Plasma concentration of TMAO is an independent predictor of adverse outcomes in patients after acute myocardial infarction

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Research Article

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Abstract

Background: Plasma concentrations of gut microbial metabolites are associated with cardiomyocytes viability and platelet reactivity. We hypothesised that increased concentrations of gut metabolites may predict major adverse cardiovascular events (MACE) after acute myocardial infarction (AMI).

Methods: We compared plasma concentrations of gut metabolites (trimethylamine-N-oxide, TMAO and indoxyl sulphate, IS) and platelet reactivity in 60 patients with AMI and 27 healthy controls. We assessed the predictive value of gut metabolites for MACE (stroke, recurrent AMI, death) over a median of 3.5-years.

Results: The concentrations of TMAO and IS did not differ between AMI patients and controls. The concentrations of TMAO and IS were higher in patients who developed MACE, compared to those who did not ($p \leq 0.015$ for all). The concentration of TMAO was the only independent predictor of MACE in a multivariate analysis (OR 35.041, CI 1.269-967.307, p=0.036). Patients with the concentration of TMAO and indoxyl sulphate above the cut-off value predictive of MACE had higher platelet activity ($p \leq 0.149$ for all).

Conclusions: Increased plasma concentration of TMAO is the independent predictor of MACE and may contribute to post-AMI cardiac dysfunction.

1. Background

Cardiovascular disease (CVD) including acute myocardial infarction (AMI) are the leading cause of death worldwide [1]. In 2009, the global healthcare costs for CVD were estimated at €106 billion [2]. Despite the progress in the pharmacological and interventional treatment of AMI, recurrent ischaemic events such as cardiovascular death, recurrent AMI or stroke occur in ~10% of patients within one year after the initial AMI [3]. At present, there is no tool to predict recurrent major adverse cardiovascular events (MACE) after AMI. Accumulating data shows that gut microbiome plays an important role in the pathogenesis of CVD [4–6]. Among gut microbial metabolites, especially trimethylamine-N-oxide (TMAO) and indoxyl sulphate (IS) are the focus of extensive research in CVD [7,8]. TMAO originates from the liver, which oxidizes trimethylamine (TMA), TMAO precursor produced by conversion of carnitine, betaine and choline by intestinal symbiotic bacteria [9]. IS, in turn, is a metabolite of dietary tryptophan that acts as a cardiotoxin and uremic toxin [10]. For example, TMAO was showed to have a dose-dependent association with platelet reactivity and cumulative incidence of thrombotic events in a cohort of over 4,000 patients presenting for elective cardiac evaluations [11]. TMAO was showed to enhance platelet responsiveness to multiple agonists (adenosine diphosphate, thrombin, collagen) by enhancing the release of calcium from intracellular stores [11]. Elevated serum TMAO levels were also predictive of thrombus formation in atrial fibrillation patients [12]. Similarly, IS was showed to promote arterial thrombosis in a rat model [13] and induce platelet hyperactivity thus contributing to chronic kidney disease (CKD)–associated thrombosis in mice [14]. Further, IS exacerbated fibrosis and proliferation of cardiomyocytes [15].

We hypothesized that plasma concentrations of gut microbial metabolites differ between patients with AMI and healthy volunteers, and plasma concentrations of metabolites may be used as biomarkers to predict MACE after AMI. The goal of this study was to (i) compare plasma concentrations of TMAO and IS in patients with AMI and healthy volunteers, as well as in patients with AMI who did and who did not experience MACE during the median follow-up of 3.5 years, (ii) evaluate the predictive value of TMAO and IS for MACE, and (iii) assess the correlation between TMAO and IS and platelet reactivity.

2. Methods

2.1. Study design

This was a prospective, observational study including patients participating in the AFFECT EV Metabolite Substudy. AFFECT EV was an investigator-initiated, prospective study conducted at the 1 st Chair and Department of Cardiology, Medical University of Warsaw, Poland [16]. The study protocol, designed in compliance with the Declaration of Helsinki, was approved by the Ethics Committee of the Medical University of Warsaw (approval number KB/112/2016), registered in the Clinical Trials database (NCT02931045), and published previously [17]. All participants provided written informed consent.

2.2. Study participants

Study inclusion and exclusion criteria are listed in Table 1. Patients were eligible for enrolment if they were (i) admitted to the hospital due to the first ST-segment elevation of AMI (STEMI) or non-STEMI (NSTEMI) with an onset of symptoms during the previous 24 hours, and (ii) underwent PCI with stent implantation. STEMI was defined as persistent ST-segment elevation of at least 0.1 mV in at least two contiguous electrocardiography leads, or a new left bundle-branch block [18]. NSTEMI was diagnosed in patients with typical anginal chest pain accompanied by ST-segment changes (ST depression, T-wave changes, transient ST elevation) on electrocardiogram and an elevation of cardiac troponin concentration in the peripheral blood [19]. When the study was initiated, patients with STEMI were pre-treated with clopidogrel before hospital admission. Because antiplatelet therapy with P2Y12 inhibitors affects platelet reactivity, only patients who received clopidogrel prior to PCI were enrolled in the study to obtain a homogenous study group. Since gut metabolites are excreted by the urinary tract, patients with CKD with estimated glomerular filtration rate (eGFR) <45 ml/min/1.73 m^2, calculated using the Modification of Diet in Renal Disease (MDRD) formula, were excluded [20]. Because the intestinal metabolism is affected by the state of gastrointestinal tract and its microbiota, patients with acute or chronic gastrointestinal diseases, autoimmune disease, treated with antibiotics within the last 2 months or taking dietary supplements within the last 7 days are excluded from the study [21].

