

Association of Gut Microbiota-Dependent Metabolite Trimethylamine N-Oxide with First Ischemic Stroke

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Aim: We aimed to investigate the relationship of trimethylamine N-oxide (TMAO) concentrations with ischemic stroke in a large-scale case-control study conducted among the hospital-based general population.

Methods: We recruited 953 case-control sex- and age-matched pairs, and cases were confined to first acute ischemic stroke in this study. Fasting plasma TMAO was measured using high-performance liquid chromatography-tandem mass spectroscopy. Conditional logistic regression analysis was conducted to calculate odds ratios (OR) for the association of plasma TMAO with ischemic stroke.

Results: We found that plasma TMAO concentrations in patients with ischemic stroke were significantly higher than that in the control group (median: 2.85 $\mu\text{mol/L}$ vs. 2.33 $\mu\text{mol/L}$, $P < 0.001$). In multivariable conditional logistic regression models, higher plasma TMAO concentrations were associated with increased odds of ischemic stroke [fully adjusted OR for highest vs. lowest TMAO quartile: 1.81; 95% confidence interval (CI): 1.27, 2.59; P for trend < 0.001]. The multivariable-adjusted OR for ischemic stroke per 1 $\mu\text{mol/L}$ increment of plasma TMAO was 1.05 (95% CI: 1.02, 1.08). Additionally, the positive association also persisted in subgroups stratified by age, sex, body mass index, smoking status, alcohol habits, history of diabetes, and history of hypertension.

Conclusions: This study suggested a positive association between plasma TMAO and ischemic stroke. Further studies are required to explore the role of plasma TMAO concentrations in predicting stroke risk.

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Key words: Trimethylamine N-oxide, Case-control study, Ischemic stroke

Introduction

Stroke represents a leading cause of mortality and disability worldwide¹. Despite stable incidence rates and declining mortality rates over the past decade, the global burden of stroke is increasing, with an estimated 80.1 million prevalent cases of stroke (84.4% were ischemic stroke) and 13.7 million new stroke

cases in 2016². Beyond traditional risk factors, including age, cigarette smoking, excessive alcohol use, physical inactivity, diabetes, hypertension, and dyslipidemia^{3,4}, there is substantial interest in identifying novel modifiable risk factors to inform the primary prevention of stroke.

Recently, the interplay between dietary composition, intestinal bacteria, and microbiota-dependent

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metabolites has been intensively investigated⁵). Specifically, trimethylamine N-oxide (TMAO), a gut flora-dependent metabolite of choline, was identified as a promoter of atherosclerosis and as a novel risk factor for the development of cardiovascular diseases⁶⁻⁸). Dietary choline, phosphatidylcholine, and L-carnitine are converted by intestinal bacteria into trimethylamine, which is absorbed and subsequently oxidized to TMAO by hepatic flavin-containing monooxygenases⁹).

Elevated TMAO concentrations enhance platelet hyperreactivity and thrombosis, induce endothelial dysfunction, and affect lipid metabolism and inflammation^{8, 10}), suggesting the importance of this molecule in the cardiovascular system. In numerous studies, it has been shown that blood TMAO concentrations are positively associated with long-term mortality risk in individuals with atherosclerosis, heart failure, and chronic kidney disease^{8, 11, 12}). A subsequent meta-analysis including 17 clinical studies demonstrated a positive dose-dependent association between plasma TMAO concentrations and increased cardiovascular risk and mortality¹³). However, most studies on TMAO and cardiovascular risk were conducted in the United States and Europe, where diet, ethnicity, and patterns of gut microbiome composition are different from that of Asian countries. By far, few researches have been done to directly evaluate the relationship of TMAO with stroke, and the results are inconsistent. One nested case-control study of a hypertensive Chinese population demonstrated that higher TMAO concentrations were associated with an increased risk of first stroke¹⁴). Nevertheless, this study was confined exclusively to patients with hypertension and did not specifically focus on ischemic stroke, which accounts for the majority of stroke cases^{2, 15}). Conversely, another study of 322 patients revealed that plasma TMAO concentrations in patients with stroke or transient ischemic attack were significantly lower, rather than higher, compared with asymptomatic controls¹⁶). Herein, the purpose of this study was to investigate the association of plasma TMAO with first ischemic stroke in a large-scale case-control study conducted among the hospital-based general population.

