ORIGINAL ARTICLE

Trimethylamine-N-oxide is elevated in the acute phase after ischaemic stroke and decreases within the first days

C. Schneider[a], J. G. Okun[b], K. V. Schwarz[b], J. Hauke[b], M. Zorn[c], C. Nürnberg[a], M. Ungerer[a], P. A. Ringleb[a] and S. Mundiyanapurath[a]

[a]*Department of Neurology, Heidelberg University Hospital, Heidelberg;* [b]*Dietmar-Hopp Metabolic Center, Center for Child and Adolescent Medicine, University Hospital, Heidelberg; and* [c]*Central Laboratory, Department of Internal Medicine, University Hospital, Heidelberg, Germany*

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Background and purpose: Trimethylamine-N-oxide (TMAO) is a biomarker of the gut microbiome and correlates with the risk of cardiovascular diseases. However, conflicting data exist on the specific role of TMAO in ischaemic stroke patients. We aimed to analyze the time course of TMAO levels in stroke patients compared with controls.

Methods: In this prospective, case-control study, patients suffering from ischaemic stroke (onset < 24 h) and control patients with less than two cardiovascular risk factors were enrolled. Plasma TMAO levels were analyzed on admission, after 48 h and after 3 months. The primary endpoint was the difference in TMAO levels on admission between stroke patients and controls.

Results: A total of 196 patients with ischaemic stroke and 100 controls were included between February 2018 and April 2019. Plasma TMAO levels on admission were significantly higher in stroke patients than in controls [median value 4.09 (2.87–6.49) vs. 3.16 (2.08–5.16) μmol/L, $P = 0.001$]. There was a significant decrease in TMAO levels in stroke patients after 48 h [median at 48 h, 3.49 (2.30–5.39) μmol/L, $P = 0.027$]. TMAO levels increased again 3 months after stroke [median 4.23 (2.92–8.13) μmol/L, $P = 0.047$]. In controls, TMAO levels did not change between admission and after 48 h [median at 48 h, 3.14 (1.63–4.61) μmol/L, $P = 0.11$]. An inverse correlation between TMAO values and kidney function was found (Spearman rho −0.334, $P < 0.001$).

Conclusions: Our study emphasizes the importance of the time course of TMAO levels after ischaemic stroke. Future studies should define the time point of TMAO analysis, preferably in the acute phase (< 24 h).

Introduction

Despite impressive progress in the treatment and diagnosis of cerebrovascular disease, the prevalence and morbidity of ischaemic stroke remain high. Recent studies identified an important influence of the gut microbiome on the pathogenesis of cardiovascular and cerebrovascular disease [1]. Because the detailed analysis of the gut microbiome itself is challenging due to interindividual and intraindividual heterogeneity and methodological inconsistencies [2,3], a biomarker that correlates with the composition of the microbiome would be useful. Trimethylamine-N-oxide (TMAO) is a plasma metabolite originating from gut bacteria metabolism of dietary L-carnitine, betaine and choline, all abundant in red meat and egg yolk. Choline, betaine and L-carnitine are metabolized to trimethylamine (TMA) by gut bacteria. TMA is rapidly absorbed in the bloodstream and oxidized to TMAO by flavin-containing monooxygenase-3 and is subsequently secreted in urine [4]. In animals, elevated

Correspondence: S. Mundiyanapurath, Department of Neurology, Heidelberg University Hospital, Im Neuenheimer Feld 400, D-69120 Heidelberg, Germany (tel.: +49(0)6221 56 7504; fax: +49(0)6221 56 8243; e-mail: sibu.mundiyanapurath@med.uni-heidelberg.de).

