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Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control

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ABSTRACT

Background: After ingestion of phosphatidylcholine, L-carnitine or betaine, trimethylamine-N-oxide (TMAO) is formed by gut microbiota and liver enzymes. Elevated TMAO plasma levels were associated with increased cardiovascular risk and other diseases. Also betaine and choline itself were recently associated with increased cardiovascular risk.

Methods: A newly developed LC-HRMS method was applied to measure the plasma concentrations of TMAO, betaine and choline in a cohort of 339 patients undergoing coronary angiography for the evaluation of suspected coronary artery disease.

Results: Betaine concentrations in males were significantly higher than in females (42.0 vs. 35.9 μmol/L; $p < 0.001$). Plasma concentrations of TMAO but not of betaine or choline were higher in patients with diabetes compared to euglycemic patients (2.39 vs. 0.980 μmol/L; $p = 0.001$) as well as in patients with metabolic syndrome as compared to patients without metabolic syndrome (2.37 vs. 1.43 μmol/L; $p = 0.002$). Plasma concentrations of TMAO or choline increased significantly with decreasing renal function (Spearman's rho: −0.281; $p < 0.001$). However, plasma levels of TMAO or betaine were associated with neither a history of myocardial infarction nor the angiographically assessed presence of coronary heart disease, nor incident cardiovascular events during 8 years of follow-up. Plasma levels of choline were significantly lower in patients with a history of acute myocardial infarction as compared to those without such history (10.0 vs. 10.8 μmol/L; $p = 0.045$).

Conclusions: Plasma levels of TMAO are confounded by impaired kidney function and poor metabolic control but are not associated with the history, presence or incidence of symptoms or events of coronary heart disease.

1. Introduction

Cardiovascular diseases are the main cause of death in the industrialized world and their importance is also increasing in developing regions of the world [1]. Although several risk factors are known today, there is still considerable medical need for additional risk factors and biomarkers to improve the identification and management of individuals at increased cardiovascular risk.

In 2011, Wang et al. [2] described for the first time an independent association between plasma concentrations of

List of abbreviations: TMAO, trimethylamine-*N*-oxide; FMO, flavinmonooxigenase; LC-HRMS, liquid chromatography-high resolution mass spectrometry; CHD, coronary heart disease; HESI, heated electrospray ionization; FWHM, full width at half maximum; QC, quality control; LLOQ, lower limit of quantification; VIVIT, Vorarlberg Institute for Vascular Investigation and Treatment; eGFR, estimated glomerular filtration rate; IQR, interquartile range; ATP III, National Cholesterol Education Program-Adult Treatment Panel III; CHD, chronic heart disease; AMI, acute myocardial infarction; CKD, chronic kidney disease; CABG, coronary artery bypass grafting.

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trimethylamine-*N*-oxide (TMAO) and cardiovascular risk. TMAO is abundant in many fish where it serves as anti-freezing agent [3]. In the human body, TMAO is produced in a pathway involving both the gut microbiota and liver enzymes [4,5]: After ingestion of dietary phosphatidylcholine, L-carnitine, or – with approx. 100 fold lower efficiency – also betaine, trimethylamine is formed by gut microbiota. Subsequently, trimethylamine is oxidized to TMAO by hepatic flavinmonooxigenases (FMO), predominantely FMO3 [6]. The crucial contribution of gut microbiota to the generation of TMAO was elegantly shown in both clinical studies and animal experiments by combining phosphatidylcholine or carnitine challenges with antibiotic eradication: In subjects with intact gut flora, the ingestion of substantial amounts of phosphatidylcholine or carnitine led to pronounced increases of TMAO levels in plasma. After suppression of the gut microbiota by oral treatment with broad spectrum antibiotics, no such increase could be detected after the ingestion of the same amount of phosphatidylcholine or carnitine [4].

Previous studies observed significant positive associations of TMAO plasma levels with cardiovascular risk [2,4,5], the staging and prognosis of heart failure [7,8], impaired glucose tolerance in mice [9], and recently also colorectal cancer [10].

Mechanistic studies in mice revealed that TMAO alters sterol metabolism and promotes the pathogenesis of atherosclerosis. Notably reverse cholesterol transport as well as the pool size and composition of bile acids were changed [2,11]. Feeding of TMAO to apolipoprotein E-null mice upregulated scavenger receptors (CD36, SR-A1), and promoted the formation of foam cells as well as atherosclerotic plaques in the aortic root [2].

Also other metabolites of choline metabolism were recently associated with increased disease risk. Elevated plasma levels of choline and betaine were associated with increased risk for major adverse cardiac events [5]. However, in another study the association of betaine plasma levels with incident cardiovascular events was found to be positive in patients with diabetes but inverse in patients without diabetes [12].

In this paper, we describe the development and validation of a liquid chromatography-high resolution mass spectrometry (LC-HRMS) method for the quantification of betaine, choline and TMAO in human plasma. Using the successfully validated method, plasma concentrations of betaine, choline and TMAO were measured in a cohort of 339 patients who underwent coronary angiography for the evaluation of suspected coronary artery disease and who were followed up for 8 years. Associations of TMAO, betaine, and choline with the presence and incidence of coronary heart disease (CHD) as well as confounding with cardiovascular risk factors were tested.

