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Gut microbiota-dependent trimethylamine-N-oxide (TMAO) Shows a U-shaped Association With Mortality But Not With Recurrent Venous Thromboembolism

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Abstract

Introduction: Gut microbiota-dependent trimethylamine-N-oxide (TMAO) correlates with arterial thrombotic events including myocardial infarction and stroke, and mortality. However, the association of TMAO with recurrent venous thromboembolism (VTE) and mortality remains unknown.

Methods: TMAO plasma levels were assessed by high performance liquid chromatography in 859 patients aged ≥65 years with acute VTE and categorized into low (<2.28μmol/L), medium (2.28–6.57μmol/L), and high levels (>6.57μmol/L) based on the 25th and 75th percentile. Associations of TMAO with recurrent VTE, major or non-major bleeding, and mortality were investigated.

Results: During a mean follow-up of 28 months, 106 patients developed recurrent VTE, 259 had major or non-major bleeding events, and 179 patients died. The risk of recurrent VTE did not differ significantly between patients with low, medium (adjusted subhazard ratio [SHR], 1.38; 95% confidence interval [CI], 0.81 to 2.36; p=0.232) and high TMAO levels (SHR, 1.44; 95% CI, 0.80 to 2.58, p=0.221). Compared with low TMAO levels, the adjusted hazard ratio [HR] for mortality was 0.68 (95% CI, 0.47 to 0.98, p=0.039) in patients with medium TMAO levels and 1.02 (95% CI, 0.68 to 1.52, p=0.922) in patients with high TMAO levels. Fractional polynomial Cox-regression confirmed a U-shaped association (adjusted p=0.045), with the lowest mortality risk in patients with TMAO around 4 μmol/L. TMAO was not associated with major or non-major bleeding.

Conclusion: TMAO showed a U-shaped association with mortality in elderly patients with acute VTE and was not associated with recurrent VTE and major or non-major bleeding.

Key words: Bleeding, deep vein thrombosis, pulmonary embolism, recurrent venous thromboembolism, trimethylamine-N-oxide

Abbreviations

CI	confidence interval
HR	hazard ratio
IQ	interquartile
DVT	deep vein thrombosis
PE	pulmonary embolism
SHR	subhazard ratio
TMAO	trimethylamine-N-oxide
VTE	venous thromboembolism

Introduction

Venous thromboembolism (VTE) consists of deep vein thrombosis (DVT) and pulmonary embolism (PE); its incidence ranges between 1.4 to 2.2 per 1,000 person-years and increases exponentially with age. [1] Anticoagulation is considered standard therapy to reduce recurrent VTE; however, a residual thrombotic risk remains and importantly, bleeding risk increases substantially. [2] In the absence of long-term anticoagulation, up to 30% of patients suffer recurrent VTE in 10 years. [1] Due to the high incidence of recurrent VTE and post-thrombotic complications, [1] substantial VTE-related healthcare costs [3] and bleeding complications, [2] further strategies are needed to reduce the burden of VTE.

Trimethylamine-N-oxide (TMAO), a gut microbiota-dependent metabolite of dietary phosphatidylcholine [4] and L-carnitine, [5] has been recently shown to increase the risk of arterial thrombotic events, such as myocardial infarction and stroke and consequently, overall mortality. [6, 7] Potential mechanisms underlying these observations include the propagation of atherosclerosis through activated monocytes/macrophages [4] as well as increased platelet activation and subsequent thrombotic arterial occlusion. [8] TMAO increases cholesterol uptake in macrophages leading to foam cell formation and thereby augments atherosclerotic lesion size converting arterial walls thrombogenic. [4] Further, it increases platelet aggregation and arterial thrombosis in rodents as well as human platelet adhesion and aggregation. [8] Both monocytes and platelets play a crucial role in the development of VTE. [9] During the initiation of venous thrombosis, monocytes and neutrophils are the dominant leukocytes adhering to venous vessel walls; likewise, platelets adhere to the endothelium and enhance leukocyte recruitment and neutrophil extracellular trap formation, [9] which is considered a crucial scaffold for venous thrombogenesis and in return activates platelet aggregation. [10-12] In line with this, platelet inhibition next to anticoagulation is recommended to prevent recurrent VTE. [2]

To date, the role of TMAO in venous thrombosis remains unknown. We therefore examined the association of TMAO plasma levels with recurrent VTE, major or non-major bleeding, and mortality in a prospective multicenter cohort study of 859 elderly patients with acute VTE.

