

Diabetes is Associated with Higher Trimethylamine N-oxide Plasma Levels

Authors

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Key words

- cardiovascular
- diabetes
- L-carnitine
- trimethylamine N-oxide

Abstract

Recent studies have revealed strong associations between systemic trimethylamine N-oxide (TMAO) levels, atherosclerosis and cardiovascular risk. In addition, plasma L-carnitine levels in patients with high TMAO concentrations predicted an increased risk for cardiovascular disease and incident major adverse cardiac events. The aim of the present study was to investigate the relation between TMAO and L-carnitine plasma levels and diabetes. Blood plasma samples were collected from 12 and 20 weeks old db/db mice and patients undergoing percutaneous coronary intervention. Diabetic compared to non-diabetic db/L mice presented 10-fold higher TMAO, but lower L-carnitine plasma concentrations at 12 weeks of age. After 8 weeks of observation, diabetic db/db mice had significantly increased body weight, insulin resist-

ance and TMAO concentration in comparison to non-diabetic control. In 191 patients undergoing percutaneous coronary intervention the median (interquartile range) plasma concentration of TMAO was 1.8 (1.2–2.6) $\mu\text{mol/L}$. Analysis of the samples showed a bivariate association of TMAO level with age, total cholesterol and L-carnitine. The multivariate linear regression analysis revealed that, in addition to L-carnitine as the strongest predictor of log transformed TMAO ($p < 0.001$), the parameters of age, diabetes status and body mass index (BMI) were independently associated with increased log transformed TMAO levels ($p < 0.01$).

Our data provide evidence that age, diabetes and BMI are associated with higher TMAO levels independently of L-carnitine. These data support the hypothesis of TMAO as a cardiovascular risk marker and warrant further investigation of TMAO for diabetes research applications.

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Abbreviations

TMAO	trimethylamine N-oxide
TMA	trimethylamine
OCTN	L-carnitine/organic cation transporter
BMI	body mass index
HDL	high density lipoprotein
SD	standard deviation

Introduction

Trimethylamine-N-oxide (TMAO) is a metabolite associated with atherosclerosis and cardiovascular risk [1]. Recent studies have demonstrated strong associations between systemic TMAO levels and angiographic measures of both coronary artery atherosclerosis and cardiovascular risk [1,2]. Also in diabetic patients, high TMAO was found to be a strong marker of all cardiovascular events [3]. In addition, plasma L-carnitine levels

in patients with high TMAO concentrations have predicted an increased risk of cardiovascular disease and an increased incidence of major adverse cardiac events [4]. Similarly, elevated plasma levels of choline and betaine only indicated increased cardiovascular risk when associated with concomitant TMAO elevation [5]. These studies reinforced the importance of diet and microbiota in cardio-metabolic health, with the TMAO level emerging as a possible target for therapeutic interventions.

TMAO is generated from dietary compounds containing a trimethylammonium moiety, including choline and L-carnitine, which undergo metabolism by gut microbiota [2,6]. After digestion in the intestine, L-carnitine is degraded into trimethylamine (TMA) [7,8], which after absorption into the circulation is subjected to metabolism by liver flavin-containing monooxygenase to result in TMAO [9]. Large perturbations in TMAO levels may result from dietary differences,

and intestinal microbiota are suggested as playing a prominent role in the variation of TMAO levels [10]. Transporter systems involved in the biodistribution of TMAO have not been characterized; currently, there is some evidence that L-carnitine/organic cation transporter (OCTN) 2 is not a TMAO transporter [11], which is different from the characteristic transport pathway for L-carnitine [12]. A comparative genome-wide association study recently concluded that genes play a marginal role and that any variation in TMAO levels determined by genetic effects results in relatively weak and complex correlations [13]. Thus, the factors that regulate circulating levels of TMAO are mostly of a dietary and microbiota-derived nature.

