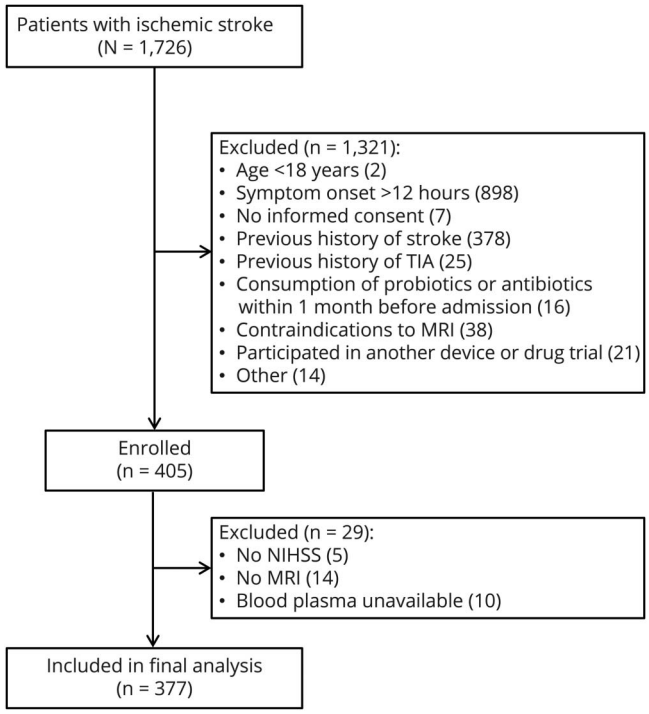
The area under ROC curve for distinguishing patients with acute ischemic stroke from healthy controls was 0.729 (95% confidence interval [CI] 0.673–0.784; $p < 0.001$) (figure 3A). A cutoff value of 4.95 μmol/L could identify stroke with a sensitivity of 49.1%, specificity of 94.0%, positive predictive value of 98.4%, negative predictive value of 19.7%, positive likelihood ratio of 8.17, and negative likelihood ratio of 0.54. After adjustment for age, sex, family history of stroke, hypertension, and systolic blood pressure, TMAO levels ≥4.95 μmol/L were found to be independent predictors of acute ischemic stroke (adjusted odds ratio [OR] 17.64, 95% CI 5.18–60.12; $p < 0.001$).

TMAO level and stroke severity

Results from the Spearman rank correlation indicated a significant association between plasma TMAO level and initial NIHSS score ($\rho = 0.557$; $p < 0.001$) (figure 4A). In the median regression model, a statistically significant increase in NIHSS score of 1.25 points for every 1–μmol/L increase in plasma TMAO levels (95% CI 1.12–1.38; $p < 0.001$) was observed. This translates to an approximate median increase of 1 point in NIHSS score for every 0.80–μmol/L increase in plasma TMAO level. This significance also existed after adjustment for age, sex, hypertension, coronary artery disease, atrial fibrillation, current smoking, peripheral artery disease, systolic blood pressure, body mass index, serum levels of glucose, low-density lipoprotein (LDL), eGFR, MRI acquired after alteplase or endovascular therapy, and time from stroke onset to MRI ($\beta = 1.13$, 95% CI 1.05–1.31; $p < 0.001$). There was no significant difference in the NIHSS scores between the various etiologies of stroke as defined by TOAST criteria ($p = 0.91$). Furthermore, the NIHSS score was also comparable when the etiology of stroke was dichotomized into cardioembolic and noncardioembolic strokes ($p = 0.54$).

At admission, 159 patients (42.2%) had mild stroke. The median plasma TMAO levels were 3.6 μmol/L in patients with mild stroke and 6.5 μmol/L in patients with moderate to severe stroke ($p < 0.001$) (figure 2). The group of patients with mild stroke had a greater number of cases of hypertension, higher baseline systolic blood pressure, higher baseline LDL levels, and smaller infarct volume and were less likely to receive alteplase or endovascular therapy, to undergo MRI scans after alteplase or endovascular therapy, or to have coronary artery disease (all $p < 0.05$). Details of baseline characteristics of the 2 groups are summarized in table 1.