Methods

Study Population and Data Collection

This was a hospital-based case-control study involving 953 ischemic stroke cases and 953 control subjects. The flowchart of participant recruitment and case-control selection is shown in [Supplemental Fig. 1](#). From October 2012 to June 2017, patients with

first acute ischemic stroke who were admitted to People's Hospital of Shenzhen, Guangdong, China, were consecutively recruited. Concomitantly, control subjects were recruited from the general population who attended an annual health examination at the hospital physical examination center. Controls were free of diagnosis of stroke and were 1:1 matched with cases for sex and age (± 5 years). According to the WHO criteria, the definition of stroke was clinical and based on the sudden onset of neurologic deficit lasting longer than 24 h or leading to death, with no apparent cause other than that of vascular origin¹⁷). Ischemic stroke was further confirmed by the results of full neurologic examination, computed head tomography, and/or magnetic resonance imaging, according to the International Classification of Disease (9th revision, codes 430–438). The inclusion criteria of cases and controls in this study were as follows: age ≥ 35 years, body mass index (BMI) < 40 kg/m², Chinese Han ethnicity, and no history of a diagnosis of cerebrovascular disease. Subjects with myocardial infarction, heart failure, malignant tumor, other systemic diseases, or who are using antibiotics within 3 months were also excluded from the study. The study was reviewed and approved by the Ethics Committee of Shenzhen Center for Disease Control and Prevention, and written informed consents were obtained from all involved participants.

Data on basic demographics, lifestyle, and health-related information were collected using standardized questionnaires. Interviewers were required to conduct face-to-face surveys with all participants involved, and proxy respondents were used for patients who were unable to communicate adequately. Current smoking (≥ 1 cigarette/day) and current drinking (drink alcoholic beverages ≥ 1 time/week) were defined by the participants' self-report. Body weight and height were measured through a standardized protocol, and BMI was calculated by dividing weight in kg to the square of height in meters.

Laboratory Assay

The fasting blood samples were drawn from all subjects on the first morning after admission, and plasma aliquots were immediately centrifuged and stored at -80°C until analysis. Routine biochemical measurements such as fasting plasma glucose, triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-cholesterol), and high-density lipoprotein cholesterol (HDL-cholesterol) were performed using automated enzymatic procedure.

Plasma TMAO concentrations were quantified by stable isotope dilution high-performance liquid chromatography-tandem mass spectroscopy (LC-MS/

Table 1. Baseline characteristics of study participants

Parameters	Cases (<i>n</i> = 953)	Controls (<i>n</i> = 953)	<i>P</i> -value
Age (years)	63.14 (9.26)	63.32 (8.47)	0.650
Male, <i>n</i> (%)	544 (57.08)	544 (57.08)	1.000
BMI (kg/m ²)	23.68 (2.98)	23.84 (2.86)	0.224
Fasting glucose (mmol/L)	6.43 (2.54)	5.92 (1.43)	<0.001
Triglycerides (mmol/L)	1.35 (1.02-1.89)	1.27 (0.89-1.86)	0.001
Total cholesterol (mmol/L)	4.96 (4.19-5.71)	5.35 (4.58-6.11)	<0.001
HDL-cholesterol (mmol/L)	1.07 (0.92-1.26)	1.39 (1.16-1.62)	<0.001
LDL-cholesterol (mmol/L)	2.89 (2.34-3.53)	2.99 (2.39-3.68)	0.037
Hypertension, <i>n</i> (%)	650 (68.21%)	376 (39.45%)	<0.001
Type 2 diabetes, <i>n</i> (%)	272 (28.54%)	132 (13.85%)	<0.001
Current smoking, <i>n</i> (%)	163 (17.10%)	117 (12.28%)	0.003
Current drinking, <i>n</i> (%)	63 (6.61%)	113 (11.86%)	<0.001
Plasma TMAO (μmol/L)	2.85 (1.74-4.59)	2.33 (1.53-3.83)	<0.001

Data are expressed as *n* (%) for categorical data, mean (standard deviation) for parametrically distributed data, or median (interquartile range) for nonparametrically distributed data. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TMAO, trimethylamine N-oxide.