Methods

Study population

The current study is a sub-study of the Swiss Cohort of Elderly Patients with Venous Thromboembolism (SWITCO65+; ClinicalTrials.gov Identifier: NCT00973596), a prospective multicenter cohort study of elderly patients with acute VTE. [13] Patients were recruited from September 2009 to March 2012 (n=1003). Inclusion criteria were age above 65 years and symptomatic, objectively confirmed VTE (proximal and/or distal DVT and/or PE). [13] Symptomatic, objectively confirmed DVT was defined as proximal and/or distal DVT detected by an objective imaging exam (ultrasonography, spiral computed tomography or magnetic resonance imaging venography) in a patient with symptoms. [13] Symptomatic, objectively confirmed PE was defined as a PE detected by spiral computed tomography, a high-probability ventilation-perfusion scintigraphy, pulmonary angiography or in the presence of objectively confirmed, symptomatic or asymptomatic proximal DVT in a patient with symptoms. [13] Exclusion criteria were inability to provide informed consent, conditions incompatible with follow-up, language barriers, thrombosis at a different site than lower limb, catheter-related thrombosis, or previous enrolment in the cohort. [13] After exclusion of 860 patients (no consent, n = 398; inability to provide consent, n = 285; follow-up not possible, n = 192; other site than lower limb, n = 21; catheter-related thrombosis, n = 7; language barriers, n = 51; multiple reasons for exclusion may apply), 1003 patients were enrolled and 144 patients were excluded from the current analysis (patients denying use of data, n = 8;, no biobank samples, n = 129; no TMAO measurement, n = 7); finally, TMAO was analyzed in 859 patients. The study received ethical approval from every participated center and eligible patients provided informed consent.

Baseline data collection and follow-up

At baseline, demographic information, comorbidities, laboratory findings, VTE-related treatment before and after the event, and concomitant antiplatelet therapy were collected using standardized data collection forms. Follow-up contact included one telephone interview

and two face-to-face evaluations during the first year followed by semi-annual contacts and periodic reviews of the patient's hospital chart. Patients were followed-up for 28 months on average.

Blood sampling and determination of TMAO

At baseline, heparin-anticoagulated blood was drawn after minimal venostasis; aliquots of samples were cryovialed in 3 ml polypropylene tubes and temporarily stored at -80°C before they were transported to and stored at the SWITCO65+ biobank at the Central Laboratory of Hematology of Lausanne University hospital, Switzerland.

For the determination of TMAO, plasma samples were shipped to the Institute of Clinical Chemistry, University Hospital Zurich (Zurich, Switzerland). Levels of TMAO were quantified by liquid chromatography-mass spectrometry, similar as previously described. [14] Briefly, 400 µL of an internal standard mixture containing TMAO-d9, at 1 µmol/L was added, vortexed and centrifuged (11'700 g, 10 min, 4 °C) and supernatant was analyzed. Separation was achieved on an Accucore HILIC column (50 x 2.1 mm, 2.6 µm particle size, Thermo Fisher Scientific, Reinach, Switzerland) under acidic conditions. As mass spectrometer, a QTrap 6500 hybrid instrument (Sciex) was used, that acquired chromatograms in positive electrospray ionization multi-reaction monitoring mode. The following transitions were used: 76.1 → 59.1 (quantifier), 76.1 → 42.1 (qualifier 1), 76.1 → 56.2 (qualifier 2) for TMAO and 85.1 → 68.1 for TMAO-d9. The modified method was validated and showed a good comparability to the original method described in [14]. All researchers involved in TMAO determination were blinded to patient characteristics and outcomes.

End points

The endpoints of the current study were recurrent VTE, major or non-major bleeding, and mortality. Recurrent VTE was defined as new fatal or new non-fatal PE, or new DVT (proximal and/or distal). [13] Diagnosis of recurrent DVT was performed by abnormal results on ultrasonography; recurrent PE was diagnosed by computed tomography, angiography, or

ventilation-perfusion lung scan; a new proximal DVT, associated with new PE symptom(s) was also considered as recurrent PE. [13] Major bleeding was defined as a fatal bleeding, symptomatic bleeding in a critical organ (intracranial, intraspinal, intraocular, retroperitoneal, intraarticular, pericardial, or intramuscular with compartment syndrome), a reduction of hemoglobin ≥ 20 g/L or transfusion ≥ 2 units of packed red blood cells. [13] Non-major bleeding was defined as bleedings that did not fulfill the criteria for major bleeding but required medical attention. [13]

Statistical analysis

TMAO plasma levels were categorized into low, medium, and high levels based on the 25th and 75th percentile (low, <2.28 μmol/L; medium, 2.28 – 6.57 μmol/L; high, >6.57 μmol/L). We compared baseline characteristics of patients with different levels of plasma concentration of TMAO using the chi-squared test and the non-parametric Kruskal-Wallis test as appropriate. We estimated the cumulative incidence of a first VTE recurrence, a first major or non-major bleeding, and death during the first three years using the Kaplan-Meier method and compared survivor functions between groups by the log-rank test. Associations between plasma concentration of TMAO levels and the time to a first VTE recurrence and a first major or non-major bleeding were assessed by competing risk regression accounting for death as a competing event, according to the method of Fine and Gray [15]. The method yields subhazard ratios (SHR) with corresponding 95% confidence intervals (CIs) and p-values for the failure event of primary interest. For death, an ordinary Cox-regression was calculated. We adjusted the models for risk factors that had previously been shown to be associated with VTE recurrence, major or non-major bleeding, or mortality. VTE recurrence was adjusted for age, gender, overt PE, prior VTE, provoked index VTE and periods of anticoagulation. Mortality was adjusted for age, gender, overt PE, active cancer, immobilization during the last 3 months, chronic or acute heart failure, chronic lung disease and periods of anticoagulation. Bleeding was adjusted for age, active cancer, history of major bleeding, antiplatelet therapy, overt PE, and periods of anticoagulation. We verified