L-carnitine is essential for the mitochondrial metabolism of long chain fatty acids, and some studies have provided evidence for improvement in glycaemia and plasma lipids after the administration of L-carnitine in type 2 diabetes mellitus [14,15]. However, other studies demonstrate the increased risk of diabetic complications in patients with higher concentrations of circulating L-carnitine [16]. The role of L-carnitine in cardiovascular health was recognized after the discovery of the proatherogenic nature of TMAO and its relation to L-carnitine metabolism [4]. The measurements of L-carnitine in combination with TMAO are expected to provide further clinical evidence for possible associations of plasma concentrations of both compounds with different cardiovascular conditions, including cardiovascular complications in diabetic patients.

In the present study we used db/db mice as a rodent model of type 2 diabetes and compared the circulating TMAO levels to those in lean control mice. In addition, TMAO levels were measured in patients undergoing percutaneous coronary intervention, because TMAO has been associated to cardiovascular risks and cardiovascular diseases are the most common cause of death in people with diabetes [17,18]. Our results support the hypothesis of TMAO as a cardiovascular risk marker and warrant further investigation into the application of TMAO in diabetes research.

Materials and Methods



Animals

10 male db/db (10 weeks old, Harlan Laboratories BV, Netherlands) and 10 age matched non-diabetic db/L mice weighing 36 g were housed under standard conditions (21–23°C, 12 h light-dark cycle) with unlimited access to food (R70 diet, Lantmännen, Sweden) and water. The experimental procedures were carried out in accordance with the guidelines of the European Community, local laws and policies and were approved by the Latvian Animal Protection Ethical Committee, Food and Veterinary Service, Riga, Latvia.

Mice were adapted to local conditions for 2 weeks before the start of blood sampling and insulin tolerance test. Animals were weighted 2 times per week. Blood samples were collected at the age of 12 and 20 weeks into heparin containing tubes and centrifuged. The obtained plasma samples were stored frozen (–80°C) until analysis.

Determination of biochemical measures

Glucose, free fatty acids and triglyceride (TG) in plasma samples were measured by Wako (Germany) and Instrumentation Laboratory (Italy) enzymatic kits. Plasma insulin concentrations were determined with a RIA kit (Millipore, USA).

For insulin tolerance test experiment, mice were *i/p* administered an insulin solution (0.2 mU), and then blood glucose was measured 0.5, 1, 2, 4 and 24 h after administration. Blood glucose was measured using a MediSense Optium blood glucose meter and corresponding strips.

Patient samples

This study was performed with the approval of the Central Medical Ethics Committee (Riga, Latvia), and written informed consent was obtained from all subjects. The study included patients undergoing percutaneous coronary intervention (PCI) with implantation of a drug-eluting stent due to clinical indications to treat a coronary artery stenosis. To characterize the severity of the coronary atherosclerosis all patients were further classified into one-, 2- or 3-vessel disease group based on the presence of stenosis in major coronary arteries or their branches: left anterior descending artery, left circumflex artery and right coronary artery. The definition of a significant coronary artery stenosis was 50% or more as judged by the interventional cardiologist or previous stent implantation in the corresponding artery. Patients with left main disease were classified as 2-vessel disease if there was no stenosis in the right coronary artery or as 3-vessel disease if there was a significant stenosis in the right coronary artery. The history of previous myocardial infarction, age at which myocardial infarction had first developed as well as the age of onset of the first coronary symptoms was recorded.

The data collected at the study inception included age, medical history, and anthropometric indices (height, weight and waist circumference) as well as pre-hospital and in-hospital treatment. Routine biochemistry tests (including lipids, blood glucose and creatinine) and full blood count were performed before PCI. Plasma samples were obtained from venous blood by venipuncture on the day after PCI and stored at –20°C prior to the analysis of L-carnitine and TMAO. The duration of the fasting state was recorded for the biochemistry tests and plasma samples.

In accordance with the guidelines of the World Health Organization [19], diabetes was diagnosed at fasting plasma glucose levels of ≥ 7 mmol/L and/or at non-fasting plasma glucose levels of ≥ 11.1 mmol/L and/or treatment with antidiabetic medication (oral medication or insulin) and/or a diagnosis of diabetes as registered by a general practitioner. Pre-diabetes was defined as having an impaired fasting plasma glucose level of 6.1–6.9 mmol/L [20].