Figure 1 Flowchart of the study



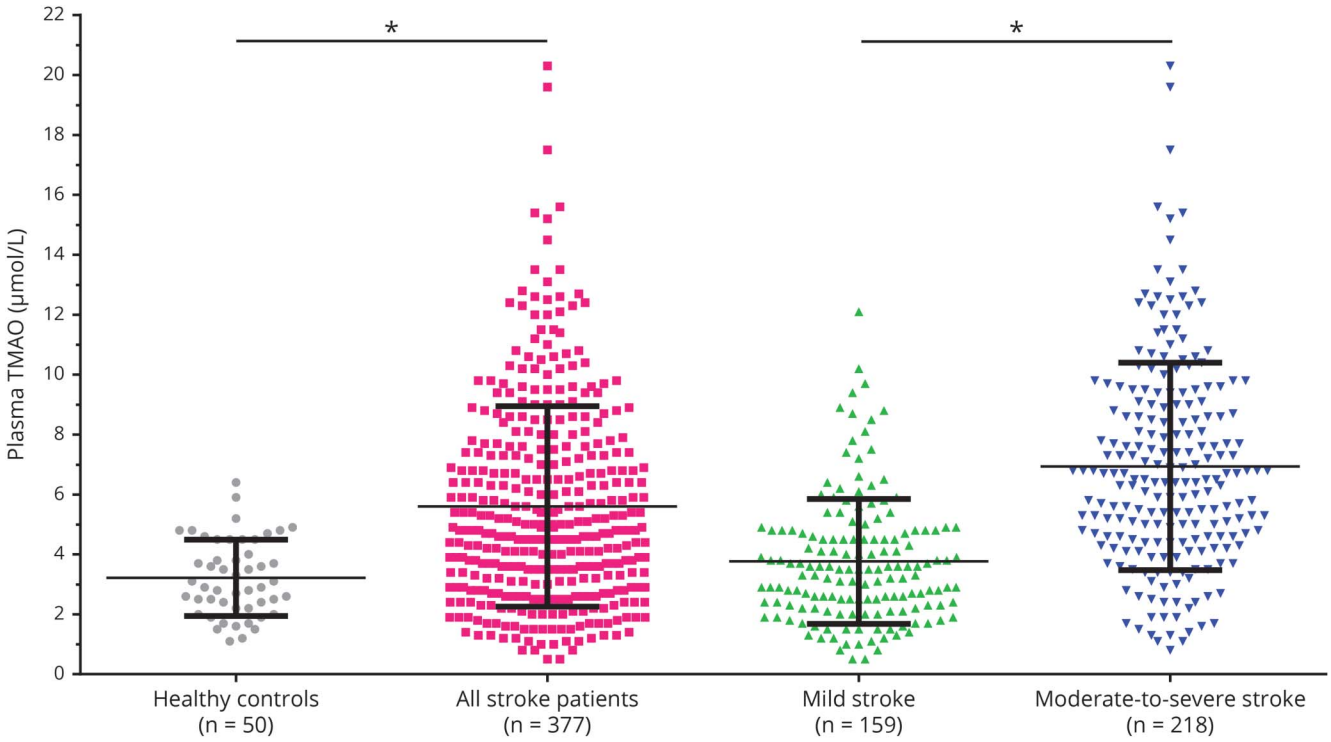
NIHSS = NIH Stroke Scale.

