Trimethylamine-N-oxide (TMAO) predicts fatal outcomes in community-acquired pneumonia patients without evident coronary artery disease

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Abstract

Introduction: The pro-atherosclerotic metabolite trimethylamine N-oxide (TMAO) is a risk factor for incident cardiovascular events and a potentially modifiable mediator of chronic inflammation through broad-spectrum antibiotic treatment by changing the microbiome. Whether TMAO is associated with adverse clinical outcomes in acute inflammatory community-acquired pneumonia (CAP) patients is unknown.

Methods: A total of 317 CAP patients from a previous Swiss multicenter trial were prospectively followed for a median of 6.1 years. TMAO plasma levels were measured by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). We used cox regression models to investigate associations between baseline TMAO levels and all-cause mortality.

Results: Six-year mortality was 45.1%, and 18.9% of the patients had CAD. Median admission TMAO ($\mu\text{mol L}^{-1}$) levels were significantly higher in non-survivors compared to survivors (4.1 [interquartile range (IQR), 2.2-7.2] vs. 2.5 [IQR, 1.5-4.1]; $p < 0.001$). A strong association between TMAO and 6-year all-cause mortality was found for patients without CAD (adjusted hazard ratio (HR) 1.9 [95%CI 1.2-3.1]; $p < 0.05$). In patients with known CAD, no such association was found (adjusted HR 0.6 (0.2-1.6); $p = 0.309$, interaction $p = 0.009$). In patients without antibiotic pretreatment receiving antibiotic treatment, TMAO significantly decreased from admission to day seven (median, 3.9 [IQR, 2.1-7.5] vs. 3.1 [IQR, 1.4-6.6]; $p < 0.01$).

Conclusions: TMAO is associated with long-term fatal outcomes in CAP patients without evident CAD and modifiable through antibiotic treatment. Whether chronic modulation of TMAO by targeting the microbiome reduces mortality risk needs to be evaluated in future interventional trials.

Keywords: biomarker, coronary artery disease, community-acquired pneumonia, mortality prediction, trimethylamine-N-oxide.

Introduction

The intestinal microbiota-dependent and pro-inflammatory metabolite trimethylamine-N-oxide (TMAO) has emerged as a new dietary-associated risk factor for incident atherosclerosis and cardiovascular events [1-3]. In addition, TMAO was independently associated with 5-year all-cause mortality in subjects with congestive heart failure (CHF) and chronic kidney disease (CKD) [4-6]. Also, patients with diabetes mellitus, colorectal cancer, CHF, advanced left ventricular diastolic dysfunction and CKD stage 3 or beyond showed significant higher TMAO plasma levels compared with controls [4-8]. Importantly, TMAO levels can be influenced by dietary factors and depend on the composition of the microbiome [2], which opens the door for therapeutic interventions that reduce TMAO and associated risks.

Synthesis of TMAO depends on dietary L-carnitine and phosphatidylcholine (PC) intake. PC is the major dietary source of the semi-essential nutrient choline [9-13]. Eggs, red meat, fish, bacon, beef and chicken liver, wheat germ, dried soya beans and vegetables such as cauliflower and cabbage contain high concentration of total choline [14, 15]. Choline and other quaternary ammonium compounds are metabolized to the gas trimethylamine (TMA) by intestinal microbes [1, 16-18]. TMA is reabsorbed efficiently into the circulation and transported into human liver, where hepatic flavin-containing monooxygenases (FMOs) oxidize TMA to TMAO [19, 20]. Thus a functional intestinal microflora is essential for the production of TMAO [3, 16]. Interestingly, a dietary challenge with choline, L-carnitine, and PC led to an increase in the concentration of plasma TMAO [2, 20, 21], whereas the production of TMAO was suppressible through broad-spectrum antibiotic treatment by changing the microbiome [1-3]. A high correlation between plasma and urine levels of TMAO is evident, which indicates an effective urinary clearance of this metabolite [2].