MS; AB Sciex Q-Trap 4500; Applied Biosystems) as previously described¹⁸. Briefly, plasma sample (50 μL) was combined with 20 μL of a 500 μg/L d9-(trimethyl)-labeled internal standard in acetonitrile, and then the sample was extracted with 200 μL acetonitrile by vortex for 1 min. The supernatant was collected after centrifugation at 20,238 g at 4°C for 5 min, and the procedure was repeated once. The combined supernatant was evaporated to dryness under vacuum at 45°C and was reconstituted with 50 μL solvent (acetonitrile/water, 1:1, vol:vol). The sample was vortexed for 1 min and then centrifuged at 20,238 g at 4°C for 5 min. Finally, 5 μL of the clear supernatant was analyzed with LC-MS/MS. For quality check, pooled plasma reference samples were inserted every 15 samples as a quality control. The intra- and interassay coefficients of variation for the quality control samples were both less than 10%.

Statistical Analysis

Continuous covariates were summarized as mean (standard deviation) or median (interquartile ranges) and compared between groups using Student's *t* test or the Mann-Whitney *U* test when appropriate. Categorical covariates were summarized as number (%) and compared with chi-squared test. Conditional multivariate logistic regression models were performed to estimate the odds ratios and 95% confidence interval for ischemic stroke for each quartile of plasma TMAO (based on the distribution of controls). *P*-values for trend were tested by treating the median value of each quartile as a continuous variable in the conditional logistic regression models. The fully adjusted

model included age, sex, BMI, smoking status, alcohol habits, history of diabetes and hypertension, triglycerides, LDL-cholesterol, and HDL-cholesterol. We explored a potential nonlinear relationship between plasma TMAO and ischemic stroke by fitting restricted cubic splines with four knots (placed at the 20th, 40th, 60th, and 80th percentiles of plasma TMAO concentrations) to logistic regression models, excluding the values outside the 95th percentiles, to make the graph more stable. We further carried out subgroup analyses and interaction tests by age (<65, ≥ 65 years), sex, BMI (<24, ≥ 24 kg/m²)¹⁹, smoking status, alcohol habits, history of diabetes, and history of hypertension. Our analyses used all participants for whom the major variables were available. All statistical analyses were done with Stata/SE 12.0 software (StataCorp LP).

Results

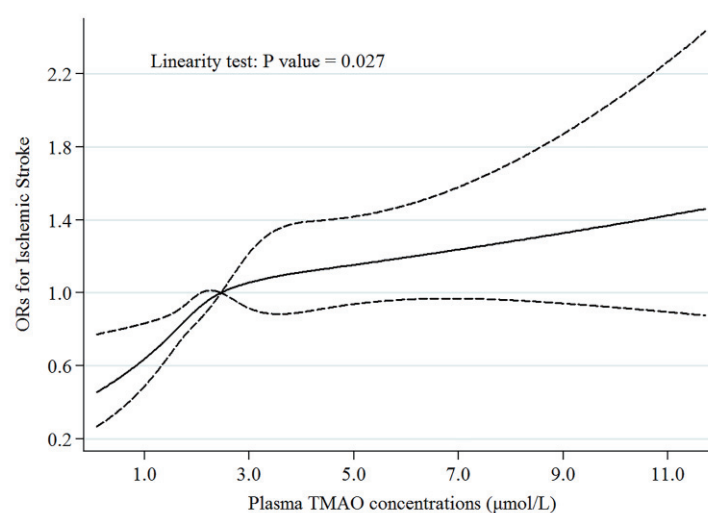
Table 1 summarizes the descriptive characteristics of the participants included in our study. Compared to controls, plasma TMAO concentrations were significantly higher in patients with ischemic stroke [median: 2.85 μmol/L (interquartile range: 1.74, 4.59) vs. 2.33 μmol/L (interquartile range: 1.53, 3.83), *P* < 0.001]. Additionally, ischemic stroke cases had higher concentrations of fasting glucose and triglycerides, lower HDL-cholesterol concentrations, and higher rates of diabetes and hypertension and were more likely to be current smokers than controls.

