Lifestyle factors and hand eczema: A systematic review and meta-analysis of observational studies

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High Plasma Levels of Betaine, a Trimethylamine N-Oxide Related Metabolite, are Associated with Severity of Cirrhosis

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Abbreviations

AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHMT, betaine homocysteine methyltransferase; BMI, body mass index; CPT, Child Pugh Turcotte; CPMG, Carr-Purcell-Meiboom-Gill; CV, coefficient of variation; CVD, cardiovascular disease; EDTA, ethylenediaminetetraacetic acid; GGT, gamma-glutamyl transferase; HR, hazard ratio; HDL, high density lipoproteins; INR, international normalized ratio; IQR, interquartile range; LDL, low density lipoproteins; MAFLD, metabolic dysfunction-associated fatty liver disease; MELD, Model for End-stage Liver Disease; NMR, nuclear magnetic resonance; OLT, orthotopic liver transplantation; PBC, primary biliary cholangitis; PREVEND, Prevention of Renal and Vascular ENd-stage Disease; PSC, primary sclerosing cholangitis; SD, standard deviation; STROBE: Strengthening the Reporting of Observational Studies in Epidemiology; T2D, type 2 diabetes.

Conflict of interest

EG and MAC are employees of Labcorp, carried out the NMR measurements and assisted with interpretation of the data. Labcorp was not involved in the study design, data analysis, nor with the decision to publish the results. VM reports a VENI research grant by the Netherlands Organization for Scientific Research (NWO; grant #09150161810030), a Research grant from the Dutch Ministry of Economic Affairs (Health~Holland Public Private Partnership grant #PPP-2019-024), and a Research grant from the Dutch Society for Gastroenterology (NVGE; #01-2021), all outside the submitted work. All other authors declare no conflicts of interest.

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Author contributions

Conception and design of study: EB, JLFG and RD. Data collection: EB, JLFG, EG, MAC and RD. Data analysis: EB, JLFG and RD. Interpretation of data: EB, JLFG, EG, MAC, VM, SB, HB an RD. Drafting the manuscript: EB, JLFG and RD. All authors have revised and approved the submitted manuscript.

Abstract

Background and aims: The gut microbiome-related metabolites betaine and trimethylamine N-oxide (TMAO) affect major health issues. In cirrhosis, betaine metabolism may be diminished due to impaired hepatic betaine homocysteine methyltransferase activity, whereas TMAO generation from trimethylamine may be altered due to impaired hepatic flavin monooxygenase expression. Here we determined plasma betaine and TMAO levels in patients with end-stage liver disease and assessed their relationships with liver disease severity.

Methods: Plasma betaine and TMAO concentrations were measured by nuclear magnetic resonance spectroscopy in 129 cirrhotic patients (TransplantLines cohort study; NCT03272841) and compared with levels from 4,837 participants of the PREVEND cohort study. Disease severity was assessed by Child-Pugh-Turcotte (CPT) classification and Model for End-stage Liver Disease (MELD) score.

Results: Plasma betaine was on average 60% higher (P<0.001), whereas TMAO was not significantly lower in cirrhotic patients *vs.* PREVEND population (P=0.44). After liver transplantation (n=13), betaine decreased (P=0.017; P=0.36 vs. PREVEND population), whereas TMAO levels tended to increase (P=0.085) to higher levels than in the PREVEND population (P=0.003). Betaine levels were positively associated with CPT stage and MELD score (both P<0.001). The association with MELD score remained in fully adjusted analysis (P<0.001). The association of TMAO with MELD score did not reach significance (P=0.11). Neither betaine, nor TMAO levels were associated with mortality on the waiting list for liver transplantation (adjusted P=0.78 and P=0.44, respectively).

Conclusion: Plasma betaine levels are elevated in cirrhotic patients in parallel with disease severity, and decrease after liver transplantation.

Key words: betaine, CPT classification, liver cirrhosis, orthotopic liver transplantation, MELD score, NMR spectroscopy, TMAO

Key Points

• In cirrhosis, impaired hepatic betaine homocysteine methyltransferase activity may diminish betaine metabolism; trimethylamine N-oxide generation from trimethylamine may be diminished due to impaired hepatic flavin monooxygenase expression.

• In 129 cirrhotic patient's plasma betaine was elevated whereas trimethylamine N-oxide tended to be reduced. In 13 patients who were restudied after orthotopic liver transplantation these abnormalities were reversed.

• Higher plasma betaine was independently associated with disease severity (MELD score).

Introduction

Chronic liver diseases represent major health issues and can progress to liver cirrhosis which carries a high mortality risk [1,2]. Death from chronic liver diseases is globally increasing in the last few decades [3]. Recently, evidence is accumulating that the gut microbiome plays a major role in the development of chronic liver diseases and decompensated cirrhosis via translocation of gut microbiota components secondary to increased gut permeability and portal hypertension [4,5]. Gut microbiota metabolize nutrients including choline to generate betaine (N,N,N-trimethylglycine), and choline and L-carnitine to generate trimethylamine (TMA, *N,N*-dimethylmethanamine) [5,6]. In the liver, TMA is converted to trimethylamine N-oxide (TMAO, *N,N*-dimethylmethanamine *N*-oxide) by flavin monooxygenase3 (FMO3) [5-8].