proportional-hazards assumptions using Schoenfeld residuals and time interactions. There was no significant interaction between time and TMAO levels, i.e. the effect of TMAO on outcomes did not change over time. Because a fractional polynomial Cox-regression suggested a U-shaped relationship between TMAO and death, we further investigated a quadratic relationship between log-transformed TMAO and death using a Cox model with a linear and quadratic term. Number of events and incidence rates per 100 person-years were displayed at 36 months for the total cohort and different TMAO levels. All analyses were done using Stata 14 (Stata Corporation, College Station, Texas). STROBE guidelines have been the basis for reporting our results. [16]

Results

Study population

The mean age was 75 years, and 45 % patients were female. The mean TMAO level was 6.51 µmol/L. Patients' baseline characteristics were largely comparable across TMAO categories. However, patients with high TMAO levels were slightly older, had less provoked index VTEs (defined as presence of ≥1 of the following factors: major surgery, oestrogen therapy, or immobilization during the last 3 months), had reduced glomerular filtration rates and were more likely to be hypertensive (Table 1). Mean follow-up was 28 months.

Recurrent VTE

During follow-up, 106 patients developed recurrent VTE. Patients with medium and high levels of TMAO were more likely to develop recurrent VTE after 3 years (low, 11.0 %; medium, 15.8 %; high, 16.1 %; p = 0.393) (Figure 1). After adjustment, medium TMAO levels were associated with a 38 % (adjusted SHR, 1.38; 95% CI, 0.81 to 2.36; p = 0.232) and high levels with a 44 % (adjusted SHR, 1.44; 95% CI, 0.80 to 2.58; p = 0.221) higher risk of developing recurrent VTE compared to low levels of TMAO; however, these observations did not reach statistical significance (Table 2).

Mortality

Death occurred in 179 patients during follow-up. The cumulative incidence of mortality differed significantly between TMAO levels (low, 22.5 %; medium, 17.4 %; high, 27.7 %; p = 0.030) (Figure 2). After adjustment, the risk of mortality was significantly lower in patients with medium, as compared to patients with low levels of TMAO (adjusted hazard ratio [HR], 0.68; 95% CI, 0.47 to 0.98; p = 0.039) (Table 2). On the other hand, patients with high TMAO levels had a comparable risk to patients with low TMAO levels for dying (adjusted HR, 1.02; 95% CI, 0.68 to 1.52; p = 0.922) (Table 2) suggesting a U-shaped relationship between TMAO and mortality, which was confirmed in a fractional polynomial Cox-regression (Figure

3). In a model with a linear and quadratic term, we found a significant quadratic relationship between log-transformed TMAO and death (adjusted joint p = 0.045) (Table 3). The lowest number of events was observed in patients with TMAO around 4 µmol/L (Figure 3). At 3 years, a total of 179 deaths were observed: 60 deaths (33.5 %) were attributable to cancer, 33 (18.4 %) to PE (7 [3.9 %] PE related, 26 [14.5 %] possibly PE related), 16 (8.9 %) to sepsis, 14 (7.8%) to infection, 12 (6.7%) to left ventricular failure, 11 (6.2 %) to bleeding, 6 (3.4 %) to pulmonary causes other than PE, 3 (1.7 %) to acute coronary syndrome, 2 (1.1 %) to stroke, 1 (0.6 %) to suicide, 5 (2.8 %) to others, and 16 (8.9 %) to unknown causes.

Major or non-major bleeding

The endpoint major or non-major bleeding occurred in 259 patients during follow-up. The cumulative incidence of major or non-major bleeding was comparable among different levels of TMAO (low, 36.0 %; medium, 33.5 %; high, 36.3 %, p = 0.817) (Figure 4). Likewise, after adjustment, the risk of major or non-major bleeding in patients with medium (adjusted SHR, 0.96; 95% CI, 0.72 to 1.28; p = 0.775) and high TMAO levels (adjusted SHR, 0.94; 95% CI, 0.67 to 1.32; p = 0.732) were comparable to low levels of TMAO (Table 2).

Subgroup analysis for mortality

In general, patients with PE are at higher risk of death, compared to patients with DVT. [1] In order to investigate whether the observed U-shaped association of TMAO and mortality was different in patients with PE compared to patients with DVT only, we performed a subgroup analysis. In our study, we found a comparable mortality between the two groups (Supplemental Table 1). The U-shaped association (i.e. lower risk in patients with medium as compared to low and high TMAO levels) was more pronounced in patients with PE than in patients with DVT only. However, a test for interaction between VTE type (PE versus DVT only) and TMAO levels was not significant.