Community-acquired pneumonia (CAP) is to date the leading cause of infectious death [22], and responsible for high costs, morbidity and mortality worldwide [23, 24]. Recent research found CAP to be associated with an increased risk of cardiovascular events [25, 26]. CAP is thus considered an independent cardiovascular risk factor.

Whether the pro-inflammatory metabolite TMAO has also a role in CAP-associated adverse clinical outcomes remains undefined. Herein, our aim was to evaluate the role of TMAO in a well-characterized CAP patient population with a follow-up over more than six year.

Methods

Study design

A total of 317 patients with a definite diagnosis of CAP and available blood specimens from a previous Swiss multicenter trial [27, 28] were included in this analysis. The initial trial included 925 (68%) CAP patients with an all-cause mortality of 45% over a follow-up period of six years [29].

The initial trial was a randomized controlled, antibiotic stewardship trial performed between October 2006 and March 2008 at six Swiss secondary and tertiary care centers [27]. The primary goal of the study was to verify the efficacy and safety of using procalcitonin (PCT) levels to guide the initiation and duration of antibiotic therapy in patients with lower respiratory tract infections [27, 28]. Patients ≥ 18 years of age presenting from the community or a nursing home to the emergency department (ED) were included if they fulfilled at least one of the following criteria: cough, dyspnea, pleural pain, sputum production or tachypnea and one infectious sign (core body temperature $>38^{\circ}\text{C}$, shivering, white blood cell count >10 or <4 cells $\times 10^9 \text{ L}^{-1}$) or one finding during auscultation (rales or crepitation). In all patients with CAP an infiltrate was radiologically confirmed. Patients with language restriction or dementia that precluded from providing written informed consent, intravenous drug use, terminal condition or hospital-acquired pneumonia were excluded. Participants were allowed to receive corticosteroids or short-term antibiotic pretreatment, but were excluded if they suffered from severe immunosuppression or received long-term antibiotic pretreatment at ED presentation.

The ethics committee of the University of Basel approved the initial study protocol and all patients provided written informed consent for the study including consent for the use of their data in secondary blood marker analyses and follow-up. The ProHOSP trial was registered at <http://controlled-trials.com> (identifier ISRCTN95122877).

Analysis of blood biomarker

Blood samples from each patient were collected and frozen upon ED admission for later measurement of different biomarkers. Baseline TMAO levels were measured in

blood samples of all 317 included CAP patients with 141 of them also having blood specimens at day seven of hospitalization for TMAO measurements.

The analysis was performed as described elsewhere [30]. In short, all experiments were done using a Thermo Fischer Ultimate 3000 UHPLC (Thermo Fisher, San Jose, California, USA) system coupled to an ABSciex 5500 QTtrap linear ion trap (LIT) quadrupole mass spectrometer (ABSciex, Darmstadt, Germany) operating in the positive ESI mode. Chromatographic separations were performed using gradient elution with 10 mM ammonium formate in 90% ACN (A) and 10 mM ammonium formate in water pH 3 (B) at a flow rate of 0.75 mL/min. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Plasma samples were prepared by simple protein precipitation with methanol.

Main outcome measurements

The primary endpoint was all-cause mortality after a follow-up period of six years. We also reported mortality at day 30, 1-year, and 3-years. Vital status was ascertained through structured phone interviews by trained medical students at day 30, 180 and 540 and at six years [29, 31]. If patients or their household members could not be contacted, the treating general practitioners were reached.