Betaine is an osmolyte and serves as a methyl donor in a reaction catalyzed by betaine homocysteine methyltransferase (BHMT), which converts homocysteine to methionine [9]. Plasma betaine was found to be inversely associated with adiposity, blood pressure and plasma triglycerides and positively with high density lipoprotein (HDL) cholesterol [10-12]. Low plasma betaine may predict future development of type 2 diabetes (T2D) [12]. Low plasma betaine concentrations were also found in metabolic dysfunction-associated fatty liver disease (MAFLD) and non-alcoholic steatohepatitis (NASH) [13,14]. Betaine may improve oxidative stress, inflammation and apoptosis in an experimental model of MAFLD, probably via its action to decrease homocysteine, and ameliorates hepatic fat accumulation by decreasing homocysteine [9,15,16] Betaine may also reduce stellate cell activation in rats with alcoholic liver fibrosis [17]. Consequently, betaine supplementation could ameliorate hepatic steatosis, although the association of betaine with cardiometabolic disorders is not always straightforward and intervention studies so far have not allowed for a definite conclusion [18]. In marked contrast, one study suggested plasma betaine to be elevated shortly after liver transplantation [19]. As a contributing mechanism to explain such betaine elevations, BHMT, which converts betaine to dimethylglycine, was found to be impaired in a rat model of liver cirrhosis [20].

TMAO has been proposed to be implicated in adverse effects on several health issues [6,21]. Plasma TMAO has been reported to be associated with all-cause mortality in the general population, particularly in subjects with lower kidney function, and with cardiovascular mortality in T2D patients [22,23]. Moreover, we found that plasma TMAO predicts graft failure in kidney transplant recipients [24]. On the other hand, circulating TMAO was not found to be associated with (cardiovascular) mortality in haemodialysis patients [25] and was not associated with an adverse cardiometabolic risk profile in children [26]. Remarkably little is known about the role of TMAO in chronic liver disease. Plasma TMAO independently predicted all-cause

mortality in subjects with MAFLD [27], but to our knowledge there are no studies on circulating TMAO in patients with cirrhotic liver disease. FMO3 protein expression in microsomes from human cirrhotic livers is reduced in relation to increasing disease severity as judged by Child Pugh Turcotte (CPT) classification [28]. It is, therefore, plausible to postulate that generation of TMAO from TMA is impaired in end-stage cirrhotic liver disease.

Nuclear magnetic resonance (NMR) spectroscopy-based assays for betaine and TMAO have been recently developed which allow for their large-scale measurement in human plasma [12,29]. So far, effects of end-stage liver disease on circulating levels of betaine and TMAO have been scarcely reported. We, therefore, initiated the present study on plasma concentrations of betaine and TMAO in patients with end-stage liver disease on the waiting list for orthotopic liver transplantation (OLT). Our aims were to determine: 1) to which extent plasma betaine and TMAO, assayed by NMR spectroscopy, are altered in end-stage liver disease and to document the effect of OLT, 2) associations of plasma betaine and TMAO with disease severity, and 3) associations of betaine and TMAO with mortality while on the waiting list for OLT.

Materials and Methods

Study populations

This report follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline. Participants of the TransplantLines cohort study were used as a group of patients with end-stage liver disease. TransplantLines is a transplant biobank and cohort study carried out in the University Medical Center Groningen, The Netherlands (NCT03272841) [30]. The study was approved by the Medical Ethics Committee of the University Medical Center Groningen (METc 2014/077) and is performed in accordance with Declaration of Helsinki guidelines. All participants provided written informed consent. Exclusion criteria were no mastery of the Dutch language and inability to intellectually understand questionnaires or physical tests [30]. There were 129 patients with cirrhotic end-stage liver disease who were screened for OLT and in whom NMR-determined betaine and TMAO measurements were available. Thirteen of these patients were restudied 3 to 6 months after OLT.

As a control group, the study population of the Prevention of REnal and Vascular ENd-stage Disease (PREVEND) cohort study was used [31,32]. The PREVEND study was approved by the Medical Ethics Committee of the University Medical Centre Groningen (MEC96/01/022) and is performed in accordance with Declaration of Helsinki guidelines. Written informed consent was obtained from all participants. PREVEND is a large prospective general population-based study, which was initiated to investigate cardiovascular and renal disease. All inhabitants (28 to 75 years old) of Groningen, the Netherlands were sent a questionnaire on demographics and cardiovascular morbidity. Pregnant women, type 1 diabetic subjects and T2D subjects using insulin were excluded from participation [31,32]. We used data of participants who completed the second screening round (2001-2003) and who were free from liver disease assessed by questionnaire (n=6,894). Participants with missing measurements for plasma betaine and TMAO were excluded, leaving 4,837 PREVEND participants.