Healthy volunteers were recruited among the hospital stuff and included people aged 18–99 years, without any medical history of chronic diseases, chronic pharmacotherapy, acute gastrointestinal disease within the last month, antibiotic therapy within the last 2 months and dietary supplements within the last 7 days.

Table 1. Eligibility criteria for the study.

Inclusion criteria	Age ≥ 18 years
	Informed consent
	First acute myocardial infarction treated with percutaneous coronary intervention with stent implantation
Exclusion criteria	Chronic kidney disease (estimated glomerular filtration rate < 45 mL/min)
	Chronic inflammatory disease
	Chronic intestinal disease
	Acute gastrointestinal disease within the last month
	Antibiotic administration in the last 2 months
	Dietary supplements in the last 7 days
	Autoimmune disease
	Active neoplasm
	Pregnancy or breast-feeding

2.3. Trial schedule and blinding

The trial schedule is presented in Figure 1. Blood was collected from patients by an independent operator (CE), who was otherwise not involved in sample analysis. Aggregometry was conducted by an independent operator (AG). During the trial, participants were identified by an individual number, and samples were coded with a sample number. Bacterial metabolite concentration analysis was performed by an independent operator, blinded to clinical data (MU). Statistical analysis was performed by independent operators (PS, KJ) [22].

2.4. Treatment

All patients received standard treatment after AMI according to the guidelines, including double antiplatelet therapy, β-blocker, angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, aldosterone receptor antagonist, and protein pump inhibitor [18,19].

2.5. Clinical data collection

Data collected at baseline include demographics (age, gender), body mass index, initial diagnosis, and cardiovascular risk factors, including arterial hypertension, diabetes, hyperlipidaemia, and smoking. In addition, routine laboratory parameters were recorded. At discharge, pharmacotherapy was recorded.

Data regarding MACE (recurrent AMI, stroke, cardiovascular death, all-cause death) were collected during the phone-call at the median of 3.5-year follow-up call [22].

2.6. Blood collection and handling

Peripheral venous blood samples were collected from fasting patients at a single time-point (within 24 hours after AMI). Briefly, blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes for metabolites analysis and into hirudin tubes for platelet reactivity analysis. The first 2 mL were disposed of to avoid pre-activation of platelets. Within a maximum of 15 minutes from blood collection, EDTA samples were centrifuged for 15 minutes at 2,500 g. Plasma was frozen at −80°C until analysed [22].

2.*7. Evaluation of gut bacterial metabolite concentration*

The concentrations of TMAO and IS were measured using a Waters Acquity Ultra Performance Liquid Chromatograph coupled with a Waters TQ-S Triple-Quadrupole Mass Spectrometer. The mass spectrometer operated in the multiple-reaction monitoring (MRM)-positive electrospray ionization (ESI) mode, as we have previously described [23].

2.8. Platelet reactivity

Platelet reactivity was assessed by multiple electrode aggregometry using the adenosine diphosphate test (ADP, 6.5 µmol/L) and thrombin receptor-activating peptide-6 (SFLLRN) test (TRAP, 32 µmol/L) was used as a positive control. Unstimulated whole blood was used as a negative control.

2.9. Endpoints

The primary end-point of the study were the differences in plasma TMAO and IS concentrations between patients with AMI and healthy volunteers. The secondary end-point was the prognostic value of TMAO and IS for the occurrence of MACE during the median 3.5-year follow-up time. The exploratory end-points was the correlation between TMAO and IS and platelet reactivity [22].

2.10. Statistical analysis

Statistical analysis was done using IBM SPSS Statistics, version 24.0 (IBM). Qualitative variables were presented as numbers and percentages and compared with the use of Fischer's exact test. A Shapiro–Wilk test was used to assess the normal distribution of continuous variables. Continuous variables were presented as median with interquartile range or as mean and SD and compared using Mann-Whitney U test or an unpaired t-test. The diagnostic ability of gut microbial metabolites to discriminate between patients with and without MACE and the cut-offs were calculated using a receiver operating characteristic (ROC) curve. Logistic regression model incorporating the gut microbial metabolites with significant sensitivity and specificity (area under the ROC curve, AUC) and clinical characteristics were used to determine the best model for MACE prediction. Mortality and other adverse events were reported depictively. A p-value below 0.05 was considered significant [22].

3. Results

Between January 2017 and July 2018, 60 patients were enrolled. Due to withdrawal of permission to participate in the study by 3 patients, 57 patients attended the final analysis and complete follow-up. Control group consisted of 27 age- and gender-matched healthy persons. MACE was developed by five patients (8.8%) during the follow-up: four deaths (two patients from unknown cause, two from cardiovascular cause) and a relapse of AMI.

3.1. Metabolites concentrations in patients with AMI and healthy controls

The concentrations of IS and TMAO concentrations were similar within patients with AMI and healthy controls (Figure 2).