Statistical analyses

All statistical analyses were performed using STATA 12.1 (Stata Corp, College Station, TX, USA) and $p < 0.05$ was considered to indicate statistical significance. Unless stated otherwise, categorical variables are expressed as percentages (numbers) and continuous variables as medians [interquartile range (IQR)]. The distribution of the biomarker TMAO was skewed. After logarithmic transformation with a base of 10, the distribution of the biomarker data approximated a normal distribution. Chi-square (Wald) tests were used for frequency comparisons, and nonparametric (Mann-Whitney-U) tests were performed for two-group comparisons. Differences in TMAO concentrations between admission and day seven were evaluated using paired t-tests of log-transformed levels. We used univariate and multivariate linear regression models to examine the association of comorbidities with initial TMAO levels. Univariate and multivariate Cox regression models were used to investigate associations between TMAO levels at baseline and all-cause mortality. These associations are reported as hazard ratios (HRs) with 95% confidence

intervals (CIs) and significance levels for the chi-square (Wald) test. Area under the receiver operating characteristics curves (AUCs) with 95% CIs are presented to illustrate discrimination and predictive power. Kaplan-Meier curves were used to illustrate mortality based on TMAO quartiles (highest versus lower three). All analysis were done in the overall population, and stratified based on past medical history of CAD. To study subgroup effects (effect modification) we included interaction terms into the statistical cox regression models.

Results

Patient characteristics

Baseline characteristics of the entire cohort (n=317) as well as for patients stratified by 6-year vital status are presented in table 1. The median age of the entire cohort was 72 years and 41.3% were female. There was a high burden of comorbidities with 18.9% having a past medical history of CAD. A total of 25.9% of patients had antibiotic pretreatment. Median duration of antibiotic treatment during the hospital stay was 9 days and penicillin (mainly amoxicillin and clavulanate potassium) was used in the majority of patients (63.7%) whereas cephalosporins (mainly ceftriaxone) were used in 29.3% of patients. In 34.7% of patients a combination of two or more antibiotics was used.

Association between TMAO and mortality

Mortality at day 30 was 4.4% (n=14) and increased to 29% (n=92) after three years and 45.1% (n=143) after six years of follow-up. In the overall population, baseline levels of TMAO were increased in 6-year non-survivors compared to survivors (median, 4.1 $\mu\text{mol L}^{-1}$ [IQR, 2.3-7.2] vs. 2.3 $\mu\text{mol L}^{-1}$ [IQR, 1.4-4.2]; $p<0.001$). Also, a step-wise increase in mortality was found with increasing quartiles of TMAO (Table 2). Figure 1 shows Kaplan-Meier curves for 6-year all-cause mortality based on the highest TMAO quartile compared to lower quartiles in the overall cohort and in patients stratified by CAD.

In Cox regression analysis, TMAO was found to be associated with mortality (Table 2). These associations remained robust after 30 days and 6 years when adjusting for different comorbidities, but not at 1 and 3 years. Further analyses revealed an AUC of 0.66 (95%CI, 0.60-0.72) for long-term mortality.

We also investigated whether the association of TMAO and mortality would change among different subgroups, namely based on patient characteristics (e.g. gender, age, body mass index (BMI), smoking status) and comorbidities (e.g. CAD, CHF, CKD, diabetes mellitus, malignant tumor, chronic obstructive pulmonary disease (COPD), cerebrovascular disease (CVD)). As illustrated in Figure 2, there was significant effect modification by the past medical history of CAD with strong associations only in subjects without CAD history (p for interaction=0.009).

Based on these findings, we stratified the analysis based on CAD history (Table 3). No association of TMAO and all-cause mortality was found in patients with known CAD, whereas results in the subgroup of patients with no history of CAD were similar to the entire cohort findings. This is also illustrated in Kaplan-Meier curves (Figure 1).

Additionally, antibiotic pretreatment before hospital admission did not influence the prognostic performance of TMAO (p for interaction=0.931). Similar to the overall cohort, in patients with antibiotic pretreatment, baseline levels of TMAO ($\mu\text{mol L}^{-1}$) significantly predicted 6-year mortality resulting in an adjusted HR of 1.8 [(95% CI, 1.1-3.0); $p=0.028$].