Sample preparation and UPLC/MS/MS analysis

To a 40- μ L volume of each sample or calibrator solution, 900 μ L of internal standard solution (200 ng/mL) in acetonitrile/methanol mixture (3/1) was added. Samples were thoroughly mixed and centrifuged for 10 min at 13000 g and supernatants were subjected to UPLC/MS/MS analysis [21].

The separation of analytes was performed by using an Acquity UPLC system (Waters) on an Acquity HILIC BEH column (2.1 mm \times 50 mm, 1.7 μ m, Waters). The analytes were ionized by electrospray ionization in positive ion mode on a Quattro Micro triple-quadrupole mass spectrometer (Waters). The TMAO and L-carnitine analysis was performed in multiple reaction mode (MRM). Data acquisition and processing were performed using MassLynx V4.1 and QuanLynx V4.1 software. The plasma extracts were kept at 10°C in the autosampler.

Statistical analyses and calculations

Data from animal experiment are presented as means \pm s.e.m. Statistically significant differences in the mean values were

tested by ANOVA and the Tukey's test, using db/L as a reference group. The differences were considered significant when $P < 0.05$. The data were analyzed using Graph Pad Prism 3.0 statistical software (Graph Pad Inc., USA).

Because the measured TMAO levels were not normally distributed, the associations of TMAO with patient characteristics and biomarkers were tested by a Spearman's rank correlation analysis in the case of continuous variables, and by a Mann-Whitney U test or Kruskal-Wallis test in the case of categorical variables with 2 or more categories, respectively.

A multivariate linear backwards regression model included the logarithmically transformed TMAO levels as the dependent variable. Covariates were the patient's age, sex, smoking status (current smokers vs. non-smokers), diabetes status (as an ordinal variable with subsequent categories "no diabetes", "pre-diabetes" and "diabetes"), hypertension (yes vs. no), body mass index, total and high density lipoprotein cholesterol, L-carnitine and pre-hospital use of statins. Covariates retaining significant association with the log-TMAO levels in the final model of regression analysis were further used in an ANCOVA model to explore the adjusted log-TMAO levels in the 3 subgroups of diabetes status. The Bonferroni method was used in a post-hoc analysis to correct for multiple comparisons. A P -value < 0.05 was considered as statistically significant. Statistical analysis was performed using the IBM SPSS Statistics 20 software program.

Results

Measurements in non-diabetic db/L and diabetic db/db mice

Diabetic db/db mice were significantly overweighted compared to db/L mice and during 8 week observation period (from 12 to 20 weeks of age) more than 3-times higher body weight gain was observed for db/db compared to db/L mice (Suppl Fig. 1). As seen in Fig. 1a, at the age of 12 weeks the L-carnitine concentration in db/db mice plasma was 34% lower than in non-diabetic db/L mice. However, in 20 week old mice the difference in L-carnitine concentration between diabetic and non-diabetic animals was not significant any more (Fig. 1a). Concentration of TMAO in 12 weeks old db/L mice was $0.9 \pm 0.1 \mu\text{mol/L}$.

Already in 12 weeks old db/db mice TMAO plasma concentration was 10-times higher than that in db/L mice, and TMAO levels increased further during the course of experiment in both db/L and db/db mice (Fig. 1b). Thus, at the age of 20 weeks in db/db mice 25% higher TMAO concentration and in db/L mice a significant 2-fold increase in TMAO concentration were observed in comparison to TMAO levels in respective groups at 12 weeks of age (Fig. 1b).

Db/db mice developed severe hyperglycemia already at the age of 12 weeks. During the 8 week observation period the average blood glucose concentration in fed db/db mice was 2-times higher than that in age matched db/L mice (Fig. 1c). In insulin tolerance test db/db mice were significantly less sensitive to insulin administration and insulin insensitivity progressed over the test period (Fig. 1c). Moreover, db/db mice exhibited severe hyperinsulinaemia, and in fed db/db mice plasma insulin concentration was significantly increased reaching 23 and 21 ng/ml at the ages of 12 and 20 weeks, respectively (Suppl Fig. 3).