Table 1 Baseline characteristics of patients with ischemic stroke

Baseline characteristics	Total (n = 377)	Minor stroke (n = 159)	Moderate to severe stroke (n = 218)	*p* Value
Age, y	62.5 ± 10.7	61.8 ± 10.6	63.0 ± 10.8	0.27
Women, n (%)	162 (43.0)	71 (44.7)	91 (41.7%	0.57
Prior vascular risk factors, n (%)				
Hypertension	257 (68.2)	119 (72.3)	138 (63.3)	0.018
Diabetes mellitus	114 (30.2)	46 (28.9)	68 (31.2)	0.64
Dyslipidemia	61 (16.2)	29 (18.2)	32 (14.7)	0.35
Coronary artery disease	35 (9.3)	9 (5.7)	26 (11.9)	0.038
Atrial fibrillation	68 (18.1)	26 (16.4)	42 (19.3)	0.47
Current smoking	80 (21.2)	35 (22.0)	45 (20.6)	0.75
Peripheral artery disease	24 (6.4)	12 (7.5)	12 (5.5)	0.42
Family history of stroke	121 (32.1)	47 (29.6)	74 (33.9)	0.37
Clinical findings				
Systolic blood pressure, mm Hg	137.7 ± 16.1	139.8 ± 15.4	136.2 ± 16.5	0.035
BMI, kg/m^2	24.3 ± 2.7	24.2 ± 2.8	24.5 ± 2.7	0.29
Admission NIHSS score	8 (4–13)	3 (2–4)	12 (9–15)	<0.001
Infarct volume, mL	16.6 (9.6–24.3)	11.0 (7.3–18.7)	20.9 (12.8–27.7)	<0.001
TOAST classification, n (%)				0.58
Large artery atherosclerosis	71 (18.8)	25 (15.7)	46 (21.1)	
Small vessel occlusion	146 (38.7)	67 (42.1)	79 (36.2)	
Cardioembolism	80 (21.2)	31 (19.5)	49 (22.5)	
Other determined	7 (1.9)	3 (1.9)	4 (1.8)	
Undetermined	73 (19.4)	33 (20.8)	40 (18.3)	
Laboratory findings				
Serum glucose, mmol/L	5.3 (4.6–5.9)	5.3 (4.7–5.9)	5.2 (4.6–5.8)	0.67
TG, mmol/L	1.6 (1.2–2.0)	1.6 (1.2–1.9)	1.6 (1.2–2.1)	0.97
LDL, mmol/L	2.9 (2.3–3.6)	2.8 (2.2–3.3)	2.6 (2.2–3.9)	0.03
HDL, mmol/L	1.4 (0.9–1.9)	1.5 (1.0–1.9)	1.3 (0.9–1.8)	0.28
Creatinine, μmol/L	81.0 (66.0–93.0)	80.0 (63.0–90.5)	83.0 (68.0–96.0)	0.37
Blood urea nitrogen, mmol/L	5.3 (4.6–6.2)	5.1 (4.8–6.4)	5.4 (4.5–5.9)	0.68
eGFR, mL/min per 1.73 m^2	88.2 (76.5–96.6)	89.8 (74.5–99.2)	87.1 (78.5–94.4)	0.34
TMAO, μmol/L	5.1 (3.3–7.6)	3.6 (2.3–5.2)	6.5 (4.5–9.0)	<0.001
Patients received alteplase or endovascular therapy, n (%)	69 (18.3)	18 (11.3)	51 (23.4)	0.003
MRI acquired after alteplase or endovascular therapy, n (%)	46 (12.2)	12 (7.5)	34 (15.6)	0.018
Stroke onset to blood collection, h	8.4 (6.4–10.6)	8.2 (6.3–10.7)	8.5 (6.4–10.5)	0.62
Stroke onset to MRI, h	17.8 (12.2–23.8)	17.5 (12.5–24.0)	18.2 (12.2–23.8)	0.79

Abbreviations: BMI = body mass index; eGFR = estimated glomerular filtration rate; HDL = high density lipoprotein; LDL = low density lipoprotein; NIHSS = NIH Stroke Scale; TG = triglyceride; TMAO = trimethylamine N-oxide; TOAST = Trial of Org 10,172 in Acute Stroke Treatment.

Figure 2 Blood concentrations of TMAO in different groups



TMAO = trimethylamine N-oxide. *$p < 0.001$.

The area under ROC curve for prediction of moderate to severe stroke as opposed to mild stroke was 0.794 (95% CI 0.748–0.839; $p < 0.001$) for plasma TMAO level (figure 3B). The estimated optimal cutoff value was 4.95 μmol/L. TMAO had a sensitivity of 70.2%, specificity of 79.9%, positive predictive value of 82.7%, negative predictive value of 66.1%, positive likelihood ratio of 3.49, and negative likelihood ratio of 0.37 at this cutoff value.