Association of TMAO with comorbidities and antibiotic pretreatment

We performed linear regression models to investigate predictors for increased TMAO levels. As shown in Table 4, higher age, higher BMI, CAD, CHF, CVD, CKD and diabetes mellitus were all significantly associated with higher TMAO levels. After multivariate adjustment these associations remained significant for higher age, higher body weight, CAD, CKD and diabetes mellitus. For CHF and cerebrovascular disease, the association was no longer significant. In contrast, there was an inverse association of TMAO level with antibiotic pretreatment. Patients with antibiotic pretreatment before hospital admission had significantly lower TMAO levels ($\mu\text{mol L}^{-1}$) on hospital admission compared to patients without antibiotic pretreatment (3.5 [IQR, 1.9-5.8] vs. 2.3 [IQR, 1.2-5.1], $p=0.007$).

Change in plasma TMAO levels between admission and day seven

In a subgroup analysis including patients with available admission and day seven TMAO levels ($n=141$), we found a significant reduction in median TMAO levels in patients without antibiotic pretreatment ($3.9 \mu\text{mol L}^{-1}$ [IQR, 2.1-7.5] vs $3.1 \mu\text{mol L}^{-1}$

[IQR, 1.4-6.6], $p < 0.01$). In patients with antibiotic pretreatment, median TMAO were already decreased on admission and did not further decrease on day seven ($p = 0.87$) (Figure 3).

Discussion

The findings of this first study evaluating TMAO levels in CAP patients with and without evident CAD are threefold. First, high level of TMAO measured upon ED admission was a strong risk factor for long-term all-cause mortality only in patients without a medical history of CAD. No such effects were found for the CAD patient subgroup. Second, initial TMAO levels correlated with different risk factors and comorbidities namely age, body weight, CAD, CKD, and diabetes mellitus. Third, plasma TMAO levels were lower in patients with antibiotic pretreatment, and showed a significant decrease from admission to day seven in patients without antibiotic pretreatment who received antibiotics for treating their CAP episode providing evidence that this risk factor is indeed modifiable even at short term.

As demonstrated by this analysis and other cohorts, short-term [26, 32-34] and long-term mortality [25, 29, 35-37] in CAP patients is substantial. It has been hypothesized that CAP-associated mortality results from cardiovascular disease that may be triggered through the infection episode [38-40]. CAP *per se* is, thus, thought to be a cardiovascular risk factor [25]. For this reason, it is interesting to look into cardiovascular risk factor and cardiovascular markers of adverse clinical outcome. Several cohorts have found TMAO to be a risk factor for cardiovascular and adverse clinical events in healthy adults [2] and patients with CKD or chronic systolic heart failure [4, 5, 7]. Our study expands these findings to CAP patients where a strong association of TMAO and all-cause mortality was found. As a limitation, the exact cause of death was not verified through autopsies in our study. Still, we speculate that cardiovascular events may have been cause of death in a majority of our patients [41]. Interestingly, TMAO was only a risk factors in patients without known past history of CAD, which has not yet been reported in the literature. Possibly, TMAO levels are more universally increased in CAD patients [2, 3] and therefore this marker loses its predictive power in this subgroup.

The correlations between plasma TMAO level and various comorbidities such as CAD, CHF, CVD, CKD, and diabetes mellitus are in line with the results of previous observational studies [4-7]. It must be emphasized that increasing patient age was associated with higher TMAO blood levels. It is tempting to speculate that increasing age leads to an alteration in the microbial composition of the human intestine and therefore to a TMAO level rise. On the other hand, older age is associated with higher probability of cardiovascular comorbidities [42-44], which in turn are associated with higher TMAO levels. Thus, it is not possible to understand whether patient age is the cause or just a cofactor of increasing plasma TMAO levels. Furthermore, there was a positive association of TMAO with patient BMI. Although the body fat to muscle ratio was not determined in our study, it is likely that the majority of patients with a high BMI had elevated body fat levels. The increased risk for cardiovascular disease and most notably CAD [45, 46] may be an explanation for TMAO elevation in these patients. Another attempt to explain could be that patients with higher BMI have more appetite and therefore are better nourished. Thereby, more dietary substrate is available to build TMAO and in addition, nutrition contributes to the composition of the human gut microbiota [47]. These effects need verification in future studies.