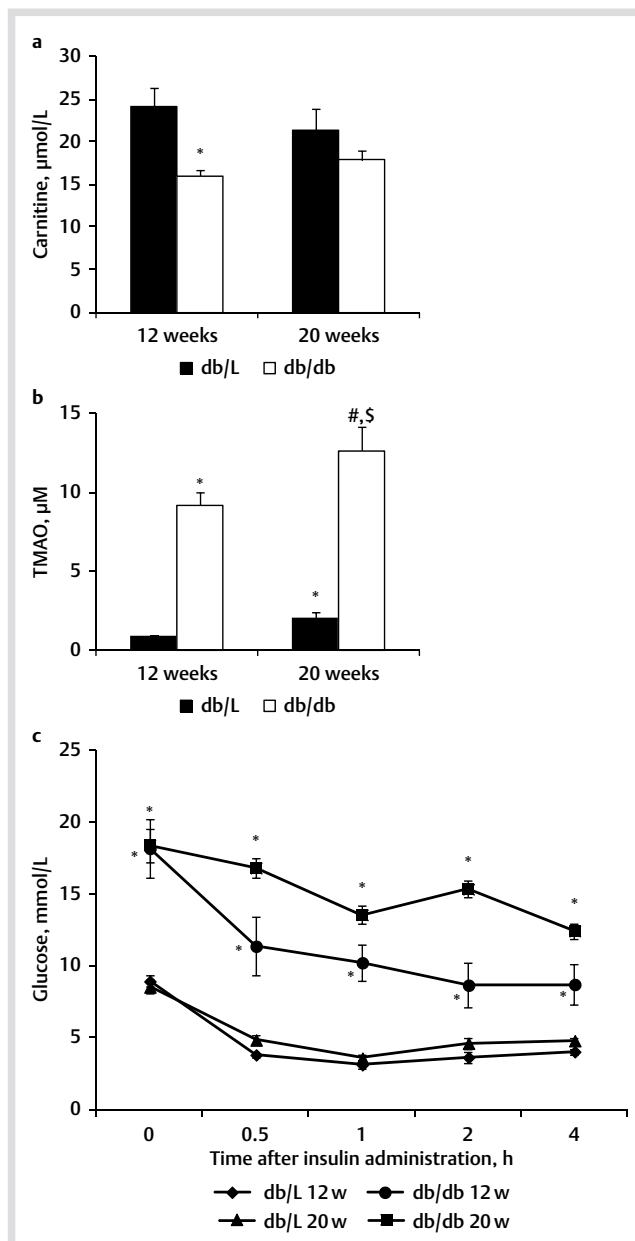


Fig. 1 Differences in db/L and db/db carnitine **a** and TMAO **b** plasma concentrations and insulin tolerance **c** at the ages of 12 and 20 weeks. Represented values \pm s.e.m. are an average of at least 8 animals. * Significantly different from 12 week db/L group (Tukey's test $P < 0.05$). # Significantly different from 20 week db/L group (Tukey's test $P < 0.05$). \$ Significantly different from 12 week db/db group (Tukey's test $P < 0.05$).

Associations of TMAO with patient characteristics and biomarkers

The patient characteristics are provided in Table 1. The study included 191 patients: mean age was 63 years, 74.9% were males, 15.2% were current smokers, 20.4% had diagnosed diabetes and 69.1% had hypertension. The fasting plasma TMAO level varied within a range of 0.4 – $11.6 \mu\text{mol/L}$ (Suppl Fig. 2), a median (IQR) of $1.8 \mu\text{mol/L}$ (1.2 – 2.6); the 0.5 percentile was 0.4 and the 99.5 percentile was $10.7 \mu\text{mol/L}$.

The TMAO concentration did not differ between men and women undergoing percutaneous coronary intervention (data

Table 1 Demographic and clinical characteristics of the patients (n = 191) undergoing percutaneous coronary intervention.