Data collection and measurements

TransplantLines has a continuous data collection which started on June 2015. For the present study data were collected up to December 2020. During visits at the outpatient clinic, questionnaires and blood samples were collected from all participants according to the TransplantLines protocol. At time of blood collection patients continued their regular medication. A standardized protocol was used to obtain anthropometric measurements. Patient information, including weight, height, body mass index (BMI),

smoking status, medication use (glucose and lipid lowering drugs) and medical history such as cardiovascular disease (CVD) and diabetes, was extracted from the TransplantLines Biobank. Additional review of all electronic patient records of study participants was performed to obtain additional data concerning the aetiology of liver disease and cirrhotic state (based on imaging, histology or transient elastography), and biochemical and clinical variables to calculate the Model for End-stage Liver Disease (MELD) and CPT scores as scores to assess the severity of end-stage liver disease. The MELD score was calculated by serum bilirubin, creatinine and international normalized ratio (INR) [33,34]. The CPT score was calculated by total bilirubin, serum albumin, INR, ascites and hepatic encephalopathy [35]. In the PREVEND cohort data were collected on demographics, lifestyle factors, anthropometric measurements, medical history and medication use, which was combined with information from a pharmacy-dispensing registry as previously described [22,32]. In both TransplantLines and PREVEND venous blood samples were drawn after an overnight fast.

Laboratory analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), total bilirubin, albumin, creatinine, high sensitivity C-reactive protein (hsCRP) and plasma glucose were analysed with standardized laboratory measurements and quality assessment control at the department of Laboratory Medicine of the University Medical Centre Groningen. Laboratory methods for PREVEND are reported as described in detail previously [31,32,36].

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated plasma samples were obtained by centrifugation at 1400x *g* for 15 min at 4^0C and then stored at -80^0C until analysis. EDTA-anticoagulated plasma samples for betaine, TMAO and lipoprotein profiles were measured using a Vantera Clinical Analyzer (Labcorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz proton (^{1}H) NMR spectroscopy platform [37]. For lipoprotein profiles, plasma samples were prepared on board the instrument, and automatically delivered to the flow probe in the NMR spectrometer's magnetic field [37]. Data acquisition on the Vantera and the spectra data processing have been reported in greater detail elsewhere [37-39]**.** Total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides were measured employing the LP4 algorithm [40,41]. Low density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation. Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol.

Plasma betaine and TMAO were quantified from one-dimensional proton Carr-Purcell-Meiboom-Gill (CPMG) spectra using a deconvolution assay as previously described [12,29]. In brief, plasma specimens were mixed with citrate/phosphate buffer (3:1 v/v) to lower the pH to 5.3, which was necessary to separate the betaine and TMAO signals that otherwise overlap at physiological pH. Betaine assay results are linear over a wide range of concentrations (26.0-1135 µmol/L). Coefficients of variation for intra- and inter-assay precision are up to 4.3% and 5.5%, respectively. Betaine as measured by the NMR assay give comparable results with mass spectrometry (MS) (R^2 = 0.94). For TMAO, linearity was demonstrated up to 3000 µmol/L. Coefficients of variation are up to 4.3% and 9.8% for intra- and interassay precision, respectively. TMAO measurements by NMR compare well with values obtained by a MS-based assay (R^2=0.98). Plasma choline in cirrhotic patients was measured by NMR from the same CPMG spectra used to generate the betaine and TMAO results. The newly developed NMR assay quantifies choline using a non-negative deconvolution algorithm [42]. Linearity was demonstrated up to 239.3 µM in plasma. Coefficients of variation are approximately 11% for intra- and interassay precision. Choline measured by the NMR assay is comparable to that of mass spectrometry (R = 0.998).

Statistical analysis

Statistical analyses were performed with IBM SPSS software (version 23.0 Armonk, NY: IBM Corp) and R language for statistical computing software (version 4.0.3 (2020), Vienna, Austria). Continuous variables are expressed as median with interquartile range (IQR) or as numbers with percentages. Normality of distribution was assessed and checked for skewness. Differences in variables between the cirrhotic patients and PREVEND participants were determined by Mann-Whitney U tests or by Chi-square tests where appropriate. Differences in betaine and TMAO according to cause of death were evaluated by Mann-Whitney U tests. Kruskal Wallis analysis of variance (ANOVA) was used to assess the relation of betaine and TMAO with CPT category. Univariate correlations were analysed by Spearman rank correlation coefficients. Multivariable logistic regression analyses were carried out to disclose the independent contribution of variables to plasma betaine and TMAO. To this end, betaine and TMAO were Ln-transformed to achieve an approximately normal distribution. Betaine and TMAO levels before and after OLT were compared by Wilcoxon test. Kaplan-Meier curves with log-rank test were performed to determine the effect of betaine and TMAO below and above their median values of the whole group of cirrhotic patients on mortality on the waiting list with the date of OLT as census date. Cox proportional hazards models were used to compute hazard ratios (HRs) and 95%CI of betaine and TMAO expressed per

1 Ln SD increment) on mortality risk at waiting list. HRs were calculated in models adjusted for age, sex and MELD score. Two-sided P-values of <0.05 were considered statistically significant.