TMAO levels in plasma directly contribute to platelet hyper-reactivity [5] and promote the development of atherosclerotic plaques [6]. Previous clinical studies demonstrated that elevated TMAO levels are found in patients with diabetes [7] and chronic renal failure [8]. Thus, TMAO might be a marker of an increased cardiovascular risk in humans [9]. Several studies revealed not only a positive association of elevated plasma TMAO levels with major adverse cardiovascular events in patients with acute coronary syndrome but also a direct correlation between TMAO levels and risk of major adverse cardiovascular event [10]. Furthermore, elevated TMAO levels predict an increased risk for ischaemic stroke in patients with carotid stenosis undergoing arterial stenting [11] and in patients with atrial fibrillation [12]. Recently, Rexidamu *et al*. demonstrated that patients with ischaemic stroke had higher TMAO levels than controls and that the TMAO concentration correlates with stroke severity [13]. However, data in stroke patients are contradictory as Yin *et al*. showed decreased TMAO levels in a cohort of stroke patients compared with healthy controls [14]. The timing of blood collection differs widely in those studies, e.g. within the first 7 days after stroke [15], within 24 h after stroke [13] and some studies do not specify the time point of analysis [11,14,16]. It remains unclear whether high TMAO levels cause ischaemic stroke and remain high or whether ischaemic stroke causes changes in the gut microbiome that could change the TMAO levels [17]. In our study, we aimed to analyze the time course of TMAO levels in stroke patients compared with controls. We hypothesized that TMAO levels are higher in stroke patients compared with controls and that they decrease due to gut dysbiosis after stroke.

Materials and methods

Study design and patient selection

This prospective, case-control cohort study included patients from February 2018 to April 2019 at the Department of Neurology, Heidelberg University Hospital. Inclusion criteria for the stroke patient group were as follows: age ≥18 years, clinical or neuroimaging proof of stroke with onset in the last 24 h (determined by a neurology consultant), admission to stroke unit and written informed consent from the patient or, if necessary, the patient's legal representative. Inclusion criteria for the control patients (controls) were as follows: patient admitted to the neurological ward, age ≥18 years, less than two cardiovascular risk factors (i.e. hypertension, atrial fibrillation, hypercholesterolemia, diabetes mellitus, smoking) and written informed consent. Exclusion criteria for all groups were antibiotic therapy in the previous 30 days, chemotherapy, probiotic treatment, severe anemia and known trimethylaminuria. In addition, patients were excluded from the control group if they had an ischaemic stroke or myocardial infarction in their medical history. The control group was composed of patients with other neurological disorders to investigate whether neurological disorders other than stroke have an effect on TMAO levels. We also chose patients with a low cardiovascular risk profile in the control group in order to prove that patients with a higher cardiovascular risk profile and ischaemic stroke have higher TMAO levels, which was challenged by a recent study [18].

In stroke patients, the first blood sampling occurred immediately on admission, which was within the first 24 h after symptom onset. The second blood sample was collected 48 (±10) h after the first. The third blood sampling was conducted 3 months after the event (±14 days). The control group was recruited during their inpatient stay in the Department of Neurology, Heidelberg University Hospital and two blood samplings were taken at any time (mostly several hours after admission) and after a period of 48 (±10) h. The third measurement of TMAO levels after 3 months was not performed in the control group, as no changes in TMAO values over time course were expected. TMAO levels and renal function were measured in all samples. The recruitment of controls was intended to achieve a similar distribution of age and sex compared with stroke patients. A 2:1 ratio was aimed for to allow for subgroup analysis within the stroke patient group.

Clinical characteristics

Participants' clinical data (e.g. vascular risk profile, hypertension, hyperlipidemia, diabetes, smoking) and patients' history (peripheral arterial occlusive disease, coronary artery disease, previous stroke) were collected in routine clinical work. National Institutes of Health Stroke Scale (NIHSS) and modified Rankin Scale (mRS) scores were recorded on admission and at discharge according to the treating physician, who had extensive training in scoring on the NIHSS and mRS. The treating physician was blinded to the TMAO levels. Stroke etiology was determined accordingly to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification by an experienced vascular neurologist [19]. Functional outcome (mRS score) after 3 months was collected either by personal examination or by telephone by one of the authors (C.S., C.N., M.U. or S.M.), blinded to TMAO levels. Blinding was accomplished by storing data of TMAO

levels in a separate building, where the analysis was performed by the members of the Dietmar-Hopp Metabolic Center. Similarly, clinical data were stored in the Department of Neurology, Heidelberg University Hospital and were not accessible to any member of the team running TMAO analysis. Patients and controls were allocated a pseudonym using a combination of numbers and letters.

