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Circulating trimethyllysine and risk of acute myocardial infarction in patients with suspected stable coronary heart disease

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Abstract. Bjørnestad EØ, Olset H, Dhar I, Løland K, Pedersen EKR, Svingen GFT, Svardal A, Berge RK, Ueland PM, Tell GS, Nilsen DWT, Nordrehaug JE, Nygaard E, Nygård O (Stavanger University Hospital, Stavanger; Haukeland University Hospital; University of Bergen, Bergen; Stavanger University Hospital, Stavanger, Norway. Circulating trimethyllysine and risk of acute myocardial infarction in patients with suspected stable coronary heart disease. *J Intern Med* 2020; **288**: 446–456.

Background. The carnitine precursor trimethyllysine (TML) is associated with progression of atherosclerosis, possibly through a relationship with trimethylamine-N-oxide (TMAO). Riboflavin is a cofactor in TMAO synthesis. We examined prospective relationships of circulating TML and TMAO with acute myocardial infarction (AMI) and potential effect modifications by riboflavin status.

Methods. By Cox modelling, risk associations were examined amongst 4098 patients (71.8% men) with suspected stable angina pectoris. Subgroup analyses were performed according to median plasma riboflavin.

Results. During a median follow-up of 4.9 years, 336 (8.2%) patients experienced an AMI. The age- and sex-adjusted hazard ratio (HR) (95% CI) comparing the 4th vs. 1st TML quartile was 2.19 (1.56–3.09). Multivariable adjustment for traditional cardiovascular risk factors and indices of renal function only slightly attenuated the risk estimates [HR (95% CI) 1.79 (1.23–2.59)], which were particularly strong amongst patients with riboflavin levels above the median (P_{int} = 0.035). Plasma TML and TMAO were strongly correlated (r_s = 0.41; P < 0.001); however, plasma TMAO was not associated with AMI risk in adjusted analyses [HR (95% CI) 0.81 (0.58–1.14)]. No interaction between TML and TMAO was observed.

Conclusion. Amongst patients with suspected stable angina pectoris, plasma TML, but not TMAO, independently predicted risk of AMI. Our results motivate further research on metabolic processes determining TML levels and their potential associations with cardiovascular disease. We did not adjust for multiple comparisons, and the subgroup analyses should be interpreted with caution.

Keywords: acute myocardial infarction, angina pectoris, atherosclerosis, biomarker, epidemiology.

Abbreviations: CI, Confidence interval; CVD, Cardiovascular disease; eGFR, Estimated glomerular filtration rate; FMO3, Flavin-containing monooxygenase 3; NRI, Net reclassification improvement; TMAO, Trimethylamine-N-oxide; TML, Trimethyllysine; WECAC, Western Norway Coronary Angiography Cohort; WENBIT, Western Norway B-Vitamin Intervention Trial; γBB, γ-butyrobetaine.

Introduction

Increasing evidence suggests that the gut microbiota contributes to atherosclerosis through metabolism of dietary components [1]. In particular,

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trimethylamine-N-oxide (TMAO) has gained attention as a metabolite potentially linking intestinal bacteria to cardiovascular disease (CVD) [2]. TMAO is a product of the hepatic oxidation of trimethylamine (TMA) by the riboflavin-dependent enzyme flavin-containing monooxygenase 3 (FMO3) [3]. TMA is generated by the gut microbiota-dependent metabolism of dietary quaternary ammoniums containing a TMA moiety, such as choline, γ-butyrobetaine (γBB) and carnitine [4]. Numerous observational studies have reported associations between circulating TMAO and future cardiovascular risk and mortality [5]; however, several controversies exist regarding the potential causal role of TMAO in CVD [6-8].

The methylated amino acid trimethyllysine (TML) can serve as a TMAO-precursor [9] and has been associated with progression of atherosclerosis [10]. TML is introduced by a variety of plant- and animal-derived dietary sources [9,11]. Further, TML is generated endogenously by post-translational methylation of lysine residues in proteins, such as histones, a process considered central in epigenetic regulation of gene transcription [12]. TML is a precursor for the endogenous production of carnitine, with γBB as an intermediate, and TML availability regulates the rate of carnitine biosynthesis [13], essential for the transport of fatty acids over the mitochondrial membrane for their subsequent β-oxidation. Despite the involvement of TML in multiple distinct pathways potentially associated with atherogenesis [14], data examining relationships between circulating TML and CVD end-points are scarce. Notably, in an untargeted metabolomics study [9], TML was strongly related to adverse cardiovascular prognosis, a finding very recently replicated amongst patients presenting with acute coronary syndrome [15].