We also performed a subgroup analysis in patients with and without cancer, because cancer was the major contributor to mortality in the current study. The U-shaped association was

more pronounced in patients with cancer. However, a test for interaction between cancer and TMAO levels was not significant. Unfortunately, we cannot draw strong conclusions from these subgroup analyses, because 95% confidence intervals were rather wide and the power to detect differences between groups rather small.

Discussion

In the current prospective multicenter cohort study we examined the association of gut microbiota-dependent TMAO with recurrent VTE, major or non-major bleeding, and mortality in patients at 65 years of age or above with acute VTE. We found that patients with medium and high TMAO levels had a 38 % and 44 % higher risk of developing recurrent VTE, respectively, compared with low levels of TMAO; however, these findings did not reach statistical significance. Interestingly, the risk of death was only reduced in patients with medium, but not in patients with high TMAO levels, as compared with low TMAO levels and we found a significant U-shaped association between log-transformed TMAO and mortality. More precisely, patients with TMAO plasma levels around 4 µmol/L had the lowest risk of death. Lastly, TMAO levels were not associated with major or non-major bleeding.

Previous studies have investigated the associations between TMAO plasma levels and arterial thrombotic events including myocardial infarction and stroke as well as mortality in patients with previous myocardial infarction or in patients at high risk of cardiovascular events. [4, 6, 7, 14, 17, 18] Most [4, 6, 7], but not all of them [14, 17, 18] found high TMAO levels to be associated with an increased risk of atherothrombotic diseases. Potential pathophysiological mechanisms include the propagation of atherosclerosis by increased activation of monocytes/macrophages [4] and augmented arterial thrombosis mediated by increased platelet activation and aggregation through TMAO. [8] Although both monocytes and platelets are crucial for VTE development [9], we did not find a significant association between TMAO and recurrent VTE. In this study, median TMAO levels in the medium and high groups were 3.9 µmol/L and 10.6 µmol/L, respectively. Previous experimental studies showed that 10 to 31 µmol/L of TMAO were required in order to affect human platelet aggregation *ex vivo* and that arterial thrombosis was increased in mice with TMAO plasma levels of 100 µmol/L; [8] further, monocyte/macrophage activation and atherosclerosis was augmented in mice with TMAO plasma levels between 20 and 70 µmol/L. [4] Thus, one explanation for the missing association between TMAO and recurrent VTE might be that TMAO in the current study was slightly too low to affect venous thrombogenesis. In addition,

the studied population is of advanced age, which per se is associated with elevated levels of coagulation factors and inflammatory mediators; [19] in addition, VTE itself triggers inflammation and coagulation [20] and these mechanisms may have played a more relevant role for the development of VTE than the effects of TMAO on monocytes and platelets.

The observation of a U-shaped association between log-transformed TMAO and mortality is novel. In contrast to our findings, previous longitudinal studies in patients with acute coronary syndrome, stable coronary heart disease, acute heart failure, chronic kidney disease or diabetes have reported linear associations between TMAO and mortality. [6, 7, 21-23] In addition, a recent meta-analysis in patients with coronary artery disease and patients at risk of cardio- and cerebrovascular disease also showed a positive direct and non-linear association of TMAO with mortality. [24] In this study we found that at baseline, patients with high TMAO levels were slightly older, had lower glomerular filtration rates and were more likely to be hypertensive. Mortality was corrected for age and the main causes of death were attributable to cancer and pulmonary embolism, which are not affected by hypertension and kidney function. TMAO is a gut microbiota-dependent metabolite and antibiotic treatment decreases TMAO levels substantially. [4, 6] Therefore, concomitant infectious diseases and the use of antibiotic therapy could explain the higher incidence of death in patients with low TMAO. Plasma levels of TMAO depend on the intake of fish as well as phosphatidylcholine and L-carnitine present in dairy products, eggs and red meat; therefore, malnutrition may be associated with both decreased TMAO levels and increased risk of mortality. [25] Body mass indices were comparable between the groups, which excludes underweight in patients with low TMAO; however, a normal body mass index does not exclude malnutrition in elderly patients. [26]

Interestingly, TMAO levels in the present study were slightly higher than in previous Central European populations [14, 27-29] possibly due the advanced age of the current population and/or the high prevalence of comorbidities such as chronic kidney disease, glucose intolerance, and inflammation, conditions that have previously been shown to be associated with higher plasma levels of TMAO. [14, 29]

Strengths of our study include the large sample size. Further, TMAO measurements were performed in a standardized and reproducible manner. [14] The current study has several limitations. First, patients were recruited in Switzerland and were 65 years or above; thus, the findings are not applicable to the general population. Further, the high but non-significant SHR and the rather wide 95% confidence interval for recurrent VTE (1.48 (95% CI 0.88 to 2.49) and 1.56 (95% CI 0.87 to 2.77) for medium and high TMAO levels, respectively) indicates a limited power to detect significant differences. The study is observational and only detects associations but not causality. Next, despite extensive adjustment, we cannot entirely exclude the possibility that the observed associations may be due to residual confounding or chance. Lastly, TMAO exhibits relevant intra-individual variation of roughly 45 % over a 1-year period [28] and single measurements may not be representative for long-term follow-up.

