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# TMAO as a Novel Predictor of Major Adverse Vascular Events and Recurrence in Patients with Large Artery Atherosclerotic Ischemic Stroke

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**Abstract**

**Objectives:** To explore the association of plasma trimethylamine N-oxide (TMAO) concentration with large artery atherosclerotic (LAA) ischemic stroke and its role in predicting neurological outcome and major vascular event recurrence.

**Materials and Methods:** We performed a case-control study that included patients with first-ever LAA stroke as cases (n = 291) and asymptomatic patients as controls (n = 235). Clinical data and venous blood samples were collected within 72 hours after stroke. All subjects were followed for 3 months. TMAO level was detected by liquid chromatography mass spectrometry (LC-MS). Logistic and Cox proportional hazard regression were performed to evaluate plasma TMAO concentration as a predictor of LAA stroke and major vascular event recurrence, respectively. Kaplan–Meier survival analysis was performed to compare major vascular event recurrence between patients with high and low TMAO concentration.

**Results:** After adjusting for traditional stroke risk factors, the plasma TMAO level was significantly higher in the LAA stroke group than the control group (OR = 1.031, 95% CI 1.024-1.037, $P < .001$). At a cutoff level of 106.9 pg/ml, TMAO had a sensitivity of 63.23% and specificity of 80.00% in discriminating the LAA stroke subjects from the controls in Receiver operator characteristic (ROC) analysis. Kaplan–Meier survival analysis demonstrated TMAO plasma concentration was significantly relevant with recurrent vascular events (Log Rank, $P = .006$). Moreover, this association was still existed after adjusting for traditional risks (adjusted HR, 3.128; 95% CI, 1.018-9.610) in Cox regression model. But TMAO plasma levels were not relevant with functional disability after 3 months of the LAA stroke.

**Conclusion:** Elevated plasma TMAO concentration was independently associated with LAA ischemic stroke. The risk of major vascular event recurrence increased by 2.128 times in the LAA stroke subjects with plasma TMAO level higher than 126.83 pg/mL. Plasma TMAO concentration might be a potential biomarker of major vascular event recurrence.

**Keywords**

trimethylamine N-oxide, ischemic stroke, atherosclerosis, large artery atherosclerotic stroke

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## Introduction

Ischemic stroke is a leading cause of disability and mortality, accounting for more than 80% of all strokes.[1,2] Stroke is a preventable and controllable disease. In addition to controlling the primary disease, there is a major need for novel effective prevention and control measures for the occurrence, development and recurrence of stroke.[3]

Trimethylamine N-oxide (TMAO) is a small molecule generated from dietary choline and carnitine.[4] Gut microbes

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metabolize these precursors into trimethylamine (TMA),[4] which is absorbed and travels via the portal circulation to the liver, where it is oxidized to TMAO by hepatic flavin-containing monooxygenases (FMOs), primarily FMO3.[5] TMAO is atherogenic[6] and has been associated with cardiovascular disease (CVD),[5,7,8] which shares common risk factors and pathophysiology with large artery atherosclerotic (LAA) ischemic stroke.[9] Since elevated plasma TMAO concentration has been associated with atherosclerosis and CVD, it is likely to be associated with LAA stroke as well.

Understanding risk factors associated with recurrent major vascular events after an initial ischemic stroke may help in the design of secondary prevention studies and allocation of limited health resources. A role of TMAO in predicting functional outcomes and major vascular event recurrence after LAA ischemic stroke has been hypothesized but not yet explored. Therefore, this study aimed to examine the relationship between plasma TMAO concentration and LAA stroke. Furthermore, we aimed to investigate plasma TMAO concentration as a predictor of outcome and major vascular event recurrence three months after stroke onset. We hypothesized that plasma TMAO concentration is elevated in LAA stroke patients and that higher levels predict a worse 3-month outcome and higher 3-month major vascular event recurrence rate.