Patients with plasma TMAO levels ≥4.95 μmol/L were significantly more likely to have a moderate to severe stroke (OR 9.34, 95% CI 5.76–15.16; $p< 0.001$) than those with plasma TMAO levels <4.95 μmol/L. The significance existed after adjustments for age, sex, hypertension, coronary artery disease, atrial fibrillation, current smoking, peripheral artery disease, body mass index, systolic blood pressure, serum levels of glucose, LDL, eGFR, MRI acquired after alteplase or endovascular therapy, and time from stroke onset to MRI (adjusted OR 9.69, 95% CI 5.42–17.21; $p< 0.001$) (table 2).

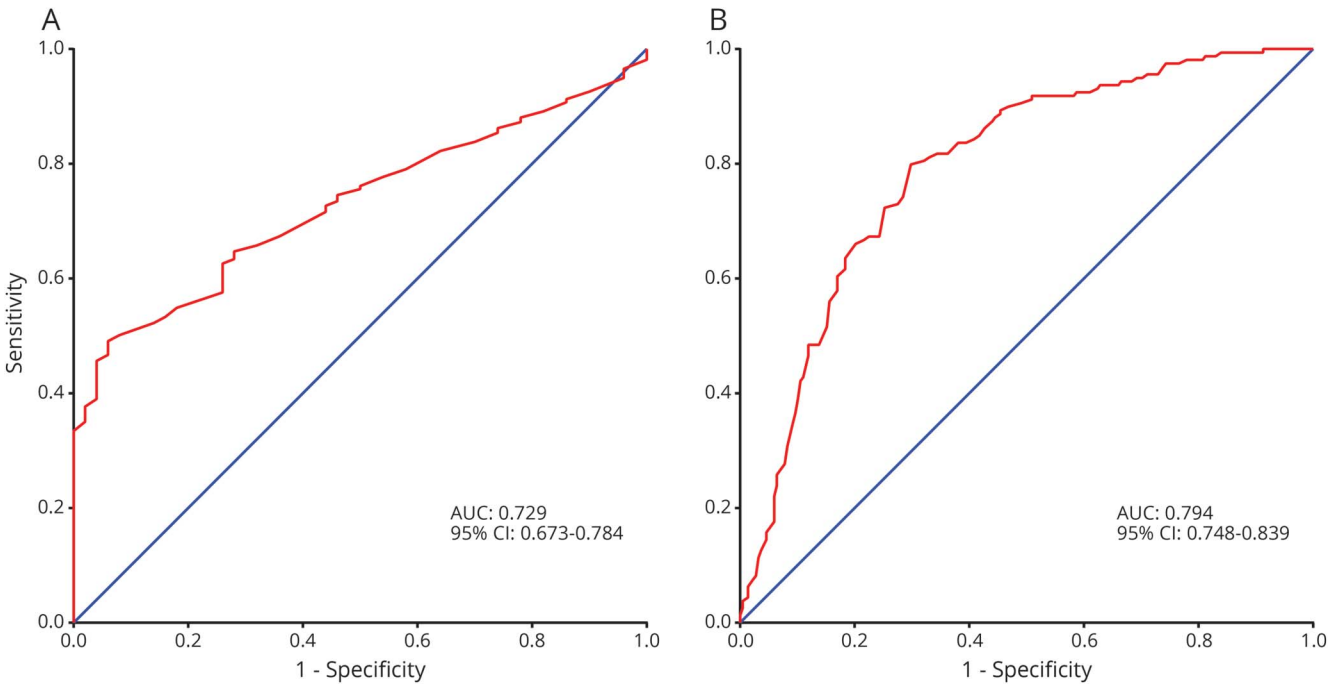
The plasma TMAO levels were divided into 4 quartiles. There was a statistically significant overall trend toward higher NIHSS scores across plasma TMAO quartiles ($p< 0.001$) (data available from Dryad, table 3, doi.10.5061/dryad.kh304mq). Being in the highest quartile (OR 21.92, 95% CI 9.89–48.58; $p < 0.001$), third quartile (OR 9.82, 95% CI 5.03–19.17; $p < 0.001$), and second quartile (OR 2.38, 95% CI 1.31–4.31; $p = 0.004$) was associated with a higher risk of moderate to severe stroke when the lowest quartile was used as a reference (table 2). In the multivariate logistic regression model, higher quartile of TMAO levels remained a significant predictor of moderate to severe stroke after adjustment for age, sex, hypertension, coronary artery disease, atrial fibrillation, current smoking, systolic blood pressure, peripheral artery disease, body mass index, serum levels of glucose, LDL, eGFR, MRI acquired after alteplase or endovascular therapy, and time from stroke onset to MRI (table 2).