2. Materials and methods

2.1. Patients

339 patients from a previously described study cohort [13] of the Vorarlberg Institute for Vascular Investigation and Treatment (VIVIT; Feldkirch, Austria) were recruited. Samples were collected from unselected white patients undergoing coronary angiography for the evaluation of suspected coronary artery disease. From every patient, written informed consent was obtained and the study was approved by the Ethics Committee of the University of Innsbruck. Patient characteristics at baseline are shown in Table 1. Patients were followed up for 8 years.

Renal function was estimated as estimated glomerular filtration rate (eGFR) according to the CKD-EPI formula from 2009 [14]. Chronic kidney disease was classified by eGFR according to the KDIGO classification [15]. Euglycemia was defined as glucose <5.6 mmol/L and HbA1c <5.6%. Increased risk for diabetes was defined as either fasting plasma glucose between 5.6 and 7 mmol/L or HbA1c between 5.6 and 6.5% as defined by the American Diabetes Association [16]. Diabetes mellitus type 2 was diagnosed by either fasting plasma glucose ≥7 mmol/L, or plasma glucose ≥11.1 mmol/L 2 h after an oral glucose tolerance test with 75 g glucose, or HbA1c ≥ 6.5%, or previously diagnosed diabetes. Metabolic syndrome was defined according to the National Cholesterol Education Program-Adult Treatment Panel III (ATP III). If the following criteria were met, non-diabetic metabolic syndrome was diagnosed: waist circumference >88 cm in women or >102 cm in men; triglycerides ≥1.7 mmol/L; HDL-cholesterol <1.3 mmol/L in women or <1.0 mmol/L in men; blood pressure ≥130/≥85 mmHg; fasting glucose ≥6.1 mmol/L but <7 mmol/L. Coronary heart disease was defined as stenosis ≥50% or diffuse coronary sclerosis. Major adverse cardiovascular events (MACE) were defined as cardiac death, myocardial infarction, or stroke.

2.2. LC-MS

Betaine, choline and TMAO were purchased from Sigma Aldrich (Steinheim, Germany). Betaine-d3, choline-d9 and TMAO-d9 were obtained from TRC (Toronto, Canada). All chemicals used were analytical grade, all solvents LC-MS grade.

The LC system consisted of a Transcend TLX-1 HTLC system including two Accela 600 pumps, a HTC PAL autosampler and a valve interface module, all controlled by Aria OS 1.6.3 (all Thermo Fisher Scientific, Reinach, Switzerland). As the stationary phase, an Accucore HILIC column (50 × 2.1 mm, 2.6 μm particle size, Thermo Fisher Scientific) was used. The composition of the mobile phase was adopted from Xiong et al. [17]. Mobile phase A consisted of 10 mM ammonium formate buffer, pH 3; mobile phase B was acetonitrile. Separation of the analytes was performed in isocratic mode with 15% mobile phase A and 85% mobile phase B during 8 min at room temperature.

As the mass spectrometer, a Q Exactive hybrid instrument was used, controlled by Tune 2.2 and XCalibur 2.2 (Thermo Fisher Scientific) and equipped with a heated electrospray ionization (HESI) source. Chromatograms were acquired in positive fullscan mode at a resolution of 70,000 full width at half maximum (FWHM). Extracted ion chromatograms of the calculated masses of the analytes with a mass window of 10 ppm were used for quantification: *m/z* 118.08626 for betaine, *m/z* 121.10509 for betaine-d3, *m/z* 104.10699 for choline, *m/z* 113.16348 for choline-d9, *m/z* 76.07569 for TMAO and *m/z* 85.13218 for TMAO-d9.

Calibrators and quality controls (QC) were prepared in charcoal stripped heparinized plasma. Calibration ranged from 5.07 to 162 μmol/L for betaine, 1.92–61.5 μmol/L for choline and 0.54–71.9 μmol/L for TMAO. QCs were prepared at concentrations of 17.1 and 85.4 μmol/L for betaine, 9.59 and 48 μmol/L for choline, and 3.33, 16.6 and 53.3 μmol/L for TMAO, respectively.

Samples were prepared by protein precipitation of 20 μl of sample with 360 μl methanol, containing the internal standards at a concentration of 1 μmol/L. After thorough vortexing and centrifugation at 11,700× *g* for 10 min at 4 °C, 10 μl of the supernatant was injected into the LC-MS system.

2.3. Method validation

Linearity was shown by analysis of the freshly prepared calibrators on five subsequent days. QCs were analyzed five times on the same day and once on five different days to calculate accuracy and imprecision within and between days, respectively. The acceptance criteria for imprecision were <15% and between 85 and 115% for accuracy. Matrix effects were evaluated by the postcolumn infusion [18] as well as the post-extraction spiking method [19].