The glomerular filtration rate (in mL/min/1.73 m^2) was calculated from serum creatinine measured in our central laboratory using the chronic kidney disease epidemiology collaboration equation (CKD-EPI) .

Sample preparation

Plasma samples were stored at −20°C until analysis. The samples were prepared according to Wang *et al.* [20] with minor modifications, i.e. 10-μL samples were pipetted into a 1.5-mL centrifuge tube. After adding 340 μL of a cold (4°C) mixture of methanol and acetonitrile (ACN) (methanol:ACN 25:75, v/v) and 50 μL of a 2 μmol/L D9-TMAO (Cambridge Isotope Laboratories, Tewksbury, MA, USA) solution in methanol, the protein was precipitated by vortex mixing for 30 s followed by 10-min incubation. The mixture was centrifuged for 5 min at 18 000 gmax and 150 μL of the supernatant were transferred to a 96-well microplate. Samples were quantified by an external nine-point calibration using the peak area ratio of TMAO to D9-TMAO. For calibrators, fetal bovine serum was used as matrix. After spiking with TMAO in the range of 0–200 μmol/L, calibrators were prepared as described above. In addition, three quality controls (3, 15 and 75 μmol/L in fetal bovine serum) were included in each sample sequence. When all calibrators, quality controls and samples of a sequence were transferred, the microplate was sealed with a pre-slit adhesive foil.

Liquid chromatography–tandem mass spectrometry analyses

Liquid chromatography–tandem mass spectrometry analyses were performed using an XEVO TQS system (Waters, Eschborn, Germany) equipped with an electrospray ion source. The instrument was controlled with MassLynx 4.1 software (Waters Corporation, Milford, MA, USA). For chromatographic separation, a Hydrophilic Interaction Chromatography column (100 × 2.1 mm, 1.7 um; Acquity UPLC BEH Amide, Waters) with a corresponding pre-column (5 × 2.1 mm, 1.7 μmol/L; Acquity UPLC BEH Amide VanGuard, Waters) was used in isocratic mode. During a 3-min chromatographic run, eluent A [10 mmol/L ammonium formate in ultrapure water (H_2OmQ) and ACN (H_2OmQ: ACN 95:5, v/v)] and eluent B (ACN) were applied with a mixing ratio of 42% eluent A and 58% eluent B at a flow rate of 0.4 mL/min. The injection volume was 1 μL. Both TMAO and its corresponding standard were analyzed using a multiple reaction monitoring experiment containing their most abundant mass transitions (TMAO, 76.1 → 59.1 Da; d9-TMAO, 85.1 → 68.1 Da; cone voltage, 40 V; collision energy, 11 V) in positive ion mode at a flow rate of 0.4 mL/min for 3 min.

Statistical analysis

We calculated absolute and relative rates for categorical variables, medians and interquartile ranges for continuous variables. The chi-squared test was performed for categorical variables and the Mann–Whitney *U*-test for continuous variables. We compared the distribution of TMAO between patients and controls by Mann–Whitney *U*-test on admission and after 48 h. We performed a Wilcoxon test to investigate the shift of TMAO levels between the blood-sampling time points. We then used crude and multivariable regression models adjusted for all other significant predictors and reported odds ratios (ORs) of the association of TMAO with stroke patients. Repeated-measures ANCOVA was performed to analyze differences in TMAO levels in the time course, controlling for renal function. Differences in TMAO according to stroke etiology were analyzed by Kruskal–Wallis test. All statistical tests were calculated on a significance value of $\alpha = 0.05$ and analyses were carried out using SPSS version 22 (SPSS, IBM, Armonk, NY, USA). All reported *P*-values are two-sided.