Importantly, we found a significant decrease of TMAO from admission to day seven in patients without antibiotic pretreatment. Conversely, in patients with antibiotic pretreatment, admission levels were lower on admission and remained low until day seven. Although a different treatment regimen was used (i.e. mainly amoxicillin/clavulanate, clarithromycin or ceftriaxone) compared to other studies, our findings are in line with previous research of Tang et al. who reported a markedly suppression of TMAO after administration of a weekly broad-spectrum antibiotic treatment (ciprofloxacin and metronidazole) [2]. Several Gram-negative bacteria from the family of Enterobacteriaceae, which are part of the normal human gut microflora, are able to metabolize choline and other quaternary ammonium compounds to TMA [17, 18, 48]. All these findings underline the possibility of modifying the pro-atherosclerotic risk factor TMAO and call for an interventional trial to study the effects of chronic antibiotic treatment on the microbiome, TMAO production and clinical outcomes.

The main strengths of our study include (I) the well-characterized cohort of patients with CAP of different severities representative for patients usually treated in EDs and hospitals, (II) the long and rigorous follow-up over a period of 6 years, (III) the large number of primary endpoints (all-cause mortality) and (IV) the high accuracy of the laboratory measurement methods. Nevertheless, our study has limitations. First, this study was conducted in several hospitals in Switzerland, and results may not be generalizable to other countries. Second, autopsies were not performed to verify the causes of death and therefore this study focused on all-cause mortality rather than cardiovascular events. Third, this was an observational study and as such just hypothesis generating. Finally, 26% of the CAP patients received antibiotic pretreatment, which could have an impact on the initial plasma TMAO levels in this subgroup.

Conclusions

In conclusion, TMAO is associated with long-term fatal outcomes in CAP patients without evident CAD. This effect was only found in patients without evident CAD, but not in patients with known CAD. Further, TMAO is modifiable through administration of antibiotic treatment. Whether modulation of TMAO by targeting the microbiome reduces mortality risk needs to be evaluated in future interventional trials.

List of abbreviations

AUC – area under the receiver operating characteristic curve

BMI – body mass index

CAD – coronary artery disease

CAP – community-acquired pneumonia

CHF – congestive heart failure

CI – confidence interval

CKD – chronic kidney disease

COPD – chronic obstructive pulmonary disease

CRP – C-reactive protein

CURB-65 – new-onset confusion, urea >7 mmol L⁻¹, respiratory rate ≥ 30 breaths per min, systolic or diastolic blood pressure <90 mmHg or ≤ 60 mmHg, respectively, age ≥ 65 years (pneumonia risk scoring system)

CVD – cerebrovascular disease

ED – emergency department

FMO – flavin-containing monooxygenases

HR – hazard ratio

IQR – interquartile range

PAOD – peripheral artery occlusive disease

PC - phosphatidylcholine

PCT – procalcitonin

TMA – trimethylamine

TMAO – trimethylamine-N-oxide

w/o – without

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Author contributions

PS, MC-C, and BM created concept and design, wrote the protocol and initiated the initial ProHOSP study. PS and MO drafted the present manuscript and performed the statistical analyses. CS and AH performed laboratory measurements of TMAO. All authors contributed to the data acquisition, interpretation and drafting of the analyses, critical review for important content, and final approval of the manuscript. PS had full access to all data in the study and takes responsibility for the integrity of the work and the accuracy of the data analysis.