In this study of more than 4000 patients with suspected stable coronary heart disease, we evaluated circulating TML and TMAO in relation to future acute myocardial infarction (AMI). As TMAO is produced using riboflavin as a cofactor, we also examined potential effect modifications by circulating riboflavin.

Material and methods

Study population

The Western Norway Coronary Angiography Cohort (WECAC) includes patients with suspected stable angina pectoris (SAP) and has been described in detail previously [16]. In short, 4164 patients underwent elective coronary angiography either at Haukeland University Hospital ($n = 3413$) or at Stavanger University Hospital ($n = 751$), Norway, during 2000 to 2004. A total of 2573 (61.8%) of the patients were enrolled in the Western Norway B-Vitamin Intervention Trial (WENBIT; ClincialTrials.gov Identifier: NCT00354081), a randomized secondary prevention trial with B-vitamin treatment [17]. Patients not enrolled in WENBIT did not receive any study intervention.

The present study excluded 66 patients due to missing baseline data on circulating TML, γBB, TMAO or riboflavin, leaving 4098 patients eligible for the final analyses.

Baseline characteristics and biochemical analyses

The collection of baseline data and biochemical analyses has been described previously [18,19]. Information on patients' lifestyle and medical history was obtained from self-administered questionnaires and verified by comparing to hospital records when available.

For patients admitted to Haukeland University Hospital, venous blood samples were drawn at baseline, usually 1–3 days before coronary angiography. At Stavanger University Hospital, samples were drawn immediately after the procedure. Routine laboratory analyses were performed at hospital laboratories at Haukeland University Hospital or Stavanger University Hospital. For study-specific analyses, serum and plasma were immediately prepared and stored in 2-mL Vacutainer tubes (Becton, Dickinson and Company, United States) at −80 °C until thawed and analysed by laboratory staff, blinded to the clinical outcome of the patients. Plasma TML, plasma TMAO, serum γBB and plasma riboflavin were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [20,21].

Clinical end-points

The primary end-point of the present study was new fatal or nonfatal AMI, classified in accordance with the International Statistical Classification of Disease Tenth Revision codes I21, I22, I46.1, R96 and R98. Events occurring within 24 h after coronary artery bypass grafting or percutaneous coronary intervention were considered procedure-related and were excluded. Information on events

was obtained from the Cause of Death registry at Statistics Norway (http://www.ssb.no) and the Western Norway Cardiovascular Registry [22]. Study end-points were assigned by an independent end-point committee. Two independent clinicians adjudicated the final diagnosis. Any disagreements were resolved by a third adjudicator. Patients were followed until 31 December 2006.

Ethics statements

The study protocol was in accordance with the Declaration of Helsinki and was approved by the Regional Committee for Medical and Health Research Ethics, Western Norway, the Norwegian Medicines Agency and the Norwegian Data Inspectorate. All patients provided written informed consent.

Statistical analyses

Continuous variables are presented as medians (25th–75th percentiles) and categorical variables are presented as counts (%). Patient baseline characteristics were assessed over TML quartiles, and trends over quartiles were tested by logistic regression for dichotomous and ordinal variables and by linear regression for continuous variables. Baseline associations were evaluated using age- and sex-adjusted Spearman rho correlation coefficients.

Event-free survival was estimated using crude Kaplan–Meier curves, and differences in survival across TML quartiles were assessed with the log-rank test. Cox-regression analyses in univariate, age- and sex-adjusted (Model 1) and multivariable adjusted [Model 2; adjusted for age, sex, diabetes mellitus, current smoking, hypertension, estimated glomerular filtration rate (eGFR), apolipoprotein (apo) B100 and apoA1] models were used to obtain hazard ratios (HRs) and 95% confidence intervals (CIs).

Results are reported according to the 4th vs. 1st quartile and per 1 standard deviation (SD) increment of log-transformed concentrations of plasma metabolites. Effect modifications according to riboflavin and TMAO levels, in addition to parameters included in the Cox model 2, were tested by introducing interaction terms to the Cox model 1. Also, we assessed the TML-AMI risk association according to left ventricular ejection fraction (LVEF).