Ethical approval and patient consent

Ethical approval for this study was obtained from the local ethics committee in Heidelberg (reference no. S-521/2017). All participants or their legal representative provided written informed consent before enrollment.

Data availability statement

All relevant data are published in the article. Additional data are available from the corresponding author upon reasonable request.

Results

Patient characteristics

A total of 303 participants were enrolled in the study and 10 patients had to be excluded [missing blood samples, $n = 6$; failure to meet inclusion criteria

(stroke mimic), $n = 3$; duplicate inclusion of one patient]. Therefore, 293 participants were available for analysis. The study population was composed of 193 patients with acute stroke (patients) and 100 control patients (controls). The baseline characteristics of both groups are presented in Table 1. Median age was 69 (60–78) years for patients and 65 (57–75) years for controls ($P = 0.052$). A total of 122 patients (63%) and 53 controls (53%) were male ($P = 0.081$). Due to the inclusion criteria, the rate of cardiovascular risk factors was higher in the patient cohort. Patients presented with a median NIHSS score on admission of 3 (2–8). A total of 76 (39%) patients received acute stroke treatment. Of those, 21 (11%) received mechanical thrombectomy only, 39 (20%) received tissue plasminogen activator only and 16 (8%) received a combined therapy. The median time from symptom onset to first blood sampling was 5.5 (2–14) h. The median time from first to second blood sampling was 46 (42–52) h. The third blood sampling in the patient group was performed after a median of 94 (90–101) days. The median mRS score at discharge was 2. Neurological disorders of the control group are shown in Table S1.

Table 1 Baseline characteristics of study population

	Patients ($n = 193$)	Controls ($n = 100$)
Male sex	122 (63%)	53 (53%)
Age (years)	69 (60–78)	65 (57–75)
Hypertonus	165 (85%)	58 (58%)
Atrial fibrillation	42 (22%)	4 (4%)
Diabetes type 2	52 (27%)	6 (6%)
Coronary artery disease	57 (30%)	4 (4%)
Peripheral artery disease	14 (7%)	0
Current smoker	73 (38%)	27 (27%)
GFR on admission	87 (74–96.5)	97 (87–110)
NIHSS score on admission	3 (1.5–8)	
Intravenous thrombolysis (IVT) (alteplase)	39 (20%)	
Mechanical thrombectomy (MT)	21 (11%)	
IVT and MT	16 (8%)	
Antibiotics < 48 h	7 (4%)	
TOAST		
Large-artery atherosclerosis	48 (25%)	
Cardioembolism	47 (24%)	
Small-vessel occlusion	24 (12%)	
Other determined etiology	8 (4%)	
Undetermined	66 (34%)	
Antithrombotic treatment		
None	5 (3%)	
ASA	104 (54%)	
Dual antiplatelet therapy	33 (17%)	
Direct oral anticoagulant	29 (15%)	
Phenprocoumon	7 (4%)	
Other	15 (8%)	

ASA, acetylsalicylic acid; GFR, glomerular filtration rate; IVT, intranvenous thrombolysis; MT, mechanical thrombectomy; NIHSS, National Institutes of Health Stroke Scale; TOAST, Trial of Org 10172 in Acute Stroke Treatment. Data are given as n (%) and median (interquartile range).