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Table 1. Study sample characteristics overall and by 6-year vital status

Characteristics	Entire cohort (n = 317)	Survivors (n = 174)	Non-survivors (n = 143)	p value
Age, years	72 [57-82]	63 [45-75]	80 [72-85]	<0.001
Female	41.3% (131)	47.7% (83)	33.6% (48)	0.011
BMI, kg m ⁻²	24.8 [22.0-28.2]	25.0 [22.5-28.4]	24.5 [21.7-27.7]	0.38
Smokers	27.7% (86)	34.5% (59)	19.4% (27)	0.003
Comorbidities*				
Coronary artery disease	18.9% (60)	8.6% (15)	31.5% (45)	<0.001
Congestive heart failure	13.2% (42)	5.2% (9)	23.1% (33)	<0.001
Cerebrovascular disease	9.5% (30)	4.0% (7)	16.1% (23)	<0.001
PAOD	5.0% (16)	2.9% (5)	7.7% (11)	0.051
Chronic kidney disease	24.0% (76)	13.2% (23)	37.1% (53)	<0.001
Diabetes mellitus	14.5% (46)	10.3% (18)	19.6% (28)	0.02
Neoplastic disease	12.6% (40)	6.9% (12)	19.6% (28)	<0.001
COPD	26.8% (85)	23.0% (40)	31.5% (45)	0.09
Clinical history				
Fever	66.1% (209)	75.3% (131)	54.9% (78)	<0.001
Chills	36.1% (101)	44.0% (70)	25.6% (31)	0.001
Clinical findings				
Confusion	6.9% (22)	3.4% (6)	11.2% (16)	0.007
Body temperature, °C	38 [37.2-38.9]	38.2 [37.3-39]	38 [37-38.8]	0.13
Heart rate, beats per min	95 [82-107]	95 [84-108]	95 [80-104]	0.20
Systolic blood pressure, mmHg	130 [119-148]	135 [120-148]	130 [111-150]	0.06
Arterial pH	7.45 (7.43, 7.49)	7.46 [7.43-7.49]	7.44 [7.41-7.48]	0.014
CURB-65 score				
0	24.0% (76)	36.8% (64)	8.4% (12)	<0.001
1	21.5% (68)	25.3% (44)	16.8% (24)	
2	30.6% (97)	20.7% (36)	42.7% (61)	
3	19.6% (62)	14.4% (25)	25.9% (37)	
4/5	4.4% (14)	2.9% (5)	6.3% (9)	
Initial laboratory findings				
CRP, mg L ⁻¹	140 [72-252]	153 [90-271]	131 [60-228]	0.016
PCT, µg L ⁻¹	0.45 [0.15-3.12]	0.63 [0.16-3.77]	0.38 [0.15-1.96]	0.21
TMAO, µmol L ⁻¹	3.0 [1.7-5.4]	2.3 [1.4-4.2]	4.1 [2.3-7.2]	<0.001
Outcomes				
Length of stay, days	8 [5-12]	7 [4-11]	9 [6-13]	<0.001
Antibiotic treatment, days	9 [5-11]	9 [6-11]	8 [5-10]	0.037
Antibiotic pretreatment	25.9% (82)	28.2% (49)	23.3% (33)	0.32

Data are presented as median [interquartile range] or percentage (n). Bolded p values are statistically significant at p<0.05. BMI, body mass index; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; CURB-65, new-onset confusion, urea >7 mmol L⁻¹, respiratory rate ≥30 breaths per min, systolic or diastolic blood pressure <90mmHg or ≤60mmHg, respectively, age ≥65 years (pneumonia risk scoring system); PAOD, peripheral artery occlusive disease; PCT, procalcitonin; TMAO, trimethylamine-N-oxide.

*Comorbidities were identified based on medical records, patient report, or both.