3.2. Metabolites as predictors of MACE after AMI

Table 2 shows the characteristics of patients who developed major adverse cardiovascular events and persons who did not. Patients with MACE were older (p=0.031), had increased creatinine level (p=0.001), and elevated level of peak troponin I (p=0.048) at baseline, in comparison to patients who did not experience MACE. Nevertheless, the rest of cardiovascular risk factors and laboratory data were similar within the groups. Pharmacotherapy did not differ much between the patients. All of them received dual antiplatelet therapy, atorvastatin (except one person). Majority received a β-blocker, ACEI and PPI (proton pump inhibitor).

Table 2. Comparison of baseline characteristics between patients who experienced MACE and those who did not during the median follow-up of 3.5 years.

	MACE (n=5)		No MACE (n=52)		p
Characteristic					
Age, years – mean (SD)	**75.0**	**9.9**	**63.4**	**9.5**	**0.031**
Male gender – n (%)	5	100	37	71	0.162
BMI – mean (SD)	22.6	3.3	29.7	4.3	0.311
STEMI at admission – n (%)	2	40	42	81	0.072
CV risk factors – n (%)					
Arterial hypertension	4	80	32	62	0.642
Diabetes mellitus	1	20	15	29	1.000
Dyslipidaemia	2	60	35	67	0.332
Smoking	1	20	24	46	0.372
Clinical data					
CrCl, ml/min – median (IQR)	**57**	**43.50-73**	**90**	**68.50-113**	**0.007**
Hb, g/dl – mean (SD)	12.9	1.3	13.9	1.3	0.335
LDL-C – median (IQR)	136	52-188	123	91-151	0.922
NT-proBNP – median (IQR)	3307	1034-3579	764	305-1893	0.109
Plt count, 10^3/µl – mean (SD)	212	28	226	69	0.129
TnI max, ng/ml – median (IQR)	**52.5**	**23.2-104.8**	**12.1**	**3.1-36.4**	**0.048**
LVEF, % – mean (SD)	40.0	7.1	49.8	8.9	0.446
Pharmacotherapy at discharge					
Aspirin – n (%)	5	100	52	100	1.000
P2Y12 inhibitor – n (%)	5	100	52	100	1.000
Statin – n (%)	5	100	51	98	0.754
β-blocker – n (%)	5	100	47	90	0.468
ACE-inhibitor or ARB – n (%)	5	100	50	96	0.655
Diuretics – n (%)	2	40	13	25	0.467
Aldosterone antagonists – n (%)	3	60	10	19	0.072
Protein pump inhibitor – n (%)	5	100	49	94	0.581

Cardiac rehabilitation – n (%)	4	80	40	77	0.437

Abbreviations: ACE: angiotensin-converting enzyme; ARB: angiotensin-receptor blockers; BMI: body mass index; CrCl: creatinine clearance, based on the Cockcroft-Gault formula; CRP: C-reactive protein; Hb: haemoglobin; IQR: interquartile range; LDL-C: low-density lipoprotein-cholesterol; LVEF: left ventricle ejection fraction; NSTEMI: non-ST-segment elevation myocardial infarction; NT-proBNP: N-terminal pro-b-type natriuretic peptide; Plt: platelets; SD: standard deviation; STEMI: ST-segment elevation myocardial infarction.

The concentrations of intestinal microbial metabolites in plasma of patients with and without MACE after AMI during the 3.5-year follow-up are shown on Figure 3. TMAO (Fig. 3A) and IS (Fig. 3C) level were elevated in patients who experienced MACE, in contrast to those who did not develop any major adverse cardiovascular events ($p \leq 0.012$ for all), and distinguished between the groups (area under ROC curve [AUC] ≥ 0.85, $p \leq 0.011$ for all) in univariate analysis (Fig. 3B, 3D).

Table 3 indicates the statistical estimates of MACE hazard via TMAO and IS, which also include the cut-off values, set based on the ROC curves. We include TMAO and IS in a logistic regression model alongside with patients' gender and other clinical data (age, creatinine, troponin), to examine if these metabolites are independent MACE predictors. Table 4 indicates that the plasma concentration of TMAO at baseline was the only independent predictor of MACE during the observation period (OR 35.041, CI 1.269-967.307, p=0.036), whereas the baseline concentrations of IS lost statistical significance to predict MACE in multivariate analysis (OR 9.260, CI 0.287-298.409, p=0.209).

Table 3. Statistical estimates for prediction of major adverse cardiovascular events by gut microbial metabolites.

Metabolite	AUC (95% CI)	p-value	Cut-off (ng/ml)	Sensitivity	Specificity	PPV	NPV
TMAO	0.86 (0.62-1.00)	0.008	478	80%	96%	67%	98%
Indoxyl sulphate	0.85 (0.71-0.99)	0.011	962	80%	71%	21%	97%

Abbreviations: AUC: area under the curve, CI: confidence interval, PPV: positive predictive value, NPV: negative predictive value.

Table 4. Multivariate logistic regression model for prediction of major adverse cardiovascular events by gut microbial metabolites along with clinical variables.