Comparison of plasma trimethylamine-N-oxide levels between stroke patients and controls

On admission, plasma TMAO levels were significantly higher in patients than in controls [4.09 (2.87–6.49) vs. 3.16 (2.08–5.16) μmol/L, respectively, $P = 0.001$, Fig. 1]. After 48 h there was a significant decrease in median TMAO levels of patients [4.09 (2.87–6.49) μmol/L on admission vs. 3.49 (2.30–5.39) μmol/L after 48 h, $P = 0.027$], whereas levels remained unchanged in controls [3.16 (2.08–5.16) μmol/L on admission vs. 3.14 (1.63–4.61) μmol/L after 48 h, $P = 0.11$, Fig. 1]. At 3 months after ischaemic stroke, the plasma TMAO levels were significantly increased compared with the levels measured 2 days after stroke [3.49 (2.30–5.39) μmol/L after 48 h vs. 4.23 (2.92–8.13) μmol/L after 3 months, $P = 0.047$, Fig. 1] and similar to the levels on admission [4.09 (2.87–6.49) μmol/L on admission vs. 4.23 (2.92–8.13) μmol/L after 3 months, $P = 0.599$]. According to the study protocol, we did not measure TMAO levels in the control group after 3 months. Furthermore, the difference between TMAO levels on admission and after 48 h showed a significant, weak correlation with stroke severity, measured by NIHSS score at discharge (Spearman rho 0.144, $P = 0.049$). No significant difference in drop of plasma TMAO levels was detected between those patients who received acute stroke treatment and those who could not be treated [no acute treatment, −0.5 (−2.36 to 1.01) μmol/L; recanalization (by mechanical thrombectomy, intravenous thrombolysis or both), −0.67 (−2.18 to 0.48) μmol/L, $P = 0.431$]. No association could be detected between the intake of different antithrombotic agents and decrease of TMAO levels within the first 48 h [no

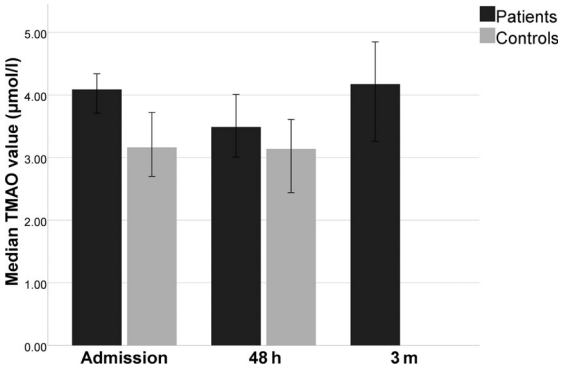

Figure 1 Time course of median trimethylamine-N-oxide (TMAO) levels in patients and controls.

antithrombotic treatment, −0.2 (−1.26 to 0.88) μmol/L; acetylsalicylic acid (ASA), −0.77 (−2.12 to 0.28) μmol/L; dual antiplatelet therapy, 0.38 (−1.83 to 1.33) μmol/L; non-vitamin K oral anticoagulation, −0.87 (−2.2 to 0.5) μmol/L; phenprocoumon, 1.91 (−8.54 to 2.21) μmol/L; other, −1.12 (−11.38 to −0.23) μmol/L, $P = 0.575$].

Trimethylamine-N-oxide levels and kidney function

Patients had worse renal function than controls [glomerular filtration rate, GFR 87 (74–97) vs. 97 (87–100), $P = 0.001$]. In both the patient and control groups renal function improved within the first 48 h [GFR 101 (83–188) and 116 (91–123), respectively]. After 3 months, renal function declined again [GFR 82 (70–94)]. We observed a significant correlation of plasma TMAO on admission with renal function in the whole study population and in stroke patients (Spearman rho −0.334, $P < 0.001$ and 0.317, $P < 0.001$, respectively). After adjustment for renal dysfunction on admission (GFR < 60) in logistic regression analysis, TMAO levels in the third and fourth quartile were significantly higher in patients compared with controls (Table 2). In a sensitivity analysis, we adjusted for GFR as a continuous parameter and found a statistical trend for higher quartiles of TMAO levels in patients compared with controls in a logistic regression model [quartile 4: OR, 2.118 (0.991–4.527), $P = 0.053$; Table 2]. The intraindividual differences in TMAO levels over time did not reach statistical significance after adjustment for renal function (admission/48 h, $P = 0.463$; 48 h/3 months, $P = 0.829$).