Table 2. Association of initial TMAO levels with all-cause mortality at different timepoints

Entire cohort (n=317)	All-cause mortality timepoint			
	30 day	1 year	3 years	6 years
TMAO quartiles	Number of deaths per quartile (%)			
I	1/80 (1.3%)	8/80 (10.0%)	13/80 (16.3%)	22/80 (27.5%)
II	3/79 (3.8%)	8/79 (10.1%)	18/79 (22.8%)	32/79 (40.5%)
III	4/79 (5.1%)	14/79 (17.7%)	23/79 (29.1%)	35/79 (44.3%)
IV	6/79 (7.6%)	22/79 (27.9%)	38/79 (48.1%)	54/79 (68.4%)
Cox regression models	HR (95% CI); <i>p</i> value			
Univariate	4.1 (1.5-11.3); 0.006	2.2 (1.2-3.9); 0.008	2.2 (1.4-3.3); <0.001	2.3 (1.7-3.3); <0.001
Multivariate*	3.8 (1.2-12.7); 0.027	1.5 (0.8-3.1); 0.235	1.6 (0.9-2.6); 0.091	1.6 (1.01-2.4); 0.029
Prognostic accuracy	AUC (95% CI)			
TMAO	0.68 (0.54-0.83)	0.64 (0.56-0.73)	0.66 (0.59-0.72)	0.66 (0.60-0.72)

Data for univariate and multivariate Cox regression models are given as hazard ratio (95% CI); *p* value. Data regarding discrimination (prognostic accuracy) are given as area under the receiver operating characteristic curve (95% CI). *P*-values in bold type are statistically significant at *p* < 0.05. AUC, area under the receiver operating characteristics curve; CI, confidence interval; HR, hazard ratio; TMAO, trimethylamine-N-oxide.

*Multivariate model: Admission concentration of TMAO adjusted for comorbidities (coronary artery disease, congestive heart failure, cerebrovascular disease, peripheral artery occlusive disease, diabetes mellitus, chronic kidney disease, neoplastic disease, and chronic obstructive pulmonary disease).

Table 3. Association of initial TMAO levels with mortality at different timepoints in patients with and without CAD

Coronary artery disease (n=60)	All-cause mortality timepoint			
	30 day	1 year	3 years	6 years
TMAO quartiles	Number of deaths per quartile (%)			
I	0/15 (0%)	1/15 (6.7%)	7/15 (46.7%)	12/15 (80%)
II	3/15 (20%)	6/15 (40%)	9/15 (60%)	12/15 (80%)
III	1/15 (6.7%)	5/15 (33.3%)	9/15 (60%)	11/15 (73.3%)
IV	1/15 (6.7%)	6/15 (40%)	7/15 (46.7%)	10/15 (66.7%)
Cox regression models	HR (95% CI); <i>p</i> value			
Univariate	1.2 (0.1-11.8); 0.875	1.6 (0.5-4.9); 0.412	0.8 (0.3-2.0); 0.651	0.7 (0.3-1.6); 0.415
Multivariate*	1.0 (0.1-15.6); 0.978	1.7 (0.5-6.0); 0.431	0.9 (0.3-2.4); 0.780	0.6 (0.2-1.6); 0.309
Prognostic accuracy	AUC (95% CI)			
TMAO	0.52 (0.29-0.75)	0.61 (0.46-0.76)	0.48 (0.33-0.63)	0.40 (0.22-0.57)

Patients without CAD (n=257)	All-cause mortality timepoint			
	30 day	1 year	3 years	6 years
TMAO quartiles	Number of deaths per quartile (%)			
I	1/66 (1.5%)	7/66 (10.6%)	12/66 (18.2%)	19/66 (28.8%)
II	2/63 (3.2%)	5/63 (7.9%)	9/63 (14.3%)	19/63 (30.2%)
III	1/64 (1.6%)	6/64 (9.4%)	11/64 (17.2%)	20/64 (31.3%)
IV	5/65 (7.8%)	16/64 (25%)	28/64 (43.8%)	40/64 (62.5%)
Cox regression models	HR (95% CI); <i>p</i> value			
Univariate	5.5 (1.6-18.8); 0.006	1.8 (0.9-3.9); 0.108	2.3 (1.3-4.0); 0.003	2.5 (1.6-3.9); <0.001
Multivariate*	6.0 (1.3-27.5); 0.021	1.2 (0.5-2.8); 0.686	1.8 (1.0-3.3); 0.065	1.9 (1.2-3.1); 0.011
Prognostic accuracy	AUC (95% CI)			
TMAO	0.69 (0.48-0.89)	0.60 (0.49-0.72)	0.64 (0.56-0.73)	0.65 (0.58-0.72)