The possible influence of unmeasured factors was evaluated by introducing E-value to the Cox model 2, as per recent recommendations of sensitivity analysis in observational studies [23]. Further, model performance of the Cox Model 2 with and without log-transformed standardized plasma TML was assessed. We tested the difference in C-statistics using Delong's test [24] in 500 stratified 10-fold cross-validations and calculated the net reclassification improvement (1/2NRI > 0) [25,26].

Since the nature of our research analysis was hypothesis-testing, we did not adjust for multiple comparisons [27] and the two-tailed significance level was set to 0.05 for all statistical models. All statistical tests were performed using SPSS for Windows (version 23; SPSS IBM, NY, USA) and R version 3.3.0 [packages 'survival' (v3.1-8), pROC (v1.16.1) and survIDNRI (v1.1-1)].

Results

Baseline characteristics

Baseline characteristics of the study population according to quartiles of plasma TML are presented in Table 1. The current cohort consisted of 71.8% men, and median (25th-75th percentile) age at inclusion was 62 (55–70) years. Median (25th-75th percentile) plasma levels of TML were 0.67 (0.54–0.87) μmol L^{-1}. There was a positive relationship between incremental TML quartiles and age and BMI, whereas a negative association was observed with eGFR and serum HDL cholesterol. A higher proportion of patients in the upper TML quartiles had hypertension, and a lower proportion were smokers. Plasma TML was not associated with baseline statin prescription status.

As presented in Table 2, plasma TML was positively correlated with TMAO as well as downstream metabolites in the carnitine biosynthesis. The strongest associations with plasma TML were observed for γBB (r_s = 0.41, P < 0.001) and TMAO (r_s = 0.41, P < 0.001).

Predictors of subsequent AMI

During median (25th-75th percentile) follow-up of 4.9 (3.6–6.0) years, 336 (8.2%) patients were registered with an AMI. Figure 1 shows increased risk of AMI with increasing TML quartiles ($p_{log\text{-}rank}$ < 0.001). Univariate and multivariable HRs (95% CI) for AMI comparing the 4th vs. 1st quartile of TML were 2.73 (1.97–3.79; P < 0.001)

Table 1. *Baseline characteristics of the total study cohort (*n = *4098) according to quartiles of plasma trimethyllysine*

