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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

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 among 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 among patients with riboflavin levels above the median ($p_{\text{int}}=0.035$). 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: Among 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

γ BB: γ -butyrobetaine

Introduction

Increasing evidence suggests that the gut microbiota contributes to atherosclerosis through metabolism of dietary components [1]. In particular, 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 (γ BB) 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 posttranslational 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 γ BB 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 endpoints are scarce. Notably, in an untargeted metabolomics study [9], TML was strongly related to adverse cardiovascular prognosis, a finding very recently replicated among 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; ClinicalTrials.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, γ BB, 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 analyzed by laboratory staff, blinded to the clinical outcome of the patients. Plasma TML, plasma TMAO, serum γ BB and plasma riboflavin were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [20, 21].

Clinical endpoints

The primary endpoint 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 hours 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 endpoints were assigned by an independent endpoint committee. Two independent clinicians adjudicated the final diagnosis. Any disagreements were resolved by a third adjudicator.

Patients were followed until December 31st, 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 (%). Patients baseline characteristics were assessed over TML quartiles, 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, 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) $\mu\text{mol/L}$. 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; $p<0.001$) 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 γ BB 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 ($p_{\text{int}}=0.035$); 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 \geq 40%.

Plasma riboflavin also modified the TMAO-AMI risk association ($p_{\text{int}}=0.010$), 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 γ BB (data not shown).

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

The addition of plasma TML as a covariate to Cox model 2 resulted in borderline significant improvements in patient reclassification ($1/2\text{NRI} > 0$ [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 γ BB; 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 among 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 among 2140 subjects with comparable follow-up time as the present cohort, a finding subsequently validated in a multi-center 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 fecal 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.

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] among 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 among 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 is considered an important contributor to CVD, likely through development of endothelial dysfunction [34]. As expected, circulating TML correlated with both downstream metabolites γ BB and carnitine. γ BB has shown proatherogenic properties in rodent models [35] and has been related to cardiovascular death in a smaller study among 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 γ BB in relation to AMI, but similar to TMAO, γ BB was not predictive of AMI risk once adjusted for potential confounders.

Notably, we recently demonstrated TML and γ BB to be oppositely related to risk of incident type 2 diabetes, possibly indicating an impaired conversion of TML to γ BB among 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 γ BB concentrations also depend on gut microbiota-dependent mechanisms [35], and thus partly reflects 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 among 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, i.e. 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 methylene tetrahydrofolate 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 endpoint 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 γ BB and TMAO; however, neither γ BB nor TMAO were 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

None declared

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References

- 1 Jonsson AL, Backhed F. Role of gut microbiota in atherosclerosis. *Nat Rev Cardiol* 2017; **14**: 79-87.
- 2 Tang WHW, Backhed F, Landmesser U, Hazen SL. Intestinal Microbiota in Cardiovascular Health and Disease: JACC State-of-the-Art Review. *J Am Coll Cardiol* 2019; **73**: 2089-105.
- 3 Shih DM, Wang Z, Lee R, *et al.* Flavin containing monooxygenase 3 exerts broad effects on glucose and lipid metabolism and atherosclerosis. *J Lipid Res* 2015; **56**: 22-37.
- 4 Wang Z, Tang WH, Buffa JA, *et al.* Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-N-oxide. *Eur Heart J* 2014; **35**: 904-10.
- 5 Schiattarella GG, Sannino A, Toscano E, *et al.* Gut microbe-generated metabolite trimethylamine-N-oxide as cardiovascular risk biomarker: a systematic review and dose-response meta-analysis. *European Heart Journal* 2017; **38**: 2948-56.
- 6 Janeiro MH, Ramirez MJ, Milagro FI, Martinez JA, Solas M. Implication of Trimethylamine N-Oxide (TMAO) in Disease: Potential Biomarker or New Therapeutic Target. *Nutrients* 2018; **10**.
- 7 Ufnal M, Zadlo A, Ostaszewski R. TMAO: A small molecule of great expectations. *Nutrition* 2015; **31**: 1317-23.