Variable	OR		95% CI	p-value
		Lower	Upper	
High TMAO	**35.041**	**1.269**	**967.307**	**0.036**
Age	1.129	0.931	1.370	0.217
Gender	0.452	0.016	12.914	0.642
TnI max	0.985	1.000	0.980	1.020
Creatinine	36.973	0.203	6739.044	0.174
High IS	**9.260**	**0.287**	**298.409**	**0.209**
Age	1.221	0.984	1.516	0.070
Gender	1.019	0.052	20.029	0.990
TnI max	0.999	0.986	1.012	0.879
Creatinine	45.814	0.730	2875.056	0.070

Abbreviations: OR: odds ratio, CI: confidence interval; TMAO: trimethylamine-N-oxide; IS: indoxyl sulphate

Figure 4 shows the Kaplan-Meier analysis of event-free survival for MACE in patients after AMI, stratified according to the plasma TMAO concentrations (low TMAO defined as TMAO concentration below the established cut-off, high TMAO defined as TMAO concentration above the established cut-off, based on the ROC curve). Patients with high baseline TMAO concentrations had substantially lower chance of event-free survival during the median follow-up of 3.5 years, compared to patients with low TMAO concentrations ($p < 0.001$ for the log-rank test).

3.3. Correlation between platelet reactivity and SDMA

Figure 5 shows the correlation between plasma metabolites concentrations and platelets reactivity in response to ADP and TRAP in patients after AMI. Although there was no significant correlation between plasma TMAO and IS concentrations and platelets reactivity (Fig. 5A, 5B), patients with the concentrations of TMAO and IS above the cut-off value predictive of MACE (“high TMAO”, “high IS”) had higher platelet reactivity, compared to patients with metabolite concentration below the cut-off value (Fig. 5C-5F). However, the statistical significance was reached only for IS in the ADP test ($p=0.042$; Fig. 5D).

4. Discussion

The main finding of our study is that the plasma concentration of TMAO is a firm and independent predictor of major adverse cardiovascular events after AMI in the course of the 3.5-year observation period, with 80% sensitivity and 96% specificity. Moreover, other studies indicate that elevated plasma level of TMAO were associated with a higher risk of MACE (death, myocardial infarction, or stroke) independent from conventional risk factors as for example chronic kidney disease, obesity or diabetes mellitus in stable patients with coronary artery disease managed with optimal medical treatment [24,25] or undergoing elective coronary angiography [26], in patients presenting to the emergency department with chest pain [27], in patients after AMI [28], with chronic heart failure [29] and those after out-of-hospital cardiac arrest [30]. The association of plasma TMAO and adverse outcomes in cardiovascular patients was also confirmed in a recent meta-analysis [31]. Nonetheless, there are also studies which showed no association between the plasma concentration of TMAO and adverse outcomes in cardiovascular patients, indicating that TMAO results tend to be confounded by impaired kidney function and poor metabolic control [32,33]. In addition, it has been suggested that TMAO precursor trimethylamine, but not TMAO itself is involved in cardiovascular pathology by exerting negative effects on cardiomyocytes, likely due to disturbing their protein structure [7]. Hence, our study adds to the discussion in the literature on this topic.

In our study, plasma concentration of IS predicted MACE after AMI in univariate analysis, but it was not an independent predictor in multivariate analysis. In contrast to our results, other authors found that IS predicted adverse cardiovascular outcomes in patients after AMI and those with CKD undergoing dialysis [34,35]. Again, further studies are required to determine the real prognostic value of IS for cardiovascular outcomes and potentially implement both TMAO and IS in daily clinical routine.

The association between plasma TMAO and IS concentrations and adverse cardiovascular events is multifactorial. First, chronic dietary L-carnitine supplementation in mice enhanced synthesis of TMA and TMAO by gut microbiota and increased atherosclerosis, thus contributing to the well-established link between high levels of red meat consumption and CVD risk [25]. There is evidence that an increased concentration of TMAO activates platelets and triggers clot formation, resulting in a higher risk of atherothrombotic events and cardiovascular death [11,36]. In our study, patients with the concentrations of TMAO above the cut-off value predictive of MACE had higher platelet reactivity, compared to patients with metabolite concentration below the cut-off value, although the result was not statistically significant. Nevertheless, based on the previous evidence from the literature [35], an association between TMAO concentration and platelet reactivity might be one of the mechanisms underlying adverse outcomes.

The uremic toxin IS, in turn, was shown to activate inflammation and coagulation signalling pathways in the rat aorta in the short-term and induce calcification in the aorta and peripheral arteries in the long-term [37]. Further, IS induced the production of reactive oxygen species and the expression of osteoblast-specific proteins in human aortic smooth muscle cells [38] and stimulated the proliferation of rat vascular smooth muscle cells [39]. These observations, derived from cell cultures and animal models, might at least partly explain the association between extensive calcification and faster progression of atherosclerosis in CKD patients. In our cohort of post-AMI patients without CKD (eGFR <45 ml/min/1.73

m^2) we could not confirm the independent predictive value of IS, suggesting that IS might be specifically used to predict adverse prognostic effects in CKD patients [40], but not I CVD patients.

5. Limitations

The main limitation of our study is the small sample size and wide confidence interval of TMAO predictive value in multivariate analysis. Therefore, we can only hypothesize rather than ultimately prove that an elevated concentration of TMAO in plasma of AMI patients is related to MACE. Accordingly, the data should be not only considered with caution but also confirmed in a larger study group.

6. Conclusions

Elevated plasma concentration of TMAO occured to be independent and firm predictor of major adverse cardiovascular events after AMI in the course of the 3.5 years follow-up. We assume that the main cause might be the correlation between TMAO concentration and increased platelet reactivity.