	Quartiles of plasma TML (μmol L^{-1})				
	Q1 (*n* = 1015) (<0.54)	Q2 (*n* = 1013) (0.54–0.67)	Q3 (*n* = 1066) (0.67–0.87)	Q4 (*n* = 1004) (>0.87)	P_{trend}
Plasma TML, μmol L^{-1}	0.5 (0.4–0.5)	0.6 (0.6–0.6)	0.8 (0.7–0.8)	1.1 (0.9–1.3)	...
Age, years	60 (53–67)	62 (55–69)	63 (55–70)	64 (56–72)	<0.001
Male sex, *n* (%)	534 (52.6)	702 (69.3)	867 (81.3)	840 (83.7)	<0.001
BMI	25 (23–28)	26 (24–28)	26 (24–29)	26 (24–29)	<0.001
Coronary risk factor, *n* (%)					
Hypertension	420 (41.4)	447 (44.1)	489 (45.9)	558 (55.6)	<0.001
Diabetes mellitus	123 (12.1)	107 (10.6)	121 (11.4)	129 (12.8)	0.52
Current smoking	354 (34.9)	333 (32.9)	310 (29.1)	304 (30.3)	0.02
HbA1c[a]	6.3 (5.6–7.2)	6.2 (5.6–6.9)	6.2 (5.5–6.9)	6.0 (5.3–6.7)	<0.001
eGFR, mL min^{-1} per $1.73m^2$	97 (88–104)	92 (83–99)	88 (78–97)	82 (65–95)	<0.001
Serum CRP, mg L^{-1}	1.7 (0.8–4.4)	1.7 (0.8–3.4)	1.9 (0.9–3.8)	1.9 (1.0–4.0)	0.46
Neopterin, nmol L^{-1}	7.7 (6.4–9.2)	8.0 (6.6–10.0)	8.3 (6.8–10.6)	8.8 (6.9–11.8)	<0.001
Serum lipids and lipoproteins					
Total cholesterol, mmol L^{-1}	4.9 (4.3–5.8)	5.0 (4.3–5.8)	4.9 (4.2–5.7)	4.8 (4.2–5.7)	0.01
LDL-C, mmol L^{-1}	2.9 (2.4–3.6)	3.0 (2.4–3.8)	2.9 (2.4–3.7)	2.9 (2.3–3.7)	0.39
HDL-C, mmol L^{-1}	1.3 (1.1–1.6)	1.2 (1.0–1.5)	1.2 (1.0–1.4)	1.2 (1.0–1.4)	<0.001
Triglycerides, mmol L^{-1}	1.4 (1.0–1.9)	1.5 (1.1–2.1)	1.5 (1.1–2.2)	1.6 (1.2–2.3)	<0.001
ApoB100, g L^{-1}	0.8 (0.7–1.0)	0.9 (0.7–1.1)	0.9 (0.7–1.1)	0.9 (0.7–1.1)	0.07
Apo A1, g L^{-1}	1.3 (1.2–1.5)	1.3 (1.1–1.5)	1.3 (1.1–1.5)	1.3 (1.1–1.4)	<0.001
Prior CVD, *n* (%)					
AMI	323 (31.8)	406 (40.1)	442 (41.5)	474 (47.2)	<0.001
PAD	66 (6.5)	82 (8.1)	106 (9.9)	115 (11.5)	<0.001
PCI	153 (15.1)	190 (18.8)	216 (20.3)	224 (22.3)	<0.001
CABG	87 (8.6)	105 (10.4)	123 (11.5)	157 (15.6)	<0.001
LVEF (%)	68 (60–70)	66 (60–70)	66 (60–70)	65 (58–70)	<0.001
Medications before angiography, *n* (%)					
Aspirin	818 (80.6)	805 (79.5)	858 (80.5)	803 (80.1)	0.89
Statins	731 (72.0)	708 (69.9)	799 (75.0)	728 (72.5)	0.29
CCB	206 (20.3)	209 (20.6)	224 (21.0)	269 (26.8)	0.001
β-Blocker	727 (71.6)	736 (72.7)	795 (74.6)	747 (74.4)	0.09
ACEI	150 (14.8)	184 (18.2)	218 (20.5)	265 (26.4)	<0.001
Medications after angiography, *n* (%)					
Aspirin	804 (79.2)	819 (80.8)	876 (82.2)	837 (83.4)	0.01
Statins	783 (77.1)	804 (79.4)	877 (82.3)	815 (81.2)	0.01
CCB	207 (20.4)	209 (20.6)	231 (21.7)	276 (27.5)	<0.001
β-Blocker	703 (69.3)	723 (71.4)	788 (73.9)	752 (74.9)	0.002
ACEI	159 (15.7)	192 (19.0)	223 (20.9)	268 (26.7)	<0.001

Table 1 (*Continued*)

	Quartiles of plasma TML (μmol L^{-1})				
	Q1 (n = 1015) (<0.54)	Q2 (n = 1013) (0.54–0.67)	Q3 (n = 1066) (0.67–0.87)	Q4 (n = 1004) (>0.87)	P_{trend}
Plasma riboflavin, nmol L^{-1}	10.9 (7.4–17.3)	11.2 (7.7–18.8)	10.9 (7.2–17.1)	11.9 (7.8–19.4)	0.02
Serum γBB, μmol L^{-1}	0.9 (0.8–1.0)	1.0 (0.9–1.1)	1.1 (0.9–1.2)	1.2 (1.0–1.3)	<0.001
Serum carnitine, μmol L^{-1}	36.7 (31.8–41.4)	38.8 (33.7–43.8)	40.1 (35.7–44.7)	40.5 (35.6–45.8)	<0.001
Plasma TMAO, μmol L^{-1}	4.1 (2.8–6.1)	5.1 (3.4–7.9)	6.0 (4.0–9.7)	9.3 (5.7–17.9)	<0.001

Continuous variables are presented as medians (25th-75th percentiles), and categorical variables are reported as counts (%). ACEIs indicates angiotensin-converting enzyme inhibitors; AMI, acute myocardial infarction; apoA1, apolipoprotein A1; apoB100, apolipoprotein B100; BMI, body mass index; CABG, coronary artery bypass grafting; CCB, calcium channel blockers; CVD, cardiovascular disease; CRP, C-reactive protein; γBB, gamma-butyrobetaine; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; LVEF, left ventricular ejection fraction; PAD, peripheral artery disease; PCI, percutaneous coronary intervention; TMAO, trimethylamine-N-oxide; TML, trimethyllysine.
[a]Data on HbA1c available only in 4054 patients.