## Methods

### Study Population

This case-control study recruited patients aged 45–80 years diagnosed with first-ever LAA ischemic stroke who were admitted within 72 hours of stroke onset from December 2016 to December 2017 as case patients. All cases in the LAA stroke group were initially diagnosed with acute ischemic stroke and considered to have large-artery atherosclerosis, by Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification.[10] The diagnosis of ischemic stroke was confirmed based on strict neurological examination, computed tomography, or magnetic resonance imaging. Subjects with a clear history of coronary atherosclerotic heart disease, ischemic stroke, or transient ischemic attack (TIA); other TOAST classification stroke subtype; severe comorbidity (heart failure, respiratory failure, renal dysfunction, hepatic impairment, pregnancy, recent severe infection, cancer, autoimmune diseases, coagulopathy or other blood disease). Asymptomatic subjects (not in any acute disease state by the report of physical examination and self-report) undergoing physical examinations served as controls. We did not use an age- and sex-matching strategy in our selection of controls and patients. Control participants were free of clinically detectable cerebrovascular disease and without any stroke history. The same exclusion criteria were applied in the control participants. All subjects completed color Doppler flow imaging, echocardiography to determine their cardiovascular conditions.

According to the criteria of the TOAST,[10] subjects with acute ischemic stroke were divided into large atherosclerosis (LAA), small-artery occlusion, cardioembolism, stroke of other determined etiology, and stroke of undetermined etiology. Adjudication of subtype was performed by 2 neurologists. Disagreements between the 2 neurologists were resolved by third reviewer to reach a consensus.

The study was performed in accordance with the Declaration of Helsinki and approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University. Written informed consent was obtained from all participants.

### Study Data

All subjects underwent a complete history, physical examination, clinical chemistry analysis and stroke severity assessment using the National Institutes of Health Stroke Scale (NHISS)[11] on admission. Plasma was separated from blood samples that were collected within 72 hours of stroke onset using ethylenediaminetetraacetic acid-containing vacutainer tubes. The first fasting samples were drawn from all participants within 24 hours after admission.

Samples were maintained at 4 °C, centrifuged at 2000 rpm for 15 minutes within 2 hours, and immediately frozen at −80 °C until analysis. Plasma TMAO concentration was quantified using liquid chromatography mass spectrometry (LC-MS). Acetonitrile (300 uL) was added to plasma (100 uL) for precipitation of proteins. After 30 seconds, samples were centrifuged for 5 minutes (15 000 rpm, 4 °C). Chromatographic separation was performed using a silica column ($2.1 \times 100$ mm, 5 μm internal diameter) and mobile phase containing acetonitrile (phase A) and ammonium formate aqueous solution (10 mmol/L) with a flow rate of 0.4 mL/min. TMAO concentration was calculated from a calibration curve generated using various known concentrations of TMAO.

The primary study outcome was neurological functional disability as determined by the modified Rankin Scale (mRS) score three months after stroke onset. Good outcome was defined as score 0–2. Poor outcome was defined as score 3–6.[12] The secondary outcome was major vascular event (TIA, recurrent ischemic stroke, acute myocardial infarction) within the first three months of stroke.

### Statistical Analysis

Continuous data with a normal distribution are presented as means with standard deviation and were compared using the t test. Continuous data with a skewed distribution are presented as medians with interquartile range and were compared using the Mann–Whitney U test. Categorical data are presented as numbers with frequency and were compared using the $\chi^2$ test. Logistic regression was used to examine plasma TMAO concentration as a predictor of LAA stroke and the 3-month post-stroke mRS score. Cox proportional hazards regression was performed to analyze plasma TMAO concentration as a predictor of major vascular event recurrence within 3 months of stroke onset. Kaplan–Meier survival analysis was performed to compare major vascular event recurrence during 3-month follow-up between patients with high and low TMAO concentration. High and low TMAO concentration was defined relative to the median concentration value. $P < .05$ was considered