Variable	Value ^a
Age, years	63.0 (9.2)
Male	143 (74.9%)
Smoking	29 (15.2%)
Diabetes status	
Diabetes	39 (20.4%)
Pre-diabetes	40 (20.9%)
No diabetes	112 (58.7%)
Hypertension	132 (69.1%)
BMI, kg/m ²	28.4 (25.8–31.8)
Total cholesterol, mmol/L	4.6 (3.9–5.5)
HDL cholesterol, mmol/L	1.3 (1.1–1.5)
Statin use	134 (70.2%)
L-Carnitine, μmol/L	52.9 (11.6)
TMAO, μmol/L	1.8 (1.2–2.6)
History of MI	57 (29.8%)
History of previous PCI	65 (34.0)
History of CABG	10 (5.2%)
2-vessel disease	69 (36.1%)
3-vessel disease	64 (33.5%)
Left-main disease	23 (12%)

^a Values are presented as the mean (SD) for normally distributed variables or median (IQR) for non-normally distributed variables, or n (%) for categorical variables. BMI, body mass index; HDL, high density lipoprotein; TMAO, trimethylamine N-oxide. MI, myocardial infarction. PCI, previous percutaneous coronary intervention. CABG, coronary artery bypass grafting

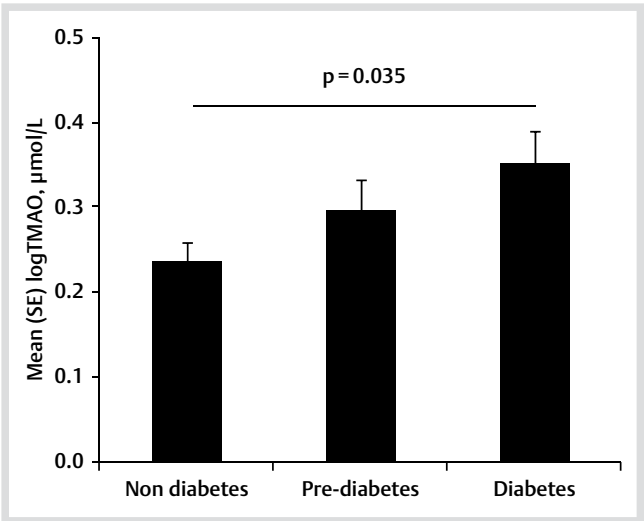


Fig. 2 Adjusted logTMAO values in the 3 categories of diabetes status. Values are represented as the averages ± SD. A significant difference was calculated against the non-diabetes group by the Bonferroni method.

not shown). Analysis of the samples showed a bivariate association of TMAO level with age, total cholesterol and L-carnitine (◉ **Table 2**). The TMAO concentrations showed significant positive correlation with L-carnitine ($r_s = 0.42$, $p < 0.001$) (◉ **Table 2**, **Suppl Fig. 2**). TMAO levels showed a weak positive correlation with age ($r_s = 0.15$, $p = 0.044$) and a weak negative correlation with total cholesterol ($r_s = -0.16$, $p = 0.030$) (◉ **Table 2**). The concentration of TMAO was lower in current smokers than in non-smokers: median (IQR) 1.4 (1.1–2.0) and 1.8 (1.2–2.8), respectively ($p = 0.033$). The TMAO levels did not differ among patients with one-, 2- and 3-vessel disease: 1.70 (1.20–2.36), 1.88 (1.45–2.58) and 1.67 (1.15–3.13), respectively ($p = 0.444$).

Table 2 Bivariate association of continuous variables with TMAO levels as obtained by Spearman's rank correlation analysis.

Patient characteristics	r_s	p
Age, years	0.146	0.044
BMI, kg/m ²	0.140	0.054
Total cholesterol, mmol/L	-0.157	0.030
HDL cholesterol, mmol/L	-0.083	0.253
L-carnitine, μmol/L	0.415	<0.001

BMI, body mass index; HDL, high density lipoprotein

The presence or absence of left main disease did not affect significance of results. Patients with or without history of myocardial infarction had similar TMAO levels: 1.74 (1.15–2.65) vs. 1.74 (1.38–2.56), respectively ($p = 0.595$). Current TMAO levels did not correlate with the age at which patients had previously developed the first myocardial infarction ($r_s = 0.083$, $p = 0.374$, $n = 57$) or had any first clinical manifestation of coronary heart disease ($r_s = 0.073$, $p = 0.316$).