As shown in **Table 2**, a significantly positive association between plasma TMAO concentrations

Table 2. Association between plasma TMAO concentrations and ischemic stroke

Variable	Quartile of plasma TMAO concentrations, $\mu\text{mol/L}$				Per 1 $\mu\text{mol/L}$ increment of plasma TMAO	<i>P</i> -trend
	Q1 (referent): ≤ 1.53	Q2: $> 1.53\text{--}2.33$	Q3: $> 2.33\text{--}3.83$	Q4: > 3.83		
Cases/control, n/n	169/239	198/235	266/239	320/240		
Crude OR (95% CI)	1	1.16 (0.88, 1.53)	1.54 (1.18, 2.00)	1.91 (1.47, 2.50)	1.06 (1.03, 1.09)	< 0.001
Adjusted OR ¹ (95% CI)	1	1.13 (0.83, 1.55)	1.77 (1.31, 2.38)	1.80 (1.34, 2.43)	1.05 (1.02, 1.08)	< 0.001
Adjusted OR ² (95% CI)	1	0.94 (0.65, 1.37)	1.64 (1.16, 2.33)	1.81 (1.27, 2.59)	1.05 (1.01, 1.09)	< 0.001

¹Model 1, adjusted for age, sex, BMI, smoking status, alcohol habit, history of hypertension, and history of diabetes; ²Model 2, additionally adjusted for triglycerides, LDL-cholesterol, and HDL-cholesterol. CI, confidence interval; OR, odd ratio; Q, quartile; TMAO, trimethylamine N-oxide.

**Fig. 1.** Association of plasma trimethylamine N-oxide (TMAO) concentrations with ischemic stroke

The restricted cubic spline regression was conducted with the use of four knots (placed at the 20th, 40th, 60th, and 80th percentiles of plasma TMAO concentrations) and adjusted for age, sex, BMI, smoking status, alcohol habit, history of hypertension, and history of diabetes. Solid lines are ORs, and dashed lines are 95% CI.

and ischemic stroke was observed in this study. After adjustment for age, sex, BMI, smoking status, alcohol habits, history of diabetes, and history of hypertension, the ORs (95% CIs) of ischemic stroke from the lowest to highest quartiles were 1 (referent), 1.13 (0.83, 1.55), 1.77 (1.31, 2.38), and 1.80 (1.34, 2.43), respectively (P for trend < 0.001). The multivariable-adjusted OR for ischemic stroke per 1 $\mu\text{mol/L}$ increment of plasma TMAO was 1.05 (95% CI: 1.02, 1.08). Further adjustment for triglycerides, LDL-cholesterol, and HDL-cholesterol concentrations did not substantially alter the above results.

The restricted cubic spline regression model revealed a nonlinear positive association between plasma TMAO and the odds of ischemic stroke, with a steeper increment at less than 2.46 $\mu\text{mol/L}$ of plasma TMAO (Fig. 1). Subsequently, we further performed

subgroup analyses stratified according to age (< 65 , ≥ 65 years), sex, BMI (< 24 , ≥ 24 kg/m^2), smoking status, alcohol habits, history of diabetes, and history of hypertension. The positive association between plasma TMAO concentrations and ischemic stroke also persisted across different subgroups, and none of the tests for interaction between subgroups was statistically significant (Table 3).