TMAO level and infarct volume

Elevated levels of plasma TMAO at admission strongly correlated with larger infarct volume ($\rho = 0.558$; $p < 0.001$) (figure 4B). In the median regression model, a statistically significant increase in infarct volume of 1.62 mL for every 1–μmol/L increase in plasma TMAO level (95% CI 1.27–1.97; $p < 0.001$) was observed. This translated to an approximate median increase of 1 mL in infarct volume for every 0.62–μmol/L increase in TMAO level. After adjustment for age, sex, hypertension, coronary artery disease, atrial fibrillation, systolic blood pressure, current smoking, peripheral artery disease, body mass index, serum levels of glucose, LDL, eGFR, MRI acquired after alteplase or endovascular therapy, and time from stroke onset to MRI, elevated plasma TMAO levels remained associated with increased infarct volume ($\beta = 1.69$, 95% CI 1.31–2.23; $p < 0.001$).

Next, the plasma TMAO levels were divided into 4 quartiles. The median infarct volumes were 10.2, 13.9, 19.3, and 24.5

Figure 3 Receiver operating characteristic curves of TMAO



(A) All patients with acute ischemic stroke vs healthy controls. (B) Those with moderate to severe stroke vs minor stroke. AUC = area under the receiver operating curve; CI = confidence interval; TMAO = trimethylamine N-oxide.

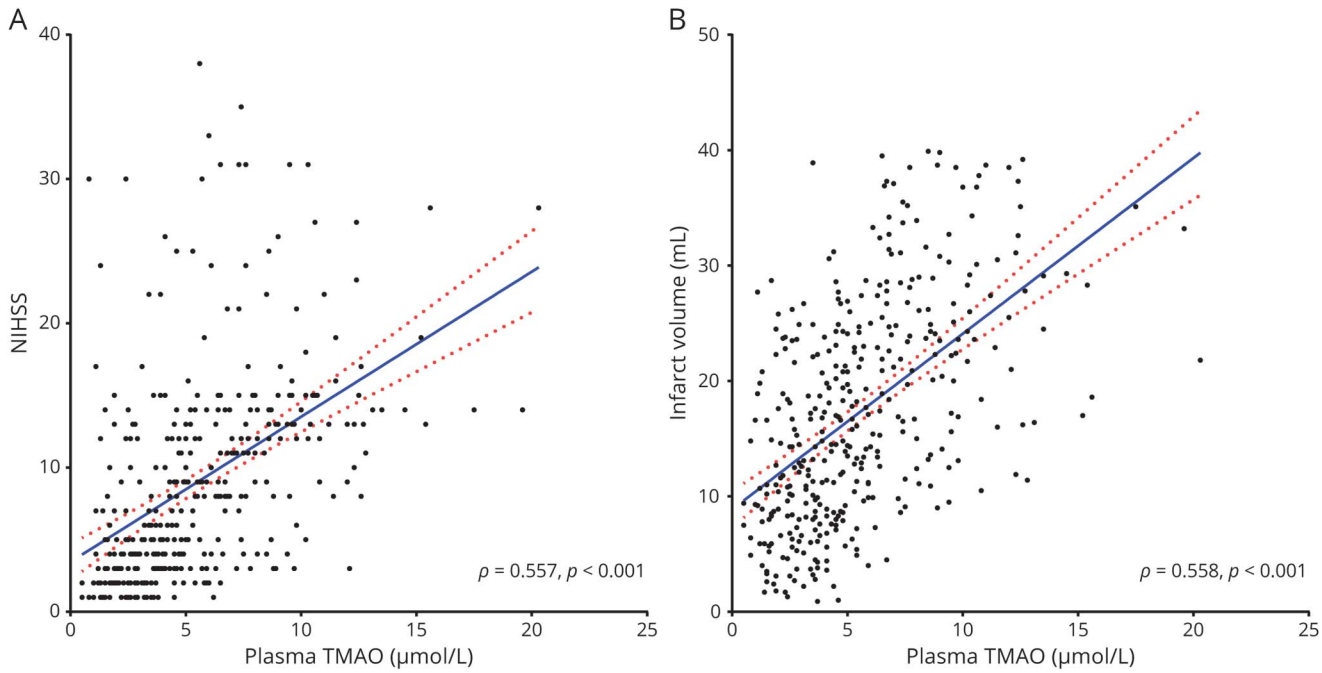
mL from the lowest to the highest quartiles ($p < 0.001$) (data available from Dryad, table 4, doi.10.5061/dryad.kh304mq). Patients in the highest quartile of plasma TMAO level (unadjusted β for highest quartile vs lowest quartile = 4.46, 95% CI 3.68–5.23; $p < 0.001$), third quartile (unadjusted β for third quartile vs lowest quartile =4.26, 95% CI 3.11–5.42]; $p < 0.001$), and second quartile (unadjusted β for second quartile vs lowest quartile = 2.93, 95% CI 0.70–5.17; $p = 0.01$) had a statistically significant larger infarct volume. In the multivariable model adjusted for age, sex, hypertension, coronary artery disease, atrial fibrillation, body mass index, current smoking, peripheral artery disease, systolic blood pressure, serum levels of glucose, LDL, eGFR, MRI acquired after alteplase or endovascular therapy, and time from stroke onset to MRI, elevated plasma TMAO levels remained significantly associated with increased infarct volume (table 3).