Results

There were 129 patients with cirrhosis who participated in the study. Their clinical and laboratory characteristics were compared with 4,837 healthy controls of the PREVEND study (Table 1). Mean age as well as a history of cardiovascular disease and T2D were not different between the groups. Among cirrhotic patients relatively more men were included and more participants had a history of diabetes. BMI was higher and blood pressure was lower in the cirrhotic patients. Bilirubin, liver enzymes and hsCRP levels were all higher in cirrhotic patients. Plasma total cholesterol, non-HDL cholesterol, LDL cholesterol and triglycerides were all lower in cirrhotic patients. Plasma betaine was on average 60% higher ($P<0.001$), whereas TMAO was not significantly lower in cirrhotic patients ($P=0.44$) compared to the values in the PREVEND cohort. Consequently, the betaine/TMAO ratio was on average 80% higher in cirrhotic patients ($P<0.001$) (Table 1). Plasma betaine ($P<0.001$) and the betaine/TMAO ratio ($P=0.050$) remained higher in cirrhotic patients after adjustment for age, sex, CVD, diabetes history and medication use. Also, the difference in plasma TMAO did not reach significance after these adjustments ($P=0.22$). There was no significant correlation between plasma betaine and TMAO in cirrhotic patients ($r=-0.042$, $P=0.64$). In the PREVEND group this correlation was of similar magnitude but reached significance ($r=-0.038$; $P=0.007$) (data not shown). As provided in Table 2, plasma betaine and TMAO concentrations did not significantly vary according to liver disease aetiology, except for plasma TMAO which tended to be lower in cirrhosis due to MAFLD and cholestatic liver disease.

Of the end-stage cirrhotic patients, 13 were again studied 3 to 6 months after OLT. In these patients, plasma betaine decreased from 59.7 (39.8-116.0) to 38.1 (31.1-50.4) µmol/L ($P=0.017$). In turn, plasma TMAO changed from 3.0 (1.25-8.1) to 7.4 (1.6-14.4) µmol/L ($P=0.085$). The betaine levels attained after follow-up were not different from those in the PREVEND population (Table 1) ($P=0.36$), whereas TMAO was higher in the OLT recipients than the values in the PREVEND population (Table 1) ($P=0.003$). In this subset, plasma choline measurements were available and increased from 5.80 (3.75-7.65) to 9.50 (7.05-12.15) µmol/L after OLT ($P=0.002$). Furthermore, plasma betaine was not correlated with choline in these 13 cirrhotic patients before OLT ($r=0.066$, $P=0.83$) but did correlate with choline after transplantation ($r=0.615$, $P=0.025$). Plasma TMAO was unrelated to choline before ($r=0.014$, $P=0.96$) and after OLT ($r=-0.022$, $P=0.94$).

In univariable analysis, plasma betaine was associated with CPT stage as well as with MELD score (Fig. 1 A and B), but TMAO was not significantly associated with CPT stage and only modestly with MELD score (Fig. 2 A and B). Multivariable logistic regression analysis demonstrated that the association of betaine with the MELD score was stronger than that with the CPT classification. This association remained significant after adjustment for underlying liver disease aetiology, plasma lipids, hsCRP and the use of lipid lowering and glucose lowering drugs (Table 3A). There was a modest association of TMAO with the MELD score which lost formal significance after full adjustment (P=0.11, Table 3B).

Median follow-up on the waiting list was 140 (IQR 52-381) days. Twenty-nine (22.5%) of patients died while on the waiting list for liver transplantation (Table 1), of whom 12 died from multi-organ failure, 5 patients from end-stage liver disease related complications, 4 from malignancy, 3 from cardiovascular disease and the rest from other causes. Kaplan Meier curves showed that mortality on the waiting list was not significantly different between patients with plasma betaine or TMAO above and below the median value of the whole cirrhotic group (Fig.3). As shown in Table 4 Cox regression analysis demonstrated that the association of plasma betaine (expressed per 1 SD increase) with mortality on the waiting list was not significant. There was a trend towards a positive association of plasma TMAO with mortality on the waiting list in crude and age- and sex-adjusted analysis, which was lost after additional adjustment for MELD score. There were no differences in plasma betaine and TMAO concentrations according to cause of death except for lower plasma betaine levels among those who died from multi-organ failure compared to other causes (P=0.024, data not shown)

Discussion

The present study demonstrates that plasma betaine concentrations are considerably increased whereas TMAO concentrations tend to be decreased in patients with cirrhosis compared to the levels found in the general population. In a small subset of patients who were followed after OLT, plasma betaine returned to levels that were not different from those in the general population. TMAO levels after liver transplantation appeared to be higher than those in the general population. Plasma betaine elevations were found across all the various aetiologies underlying end-stage lever disease. Higher plasma betaine concentrations were associated with higher CPT category and were strongly associated with the MELD score. The positive association with MELD score remained in fully adjusted analysis. On the other hand, plasma TMAO levels were not independently associated with disease severity. Combined, these results suggest that end-stage liver cirrhotic liver disease may be implicated in plasma betaine elevations.