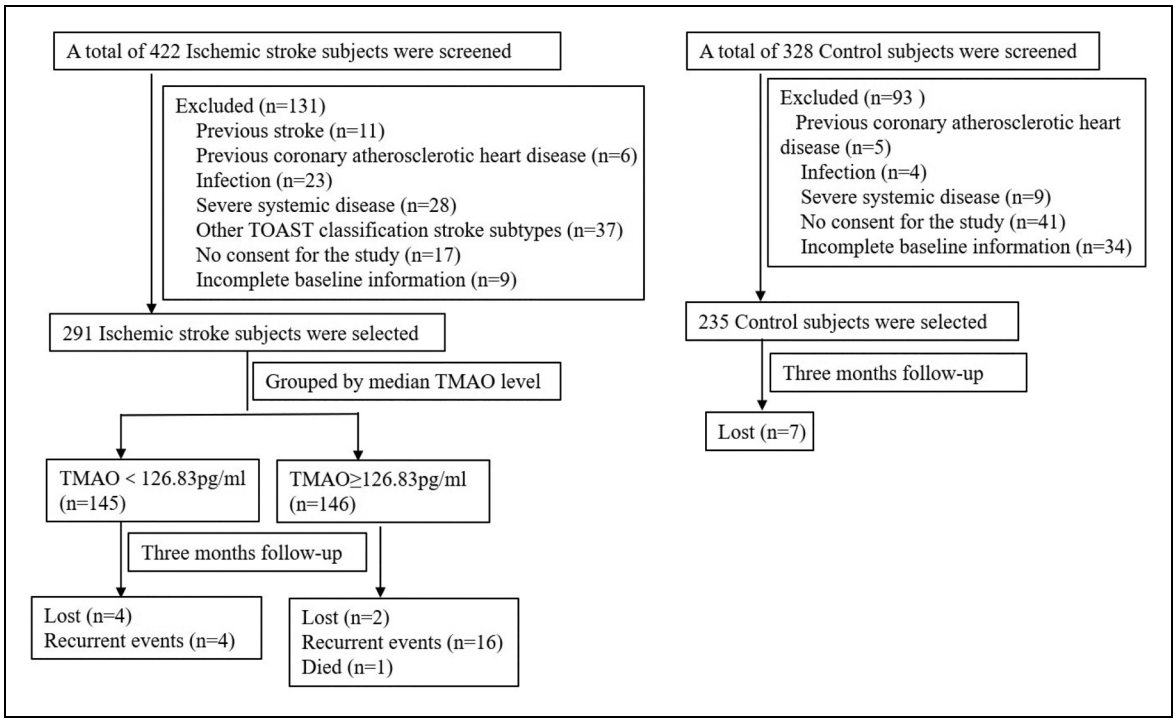


**Figure 1.** Study of flow chart.

**Table 1.** Baseline patient characteristics.

| Variable | Control group (n = 235) | LAA-stroke group (n = 291) | $t/\chi^2$ | *P* value |
|---|---|---|---|---|
| Age,y | 59.71 ± 7.67 | 61.68 ± 7.27 | 3.005 | .003 |
| Female, N (%) | 98 (41.7) | 132 (45.4) | 0.707 | .427 |
| Hypertension, N (%) | 108 (46) | 221 (64.6) | 49.09 | <.001 |
| Diabetes mellitus, N (%) | 64 (27.2) | 132 (45.4) | 18.273 | <.001 |
| Smoker, N (%) | 94 (40.0) | 85 (29.2) | 6.73 | .009 |
| Cre (umol/L) | 66.29 ± 16.21 | 73.98 ± 21.18 | 15.059 | <.001 |
| LDL-C (mmol/L) | 2.75 ± 0.92 | 2.84 ± 1.88 | 0.18 | .507 |
| TMAO (pg/ml) | 85.15 ± 32.11 | 129. 65 ± 46.24 | 29.39 | <.001 |

Abbreviations: Cre, creatinine; LDL-C, low-density lipoprotein-cholesterol; TMAO, trimethylamine N-oxide.
Continuous data are presented as means ± standard deviation.
Categorical data are presented as numbers (%).

significant. Statistical analyzes were performed using SPSS software version 15.0 (IBM Corp., Armonk, NY, United States).

## Results

### *Baseline Characteristics of this Cohorts*

In our study, the LAA stroke and control group comprised 291 and 235 patients, respectively (Figure 1). Baseline patient characteristics are shown in Table 1. Mean patient age significantly differed between the LAA stroke (61.68 ± 7.27 years) and the control group (59.71 ± 7.67 years) ($P = .003$). The proportions of patients with hypertension was significantly higher in the LAA stroke group (64.6%) versus the control group (46%) ($P < .001$). Diabetes was found significantly higher in the LAA stroke group (45.4%) than the control group (27.2%)