Discussion

We demonstrated an association between plasma TMAO levels and ischemic stroke injury. Compared with healthy controls, patients with acute ischemic stroke have elevated plasma TMAO levels. Patients with increased plasma TMAO levels at admission are more likely to have sustained more severe ischemic stroke and to have a larger infarct volume at the time of their index stroke than patients without increased plasma levels of TMAO. Plasma TMAO level was a predictor of severe stroke and larger infarct volume, with a median increase of 1.13 points in NIHSS score and 1.69 mL in infarct volume for every 1–μmol/L increase in plasma TMAO levels after adjustment for confounders.

Our findings are partly in line with a recently published meta-analysis that included 19 prospective studies and reported that elevated serum concentrations of TMAO levels were independent risk factors for major adverse cardiovascular events and all-cause mortality.[13] Although most studies focus on cardiovascular events, some studies include cerebrovascular disease in their outcomes. A recently published nested case-control study in a sample population from the China Stroke Primary Prevention Trial found that the median plasma TMAO concentration in Chinese patients with hypertension was 2.4 μmol/L,[14] which is lower than the levels found in the healthy controls (3.0 μmol/L) of the present study. This difference may be due to the effects of different dietary patterns between hypertensive patients reported in a randomized trial[3,15] and was found to influence plasma TMAO levels.[16] The differences of TMAO levels noted between patients with stroke and healthy controls in the current study are possibly but unlikely attributed to different dietary patterns because our study included only patients with a first episode of stroke (12-hour window). In addition, most healthy controls were spouses of the patients who may have similar dietary patterns. However, no direct measures of dietary intake were available in the present study

Figure 4 Association of TMAO levels with NIHSS scores and infarct volume



Linear fit (solid line) and 95% confidence intervals (dashed lines) are shown in color. NIHSS = NIH Stroke Scale; TMAO = trimethylamine N-oxide.

to address the potential threat to validity. The results of our trial partly differ from that of a previous study that found that blood TMAO levels are lower in patients with large artery atherosclerotic stroke and TIA.[17] Different spectrums of patients and different protocols for blood collection may explain this difference. Our study did not exclude patients with small vessel occlusion, cardioembolism, or other etiologies of stroke. Hence, our findings can be considered representative of the spectrum of ischemic stroke.