Discussion

In this large-scale case-control study conducted among a hospital-based population, we demonstrated that elevated concentrations of plasma TMAO were associated, in a dose-dependent manner, with increased odds of first ischemic stroke. Following full multivariate adjustment, plasma TMAO concentra-

Table 3. Stratified analyses of ischemic stroke and plasma TMAO concentrations by age, sex, BMI, smoking status, alcohol habit, history of diabetes, and history of hypertension

Groups	Quartile of plasma TMAO concentrations, $\mu\text{mol/L}$				<i>P</i> for interaction
	Q1 (referent): ≤ 1.53	Q2: $>1.53\text{--}2.33$	Q3: $>2.33\text{--}3.83$	Q4: >3.83	
Sex					0.501
Male ($n = 1,088$)	1	1.06 (0.72, 1.56)	1.74 (1.20, 2.54)	1.55 (1.08, 2.24)	
Female ($n = 818$)	1	1.23 (0.79, 1.91)	1.37 (0.90, 2.10)	1.73 (1.14, 2.65)	
Age					0.399
< 65 y ($n = 1,047$)	1	1.21 (0.82, 1.78)	1.43 (0.98, 2.09)	1.44 (0.99, 2.09)	
≥ 65 y ($n = 859$)	1	1.05 (0.67, 1.65)	1.77 (1.15, 2.72)	1.86 (1.23, 2.83)	
BMI					0.715
< 24 ($n = 1,039$)	1	1.32 (0.90, 1.94)	1.64 (1.12, 2.40)	1.76 (1.22, 2.55)	
≥ 24 ($n = 867$)	1	0.92 (0.58, 1.44)	1.44 (0.94, 2.20)	1.46 (0.96, 2.23)	
Current smoking					0.159
Yes ($n = 280$)	1	0.56 (0.25, 1.26)	1.50 (0.71, 3.16)	1.28 (0.64, 2.56)	
No ($n = 1,626$)	1	1.27 (0.92, 1.74)	1.61 (1.18, 2.19)	1.73 (1.28, 2.35)	
Current drinking					0.545
Yes ($n = 176$)	1	0.69 (0.21, 2.28)	1.80 (0.69, 4.73)	1.49 (0.56, 3.99)	
No ($n = 1,730$)	1	1.19 (0.88, 1.61)	1.58 (1.17, 2.12)	1.68 (1.26, 2.24)	
History of diabetes					0.794
Yes ($n = 404$)	1	1.50 (0.74, 3.04)	2.15 (1.06, 4.37)	1.98 (1.03, 3.83)	
No ($n = 1,502$)	1	1.07 (0.78, 1.47)	1.48 (1.09, 2.01)	1.54 (1.13, 2.08)	
History of hypertension					0.607
Yes ($n = 1,026$)	1	1.29 (0.86, 1.93)	1.73 (1.17, 2.56)	1.95 (1.34, 2.84)	
No ($n = 880$)	1	0.98 (0.64, 1.49)	1.41 (0.95, 2.11)	1.34 (0.89, 2.01)	

Data are presented as OR (95% CIs). The multivariate model was adjusted for age, sex, BMI, smoking status, alcohol habit, history of hypertension, and history of diabetes. Q, quartile; TMAO, trimethylamine N-oxide.

tions remained positively associated with the odds of ischemic stroke. Furthermore, the association of plasma TMAO with the odds of ischemic stroke was also consistent across examined subgroups. Collectively, our findings suggested a positive association between plasma TMAO concentrations and ischemic stroke.