The association of TMAO with venous thrombosis remained unknown so far. In the current study we found a trend for an increased risk of recurrent VTE in the elderly, which, however, was not statistically significant. Whether a larger sample size may had reached significant results remains to be determined. Further, the association of TMAO with recurrent VTE in younger patients or in patients with primary rather than recurrent VTE should be investigated in future studies. A U-shaped relationship of TMAO with mortality has not been reported earlier and may be specific to patients with VTE or to an elderly population due to comorbidities and/or nutritional habits. Whether pharmacological changes of TMAO affect recurrent VTE, bleeding or mortality remains to be determined in randomized controlled intervention trials.

In conclusion, TMAO showed a significant U-shaped association with mortality in elderly patients with acute VTE. The lowest number of events was observed in patients with TMAO around 4 μmol/L. Contrary to arterial thrombosis, TMAO was not associated with recurrent VTE and TMAO did not correlate with major or non-major bleeding.

Addendum

MFR, DM, OS, AL, MM, NR, DA, AAS, TFL, AvE and JHB designed the study. MFR, DM, SG, OS, AL, MM, NR, DA, AAS, and JHB performed measurements and analysis. MFR, DM, SG, OS, AL, NRB, LP, MM, NR, DA, AAS, CMM, TFL, GGC, AvE and JHB interpreted results. MFR, DM, SG, OS, AL, NRB, LP, MM, NR, DA, AAS, CMM, TFL, GGC, AvE and JHB wrote the manuscript. All authors critically read and revised the manuscript and approved the final version to be published.

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Disclosure of Conflicts of Interest

The authors have nothing to disclose.

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Legends to Figures and Tables

Figure 1. Kaplan–Meier estimates of recurrent VTE by TMAO levels. After 3 years, the cumulative incidence of recurrent VTE was higher in patients with medium and high levels of TMAO than in patients with low levels, but differences were not statistically significant (low, 11.0 %; medium, 15.8 %; high, 16.1 %; p = 0.393).

Figure 2. Kaplan–Meier estimates to mortality by TMAO levels. After 3 years, the cumulative incidence of mortality varied significantly between patients with different levels of TMAO (low, 22.5 %; medium, 17.4 %; high, 27.7 %; p = 0.030).

Figure 3. Relative hazards for mortality according to TMAO levels. Fractional polynomial Cox-regression showed a U-shaped association between log-transformed TMAO and death. The lowest risk of death was observed in patients presenting with TMAO around 4 µmol/L.

Figure 4. Kaplan–Meier estimates of major or non-major bleeding by TMAO levels. After 3 years, the cumulative incidence of major or non-major bleeding was comparable between different levels of TMAO (low, 36.0 %; medium, 33.5 %; high, 36.3 %, p = 0.817).

Table 1. Baseline characteristics by TMAO levels.

Values were missing in body mass index (1 %), systolic blood pressure (2 %), GFR (8 %), anaemia (6 %), and platelet count (6 %). Provoked VTE was defined as presence of ≥1 of the following factors: major surgery, oestrogen therapy, or immobilization during the last 3 months; polypharmacy was defined as prescription of more than four different drugs. GFR = glomerular filtration rate, IQ = interquartile, TMAO = trimethylamine-N-oxide; PE = pulmonary embolism, TIA = transient ischemic attack, VTE = venous thromboembolism.

Table 2. Association of TMAO with clinical end points after entire follow-up.

VTE recurrence was adjusted for age, gender, overt PE, prior VTE, provoked index VTE and periods of anticoagulation. Mortality was adjusted for age, gender, overt PE, active cancer, immobilization during the last 3 months, chronic or acute heart failure, chronic lung disease and periods of anticoagulation. Bleeding was adjusted age, active cancer, history of major bleeding, antiplatelet therapy, overt PE, and periods of anticoagulation. HR = hazard ratio, IR = incidence rate (per 100 patient-years), PE = pulmonary embolism, SHR = subhazard ratio, TMAO = Trimethylamine-N-oxide, VTE = venous thromboembolism.

Table 3. Association of TMAO with Mortality – quadratic modeling.

The adjusted joint p-value for both the linear and quadratic term was 0.045. Mortality was adjusted for age, gender, overt PE, active cancer, immobilization during the last 3 months, chronic or acute heart failure, chronic lung disease and periods of anticoagulation. HR = hazard ratio, IR = incidence rate, PE = pulmonary embolism, TMAO = Trimethylamine-N-oxide, VTE = venous thromboembolism.

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Tables

Table 1. Baseline characteristics by TMAO levels.