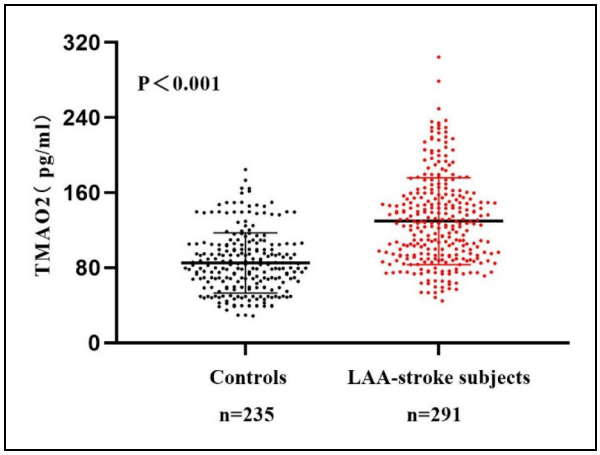


**Figure 2.** Comparison of plasma Trimethylamine N-oxide (TMAO) levels between the control subjects and the LAA-stroke subjects.

**Table 2.** Logistic regression analyzes of plasma TMAO concentration as a predictor of large artery atherosclerotic stroke.

| Model | Odds ratio (95% CI) | *P* value |
|---|---|---|
| Unadjusted | 1.030 (1.024-1.037) | <.001 |
| Model 1 | 1.031 (1.024-1.037) | <.001 |
| Model 2 | 1.032 (1.025-1.039) | <.001 |

Model 1 was adjusted for age and sex.
Model 2 was adjusted for age, sex, hypertension, diabetes mellitus, smoking, and creatinine.

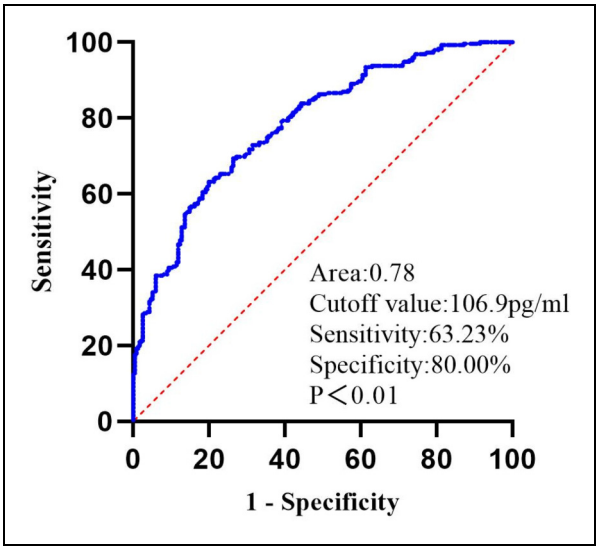


**Figure 3.** Receiver operator characteristic (ROC) curve of plasma TMAO level for discriminating between the control group a nd the LAA-stroke group.

($P < .001$). Mean plasma TMAO concentration was significantly higher in the LAA-stroke group (129. 65 ± 46.24 pg/ml) than in the control group (85.15 ± 32.11 pg/ml) ($P < .001$; Table 1, Figure 2). Plasma TMAO concentration was significantly elevated in the LAA stroke in unadjusted regression models ($P < .001$; Table 2). Moreover, the significant value of plasma TMAO concentration preserved after adjusting for age, sex, hypertension, diabetes mellitus, smoking, and creatinine ($P < .001$; Table 2).

## *Diagnostic Value of Plasma TMAO Level*

The diagnostic value of plasma TMAO in distinguishing the LAA stroke group and control group was evaluated with Receiver operator characteristic (ROC) curves analysis. The best cutoff plasma level of TMAO was 106.90 pg/ml to generate the maximum summation of sensitivity and specificity in discriminating the LAA stroke and control group. The area under the ROC curve was 0.78 (95% CI: 74.65%-82.31%, sensitivity = 0.63, specificity = 0.80) (Figure 3), suggesting that the plasma TMAO concentrations could be applied to discriminate the LAA stroke subjects from the asymptomatic subjects.