A recent large clinical study including 431 white and 801 black patients on hemodialysis found a significant correlation between TMAO levels and cardiovascular events. Although there was no significant difference in TMAO levels between white and black people, the correlation of TMAO and cardiovascular events differed by race. In the white population, higher TMAO levels were significantly associated with higher risk of cardiac death, sudden cardiac death, first cardiovascular event, and all-cause death. However, only cardiac death was found to be significantly associated with TMAO levels below the median in black patients.[18] Therefore, the association between plasma TMAO levels and outcomes might differ according to race and ethnic groups. Our study further demonstrates that this association may also exist in Asians; however, the results of the present study should be interpreted cautiously for other races and ethnic groups. The results of this study need to be confirmed in the future with a larger sample size and in different ethnic groups to generalize the results.

Metabolism of choline and phosphatidylcholine by the intestinal microbiota produces trimethylamine by cutting the choline portion of lecithin at the carbon-nitrogen bond. Trimethylamine metabolizes to TMAO in the liver. TMAO has been shown to be atherogenic.[3] Animal studies have shown that TMAO can increase the expression of proatherogenic scavenger receptors and CD36 on the surface of macrophages. This process promotes the accumulation of cholesterol and foam cell formation in macrophages in a microbial-dependent manner, leading to atherosclerosis.[2,19,20] TMAO interacts with platelets, increases the platelet reactivity, and promotes thrombosis.[9] It also promotes thrombosis through increasing tissue factor expression and activity in primary human coronary artery endothelial cells.[21] Inhibiting thrombosis is essential for stroke treatment and prevention. Future studies should focus on the relationship between TMAO and thrombosis, which may reveal whether regulating the level of TMAO can have an antithrombotic effect. In addition, in an animal study, mice were fed a high-fat diet and TMAO. TMAO increased the fasting insulin level by regulating the gene expression of insulin signal transduction pathway in the liver, which could aggravate impaired glucose tolerance and induce insulin resistance.[22] These factors can also lead to stroke. Another clinical study further found that patients with different severities of stroke had different diversity of gut microbiota,[17] which may explain the mechanism of the results observed in the present study. However, future studies are warranted to examine the underlying mechanisms.

Table 2 Relationship between TMAO levels and moderate to severe stroke

	OR (95% CI)			
TMAO level, μmol/L	**Unadjusted**	***p* Value**	**Adjusted[a]**	***p* Value**
Quartile of TMAO level				
1 (<3.3)	1		1	
2 (3.3–5.1)	2.38 (1.31–4.31)	0.004	2.66 (1.33–5.48)	0.003
3 (5.2–7.6)	9.82 (5.03–19.17)	<0.001	10.11 (5.56–23.41)	<0.001
4 (>7.7)	21.92 (9.89–48.58)	<0.001	24.78 (11.57–56.21)	<0.001
Continuous TMAO level				
<4.95	1		1	
≥4.95	9.34 (5.76–15.16)	<0.001	9.69 (5.42–17.21)	<0.001

Abbreviations: CI = confidence interval; OR = odds ratio; TMAO = trimethylamine N-oxide.
[a] ORs were adjusted for age, sex, hypertension, coronary artery disease, atrial fibrillation, current smoking, peripheral artery disease, systolic blood pressure, body mass index, serum levels of glucose, low-density lipoprotein, estimated glomerular filtration rate, MRI acquired after alteplase or endovascular therapy, and time from stroke onset to MRI.

Although it is not clear from this study whether the changes of TMAO occur before or after stroke, numerous case-control and longitudinal human studies have shown that there may be a causal relationship between elevated TMAO levels and increased risk of major adverse cardiovascular events. The Cleveland Cohort Study prospectively included 4,007 adults who underwent elective coronary angiography. The results showed that increased TMAO at baseline was an independent predictor of major adverse cardiovascular events after adjustment for traditional cardiovascular risk factors during the 3-year follow-up.[1] Another study conducted in China also demonstrated that higher TMAO levels in hypertensive patients were significantly associated with the risk of first stroke.[14] The present study further demonstrates that elevated TMAO level may be associated with increased stroke injury as measured by the NIHSS and infarct volume. In addition, some studies have found that flavin monooxygenase-3 can convert trimethylamine produced by microorganisms into TMAO. Using an antisense oligonucleotide method to knock down flavin monooxygenase-3 can inhibit production of TMAO and the formation of atherosclerosis in animal models.[23–26] The trimethylamine/flavin monooxygenase-3/TMAO pathway is a central regulator of lipid metabolism and cholesterol balance.[26] These studies suggest that TMAO not only is associated with stroke injury but also is involved in the pathophysiologic process of stroke. TMAO and its production pathway may be a potential new therapeutic target for improving the prognosis of patients with ischemic stroke. Preclinical studies have found that single-dose oral trimethylamine-generating enzyme (CutC and CutD) inhibitor could inhibit the increase of TMAO within 3 days and reverse diet-induced platelet hyperresponsiveness and thrombosis. At the same time, the inhibitor did not increase the risk of bleeding, and no toxicity was observed.[27] Future studies should also include dietary intake or biochemical precursors of TMAO (choline and microbiome). This might refine the hypothesis and target the pathway (upstream precursors or TMAO directly).