- 8 Zeisel SH, Warrier M. Trimethylamine N-Oxide, the Microbiome, and Heart and Kidney Disease. *Annu Rev Nutr* 2017; **37**: 157-81.
- 9 Li XS, Wang Z, Cajka T, *et al.* Untargeted metabolomics identifies trimethyllysine, a TMAO-producing nutrient precursor, as a predictor of incident cardiovascular disease risk. *JCI Insight* 2018; **3**.
- 10 Loland KH, Bleie O, Borgeraas H, *et al.* The association between progression of atherosclerosis and the methylated amino acids asymmetric dimethylarginine and trimethyllysine. *PLoS One* 2013; **8**: e64774.
- 11 Servillo L, Giovane A, Cautela D, Castaldo D, Balestrieri ML. Where does N(epsilon)-trimethyllysine for the carnitine biosynthesis in mammals come from? *PLoS One* 2014; **9**: e84589.
- 12 Handy DE, Castro R, Loscalzo J. Epigenetic modifications: basic mechanisms and role in cardiovascular disease. *Circulation* 2011; **123**: 2145-56.
- 13 Vaz FM, Wanders RJ. Carnitine biosynthesis in mammals. *Biochem J* 2002; **361**: 417-29.
- 14 Schiattarella GG, Trimarco B. Microbial metabolites as predictive biomarkers: a paradigm shift for cardiovascular risk stratification. *Eur Heart J* 2019; **40**: 2710-2.
- 15 Li XS, Obeid S, Wang Z, *et al.* Trimethyllysine, a trimethylamine N-oxide precursor, provides near- and long-term prognostic value in patients presenting with acute coronary syndromes. *European Heart Journal* 2019; **40**: 2700-9.
- 16 Svingen GF, Ueland PM, Pedersen EK, *et al.* Plasma dimethylglycine and risk of incident acute myocardial infarction in patients with stable angina pectoris. *Arterioscler Thromb Vasc Biol* 2013; **33**: 2041-8.
- 17 Ebbing M, Bleie O, Ueland PM, *et al.* Mortality and cardiovascular events in patients treated with homocysteine-lowering B vitamins after coronary angiography: a randomized controlled trial. *Jama* 2008; **300**: 795-804.
- 18 Pedersen ER, Tuseth N, Eussen SJ, *et al.* Associations of plasma kynurenines with risk of acute myocardial infarction in patients with stable angina pectoris. *Arterioscler Thromb Vasc Biol* 2015; **35**: 455-62.
- 19 Bjornestad EO, Borsholm RA, Svingen GFT, *et al.* Neopterin as an Effect Modifier of the Cardiovascular Risk Predicted by Total Homocysteine: A Prospective 2-Cohort Study. *J Am Heart Assoc* 2017; **6**.

- 20 Midttun O, Kvalheim G, Ueland PM. High-throughput, low-volume, multianalyte quantification of plasma metabolites related to one-carbon metabolism using HPLC-MS/MS. *Anal Bioanal Chem* 2013; **405**: 2009-17.
- 21 Strand E, Rebnord EW, Flygel MR, *et al.* Serum Carnitine Metabolites and Incident Type 2 Diabetes Mellitus in Patients With Suspected Stable Angina Pectoris. *J Clin Endocrinol Metab* 2018; **103**: 1033-41.
- 22 Oyen N, Nygard O, Igland J, *et al.* [Hospital admission rates for cardiovascular diseases in Western Norway, 1992-2001]. *Tidsskr Nor Laegeforen* 2008; **128**: 17-23.
- 23 VanderWeele TJ, Ding P. Sensitivity Analysis in Observational Research: Introducing the E-Value. *Ann Intern Med* 2017; **167**: 268-74.
- 24 Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Muller M. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 2011; **12**: 77.
- 25 Pencina MJ, D'Agostino RB, Sr., Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. *Stat Med* 2011; **30**: 11-21.
- 26 Uno H, Tian L, Cai T, Kohane IS, Wei LJ. A unified inference procedure for a class of measures to assess improvement in risk prediction systems with survival data. *Stat Med* 2013; **32**: 2430-42.
- 27 Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology* 1990; **1**: 43-6.
- 28 Skagen K, Troseid M, Ueland T, *et al.* The Carnitine-butYRObetaine-trimethylamine-N-oxide pathway and its association with cardiovascular mortality in patients with carotid atherosclerosis. *Atherosclerosis* 2016; **247**: 64-9.
- 29 Mueller DM, Allenspach M, Othman A, *et al.* Plasma levels of trimethylamine-N-oxide are confounded by impaired kidney function and poor metabolic control. *Atherosclerosis* 2015; **243**: 638-44.
- 30 Collins HL, Drazul-Schrader D, Sulpizio AC, *et al.* L-Carnitine intake and high trimethylamine N-oxide plasma levels correlate with low aortic lesions in ApoE(-/-) transgenic mice expressing CETP. *Atherosclerosis* 2016; **244**: 29-37.
- 31 Meyer KA, Shea JW. Dietary Choline and Betaine and Risk of CVD: A Systematic Review and Meta-Analysis of Prospective Studies. *Nutrients* 2017; **9**.