Association of plasma trimethylamine-N-oxide levels, cardiovascular risk factors and stroke etiology

When comparing the plasma levels of TMAO according to stroke etiology (according to TOAST classification), a weak but significant correlation was observed between TMAO levels and the number of vascular risk factors (Spearman rho $r = 0.170$, $P = 0.018$). Higher TMAO levels were independent predictors of coronary artery disease and diabetes in multivariable regression, adjusting for GFR (see Table 3). No association to other cardiovascular risk factors was found. When comparing the plasma levels of TMAO according to stroke etiology (TOAST classification), no significant association was found ($P = 0.45$). Moreover, no significant difference was detected between the median TMAO levels in patients with macroangiopathic vs. cardioembolic etiology [3.68 (2.32–6.45) vs. 4.18 (3.1–8.11) μmol/L, respectively, $P = 0.304$].

Association of plasma trimethylamine-N-oxide levels and functional outcome

We further investigated the association between plasma TMAO level on admission and functional outcome. Median mRS score after 3 months was 2 (1–3). TMAO levels had no association with functional outcome at discharge, either in crude regression analysis [OR, 0.999 (0.961–1.037)] or in a regression model adjusted for age, renal function, NIHSS score on admission and recanalization therapy [OR, 1.022 (0.959–1.048)]. Furthermore, TMAO levels on admission did not show an association with functional outcome after 3 months [OR, 0.976 (0.938–1.015)].

A total of 62 (32%) patients completed the 3-month follow-up. These participants were slightly younger [66 (58–72) vs. 72 (60–80) years, $P = 0.023$], less affected by stroke on admission [median NIHSS score 3 (1–5) vs. 4 (2–8), $P = 0.017$] and had a better functional outcome at discharge [mRS score 1 (1–2) vs. 2 (1–3), $P = 0.001$] compared with those patients who were lost to follow-up (see Table S2). Plasma TMAO levels on admission and after 48 h showed no relevant differences between patients who completed the 3-month follow-up and those who were lost to follow-up (Table S3).

Table 2 Differences in trimethylamine-N-oxide (TMAO) levels between patients and controls on admission in a logistic regression model

TMAO level	Dependent variable: patients; adjusted			
	OR (95% CI)[a]	*P*-value	OR (95% CI)[b]	*P*-value
Quartile 1	Ref.		Ref.	
Quartile 2	1.410 (0.730–2.725)	0.306	1.228 (0.629–2.397)	0.547
Quartile 3	2.001 (1.006–3.979)	**0.048**	1.845 (0.918–3.708)	0.085
Quartile 4	2.533 (1.216–5.279)	**0.013**	2.118 (0.991–4.527)	**0.053**

Significant *P*-values depicted in bold numbers. CI, confidence intervals; GFR, glomerular filtration rate; OR, odds ratio. [a]Adjusted for kidney failure (GFR < 60). [b]Adjusted for GFR.

Table 3 Association of trimethylamine-N-oxide (TMAO) with coronary artery disease and diabetes in logistic regression

TMAO level	Dependent variable: (a) coronary artery disease; (b) diabetes mellitus			
	OR (95% CI)[a]	*P*-value	OR (95% CI)[b]	*P*-value
Quartile 1	Ref.		Ref.	
Quartile 2	3.746 (1.163–12.072)	**0.027**	2.723 (0.810–9.147)	0.105
Quartile 3	4.154 (1.299–13.284)	**0.016**	4.038 (1.252–13.025)	**0.019**
Quartile 4	7.153 (2.256–22.676)	**0.001**	11.714 (3.712–36.966)	**< 0.001**

Significant *P*-values depicted in bold numbers. CI, confidence intervals; OR, odds ratio. (a) and (b) adjusted for glomerular filtration rate.