Plasma betaine was on average 60% higher in end-stage liver cirrhosis. Plasma betaine elevations as documented here appear to be in line with recent findings suggesting high plasma betaine levels among 74 Chinese patients measured one day after a living donor liver transplantation procedure, although the precise concentrations were not reported in that study [19]. Choline is an important precursor of betaine. Indeed, plasma betaine was positively correlated with choline in the small subset of patients who were restudied after liver transplantation, while no such relationship was observed before transplantation. Enhanced choline availability is an unlikely mechanism responsible for high plasma betaine in cirrhosis, since choline deficiency is common in cirrhotic patients who are often malnourished [43], and choline supplementation seems justified in this patient category [44]. Instead, it is plausible that cirrhosis-induced BHMT deficiency, as shown both at the mRNA level and expressed as protein activity in a rat model of bile duct ligation-induced liver cirrhosis [20], is at least in part responsible for plasma betaine elevations in cirrhosis. In line with this possibility, plasma betaine was strongly and positively related to disease severity as demonstrated by its association with the CPT category and the MELD score, the latter association being independent of relevant clinical and laboratory characteristics including lipids and lipoproteins (deemed necessary to adjust for given their relationships with betaine [10-12]). In addition, plasma betaine decreased to levels as found in the general population in those patients who could be followed after liver transplantation, further corroborating the impact of cirrhosis on betaine metabolism.

In contrast to high plasma betaine, TMAO tended to be lower in cirrhotic patients. As a result, the betaine/TMAO ratio was on average 80% higher in cirrhotic patients. Plasma TMAO tended to increase after liver transplantation to levels that were higher than those in the general population. We found only

very modest relationships between TMAO and betaine in the cirrhotic group as well as in the PREVEND population in accordance with previous findings [12]. A diet intervention study in rats has suggested that betaine provides only a minor source of TMAO [45]. Choline deficiency could contribute to low plasma TMAO in liver cirrhosis, although in our study TMAO was unrelated to choline before and after OLT. Impaired conversion of TMA to TMAO in the liver consequent to hepatic FMO3 deficiency could also play a role [28].

Using mortality on the waiting list for OLT as clinical end-point we did not find a significant association with plasma betaine in crude nor in adjusted analysis. The reason for the lack of effect of plasma betaine on waiting list mortality is not known. Betaine may have beneficial effects on oxidative and inflammatory processes involved in liver injury but this effect of betaine is most likely attributable to its action to lower homocysteine via BHMT activity pathway [9,15,16], the pathway which we specifically propose to be disturbed in cirrhosis. In comparison, in the post liver transplantation study from China, early graft dysfunction was to a minor extent predicted by higher plasma betaine levels alone, but its predictive ability was improved when combined with other phospholipid and fatty acid biomarkers [19]. On the other hand, higher plasma TMAO tended to be positively associated with waiting-list mortality in crude Cox regression analysis which was downsized after adjustment for the MELD score. This would raise the possibility of a dual effect of TMAO. A putative detrimental effect of circulating TMAO *per se,* in keeping with the notion that TMAO could adversely affect health [6,21], combined with lower TMAO generation, consequent to cirrhosis severity. Additionally, betaine appeared to be lower in subjects who died from multi-organ failure, conceivably as a consequence of more advanced disease severity. Our findings should be regarded as preliminary. Besides absence of formal statistical significance of the associations of betaine and TMAO with mortality on the waiting list, it is not clear whether betaine and TMAO could be regarded as mere markers of liver disease severity rather than as causative factors implicated in prognosis.

Strengths of this study include combined betaine and TMAO measurements in a considerable group of cirrhotic patients with detailed and standardized assessment of clinical and laboratory characteristics consequent to the TransplantLines Biobank and Cohort study set-up [30] in comparison with data from the PREVEND study [12,27], which served as a large community-dwelling control cohort. Limitations of our study include absence of detailed information regarding dietary intake and composition, gut microbiome data, as well as absence of plasma choline measurements in the whole cirrhotic group and in the PREVEND population. Plasma TMA measurements were not available because its concentration is too low to allow for its accurate assay using NMR spectroscopy. Furthermore, we carried out a single-centre study which can limit its external

validity. Also, only a limited number of participants could be studied after liver transplantation. In fact, if such plasma TMAO elevations are confirmed in a larger liver transplantation cohort, this would warrant diet intervention in this patient category.

In conclusion, plasma betaine is elevated in cirrhotic patients, likely returns to levels found in the general population after OLT and is positively associated with disease severity. In contrast, plasma TMAO tends to be lower in cirrhosis and could be elevated after OLT. A possible association of betaine and TMAO with cirrhosis-related mortality deserves further study.

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Tables and Figures

Table 1. Clinical and laboratory characteristics including betaine and TMAO in 129 patients with end-stage cirrhotic liver disease and 4,837 PREVEND participants