	All	Low <2.28 µmol/L	Medium 2.28-6.57 µmol/L	High >6.57 µmol/L	p-value
	n (%) or median (IQ-range)	n (%) or median (IQ-range)	n (%) or median (IQ-range)	n (%) or median (IQ-range)	
Total N	859	214	430	215	
Age	75.0 (69.0; 81.0)	74.0 (69.0; 80.0)	74.0 (69.0; 80.0)	76.0 (71.0; 83.0)	0.005
Female	386 (45%)	104 (49%)	185 (43%)	97 (45%)	0.407
BMI	26.6 (24.1; 29.8)	26.2 (23.8; 29.4)	26.7 (24.2; 29.8)	27.0 (23.8; 30.2)	0.222
Overt PE	599 (70%)	164 (77%)	293 (68%)	142 (66%)	0.035
Prior VTE	251 (29%)	54 (25%)	134 (31%)	63 (29%)	0.297
Major surgery during the last 3 months	130 (15%)	40 (19%)	67 (16%)	23 (11%)	0.065
Current oestrogen therapy during the last 3 months	27 (3%)	6 (3%)	14 (3%)	7 (3%)	0.951
Immobilization during the last 3 months	185 (22%)	54 (25%)	95 (22%)	36 (17%)	0.094
Provoked index VTE	252 (29%)	73 (34%)	132 (31%)	47 (22%)	0.014
Active cancer	157 (18%)	43 (20%)	73 (17%)	41 (19%)	0.592
History of major bleeding	89 (10%)	23 (11%)	38 (9%)	28 (13%)	0.257
Arterial hypertension	549 (64%)	123 (57%)	271 (63%)	155 (72%)	0.006
Systolic blood pressure	135.0 (120.0; 150.0)	130.0 (119.5; 148.5)	138.0 (125.0; 150.0)	134.0 (120.5; 146.5)	0.028
Chronic/acute heart failure	102 (12%)	27 (13%)	47 (11%)	28 (13%)	0.687
Diabetes	190 (22%)	43 (20%)	88 (20%)	59 (27%)	0.094
HbA1c [%]	5.7 (5.4; 6.1)	5.7 (5.4; 6.1)	5.7 (5.4; 6.1)	5.8 (5.5; 6.2)	0.088
Cerebrovascular disease (stroke, TIA)	82 (10%)	16 (7%)	39 (9%)	27 (13%)	0.180
Chronic renal disease	156 (18%)	24 (11%)	70 (16%)	62 (29%)	<0.001
GFR [mL/min/1.73m^2]	58.1 (47.4; 73.6)	66.2 (53.6; 82.2)	58.5 (49.3; 74.1)	49.6 (36.7; 62.0)	<0.001
Anaemia	333 (39%)	94 (44%)	156 (36%)	83 (39%)	0.378
Platelet count <150 G/l	130 (15%)	25 (12%)	69 (16%)	36 (17%)	0.171
Polypharmacy	439 (51%)	108 (50%)	214 (50%)	117 (54%)	0.525
Anticoagulation prior to index VTE	41 (5%)	13 (6%)	20 (5%)	8 (4%)	0.513
Type of initial parenteral anticoagulation					0.094
LMWH	410 (48%)	97 (45%)	212 (49%)	101 (47%)	
UFH	272 (32%)	79 (37%)	123 (29%)	70 (33%)	
Fondaparinux	147 (17%)	27 (13%)	81 (19%)	39 (18%)	
Danaparoid	1 (0%)	0 (0%)	0 (0%)	1 (0%)	

None	29 (3%)	11 (5%)	14 (3%)	4 (2%)	
Initial VKA therapy	746 (87%)	178 (83%)	380 (88%)	188 (87%)	0.177
Antiplatelet therapy	275 (32%)	62 (29%)	138 (32%)	75 (35%)	0.422
Aspirin	243 (28%)	57 (27%)	120 (28%)	66 (31%)	0.627

Table 2. Association of TMAO with clinical end points after entire follow-up.

End point	TMAO levels	N° of patients	N° events/ patient-years	IR (95% CI)	Crude SHR or HR (95% CI)	p-value	Adjusted SHR or HR (95%-CI)	p-value
VTE recurrence	All	859	106 / 1809.1	5.9 (4.8 to 7.1)				
	Low	214	19 / 446.6	4.3 (2.7 to 6.7)	1 (reference)		1 (reference)	
	Medium	430	57 / 924.3	6.2 (4.8 to 8.0)	1.48 (0.88 to 2.49)	0.142	1.38 (0.81 to 2.36)	0.232
	High	215	30 / 438.2	6.8 (4.8 to 9.8)	1.56 (0.87 to 2.77)	0.133	1.44 (0.80 to 2.58)	0.221
Mortality	All	859	179 / 1921.2	9.3 (8.0 to 10.8)				
	Low	214	46 / 456.0	10.1 (7.6 to 13.5)	1 (reference)		1 (reference	
	Medium	430	75 / 1003.4	7.5 (6.0 to 9.4)	0.75 (0.52 to 1.09)	0.130	0.68 (0.47 to 0.98)	0.039
	High	215	58 / 461.7	12.6 (9.7 to 16.2)	1.21 (0.82 to 1.78)	0.340	1.02 (0.68 to 1.52)	0.922
Major or non-major bleeding	All	859	259 / 1565.1	16.5 (14.7 to 18.7)				
	Low	214	66 / 374.2	17.6 (13.9 to 22.4)	1 (reference)		1 (reference)	
	Medium	430	126 / 799.2	15.8 (13.2 to 18.8)	0.95 (0.71 to 1.28)	0.753	0.96 (0.72 to 1.28)	0.775
	High	215	67 / 391.7	17.1 (13.5 to 21.7)	1.01 (0.72 to 1.42)	0.933	0.94 (0.67 to 1.32)	0.732

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Table 3. Association of TMAO with Mortality – quadratic modeling.