Discussion

In our study, we found that plasma TMAO levels are increased in stroke patients compared with non-stroke controls on admission. Remarkably, TMAO levels decrease in stroke patients within the first 2 days after stroke onset, without any significant difference as compared with non-stroke control patients at 48 h and are elevated again 3 months after stroke.

Our results offer an explanation for the inconsistent results on TMAO levels in stroke patients. Although most studies found elevated TMAO levels, one study reported lower TMAO levels in stroke patients [14]. As the time point of TMAO measurement has not been standardized and is sometimes not even reported, the results cannot be compared. From our results, it seems reasonable to use the level on admission (symptom-onset-to-admission time < 24 h) or after 3 months, although we are not able to pinpoint the peak and minimum of TMAO levels during this period from our data. One possible reason for the decrease in TMAO levels after 48 h could be gut dysbiosis due to ischaemic stroke. Previous studies have shown that severe dysbiosis can occur within days after stroke [21]. The authors showed that these changes occurred after large infarctions but not after small cortical infarcts. The significant correlation of the NIHSS score at discharge (relating to final infarct size) with the decrease in TMAO would support this explanation. The increase after 3 months might be a sign of a restored microbiome, although this remains speculative at present. The higher rate of cardiovascular risk factors in the patient group might contribute to higher TMAO levels in stroke patients compared with the control group. Nevertheless, as the number of cardiovascular risk factors does not change in the short time course, it cannot serve as an explanation for the change in TMAO levels in stroke patients.

Another explanation for the fluctuation of TMAO levels could be the change in renal function. TMAO shows a strong association with kidney function. Significant increases of GFR after admission in stroke patients have been reported previously [22], which might have been an additional cause of the differences in TMAO values between patients and controls and the decrease in TMAO in our patients after 48 h. We believe that the difference in TMAO levels between patients and controls and the drop in TMAO after 48 h are probably not solely caused by differences in kidney function for several reasons. Firstly, in the logistic regression analysis of our study, TMAO levels were higher in stroke patients after adjustment for renal insufficiency (GFR < 60). Second, although GFR increased similarly in patients and controls after 48 h (14 vs. 19 mL/min/1.73 m^2), TMAO levels only decreased in patients and not in controls. Third, larger trials have shown that the effect of TMAO on major cardiovascular events is independent of kidney function [10]. Independent of the time course, experimental studies showed a proatherogenic effect of TMAO by activating platelets and increasing thrombus formation and endothelial cell activation [6]. Although the elevation of TMAO might be associated with kidney function, its pathological effects could be independent of kidney function. Some authors believe that substances such as TMAO may be 'gut-derived uremic toxins' that mediate the deleterious effects of kidney failure [23]. Nonetheless, due to the strong association [24], future analyses of TMAO should report a measurement of kidney function. It might be beneficial to use more precise estimations of kidney function, such as cystatin C-based GFR [25].

Acute stroke treatment might also have influenced the course of TMAO, although we did not find any statistically significant difference in TMAO levels between different types of treatment.

As TMAO is associated with atherosclerosis, we expected to find higher TMAO levels in the patients with atherosclerotic etiology than in those with cardioembolic etiology, which was not the case in our study. One explanation might be that many patients in our group had overlapping risk profiles. Patients with suspected cardioembolic stroke suffered from hypertension (77%), diabetes (25%) and coronary artery disease (33%). Our results show that TMAO is elevated in patients with diabetes and coronary artery disease, which makes it less likely that a difference will be found between cardioembolic and atherosclerotic strokes if there is a substantial proportion of patients with these disorders in both groups. Stroke patients might also have an elevated TMAO level in general, independent of suspected etiology, which would be in line with a recent study demonstrating elevated plasma TMAO concentrations in patients with ischaemic stroke and atrial fibrillation [12]. More research is needed to answer the question of why stroke etiology is not associated with TMAO levels. It might be possible to examine this aspect with a cohort of patients with atrial fibrillation without any other cardiovascular risk factors.