Table 2. *Spearman correlation coefficients between plasma TML, downstream carnitine metabolites and TMAO*

	TML	γBB	Carnitine	TMAO
TML	*	0.41	0.21	0.41
γBB	0.41	*	0.44	0.23
Carnitine	0.21	0.44	*	0.08
TMAO	0.41	0.23	0.08	*

TML indicates trimethyllysine; TMAO, trimethylamine-N-oxide; γBB, gamma-butyrobetaine.
Adjusted for age and sex. All correlations coefficients are statistically significant (P < 0.001).

and 1.79 (1.23–2.59; P = 0.002), respectively (Table 3).

As we observed moderate to strong correlations between plasma TML and circulating γBB, carnitine and TMAO, we also evaluated whether additional adjustment for these biomarkers influenced the TML-AMI risk association. However, including any of the biomarkers either separately (data not shown) or combined in Cox model 2 only marginally affected the results HR (95% CI) for AMI comparing the 4th vs. 1st quartile of TML 1.85 (1.27–2.69).

Similarly, addition of the combination of prior AMI, prior percutaneous coronary intervention, prior coronary artery bypass grafting and peripheral artery disease did not substantially alter the risk estimates [HR (95% CI) for AMI comparing the 4th vs. 1st quartile of TML 1.60 (1.10–2.32; P = 0.014)]. Adjustment for statin prescription status at discharge or baseline HbA1c (instead of diabetes) had minimal influence on the risk associations (data not shown).

Both TMAO and γBB showed positive relationships with AMI in crude analyses (Table 4 and Table S1). However, these associations were completely attenuated in adjusted Cox models 1 and 2.

Possible effect modifiers

The risk of AMI per 1-SD increment of log-transformed TML was particularly strong in patients with plasma riboflavin above the median (p_{int} = 0.035); otherwise, no statistically significant effect modifications were observed (Table 5). As shown in Table S2, plasma TML was predictive of AMI in both patients with LVEF < 40% and LVEF ≥ 40%.

Plasma riboflavin also modified the TMAO-AMI risk association (p_{int} = 0.010), with stronger associations observed in the high-riboflavin group (Table S3). There were no effect modifications by levels of riboflavin on the AMI risk associated with γBB (data not shown).

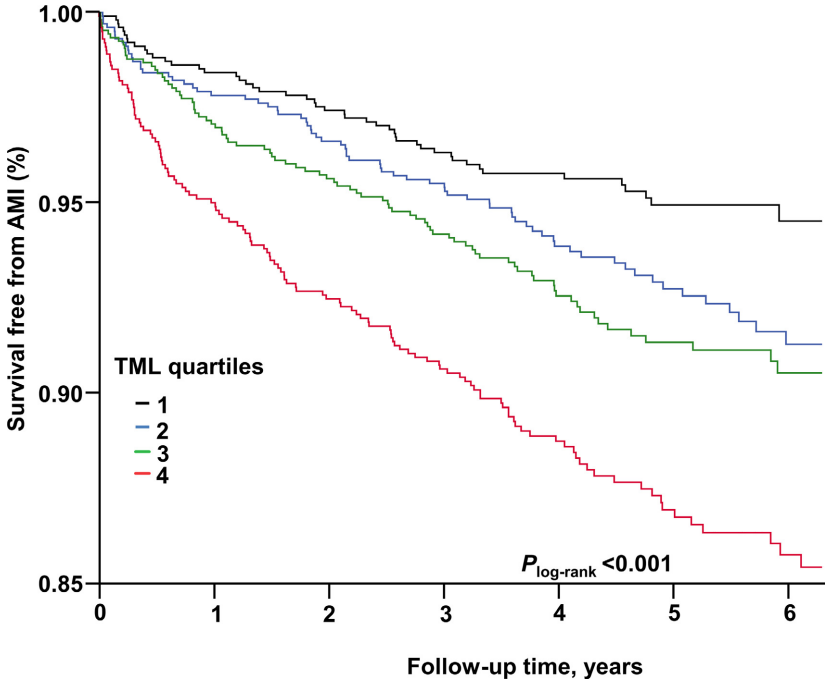

Fig. 1 *Kaplan–Meier curve showing crude event-free survival according to quartiles of plasma TML. The x-axis is trimmed at 6.5 years. AMI indicates acute myocardial infarction; TML, trimethyllysine.*