	Cirrhotic patients N = 129	PREVEND participants N = 4,837	P-value
Baseline characteristics			
Sex: men / women, *n (%)*	84 (65.1) / 45 (34.9)	2,388 (49.4) / 2,449 (50.6)	< 0.001
Age (years), *median (IQR)*	60 (52-65)	53 (44-63)	0.650
BMI (kg/m^2), *median (IQR)*	28.0 (24.2-30.9)	26.1 (23.7-28.9)	0.006
BMI			
• Normal; ≤ 25 kg/m^2, *n (%)*	28 (31.1)	1,882 (38.9)	0.132
• Overweight; 25-30 kg/m^2, *n (%)*	32 (35.6)	2,043 (42.2)	0.203
• Obese; ≥ 30 kg/m^2, *n (%)*	30 (33.3)	912 (18.9)	0.001
Systolic blood pressure (mmHg), *median (IQR)*	115 (107-130)	123 (112-137)	0.001
Diastolic blood pressure (mmHg), *median (IQR)*	65 (59-75)	72 (67-79)	<0.001
Smoking, *n (%)*	16 (18.8)	1,321 (27.3)	0.081
Child Pugh Turcotte classification			
• Child Pugh Turcotte A, *n (%)*	28 (21.7)	n.a.	n.a.
• Child Pugh Turcotte B, *n (%)*	63 (48.8)		
• Child Pugh Turcotte C, *n (%)*	38 (29.5)		
MELD score, *median (IQR)*	15 (10-19)	n.a.	n.a.
Mortality on waiting list, *n* (%)	29 (22.5)	n.a.	n.a.
History of cardiovascular disease, *n (%)*	6 (4.8)	301 (6.2)	0.514
History of diabetes, *n (%)*	36 (28.8)	294 (6.1)	<0.001
Glucose lowering drugs, *n (%)*	35 (32.1)	178 (3.7)	<0.001
Lipid lowering drugs, *n (%)*	19 (17.4)	458 (9.5)	0.005
Blood tests			
Creatinine (µmol/L), *median (IQR)*	79.0 (62.5-102.0)	83.2 (73.9-92.4)	0.163
Fasting glucose (mmol/L), *median (IQR)*	6.4 (5.0-8.5)	4.8 (4.4-5.3)	<0.001
Total cholesterol (mmol/L), *median (IQR)*	3.2 (2.5-4.2)	5.3 (4.7-6.1)	<0.001
Non-HDL cholesterol (mmol/L), *median (IQR)*	2.2 (1.7-3.0)	4.1 (3.4-4.8)	<0.001
HDL cholesterol (mmol/L), *median (IQR)*	0.9 (0.4-1.2)	1.2 (1.0-1.4)	<0.001
LDL cholesterol (mmol/L), *median (IQR)*	1.8 (1.3-2.3)	3.5 (2.9-4.1)	<0.001
Triglycerides (mmol/L), *median (IQR)*	0.7 (0.5-1.1)	1.1 (0.8-1.6)	<0.001
ALT (U/L), *median (IQR)*	40.0 (28.0-60.0)	17.0 (13.0-24.0)	<0.001
AST (U/L), *median (IQR)*	54.0 (44.0-84.0)	22.0 (19.0-26.0)	<0.001
GGT (U/L), *median (IQR)*	95.0 (48.0-151.0)	24.0 (16.0-38.0)	<0.001
ALP (U/L), *median (IQR)*	141.0 (98.0-217.0)	66.0 (55.0-79.0)	<0.001
Bilirubin total (µmol/L), *median (IQR)*	42.0 (23.0-99.3)	7.0 (5.0-9.0)	<0.001
CRP(mg/L), *median (IQR)*	10.0 (4.3-25.5)	1.3 (0.6-3.0)	<0.001
Betaine (µmol/L), *median (IQR)*	59.7 (46.9-75.6)	36.7 (30.7-43.6)	<0.001
TMAO (µmol/L), *median (IQR)*	3.0 (1.4-6.1)	3.2 (1.7-5.6)	0.44
Betaine/TMAO ratio, *median (IQR)*	21.2 (9.3-54.3)	11.6 (6.3-21.5)	<0.001

Data are given in number with percentages (%) or median with interquartile ranges (IQR). Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, gamma-glutamyltransferase; HbA1c, glycated hemoglobin; HDL, high density lipoproteins; LDL, low density lipoproteins; MELD score, model for end-stage liver disease.

Table 2. Plasma betaine and TMAO levels and betaine/TMAO ratio according to aetiology of primary liver diseases in 129 patients with end-stage cirrhotic liver disease.

	N	Betaine (µmol/L)	P-value	TMAO (µmol/L)	P-value	Betaine / TMAO ratio	P-value
Aetiology of primary liver disease							
Cholestatic liver disease (PSC/PBC)	34	53.2 (45.6-79.8)	0.38	2.9 (1.5-12.1)	0.086	21.6 (4.6-51.7)	0.98
Storage disease	4	67.4 (61.8-93.7)	0.94	2.4 (0.6-28.9)	0.26	48.7 (5.9-148.3)	0.87
MAFLD	33	57.2 (44.9-76.6)	0.25	2.7 (1.2-4.4)	0.053	30.1 (13.8-51.9	0.74
Alcohol	28	67.8 (45.0-87.8)	0.19	3.2 (1.1-6.2)	0.39	21.7 (9.1-61.2)	0.28
viral	12	53.9 (38.6-62.1)	0.34	3.4 (2.1-15.9)	0.32	13.4 (7.3-29.5)	0.24
Autoimmune hepatitis	11	71.4 (43.2-100.7)	0.61	5.3 (1.4-10.8)	0.14	12.7 (4.6-61.0)	0.71
Storage disease	4	67.4 (61.8-93.7)	0.94	2.4 (0.6-28.9)	0.26	48.7 (5.9-148.3)	0.87
Vascular	2	66.8	0.90	3.6	0.56	36.5	0.72
Other	5	47.8 (36.2-57.7)	0.20	2.1 (0.8-5.9)	0.56	31.6 (8.6-100.8)	0.72

Data are given in medians with interquartile ranges (IQR). Due to N=2 no IQR can be presented for vascular liver disease.
Abbreviations: MAFLD, metabolic associated fatty liver disease; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.
* Adjusted for age and sex and compared to other aetiologies of primary liver diseases.