As presented in ◉ **Table 3**, the factors, independently associated with a higher logTMAO in the multiple linear regression model, were L-carnitine ($p < 0.001$), patient age ($p = 0.015$), diabetes status ($p = 0.037$) and BMI ($p = 0.023$). In the backwards regression model, all the 4 factors remained significant predictors of a higher log-TMAO concentration: L-carnitine ($\beta = 0.010$, $p < 0.001$), age ($\beta = 0.006$, $p = 0.001$), BMI ($\beta = 0.010$, $p = 0.008$) and diabetes status ($\beta = 0.058$, $p = 0.009$). The mean logTMAO, as adjusted for other factors, showed a gradual increase across the subsequent categories of diabetes status (◉ **Fig. 2**). The highest mean log-TMAO value was observed in patients with diagnosed diabetes (0.352, SD=0.039) and the lowest value in non-diabetic individuals (0.237, SD=0.022). Thus, the difference was 0.115 of the logTMAO ($p = 0.035$ after Bonferroni corrections). In patients with pre-diabetes, the mean logTMAO value (0.295, SD=0.038) was higher than in patients without diabetes (difference 0.058) and was lower compared to diabetic individuals (difference 0.057), however, these differences were not statistically significant.

Discussion

Studies in mice models and cross-sectional clinical studies in humans have suggested an association of TMAO with atherosclerosis and heart failure [1,9]. Our study provides evidence for TMAO as a metabolite associated with diabetes both in animal and human samples. In db/db mice circulating TMAO levels were up to 10-fold higher than in lean control animals and higher logTMAO was independently associated with patient diabetes status. We further found that also age and BMI are associated with higher TMAO levels independently of L-carnitine. Previous research supports our findings that diabetes is a significant contributor to elevated TMAO levels. Thus, in the animal model of *Macaca mulatta*, TMAO was indicated as a possible predictor for the evaluation of impaired glucose tolerance and insulin resistance risks in the prediabetic state [22]. In a high-fat-diet mouse model, it was shown that dietary TMAO exacerbates impaired glucose tolerance, obstructs the hepatic insulin signaling pathway, and causes adipose tissue inflammation [23]. Nevertheless, TMAO treatment attenuated peripheral nerve dysfunction in streptozotocin induced diabetes model without effect on diabetic hyperglycemia [24]. TMAO has been shown to produce a proatherogenic macrophage phenotype [1] and affect

Table 3 Association of patient characteristics with logTMAO by multivariate linear regression analysis.

Variables	Full model				Backward regression final model			
	B	SE	t	p	B	SE	t	p
Age, years	0.005	0.002	2.445	0.015	0.006	0.002	3.270	0.001
Male sex	-0.015	0.047	-0.315	0.753	–	–	–	–
Smoking	-0.053	0.053	-0.992	0.322	–	–	–	–
Diabetes status	0.049	0.023	2.106	0.037	0.058	0.022	2.639	0.009
Hypertension	0.021	0.039	0.552	0.582	–	–	–	–
BMI, kg/m ²	0.009	0.004	2.290	0.023	0.010	0.004	2.664	0.008
Total cholesterol, mmol/L	-0.001	0.014	-0.089	0.929	–	–	–	–
HDL cholesterol, mmol/L	-0.064	0.051	-1.259	0.210	–	–	–	–
Statin use	-0.011	0.040	-0.277	0.782	–	–	–	–
L-carnitine, μ mol/L	0.010	0.002	6.321	<0.001	0.010	0.001	6.781	<0.001

SE, standard error; BMI, body mass index; HDL, high density lipoprotein

cholesterol and sterol metabolism in macrophages, liver, and intestine [4]. In line with these findings, in our study population, we found that diabetes and BMI are associated with higher TMAO levels, which warrants further investigation of TMAO as a cardiovascular risk marker and in diabetes research.