As a gut microbial-generated metabolite, TMAO was first identified and then shown to predict risk for cardiovascular disease in an independent large clinical cohort by Wang *et al.*⁶. Since then, numerous cohort studies showed that an increasing number of adverse outcomes are associated with elevated TMAO concentrations^{7, 8, 11, 12}. Likewise, a systematic review of 19 prospective studies found that high concentrations of TMAO and TMAO precursors were positively associated with the risk of major adverse cardiovascular events and all-cause mortality independently of tradi-

tional cardiovascular risk factors²⁰. Our findings are suggestive of a positive association between plasma TMAO and ischemic stroke, which is consistent with prior observations of cardiovascular events. To the best of our knowledge, few studies have directly examined TMAO concentrations and the risk of stroke. Nie *et al.* conducted a case-control study among hypertensive patients and observed that higher TMAO concentrations were associated with increased risk of first stroke¹⁴. Another study from Raxidamu *et al.* demonstrated that the serum concentrations of TMAO in stroke were significantly higher than in healthy volunteers²¹. Compared with these studies, our analysis here has greater precision because of much larger sample sizes and has further explored the potential nonlinear relationship between plasma TMAO and ischemic stroke. Interestingly, in another case-control study of patients with large-artery atherosclerotic ischemic

stroke or transient ischemic attack, a significant decrease, rather than an increase, in TMAO concentrations in the patients was observed as compared to the asymptomatic group¹⁶). Considering that either the preexisting stroke or the treatment may reduce TMAO concentrations and the inclusion of patients and controls was not well balanced, this result should be interpreted with caution.

As previously mentioned, the median of plasma TMAO in the current study was 2.33 $\mu\text{mol/L}$, which is comparable to other Chinese populations [2.3 (1.4–3.7) $\mu\text{mol/L}$ in subjects from the CSPPT¹⁴), 2.18 (1.34–3.90) $\mu\text{mol/L}$ in patients with STEMI²²) and 1.77 (1.09–2.80) in diabetic patients²³]. However, plasma TMAO concentrations in the above populations are significantly lower than that in Western countries [4.4 $\mu\text{mol/L}$ in the United States²⁴), 5.6 $\mu\text{mol/L}$ in the United Kingdom²⁵), 3.2 $\mu\text{mol/L}$ in the Netherlands²⁶), and 20.4 $\mu\text{mol/L}$ in Canada²⁷]. These discrepancies could be attributed to lower consumption of red meat and fat in China than in Western countries^{28, 29}). Recent studies reported that increased TMAO concentrations were dependent on consumption of dietary phosphatidylcholine and L-carnitine, which are commonly found in Western diet such as red meat and full fat dairy products^{7, 8}). Other than diet, ethnic diversity of gut microbiota in populations may also contribute to this difference³⁰). In particular, associations appeared to be stronger among the participants with hypertension or diabetes, although such differences did not attain statistical significance. It is possible to speculate that the effects of dietary differences influencing TMAO concentrations might be more apparent in patients with hypertension or diabetes. Previous evidences showed that higher concentrations of circulating TMAO were associated with increased risk of type 2 diabetes and hypertension^{31, 32}), both of which are risk factors for stroke. Whether hypertension or diabetes accounted for the association of TMAO with ischemic stroke should be further investigated.

Though the mechanisms through which TMAO promotes atherosclerosis are not fully elucidated, several adverse aspects of TMAO have been proposed, including potential interactions with cholesterol metabolism, pro-inflammatory pathways, platelet activation, and subsequent thrombosis. TMAO can modulate cholesterol and sterol metabolism that would, at least partly, contribute to the development of cardiovascular diseases^{6, 8}). TMAO is also known to function as a switch to activate pro-inflammatory cascades, causing arterial damage that allows cholesterol to enter the arterial walls and subsequent plaque formation³³). Notably, the TMAO-generating enzyme flavin mono-

oxygenase 3 (FMO3) is identified as a key regulator of lipid cholesterol and inflammation³⁴), and perturbation of FMO3 expression has profound effects on glucose and lipid metabolism and atherosclerosis³⁵). Finally, studies conducted on animal models and humans suggest that high concentrations of TMAO contribute directly to platelet hyperreactivity and enhance thrombosis risk¹⁰), and the inhibition of gut microbial trimethylamine and TMAO production reduce thrombosis potential³⁶).