7. List Of Abbreviations

ACE - angiotensin-converting enzyme,

ADP - adenosine diphosphate,

AMI - acute myocardial infarction,

ARB - angiotensin-receptor blockers,

AUC - area under the curve,

BMI - body mass index,

CKD - chronic kidney disease,

CRP - C-reactive protein,

CVD - cardiovascular disease,

EDTA - ethylenediaminetetraacetic Acid,

ESI - electrospray ionization,

Hb - haemoglobin,

IQR - interquartile range,

IS - indoxyl sulphate,

LDL-C - low-density lipoprotein-cholesterol,

LVEF - left ventricular ejection fraction,

MACE - major adverse cardiovascular events,

MDRD - modification of diet in renal disease,

MRM - multiple-reaction monitoring,

NSTEMI - non ST elevation myocardial infarction,

NT-proBNP - N-terminal pro-b-type natriuretic peptide,

ROC-receiver operating curve,

PCI - percutaneous coronary intervention,

Plt - platelets,

PPI - proton pump inhibitor,

SD - standard deviation,

SFLLRN - thrombin receptor activating peptide,

STEMI - ST elevation myocardial infarction,

TMA - trimethylamine,

TMAO - trimethylamine-N-oxide,

TRAP - thrombin receptor-activating peptide

8. Declarations

8.1 Ethics approval and consent to participate

The study protocol, designed in compliance with the Declaration of Helsinki, was approved by the Ethics Committee of the Medical University of Warsaw (approval number KB/112/2016), registered in the Clinical Trials database (NCT02931045), and published previously [17]. All participants provided written informed consent to participate in the study and to publish the results.

8.2 Consent for publication

All participants provided written informed consent to participate in the study and to publish the results.

8.3 Availability of data and materials

The dataset used and/or analysed during the current study are available upon request to the corresponding author.

8.4 Competing interests

The authors declare that they have no competing interests.

8.5 Funding

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8.6 Authors' contributions

Conceptualization, A.G., C.E., M.P., M.U.; Data curation, A.G., O.F., A.K., K.J., P.S.; Formal analysis, A.G., P.S, K.W.; Funding acquisition, M.U.; Investigation, O.F., A.K., K.J., P.S., K.W.; Methodology, A.G., C.E., M.P., K.J.F., M.U.; Project administration, A.G.; Resources, M.P., M.G., K.J.F., M.U.; Software, A.G., M.U.; Supervision, M.P., M.G., K.J.F., M.U.; Validation, A.G., M.U.; Writing—original draft, A.G., O.F., A.K., K.J., P.S.; Writing—review & editing, C.E., M.P., M.G., K.J.F., M.U. All authors have read and agreed to the published version of the manuscript.

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Figures

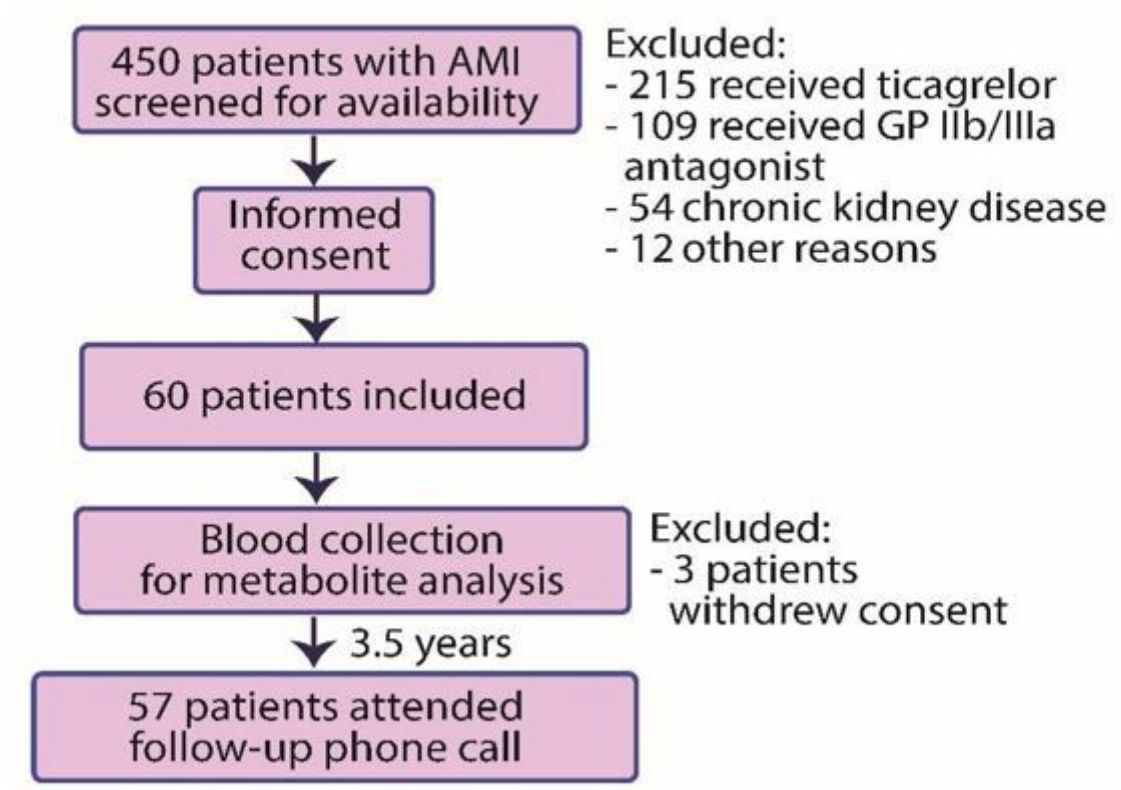

Figure 1

Inclusion and exclusion chart. AMI: acute myocardial infarction; GP: glycoprotein