Data for univariate and multivariate Cox regression models are given as hazard ratio (95% CI); *p*-value. Data regarding discrimination (prognostic accuracy) are given as area under the receiver operating characteristic curve (95% CI). Bolded *p* values are statistically significant at *p* < 0.05. AUC, area under the receiver operating characteristics curve; CAD, coronary artery disease; CI, confidence interval; HR, hazard ratio; TMAO, trimethylamine-N-oxide.

*Multivariate model: Admission concentration of TMAO adjusted for comorbidities (congestive heart failure, cerebrovascular disease, peripheral artery occlusive disease, diabetes mellitus, chronic kidney disease, neoplastic disease, and chronic obstructive pulmonary disease).

Table 4. Association of initial TMAO levels with demographic characteristics, comorbidities, and antibiotic pretreatment

	Univariate model		Multivariate model*	
	Coefficient (95% CI)	p-value	Coefficient (95% CI)	p-value
Patient characteristics				
Age	0.12 (0.09-0.15)	<0.001	0.08 (0.05-0.11)	<0.001
Male	0.98 (-0.12-2.08)	0.082	0.6 (-0.34-1.54)	0.209
BMI	0.14 (0.03-0.25)	0.012	0.11 (0.01-0.20)	0.025
Comorbidities				
Coronary artery disease	3.81 (2.48-5.14)	<0.001	1.45 (0.2-2.69)	0.023
Congestive heart failure	2.30 (0.71-3.89)	0.005	0.63 (-0.78-2.05)	0.381
Cerebrovascular disease	2.17 (0.33-4.02)	0.021	0.24 (-1.38-1.87)	0.767
PAOD	1.85 (-0.63-4.33)	0.144	(-)0.3 (-2.42-1.83)	0.784
Chronic renal failure	5.11 (3.96-6.25)	<0.001	3.6 (2.46-4.74)	<0.001
Diabetes mellitus	3.62 (2.12-5.11)	<0.001	1.62 (0.29-2.95)	0.017
Neoplastic disease	0.79 (-0.86-2.43)	0.347	(-)0.64 (-2.05-0.77)	0.374
COPD	1.17 (-0.05-2.4)	0.061	0.35 (-0.69-1.39)	0.508
Therapy				
Antibiotic pretreatment	(-)2.18 (-3.41-(-0.96))	0.001	(-)1.38 (-2.42-(-0.34))	0.009

Data for univariate and multivariate linear regression analyses are given as coefficient (95% CI). Bolded *p*-values are statistically significant at *p* < 0.05. BMI, body mass index; CI, confidence interval; COPD, chronic obstructive pulmonary disease; PAOD, peripheral artery occlusive disease.

*Multivariate model: adjusted for patient age, gender and comorbidities (coronary artery disease, congestive heart failure, cerebrovascular disease, PAOD, chronic kidney disease, diabetes mellitus, neoplastic disease, COPD)

Figure titles and legends

Figure 1. Kaplan-Meier survival estimate according to initial TMAO concentration for entire cohort and patients with or w/o coronary artery disease: 4th quartile versus 1st-3rd quartile
q, quartile; w/o, without.

Figure 2. Discrimination and predictive power of initial TMAO levels in different study subgroups.

Bolded *p* values are statistically significant at $p < 0.05$. AUC, area under the receiver operating characteristic curve; CAD, coronary artery disease; CHF, congestive heart failure; CI, confidence interval; CKD, chronic kidney disease; COPD, chronic obstructive pulmonary; CVD, cerebrovascular disease; PAOD, peripheral artery occlusive disease; w/o, without.

**p* for interaction

Figure 3. Change in plasma trimethylamine-*N*-oxide (TMAO) between admission and day seven stratified by antibiotic pretreatment.