Table 3. *Risk association between plasma trimethyllysine and acute myocardial infarction*

	Unadjusted		Model 1[a]		Model 2[b]	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Plasma TML						
Quartiles						
1st	Reference		Reference		Reference	
2nd	1.46 (1.02–2.09)	0.04	1.32 (0.91–1.90)	0.14	1.25 (0.87–1.81)	0.24
3rd	1.72 (1.21–2.43)	0.003	1.45 (1.01–2.08)	0.04	1.34 (0.93–1.94)	0.11
4th	2.73 (1.97–3.79)	<0.001	2.19 (1.56–3.09)	<0.001	1.79 (1.23–2.59)	0.002
Trend	1.38 (1.25–1.53)	<0.001	1.29 (1.16–1.44)	<0.001	1.20 (1.07–1.35)	0.002
Per 1-SD[c]	1.34 (1.22–1.46)	<0.001	1.28 (1.16–1.41)	<0.001	1.15 (1.03–1.29)	0.01

CI indicates confidence interval; HR, hazard ratio; SD, standard deviation; TML, trimethyllysine.
[a]Model 1: Adjusted for age and sex.
[b]Model 2: Adjusted for age, sex, diabetes mellitus, smoking, hypertension, estimated glomerular filtration rate, apolipoprotein A1 and apolipoprotein B100.
[c]Log-transformed.

Sensitivity analysis, model discrimination and reclassification

Application of E-value formula to the Cox model 2 revealed good strength of the observed association between plasma TML and AMI, with E-values of 2.69 and 1.54 for the effect estimate and the lower reported CI, respectively, when comparing the 4th vs. 1st TML quartile.

After exclusion of patients receiving B-vitamin treatment in WENBIT (n = 1891), we obtained numerically stronger relationships between plasma TML

Table 4. *Risk association between plasma trimethylamine-N-oxide and acute myocardial infarction*

	Unadjusted		Model 1[a]		Model 2[b]	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Plasma TMAO						
Quartiles						
1st	Reference		Reference		Reference	
2nd	1.14 (0.85–1.63)	0.43	0.98 (0.70–1.36)	0.89	0.88 (0.63–1.23)	0.47
3rd	1.38 (1.00–1.89)	0.048	1.08 (0.78–1.49)	0.64	0.94 (0.68–1.31)	0.73
4th	1.50 (1.10–2.05)	0.010	1.07 (0.77–1.47)	0.69	0.81 (0.58–1.14)	0.22
Trend	1.15 (1.05–1.27)	0.004	1.03 (0.93–1.14)	0.56	0.95 (0.85–1.05)	0.30
Per 1-SD[c]	1.21 (1.09–1.34)	<0.001	1.09 (0.98–1.21)	0.12	0.97 (0.87–1.10)	0.70

CI indicates confidence interval; HR, hazard ratio; SD, standard deviation; TMAO, trimethylamine-N-oxide.
[a]Model 1: Adjusted for age and sex.
[b]Model 2: Adjusted for age, sex, diabetes mellitus, smoking, hypertension, estimated glomerular filtration rate, apolipoprotein A1 and apolipoprotein B100.
[c]Log-transformed.

and risk of AMI (Table S4); however, there were no statistically significant interactions according to study intervention ($P_{int} \geq 0.43$) (Table S5).

The addition of plasma TML as a covariate to Cox model 2 resulted in borderline significant improvements in patient reclassification [1/2NRI> 0 (95% CI): 0.074 (−0.006–0.136), $P = 0.067$], whereas the C-statistic did not significantly improve [AUC 0.690 (with TML) vs. 0.685 (without TML), $P = 0.48$].

Discussion

Principal findings

This large, prospective cohort of patients with suspected stable coronary heart disease showed that higher circulating TML was associated with increased risk of experiencing an AMI, also after adjustment for traditional risk factors, indices of renal function and plasma TMAO. Notably, the TML-AMI risk relationship was confined to patients with elevated plasma riboflavin. TML levels were strongly related to plasma TMAO and serum γBB; however, neither of the latter two biomarkers added predictive value in adjusted analyses.