End point	TMAO levels	Crude HR (95% CI)	p-value	Adjusted HR (95%-CI)	p-value
Mortality	log (TMAO)	0.73 (0.53 to 1.00)	0.048	0.73 (0.53 to 0.98)	0.039
	$\log(TMAO)^2$	1.13 (1.04 to 1.23)	0.004	1.11 (1.02 to 1.21)	0.013

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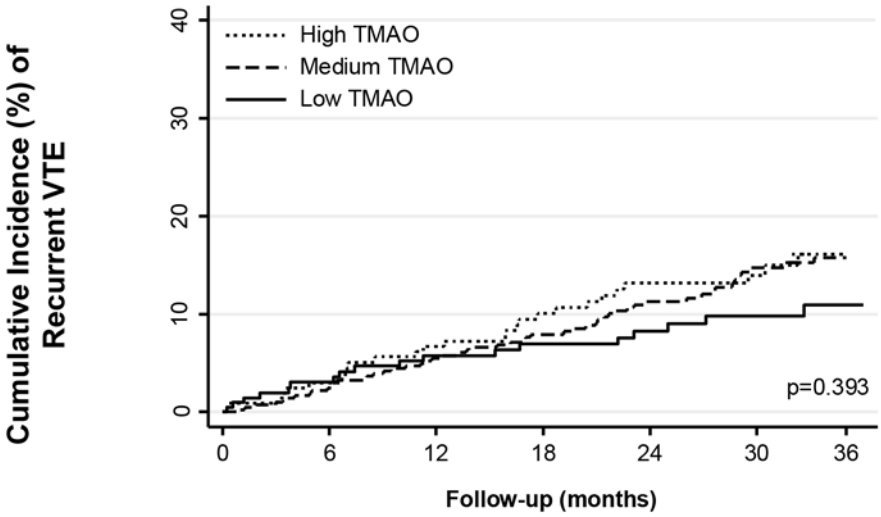
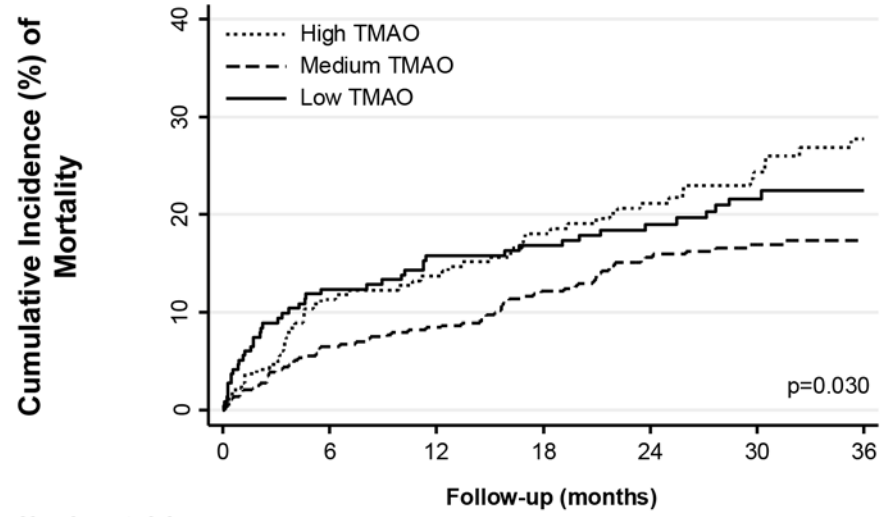
Figures

Figure 1



Number at risk							
High TMAO	215	183	171	147	125	88	51
Medium TMAO	430	386	360	323	249	182	117
Low TMAO	214	177	164	151	121	88	54

Figure 2



Number at risk							
High TMAO	215	186	180	160	139	98	61
Medium TMAO	430	392	378	347	275	211	135
Low TMAO	214	179	169	158	129	95	61