- 32 Yazaki Y, Salzano A, Nelson CP, *et al.* Geographical location affects the levels and association of trimethylamine N-oxide with heart failure mortality in BIOSTAT-CHF: a post-hoc analysis. *Eur J Heart Fail* 2019.
- 33 Cardona A, O'Brien A, Bernier MC, *et al.* Trimethylamine N-oxide and incident atherosclerotic events in high-risk individuals with diabetes: an ACCORD trial post hoc analysis. *BMJ Open Diabetes Res Care* 2019; **7**: e000718.
- 34 Sharma S, Black SM. CARNITINE HOMEOSTASIS, MITOCHONDRIAL FUNCTION, AND CARDIOVASCULAR DISEASE. *Drug Discov Today Dis Mech* 2009; **6**: e31-e9.
- 35 Koeth RA, Levison BS, Culley MK, *et al.* gamma-Butyrobetaine is a proatherogenic intermediate in gut microbial metabolism of L-carnitine to TMAO. *Cell Metab* 2014; **20**: 799-812.
- 36 Strijbis K, Vaz FM, Distel B. Enzymology of the carnitine biosynthesis pathway. *IUBMB Life* 2010; **62**: 357-62.
- 37 Warriar M, Shih DM, Burrows AC, *et al.* The TMAO-Generating Enzyme Flavin Monooxygenase 3 Is a Central Regulator of Cholesterol Balance. *Cell Rep* 2015; **10**: 326-38.
- 38 Joseph J, Loscalzo J. Methoxistasis: integrating the roles of homocysteine and folic acid in cardiovascular pathobiology. *Nutrients* 2013; **5**: 3235-56.

Figure legends

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

	Quartiles of plasma TML (μmol/L)				<i>P</i> _{trend}
	Q1 (n=1015) (<0.54)	Q2 (n=1013) (0.54-0.67)	Q3 (n=1066) (0.67-0.87)	Q4 (n=1004) (>0.87)	
Plasma TML, μmol/L	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, y	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*	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 per 1.73m ²	97 (88-104)	92 (83-99)	88 (78-97)	82 (65-95)	<0.001
Serum CRP, mg/L	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	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	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	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	1.3 (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	1.4 (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	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	1.3 (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
Plasma riboflavin, nmol/L	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 γ BB, μ mol/L	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, μ mol/L	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, $\mu\text{mol/L}$	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
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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.

*Data on HbA1c available only in 4054 patients.

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

	TML	γ BB	Carnitine	TMAO
TML	*	0.41	0.21	0.41
γ BB	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; γ BB, gamma-butyrobetaine.

Adjusted for age and sex. All correlations coefficients are statistically significant ($p < 0.001$).

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

	Unadjusted		Model 1*		Model 2†	
	HR (95% CI)	<i>P</i> -value	HR (95% CI)	<i>P</i> -value	HR (95% CI)	<i>P</i> -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‡	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.

*Model 1: Adjusted for age and sex.

†Model 2: Adjusted for age, sex, diabetes mellitus, smoking, hypertension, estimated glomerular filtration rate, apolipoprotein A1 and apolipoprotein B100.

‡Log-transformed.

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

	Unadjusted		Model 1*		Model 2†	
	HR (95% CI)	<i>P</i> -value	HR (95% CI)	<i>P</i> -value	HR (95% CI)	<i>P</i> -value
Plasma TMAO						

Quartiles	Reference		Reference		Reference	
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 [‡]	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.