TML and CVD events in previous epidemiologic studies

We previously showed that higher levels of TML were associated with progression of atherosclerosis in a sub-cohort from the current source population who underwent repeat coronary angiography [10]. In a small study amongst patients with carotid atherosclerosis [28], circulating TML was predictive of cardiovascular death. More recently, an untargeted metabolomics study [9] identified TML as a predictor of future major adverse cardiovascular events amongst 2140 subjects with comparable follow-up time as the present cohort, a finding subsequently validated in a multicentre study evaluating patients presenting with acute coronary syndrome [15]. The present cohort extends the association between plasma TML and CVD by identifying TML as a strong independent risk marker for AMI in patients with suspected stable coronary heart disease.

TML and TMAO

In agreement with two recent reports [9,15], we observed a strong correlation between circulating levels of plasma TML and TMAO at baseline. TML is converted to the TMAO-precursor TMA in a gut microbiota-dependent fashion in mouse and human faecal cultures; however, the overall TMA-generation appears to be relatively low [9]. Accordingly, dietary TML has not shown to increase circulating TMAO in mouse models [9]. In the current study, TMAO did not attenuate the TML-AMI-relationship when included in the multivariable model, nor did we observe any effect modification according to TMAO levels. Thus, the observed relationship between TML and AMI seems to reflect biological mechanisms at least partly independent of TMAO.

Table 5. *Risk association between plasma TML and acute myocardial infarction according to subgroups of traditional cardiovascular risk factors, riboflavin and TMAO*

Subgroups	HR (95% CI)[a]	*P*-value	P_{int}
Age			
≤median	1.12 (0.95–1.31)	0.19	0.06
>median	1.37 (1.22–1.55)	<0.001	
Sex			
Female	1.35 (1.11–1.65)	0.003	0.52
Male	1.27 (1.14–1.41)	<0.001	
Diabetes			
No	1.24 (1.11–1.39)	<0.001	0.61
Yes	1.31 (1.11–1.54)	0.001	
Hypertension			
No	1.36 (1.07–1.57)	<0.001	0.23
Yes	1.21 (1.07–1.37)	0.003	
Smoking			
No	1.27 (1.11–1.44)	<0.001	0.89
Yes	1.29 (1.12–1.49)	<0.001	
ApoA1			
≤median	1.27 (1.13–1.44)	<0.001	0.95
>median	1.28 (1.09–1.49)	0.001	
ApoB100			
≤median	1.29 (1.12–1.48)	0.001	0.58
>median	1.26 (1.11–1.43)	<0.001	
eGFR			
≤median	1.34 (1.19–1.49)	<0.001	0.21
>median	1.12 (0.93–1.36)	0.23	
Riboflavin			
≤median	1.12 (0.96–1.32)	0.15	0.035
>median	1.38 (1.23–1.56)	<0.001	
TMAO			
≤median	1.41 (1.16–1.71)	<0.001	0.35
>median	1.26 (1.11–1.41)	<0.001	

AMI, acute myocardial infarction; ApoA1, apolipoprotein A1; ApoB100, apolipoprotein B100; γBB: gamma-butyrobetaine; eGFR, estimated glomerular filtration rate; HR, hazard ratio; SD, standard deviation; TMAO, trimethylamine-N-oxide.
[a]Per 1SD (log-transformed). Adjusted for age and sex.

Surprisingly, plasma TMAO did not add any prognostic value in this cohort. Although the majority of experimental [2] and epidemiological [5] evidence has implicated TMAO in CVD development, whether reducing circulating TMAO actually improves CVD prognosis is not known. Indeed, TMAO is related to several potentially confounding proatherogenic conditions [8,29] and may also exert protective functions [6,30]. Further, a large meta-analysis [31] did not support an association of the dietary TMAO-precursors choline and betaine with incident CVD. Notably, our findings from a Norwegian population are in line with results from a recent *post hoc* analysis [32] amongst patients with heart failure, showing weaker relationships between TMAO and adverse prognosis in northern/western European populations compared to other European regions. The present results also support a recent study amongst high-risk individuals with type 2-diabetes [33], in which TMAO showed no predictive value for incident CVD events.

TML, carnitine biosynthesis and renal function

TML has previously mainly been evaluated for its role as a precursor for endogenous carnitine biosynthesis and hence its importance for the β-oxidation of fatty acids [13]. Impairments in carnitine homeostasis are considered an important contributor to CVD, likely through development of endothelial dysfunction [34]. As expected, circulating TML correlated with both downstream metabolites γBB and carnitine. γBB has shown proatherogenic properties in rodent models [35] and has been related to cardiovascular death in a smaller study amongst patients with carotid atherosclerosis [28]; however, the authors did not adjust for traditional CVD risk factors. To our knowledge, our study is the first to evaluate circulating γBB in relation to AMI, but similar to TMAO, γBB was not predictive of AMI risk once adjusted for potential confounders.