In a recent study of diabetic and overweight individuals (median BMI 34.6, age 60) from New Zealand the mean TMAO concentration did not change significantly over a 2 year period, and the authors noted a high intra-individual variability for TMAO concentrations [25]. 2 year period might be too short to see significant changes in TMAO levels. For example, age dependent increase in TMAO levels for less than 40 years old and more than 60 years old subject groups has been reported [26]. Increasing TMAO values with age have been presented also in our study both in mice (from 12 to 20 weeks of age) and human samples (median age 63, range from 32–83 years). The factors that shift with age are microbiome and nutrition [27], and microbiota-dependent metabolism of dietary L-carnitine and TMA underlies production of TMAO [2,6], which might result in different circulating levels of TMAO. It must be noted that TMAO levels significantly increased with age both in lean and obese mice, even though both groups consumed the same diet.

In present study the fasting plasma TMAO levels in patients undergoing percutaneous coronary intervention varied within a range of 0.4–11.6 μ mol/L. Previous studies have reported higher plasma levels of TMAO. Thus, in subjects undergoing elective cardiac evaluations, the 0.5 percentile was 0.63 μ M and the 99.5 percentile was 77.2 μ M [1]. In a follow-up study of patients undergoing elective diagnostic cardiac catheterization, the mean fasting TMAO plasma concentration was reported as 3.7 (2.4–6.2) μ M [9]. It should be noted that in a recent study of healthy volunteers plasma TMAO concentration was easily increased by more than 10-fold after one week of intake of a trimethylamine rich diet [11]. As it has been noted before, dietary factors might be the most important regulators of circulating TMAO levels [13] and somewhat lower concentrations of TMAO found in the present study could be explained by the different dietary habits of the European (Latvian) population, which is characterized by a low fish and meat intake [28]. It appears that the measured plasma concentrations may vary over different populations and clinical conditions.

Recent studies have found that plasma L-carnitine levels in subjects undergoing cardiac evaluation predicted increased risks for cardiovascular disease and an increased incidence of major adverse cardiac events (myocardial infarction, stroke or death), but only among subjects with concurrently high TMAO levels [4].

Additionally, subjects with lower TMAO levels despite high choline and betaine levels have been found to be significantly less likely to have an adverse cardiovascular event than those with higher TMAO levels [26]. However, it has been unclear whether TMAO is an independent risk factor or a risk marker [29]; and the impact of known risk factors on TMAO levels is also unclear. Our data indicate that, among the traditional cardiovascular risk factors, age, diabetes and BMI are linked to higher plasma TMAO concentrations.

We did not observe any correlations of TMAO levels with history of myocardial infarction, age of onset of coronary heart disease or severity of coronary atherosclerosis in the patient sample. This finding should, however, be interpreted with caution as all patients had significant coronary artery disease and therefore subgroup analyses were less likely to show differences in such a very high risk population. Levels of TMAO are subject to changes over time and may have been influenced by preventive measures since the onset of clinical symptoms. The fact that despite all these considerations we found a significant association of TMAO levels with age, diabetes and BMI therefore argues against a potential concern that the findings could be explained by a more advanced atherosclerosis.

In addition to the aforementioned factors, several conditions and dietary factors were shown to be associated with increased plasma TMAO concentrations. It has been demonstrated that TMAO concentrations are increased after the consumption of fish products [11,30] and eggs [31]. TMAO was shown to accumulate in patients with kidney failure [7]. The association of plasma TMAO concentrations with different dietary factors, including a popular food supplement L-carnitine, should also be further investigated. All these conclusions should be taken into account when planning future studies of TMAO concentrations in different medical and dietary conditions.

Conclusions

▼ The results of the present study provide evidence that age, diabetes and BMI is associated with higher TMAO levels independently of L-carnitine.

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Conflict of interest: The authors declared that there is no conflict of interest.

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