The strengths of this study include the prospective design and blinding of the investigators who estimated the infarct volume

Table 3 Relationship between TMAO levels and infarct volume

Quartile of TMAO level	**Univariate change in infarct volume (95% CI), mL**	***p* Value**	**Multivariate change in infarct volume (95% CI), mL[a]**	***p* Value**
1 (<2.9)	1		1	
2 (2.9–5.1)	2.93 (0.70–5.17)	0.01	3.11 (0.92–6.03)	0.007
3 (5.2–8.1)	4.26 (3.11–5.42)	<0.001	4.07 (2.81–5.54)	<0.001
4 (>8.1)	4.46 (3.68–5.23)	<0.001	4.36 (3.21–5.87)	<0.001

Abbreviations: CI = confidence interval; TMAO = trimethylamine N-oxide.
[a] Odd ratios were adjusted for age, sex, hypertension, coronary artery disease, atrial fibrillation, current smoking, peripheral artery disease, systolic blood pressure, body mass index, serum levels of glucose, low-density lipoprotein, estimated glomerular filtration rate, MRI acquired after alteplase or endovascular therapy, and time from stroke onset to MRI.

by DWI. This study has some limitations to consider. First, previous studies have shown that changes in dietary pattern can affect TMAO levels.[1,16] To avoid the impact of dietary management after admission on baseline TMAO levels, only patients who presented within 12 hours were included in the present study. This may limit the general applicability of the results. Second, on the basis of the same considerations, blood samples for testing TMAO were obtained at admission, and they were not necessarily fasting samples. The duration between the last meal and blood collection may have influenced the level of TMAO. However, there were no significant differences between the TMAO levels at admission and after fasting in the present cohort. Third, diets differ by sex in the elements that can throttle TMAO (meat vs plant based). However, sex was adjusted for in the multivariable analysis and is not an effect modifier in the present study. This may be explained by the fact that all the participants are patients with stoke in the present study, and these patients may have common risk factors for stroke (such as unhealthy diet). Fourth, the relationship between stroke severity and plasma levels of TMAO at admission may be influenced by multiple variables such as diet, gut microbiota, flavin monooxygenase enzymes, and kidney functions. Although we adjusted for potential confounders using multivariable analyses, it is possible that there were unknown confounders. Fifth, TMAO levels may be dynamic, and 1 or 2 isolated values at admission seem to be insufficient to understand the complexity of the metabolic processes in the ischemic brain. Future studies should include assessment of TMAO at multiple time points. Finally, the cross-sectional study design did not allow us to establish a cause-effect relationship between plasma TMAO levels and stroke injury. This warrants future well-designed longitudinal cohort studies and trimethylamine-generating enzyme inhibitor intervention clinical studies.

The current study provides preliminary data to demonstrate that plasma TMAO levels at admission were increased in patients with acute ischemic stroke compared to healthy controls. Plasma TMAO levels at the index ischemic stroke are independently associated with increased severity of stroke and infarct volume. More studies are warranted to include assessment of TMAO at multiple time points, to focus on the role of TMAO and intestinal microbiota in the pathogenesis of atherosclerosis, and to investigate this potential therapeutic target in patients with stroke.

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Disclosure

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Appendix *(continued)*

Name	Location	Role	Contribution
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