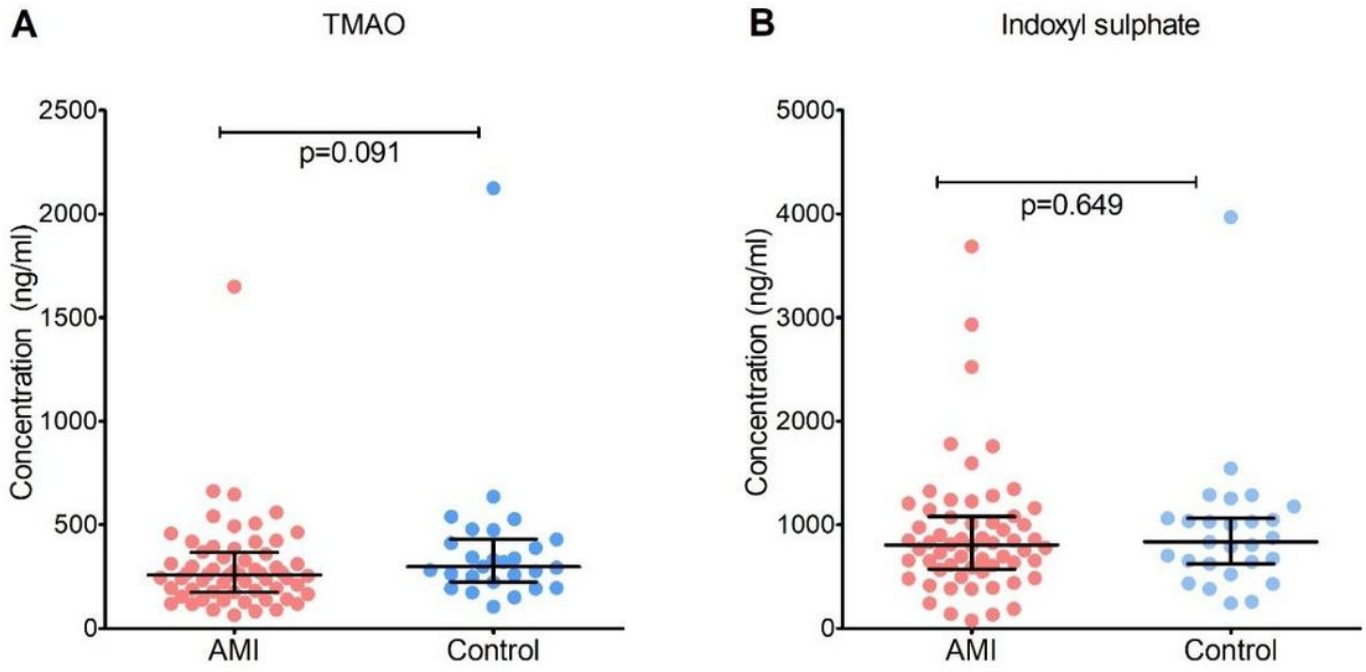

Figure 2

Intestinal microbial metabolites in plasma of patients with AMI and control group. A: Trimethylamine-N-oxide (TMAO). B: Indoxyl sulphate.