*Model 1: Adjusted for age and sex.

†Model 2: Adjusted for age, sex, diabetes mellitus, smoking, hypertension, estimated glomerular filtration rate, apolipoprotein A1 and apolipoprotein B100.

‡Log-transformed.

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)*	<i>P</i> -value	<i>P</i> _{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 indicates 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.

*Per 1SD (log transformed). Adjusted for age and sex.

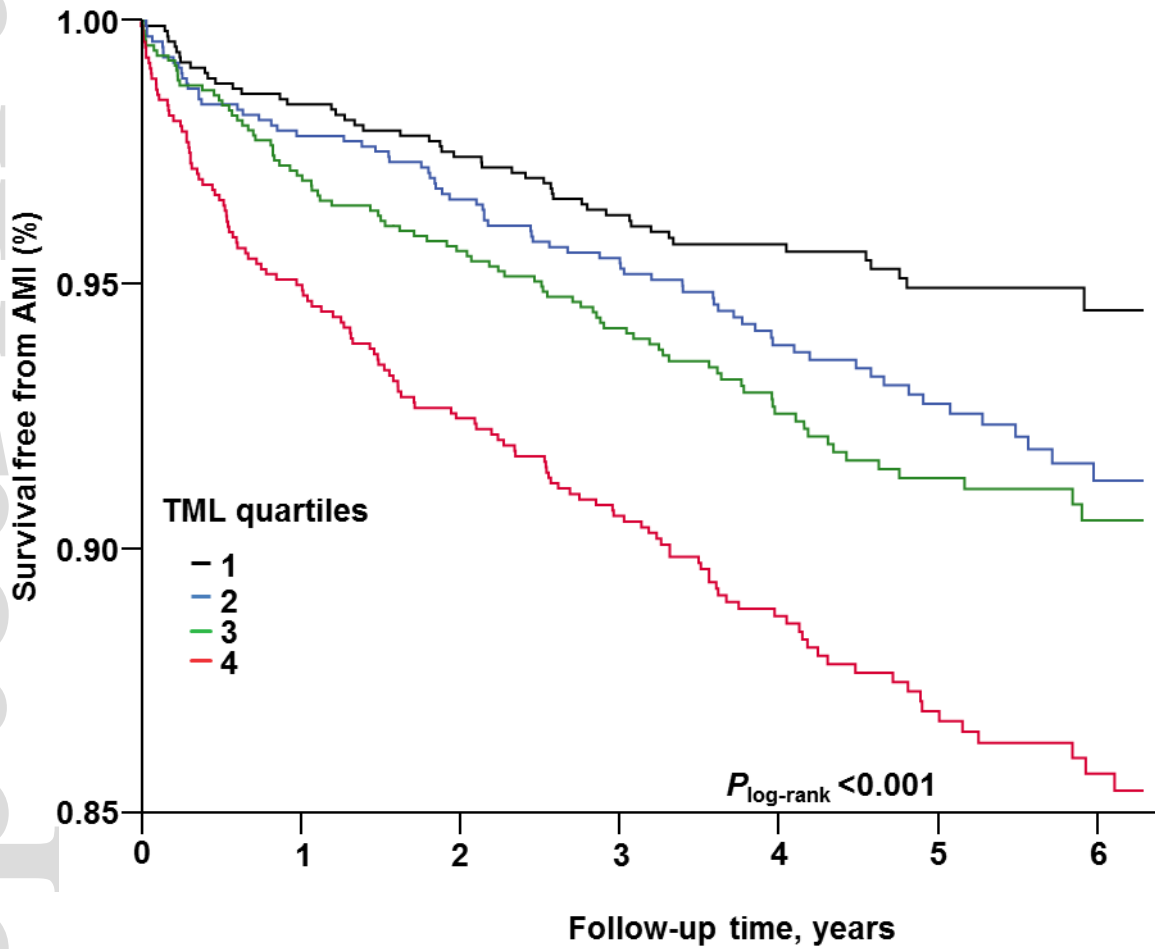


Figure 1. Kaplan–Meier curve showing crude event-free survival according to quartiles of plasma TML. The x-axis is trimmed at 6.5 years. AMI indicates acute myocardial infarction; TML, trimethyllysine.