## *Relationship between Plasma TMAO Levels and Vascular Events Recurrence*

The LAA stroke group patients were dichotomized into high and low concentration subgroups using median TMAO concentration (126.83 pg/mL) as the cutoff. Characteristics of these subgroups are shown in Table 3. The proportions of patients with hypertension and diabetes were significantly higher in the high TMAO concentration subgroup ($P < .05$). Patient age, sex, smoking status, and creatinine concentration,

**Table 3.** Characteristics of the large artery atherosclerotic stroke group patients dichotomized by median TMAO concentration.

| | TMAO (pg/ml) | | | |
|---|---|---|---|---|
| Variable | <126.83 (n = 145) | ≥126.83 (n = 146) | $t/\chi^2$ | *P* value |
| Age, y | 60.94 ± 7.16 | 62.41 ± 7.33 | 1.724 | .086 |
| Female, n % | 63 (43.4) | 69 (47.2) | 0.427 | .514 |
| Hypertension, n (%) | 120 (82.8) | 101 (69.2) | 7.344 | .007 |
| Diabetes mellitus, n (%) | 53 (36.6) | 79 (54.1) | 9.049 | .003 |
| Smoke, n (%) | 46 (31.7) | 39 (26.7) | 0.884 | .347 |
| Cre (umol/L) | 75.30 ± 21.67 | 72.67 ± 20.66 | 1.057 | .291 |
| LDL (mmol/L) | 2.71 ± 0.82 | 2.97 ± 2.53 | 1.211 | .227 |
| Antihypertension treatment, n (%) | 95 (65.5) | 82 (56.2) | 2.671 | .102 |
| Antidiabetes treatment, n (%) | 49 (33.8) | 65 (44.5) | 3.513 | .061 |
| Lipid-lowering treatment, n (%) | 68 (46.9) | 74 (50.7) | 0.418 | .518 |
| Antiplatelet treatment, n (%) | 60 (41.4) | 72 (49.3) | 1.849 | .174 |
| NHISS | 3 (2-4.5) | 4 (3-5.25) | | .079 |

Abbreviations: Cre, creatinine; LDL-C, low-density lipoprotein-cholesterol; TMAO, trimethylamine N-oxide; NIHSS, National Institutes of Health Stroke Scale
Continuous data are presented as means ± standard deviation.
Categorical data are presented as numbers (%).

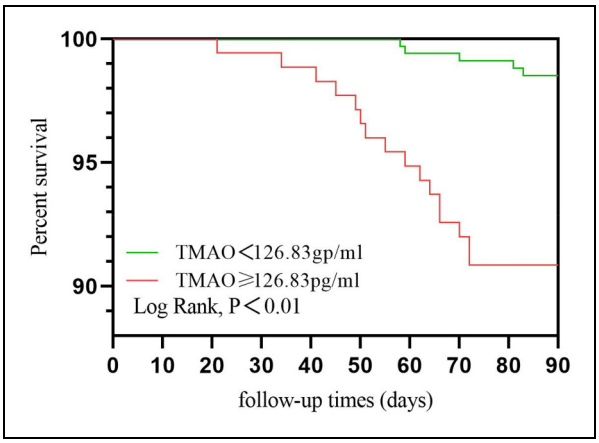


**Figure 4.** Kaplan–Meier survival curves for major vascular event recurrence in the high and low plasma TMAO concentration subgroups.

**Table 4.** Cox regression analyzes of plasma TMAO concentration as a predictor of major vascular event recurrence three months after stroke onset.

| | TMAO (pg/ml) | | |
|---|---|---|---|
| Model | <126.83 | ≥126.83 | *P* value |
| Unadjusted | 1 | 4.156 (1. 389, 12.432) | .011 |
| Model 1 | 1 | 4.131 (1.375, 12.410) | .011 |
| Model 2 | 1 | 3.128 (1.018, 9.610) | .046 |

Model 1 was adjusted for age and sex.
Model 2 was adjusted for age, sex, hypertension, diabetes mellitus, smoking, and creatinine.

antihypertension treatment, antidiabetic treatment, antiplatelet therapy and lipid-lowering treatment did not significantly differ between the subgroups. NIHSS score 72 hours after stroke onset did not significantly differ between the high and low TMAO concentration groups. Unadjusted logistic regression analysis showed that plasma TMAO concentration was not significantly associated with mRS score three months after stroke onset (odds ratio[OR] 1.10; 95% confidence interval [CI], 0.571-2.120; $P > .05$). Kaplan–Meier survival analysis demonstrated a significant difference in major vascular event recurrence between the high and low TMAO concentration subgroups (Log Rank, $P = .006$; Figure 4). Unadjusted Cox regression analysis showed that higher TMAO concentration was associated with major vascular event recurrence (hazard ratio [HR] 4.156; 95% CI, 1.389-12.432; $P = .011$). After adjusting for traditional risk factors, the association remained significant (HR 3.128; 95% CI, 1.018-9.610; $P = .046$; Table 4).