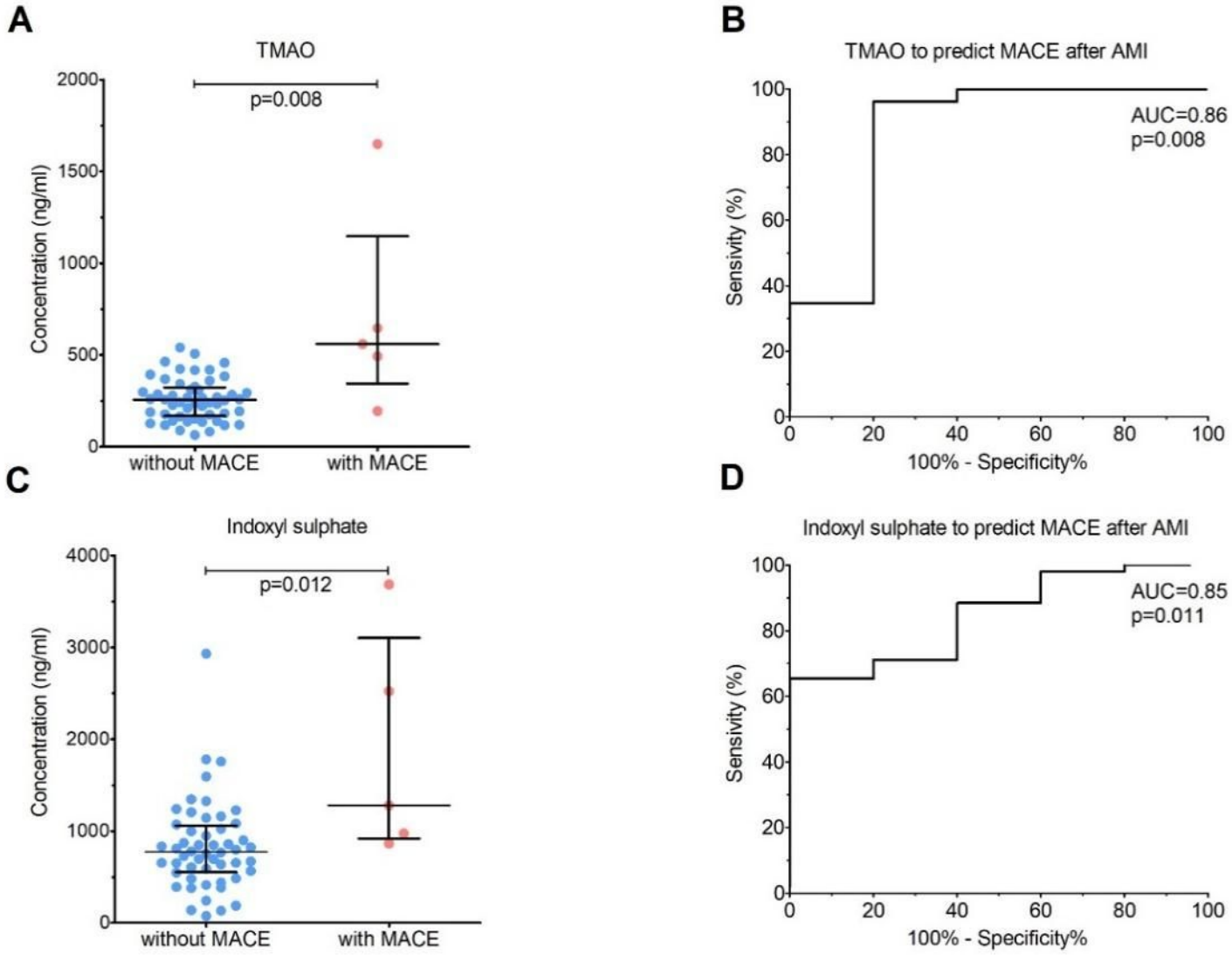

Figure 3

Gut microbial metabolites in plasma of patients with acute myocardial infarction predict major adverse cardiovascular events (MACE) during the median follow-up of 3.5 years. A, B: Trimethylamine-N-oxide (TMAO). C, D: Indoxyl sulphate.