Notably, we recently demonstrated TML and γBB to be oppositely related to risk of incident type 2 diabetes, possibly indicating an impaired conversion of TML to γBB amongst subjects prone to diabetes development [21]. Potentially, similar impairments in carnitine biosynthesis could underlie the TML-AMI-relationship observed in the present work, although adjusting for diabetes at baseline did not attenuate the risk association. Importantly, however, circulating γBB concentrations also depend on gut microbiota-dependent mechanisms [35] and thus partly reflect pathways independent of TML.

TML clearance depends on renal function [13]; accordingly, we observed an inverse correlation

between plasma TML and eGFR at baseline. Interestingly, although not statistically significant, the TML-AMI relationship was predominately present in patients with eGFR below the median. However, including eGFR in Model 2 did not substantially alter the risk estimates, suggesting additional mechanisms than impaired renal function underlying the increased AMI risk. As most subjects in the present study had adequate renal function, future studies amongst patients with renal impairment are needed to further evaluate potential interactions between circulating TML, kidney disease and CVD.

TML, TMAO, riboflavin and one-carbon metabolism

In histones, lysine is one of only two amino acids that can be methylated, that is forming TML, methyllysine and dimethyllysine. Methyl groups for this post-translational modification are provided by S-adenosyl methionine [36], generated by one-carbon metabolism. Hence, elevated circulating TML could reflect a disturbance in histone methylation due to changes in methylation potential, shown to be associated with CVD, probably due to an influence on the expression of genes associated with atherogenesis [12].

The effect modification by circulating riboflavin on the risk association between TML and AMI could potentially be due to increased TMAO production caused by upregulation of FMO3-activity when riboflavin levels are elevated. However, as TMAO did not independently predict risk of AMI, the effect modification by riboflavin is likely due to other mechanisms. Importantly, FMO3 itself is shown to have proatherogenic properties independent of TMAO [3,37]. In addition, several enzymes in the one-carbon metabolism important for regulation of methylation potential [38] are riboflavin-dependent, including methylenetetrahydrofolate reductase and methionine synthase reductase. Hence, the association between elevated TML and AMI might reflect altered flux through one or more of these enzymes when riboflavin is elevated. Indeed, future studies evaluating potential interactions between one-carbon metabolites and circulating TML could provide further insight into metabolic mechanisms relating TML to CVD.

Strengths and limitations

Major strengths of the study include the large population size, the detailed information on baseline clinical and biochemical characteristics, and the long-term follow-up with end-point data collected from a patient-administrative and a population-based registry. We cannot rule out underreporting of clinical features or diseases at baseline, or of clinical end-points. As in any observational study, residual confounding might influence the assessment of risk predictors. However, we observed high E-values of the association between TML and AMI, which reduces the potential of bias from unobserved factors and unmeasured cofounders [23]. We mainly studied white men with stable coronary heart disease, and our results may not be applicable to populations with other demographic characteristics. Lastly, we did not adjust for multiple comparisons, which increases the risk of type 1 statistical errors.

Conclusion

Elevated circulating TML was associated with increased risk of AMI in patients with suspected stable coronary heart disease, also after adjustment for traditional CVD risk factors, indices of renal function, HbA1c and TMAO. TML strongly correlated with its downstream metabolite γBB and TMAO; however, neither γBB nor TMAO was independent risk markers of AMI. Future studies should aim to examine metabolic processes determining circulating levels of TML and their potential associations with CVD risks. We did not adjust for multiple comparisons, and the subgroup analyses should be interpreted with caution.

Conflict of interest statement

None declared.

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Author contributions

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Risk association between serum γ-butyrobetaine and acute myocardial infarction.

Table S2. Risk association between plasma TML and acute myocardial infarction according to subgroups of LVEF.

Table S3. Risk association between plasma TMAO and acute myocardial infarction according to plasma riboflavin.

Table S4. Risk association between plasma trimethyllysine and acute myocardial infarction in patients not treated with B-vitamins.

Table S5. Risk association between plasma TML and acute myocardial infarction according to treatment groups among WENBIT-participants.