Our study has several strengths. In the present study, only patients with first acute ischemic stroke were included to avoid possible dietary changes before stroke onset, which may confound the association of plasma TMAO with ischemic stroke risk. Additionally, the influence of several potential confounders and traditional risk factors for stroke was carefully assessed to minimize the possible residual confounding. Several limitations also warrant consideration. Firstly, blood samples were collected at the time of diagnosis, and a single measurement in our study may not capture the long-term concentrations of TMAO. Secondly, some clinical conditions (including diabetes, hypertension, and dyslipidemia) might lead to dietary changes over time before the onset of stroke, which could diminish the ability to examine the relationship between plasma TMAO and stroke risk. However, such bias seemed to attenuate the association as patients with such symptoms probably limit intake of phosphatidylcholine-rich foods, since these foods are typically high in fat and cholesterol³⁷). Thirdly, TMAO status can be modulated by diet and the composition of gut microbiota, but neither was able to be measured in this study, limiting the ability to investigate their potential roles in the effect of TMAO on ischemic stroke. Finally, the case–control nature of this study did not allow us to infer any causal relationship between TMAO and ischemic stroke.

Conclusions

The findings of this hospital-based case–control study demonstrated a positive association between plasma TMAO concentrations and ischemic stroke, which may contribute to knowledge regarding the prevention of ischemic stroke. Further longitudinal studies are required to explore the role of plasma TMAO levels in predicting stroke risk.

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Author Contributions

Taoping Sun, Jinquan Cheng, and Liegang Liu designed the research. Yanwei Zhang, Suli Huang, Ying Wen, and Liangkai Chen performed the data collection. Taoping Sun, Jiawei Yin, Xiaobo Peng, Li Zhou, and Benfeng Cao conducted the experiments. Taoping Sun and Liegang Liu analyzed the data and wrote the manuscript. Xiaoqin Li, Wei Yang, and Aijun Tan supervised and provided critical comments on the manuscript. All authors approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

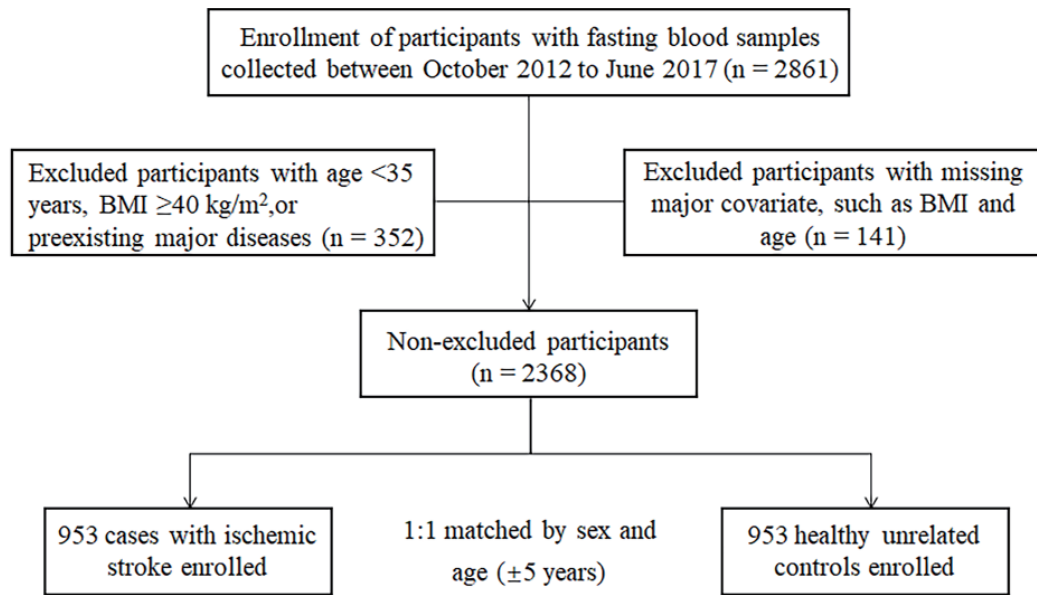
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Supplemental Fig. 1. Flowcharts of the participant recruitment and case-control selection