## Discussion

In this study, plasma TMAO concentration measured within 72 hours of stroke onset was significantly higher in patients with LAA-stroke than in normal controls, even after adjusting for age, sex, smoking, renal function, hypertension, and diabetes. Furthermore, elevated TMAO concentration seemed to be an independent predictor of major vascular event recurrence within the first 3 months after stroke after adjusting for traditional risk factors. Our study provides evidence regarding the role of plasma TMAO concentration in predicting major vascular event recurrence in LAA stroke patients. However, plasma TMAO concentration was not associated with neurological outcome three months after stroke.

Intestinal microflora play an important role in human health.[13] TMAO is a metabolite of gut microbes that is involved in atherosclerosis pathogenesis.[4,14–17] Atherosclerosis can serve as the pathological basis of ischemic infarction as well as CVD.[18–20] Recent evidence has shown that TMAO promotes atherosclerosis pathogenesis and increases cardiovascular risk.[4,16,21] Previous studies have suggested that plasma TMAO concentration is increased in people with increased risk of CVD. These patients also usually show elevated levels of TMAO precursors.[6,22,23] Furthermore, elevated plasma TMAO concentration has been associated with CVD prevalence and poor prognosis.[24] Our results from a Chinese population showed that elevated TMAO concentration is associated with LAA stroke but not NHISS score 72 hours after stroke onset.

A previous study suggested that increased concentrations of TMAO precursors are correlated with the risk of CVD, but only when TMAO concentration increased as well.[14] Our study found no difference between plasma TMAO concentration and 3-month neurological outcome in LAA stroke patients. Two possible reasons may explain this negative finding. First, the number of participants in our study was relatively small. Second, many participants were prescribed medications such as statins, antihypertensives, hypoglycemic agents, and others that may have affected plasma TMAO concentration.

The mechanism linking TMAO to atherosclerosis development and CVD remains unknown. However, elevated plasma TMAO concentration has been associated with platelet hyperreactivity, intracellular calcium release, and augmented thrombotic potential and has been proposed as an independent biomarker of thrombosis risk.[25] Several experimental studies have pointed to a strong association between TMAO and lipid homeostasis, providing evidence for TMAO causing progression of atherosclerosis and CVD.[6,26] TMAO has also been proposed to enhance atherosclerosis development by impairing cholesterol reverse transport.[27] In addition, elevated plasma TMAO concentration has been correlated with plaque rupture in coronary artery disease patients, suggesting that TMAO might be a biomarker to improve risk stratification in these patients,[17] which is similar to our findings in stroke patients.

The TMAO level showed a mechanistic link to atherosclerosis, platelet function, atherosclerosis and thrombosis risk.[28] Inflammation is an important factor in ischemic stroke[29,30] and plays a key role in atherosclerotic plaque development, progression, and rupture as well as vascular embolism.[31]

Our study suggests that elevated plasma TMAO can serve as a novel biomarker of major vascular event recurrence in the first

3 months after LAA stroke; however, the underlying mechanism remains unclear. Further study is warranted. Predicting major vascular event recurrence after initial acute ischemic cerebral infarction can assist in guiding patient management.

This study has several limitations. First, it was conducted in a single center and the number of participants was small; therefore, selection bias may have been introduced. Future large scale multicenter clinical studies are needed to confirm our findings. Second, we could not determine whether plasma TMAO concentration changed before or after stroke. The long-term effect of TMAO in stroke patients deserves further study. Third, the causal relationship between plasma TMAO concentration and major vascular event recurrence was not examined, which should be investigated in a future study.

## Conclusions

In conclusion, plasma TMAO concentration was elevated within 72 hours of LAA stroke onset. Elevated plasma TMAO concentration was independently associated with LAA ischemic stroke. The risk of major vascular event recurrence increased by 2.128 times in the LAA stroke subjects with plasma TMAO level higher than 126.83 pg/mL. To the best of our knowledge, we demonstrated for the first time an independent correlation between TMAO plasma levels and adverse vascular events recurrence after 3 months of the LAA stroke. TMAO has the potential to serve as a promising biomarker of recurrent adverse vascular events in this patient population.

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### Declaration of Conflicting Interests

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