Table 3A. Multivariable linear regression analyses demonstrating independent associations of plasma betaine in 129 end-stage cirrhotic patients

	Model 1		Model 2		Model 3		Model 4	
	β	**P-value**	**β**	**P-value**	**β**	**P-value**	**β**	**P-value**
Age (years)	-0.035	0.695	0.025	0.760	0.001	0.988	0.030	0.758
Sex (women *vs.* men)	-0.025	0.778	-0.005	0.953	-0.078	0.346	-0.077	0.378
Child Pugh classification (A/B/C)	0.265	0.003			-0.014	0.895	0.021	0.849
MELD score			0.472	<0.001	0.748	<0.001	0.782	<0.001
Total cholesterol					-0.363	0.008	-0.361	0.012
Triglycerides					0.234	0.085	0.238	0.091
HDL cholesterol					0.206	0.075	0.239	0.054
HsCRP					-0.139	0.165	-0.137	0.184
Lipid lowering drugs (yes/no)							-0.122	0.188
Glucose lowering drugs (yes/no)							0.077	0.411

All models are mutually adjusted for the variables included in the analyses. Abbreviations: HDL, high density lipoprotein, MELD score, model for end-stage liver disease score.

Table 3B. Multivariable linear regression analyses demonstrating independent associations of plasma TMAO in 129 end-stage cirrhotic patients

	Model 1		Model 2		Model 3		Model 4	
	β	**P-value**	**β**	**P-value**	**β**	**P-value**	**β**	**P-value**
Age (years)	0.201	0.027	0.221	0.015	0.246	0.037	0.322	0.012
Sex (women *vs.* men)	-0.039	0.655	-0.031	0.724	-0.020	0.851	-0.035	0.762
Child Pugh classification (A/B/C)	0.119	0.186			-0.081	0.562	-0.090	0.536
MELD score			0.182	0.043	0.195	0.199	0.256	0.108
Total cholesterol					0.256	0.143	0.237	0.199
Triglycerides					-0.194	0.263	-0.195	0.286
HDL cholesterol					-0.190	0.199	-0.145	0.364
CRP					0.141	0.275	0.139	0.300
Lipid lowering drugs (yes/no)							-0.112	0.353
Glucose lowering drugs (yes/no)							-0.033	0.789

OR: odds ratio. CI 95%: 95% confidence interval. All models are mutually adjusted for the variables included in the analyses. Abbreviations: MELD score, model for end-stage liver disease score, TMAO, Trimethylamine N-oxide.

Table 4. Association of plasma Betaine (A) and plasma TMAO (B) with mortality on the waiting list		
A. Betaine	**HR per 1 SD increase**	**P-value**
Model 1	1.08 [0.75;1.54]	0.69
Model 2	1.11 [0.76;1.61]	0.58
Model 3	0.94 [0.60;1.47]	0.78
B. TMAO	**HR per 1 SD increase**	**P-value**
Model 1	1.53 [0.97;2.41]	0.06
Model 2	1.48 [0.95;2.31]	0.08
Model 3	1.18 [0.77;1.81]	0.45
Plasma betaine and TMAO are Ln transformed Models 1: crude Models 2: adjusted for age and sex Models 3 adjusted for age, sex and MELD score Abbreviations: MELD score, model for end-stage liver disease score, TMAO, Trimethylamine N-oxide.		