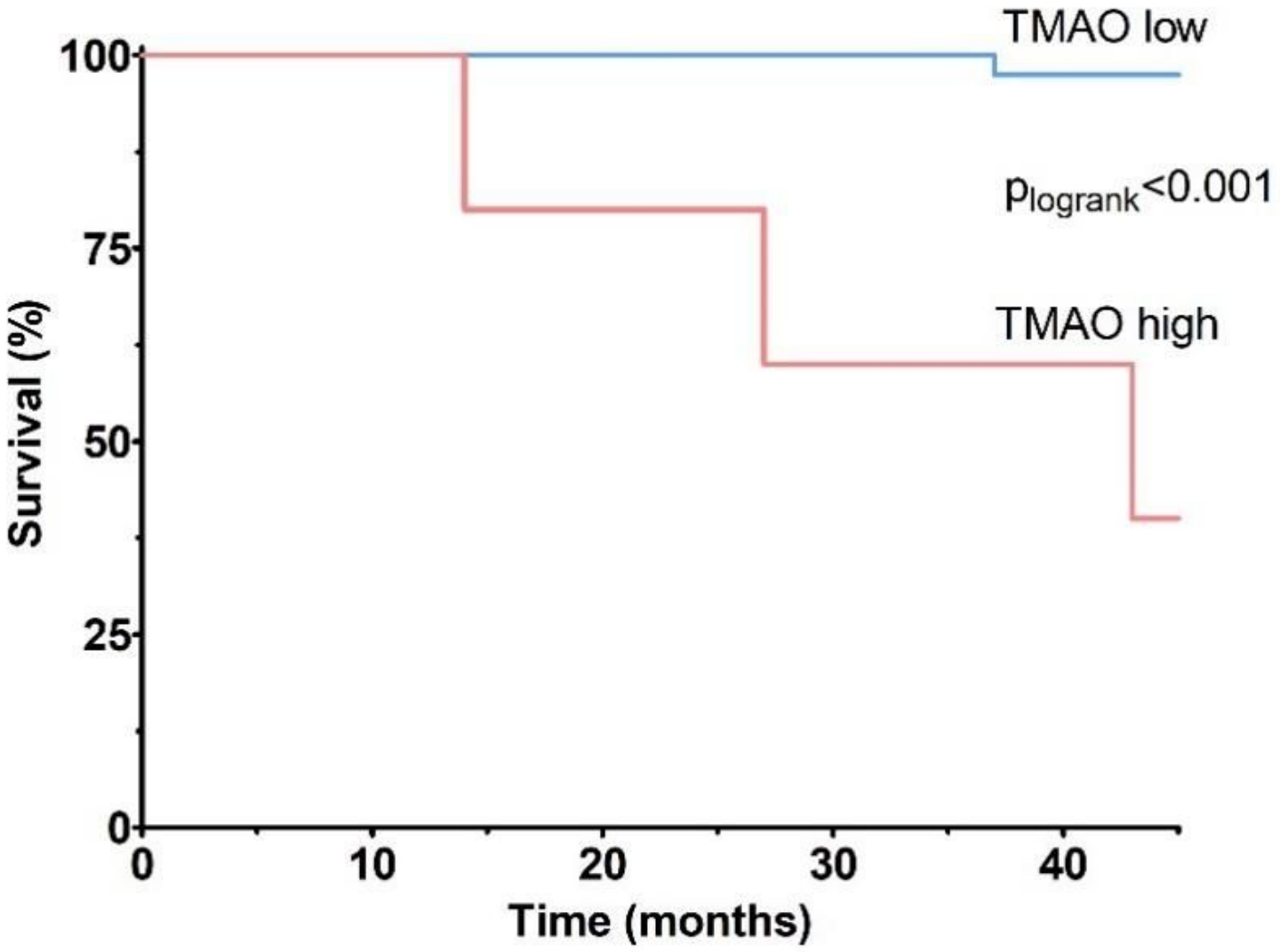

Figure 4

Kaplan–Meier analysis of event-free survival for major adverse cardiac events in patients after myocardial infarction, stratified according to the plasma trimethylamine-N-oxide levels during the median follow-up of 3.5 years.

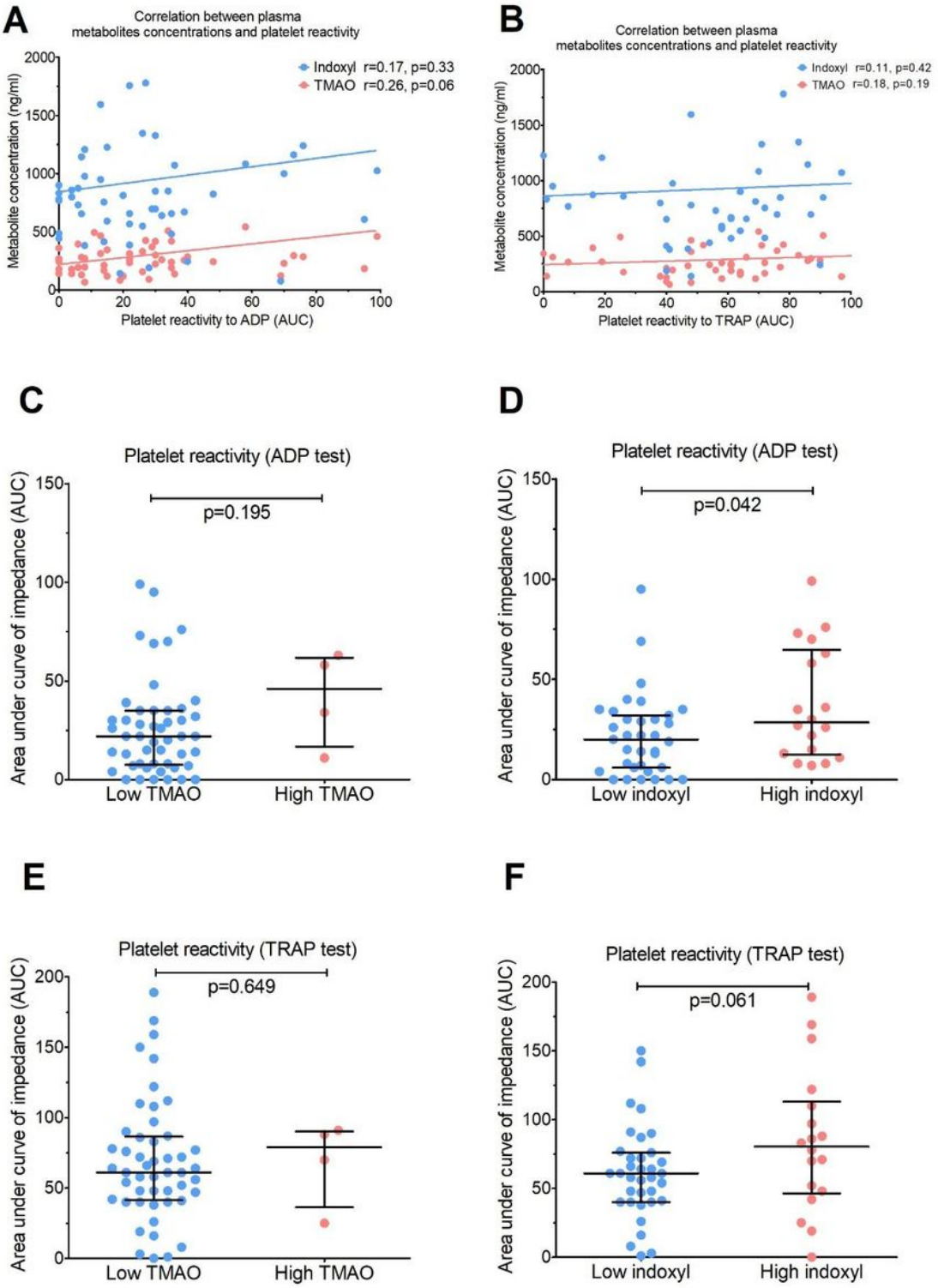

Figure 5

Correlations between platelet reactivity in response to ADP and TRAP and plasma gut microbial metabolites concentrations (5A, 5B) and comparison of platelet reactivity in patients with the concentrations of TMAO and IS above the cut-off value predictive of MACE (“high TMAO”, “high IS”), compared to those with metabolite concentration below the cut-off value (5C-5F).