Recently, Jaworska *et al.* investigated the toxic effects of TMA, a precursor of TMAO. Interestingly, they found that TMA exerts cytotoxic effects on cardiomyocytes and leads to degradation of lactate dehydrogenase and albumin, whereas this detrimental impact was not seen with TMAO. These findings suggest that TMA is an important mediator and marker for vascular diseases, whereas TMAO could be an

adaptive response to the increased TMA and might therefore be protective. In this case, TMAO would only be a surrogate parameter that was not causing the detrimental effects itself. This should be studied in future trials [18].

In our study, we found no effect of TMAO levels on outcome measured by the mRS score after 3 months. Therefore, we think that TMAO is a risk marker for ischaemic stroke that cannot be used for prognosis. However, TMAO has been described as being associated with the occurrence of major cardiovascular events [26]. The 3-month follow-up in our study might be too short to follow up these events. In a current study, TMAO was associated with mortality after 3 years [27].

Our prospective study had a representative patient cohort with median TMAO levels similar to those reported in other studies in German populations [24,28]. Nonetheless, the study also has some limitations. There was a selection bias in the study population as the median NIHSS score was 3. This might have consequences on the decrease of TMAO, which might have been larger if we had included more patients with severe stroke. Moreover, the correlation between TMAO levels and stroke severity might have been stronger if more patients with severe stroke were included. The higher rate of cardiovascular risk factors could be a reason for elevated TMAO levels independent of ischaemic stroke. This is a limitation of our study. To prove the effect of ischaemic stroke on TMAO levels, future studies should include an asymptomatic control group with an equal distribution of cardiovascular risk factors between the groups. Furthermore, the statistical analysis regarding the association of TMAO with different stroke etiologies might be limited by small sample size in each subgroup and should be investigated in a larger population, preferably with a subgroup with atrial fibrillation without other cardiovascular risk factors. We did not analyze nutrition in our study. We assume that this is not a limitation as Krüger *et al.* demonstrated that the effect of current food consumption on TMAO is small [29]. This was also the reason why we did not require fasting blood samples. Considering our study population, the impact of short-term nutrition should be negligible. Patients and controls both received approximately the same diet during their inhospital stay, which did not contain high amounts of dairy, fish or red meat. Long-term nutritional habits might be of more interest but were not the focus of this study. Furthermore, the results of our study are limited by the small number of participants with completed follow-up. For feasibility of the trial, we also did not require a blood sample from controls after 3 months. Our data show that patients with more severe stroke and worse outcome tended to miss the scheduled follow-up appointments. Our 3-month results should be interpreted accordingly. Finally, we were able to plot three time points of the TMAO course after ischaemic stroke. Obviously, there are large gaps in this time course and the lowest level of TMAO or whether there is more fluctuation cannot be determined.

In conclusion, our study provides an explanation for the contradictory role of TMAO plasma levels in patients with acute ischaemic strokes in the literature. By sequential measurements of plasma TMAO levels at different time points, we were able to show that TMAO levels decrease in patients suffering from ischaemic stroke within 48 h, arguably due to a gut microbiome dysbiosis. Our findings may help to establish TMAO as screening marker in the clinical routine in the future. Moreover, our data suggest that the time point of TMAO sampling should be standardized, preferably directly on admission, within the first 24 h after stroke onset. Kidney function should be assessed in all patients and reported to guarantee comparability.

Disclosure of conflicts of interest

Personal fees, travel support, speaker honoraria or research grants were received from Bayer (P.A.R.), BMS Pfizer (P.A.R. and S.M.), Boehringer Ingelheim (P.A.R.), Novartis (S.M.) and Deutsche Forschungsgesellschaft (P.A.R.) not related to the study. The other authors declare no financial or other conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Neurological diseases of control group.

Table S2. Comparison of follow-up participants and non-participants.

Table S3. Comparison of plasma trimethylamine-N-oxide levels between follow-up participants and non-participants.

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