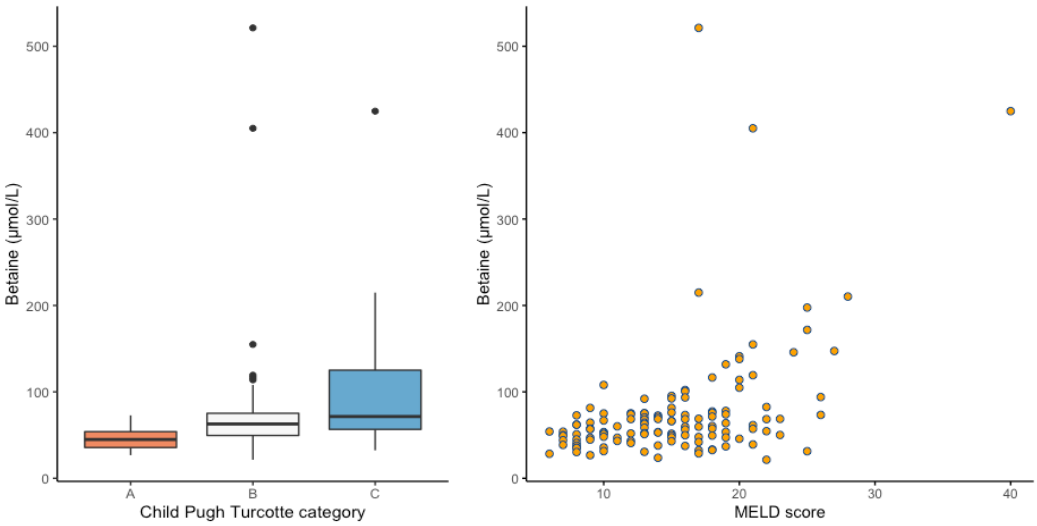

Figure 1. Plasma concentrations of betaine according to Child Pugh Turcotte stage and MELD scores in 129 cirrhotic patients. Left panel: Boxplots of plasma betaine concentrations according Child Pugh Turcotte stage (bars represent median betaine values) (P<0.001 by ANOVA). Right panel: Relationship of plasma betaine concentrations with MELD score (Spearman rank correlation coefficient: 0.46, P<0.001).

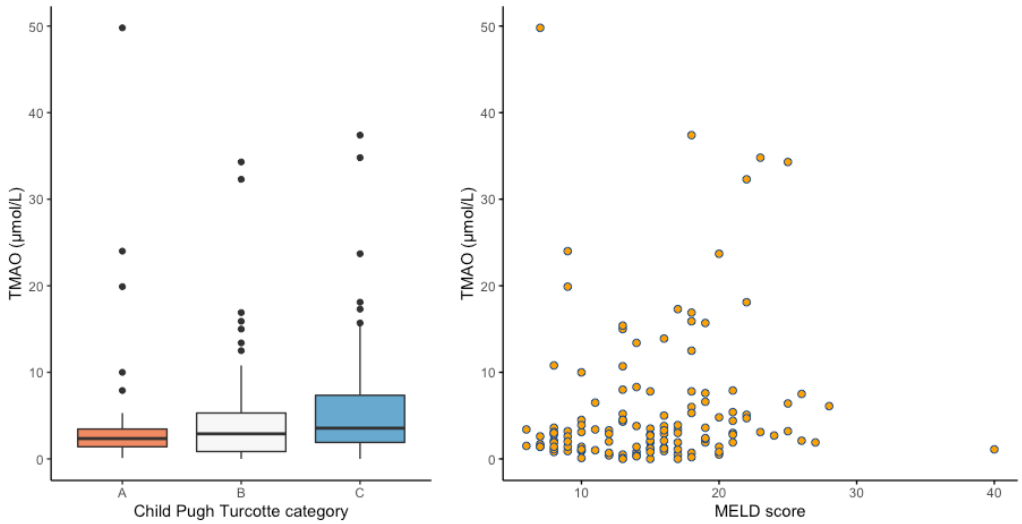

Figure 2. Plasma concentrations of TMAO according to Child Pugh Turcotte stage and MELD scores in 129 cirrhotic patients. Left panel: Boxplots of plama TMAO concentrations according Child Pugh Turcotte stage (bars represent median TMAO values) (P=0.14 by ANOVA). Right panel: Relationship of plasma TMAO concentrations with MELD score (Spearman rank correlation coefficient: 0.22, P=0.01).

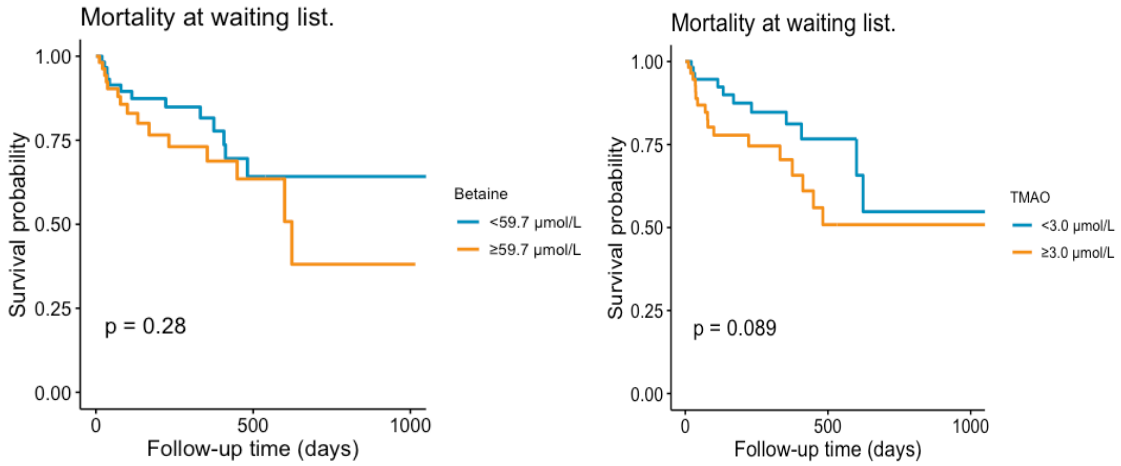

Figure 3. Kaplan-Meier plots for all-cause mortality on waiting list for liver transplantation. Left panel: comparison of participants subjects above and below the median plasma betaine concentration (P= 0.28 for log-rank test). Right panel: comparison of participants subjects above and below the median plasma betaine TMAO (P=0.089 for log-rank test).