Figure 3

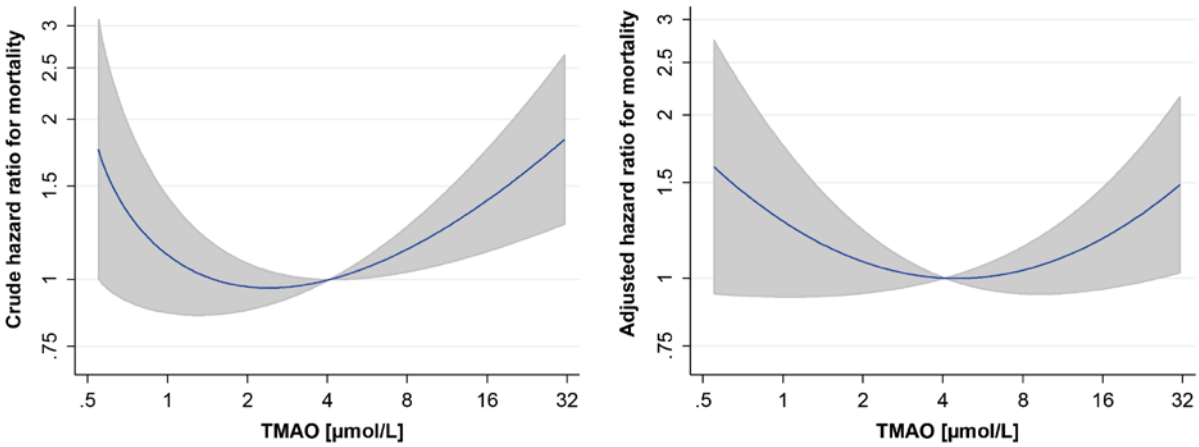
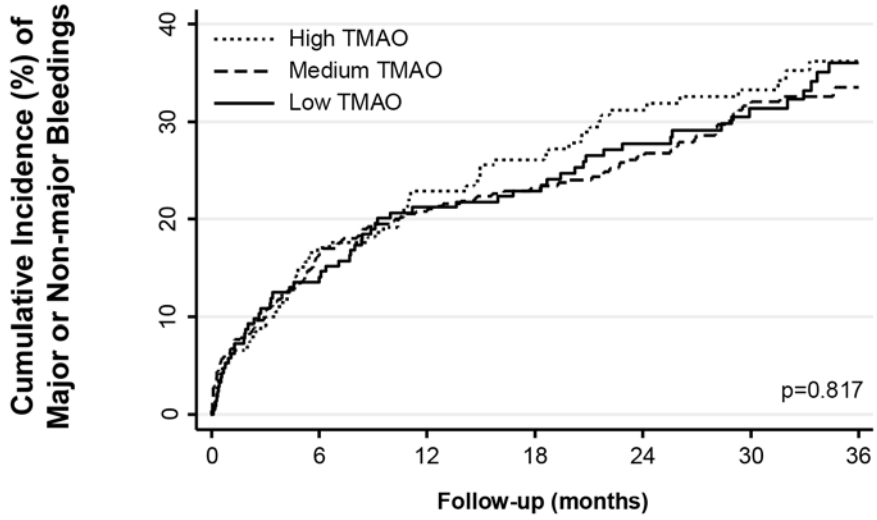


Figure 4



Number at risk							
High TMAO	215	161	146	132	110	74	44
Medium TMAO	430	336	308	279	214	155	97
Low TMAO	214	157	140	132	109	78	46

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Supplemental Table

Supplemental Table 1. Subgroup analysis for mortality.

Subgroup	TMAO levels	N° events/ N° patients	Crude HR (95% CI)	p-value	Adjusted HR (95%-CI)	p-value
Patients with overt PE	All	121/599				
	Low	37/164	1 (reference)		1 (reference)	
	Medium	49/293	0.70 (0.46 to 1.07)	0.101	0.65 (0.42 to 0.99)	0.047
	High	35/142	1.04 (0.66 to 1.66)	0.854	0.94 (0.58 to 1.51)	0.788
Patients with DVT only	All	58/260				
	Low	9/50	1 (reference)		1 (reference	
	Medium	26/137	0.95 (0.44 to 2.02)	0.887	0.92 (0.41 to 2.04)	0.829
	High	23/73	1.74 (0.80 to 3.76)	0.160	1.53 (0.67 to 3.49)	0.308
Patients without cancer	All	96/702				
	Low	22/171	1 (reference)		1 (reference	
	Medium	41/357	0.86 (0.51 to 1.45)	0.573	0.99 (0.59 to 1.68)	0.979
	High	33/174	1.46 (0.85 to 2.50)	0.172	1.37 (0.79 to 2.36)	0.266
Patients with cancer	All	83/157				
	Low	24/43	1 (reference)		1 (reference	
	Medium	34/73	0.62 (0.37 to 1.05)	0.077	0.55 (0.32 to 0.95)	0.032
	High	25/41	0.89 (0.51 to 1.57)	0.694	0.71 (0.39 to 1.30)	0.267

The adjusted HR in subgroup analysis for patients with overt PE and DVT only was corrected for age, gender, active cancer, immobilization during the last 3 months, chronic or acute heart failure, chronic lung disease and AC as time-varying covariate. The adjusted HR in subgroup analysis for patients with vs. without cancer was corrected for age, gender, overt PE, immobilization during the last 3 months, chronic or acute heart failure, chronic lung disease and AC as time-varying covariate. HR = hazard ratio, DVT = deep venous thrombosis, PE = pulmonary embolism, TMAO = Trimethylamine-N-oxide.