Atherosclerosis 247 (2016) 64–69

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

The Carnitine-butyrobetaine-trimethylamine-N-oxide pathway and its association with cardiovascular mortality in patients with carotid atherosclerosis

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ARTICLE INFO

Article history:
Received 28 August 2015
Received in revised form
12 December 2015
Accepted 20 January 2016
Available online 5 February 2016

Keywords:
Carotid atherosclerosis
Stroke
Microbiota
γBB
TMAO

ABSTRACT

Background and purpose: γ-butyrobetaine (γBB) is a metabolite from dietary Carnitine, involved in the gut microbiota-dependent conversion from Carnitine to the pro-atherogenic metabolite trimethylamine-N-oxide (TMAO). Orally ingested γBB has a pro-atherogenic effect in experimental studies, but γBB has not been studied in relation to atherosclerosis in humans. The aim of this study was to evaluate associations between serum levels of γBB, TMAO and their common precursors Carnitine and trimethyllysine (TML) and carotid atherosclerosis and adverse outcome.

Methods: Serum γBB, Carnitine, TML and TMAO were quantified by high performance liquid chromatography in patients with carotid artery atherosclerosis (n = 264) and healthy controls (n = 62).

Results: Serum γBB (p = 0.024) and Carnitine (p = 0.001), but not TMAO or TML, were increased in patients with carotid atherosclerosis. Higher levels of γBB and TML, but not TMAO or Carnitine were independently associated with cardiovascular death also after adjustment for age and eGFR (adjusted HR [95%] 3.3 [1.9–9.1], p = 0.047 and 6.0 [1.8–20.34], p = 0.026, respectively).

Conclusions: Patients with carotid atherosclerosis had increased serum levels of γBB, and elevated levels of γBB and its precursor TML were associated with cardiovascular mortality. Long-term clinical studies of γBB, as a cardiovascular risk marker, and safety studies regarding dietary supplementation of γBB, are warranted.

1. Introduction

Atherosclerosis is a chronic and progressive process with the

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http://dx.doi.org/10.1016/j.atherosclerosis.2016.01.033
0021-9150/

bidirectional interaction between lipid and inflammation as its pathogenic hallmark [1]. Clinically, atherosclerosis has a long asymptomatic phase, but over years several patients will develop symptoms of chronic ischemia in affected organs, or during plaque destabilization and thrombus formation, the manifestation of acute ischemic events such as myocardial infarction (MI) or ischemic stroke. Atherosclerotic related cardiovascular disease (CVD) is the main cause of death in developed countries [2].

Carotid atherosclerosis is associated with increased risk of cardiovascular events [3]. Traditional risk factors including inflammatory markers such as C-reactive protein (CRP) and hyperlipidemia explain only half of carotid atherosclerotic burden [4,5], and CVD in patients in the absence of the these risk factors are thought to be due to heritage. However, large-scale, genome-wide association studies only explain about 10% of CVD heritability [6], indicating the possibility of unrecognized risk factors. Recently, the gut microbiota has been suggested as a novel determinant of CVD risk [7].

The gut microbiota, i.e. the microbial inhabitants of the gastrointestinal tract, is a metabolically highly active "human organ", which has been linked to traditional, modifiable risk factors for CVD such as diabetes [8], hyperlipidemia [9] and obesity [10]. Recent studies have also demonstrated a direct connection between the gut microbiota and atherosclerosis, at least partly through the metabolite trimethylamine-N-oxide (TMAO) [11]. TMAO could potentially promote atherosclerosis by enhancing foam cell formation, and raised TMAO levels have been associated with MI, stroke and all-cause mortality in CVD patients [12], but these issues are far from clear.

TMAO has been proposed to be produced through a three-step process [13]. First by dietary intake of Carnitine or phosphatidylcholine, second by their conversion to trimethylamine (TMA) by the intestinal microbiota, and third by oxidation to TMAO by flavin-containing monooxygenases in the liver. An alternative pathway of TMAO formation from Carnitine, via the microbiota-dependent intermediate metabolite γ-butyrobetain (γBB) was recently reported [7]. In the same study animals fed with γBB displayed increased atherosclerotic lesion formation suggesting that γBB may have a pro-atherogenic effect, although a direct role of γBB in atherogenesis has been questioned [14]. In addition to the production from dietary Carnitine, γBB can be generated endogenously from trimethyllysine (TML), with further conversion from γBB to Carnitine and potentially to TMA and TMAO, illustrating the complex interaction between Carnitine-related metabolites (Fig. 1).

At present, there are no published studies evaluating γBB in relation to clinical atherosclerosis. Based on its relation to Carnitine and TMAO as well as its suggested pro-atherogenic effects, we hypothesized that γBB could be related to the presence of carotid atherosclerosis and its complications. This hypothesis was evaluated in a cohort of 264 patients with carotid atherosclerosis and 62 healthy controls where serum levels of γBB, TMAO and their common precursors Carnitine and TML were related to the presence of carotid atherosclerosis, clinical characteristics of the patient group, and outcome during follow-up.

2. Materials and methods

2.1. Patients and control subjects

Between 2004 and 2015, 264 patients with moderate (50–69%) or severe (≥70%) carotid stenosis were consecutively recruited at the Department of Neurology, Oslo University Hospital Rikshospitalet according to predefined inclusion and exclusion criteria [15]. In particular, patients with severe concomitant disease such as infections, connective tissue disease, malignancies, heart failure, and liver or kidney disease were not included. The patients in the present study constitute the total cohort of the prospective study. All patients were scheduled for carotid endarterectomy or treated conservatively. The patients with symptoms of ischemic stroke, amaurosis fugax or transient ischemic attack (TIA) ipsilateral to the stenotic carotid artery within 2 months prior to blood sampling were classified as symptomatic, and the remaining patients classified as asymptomatic. The patients who had never had symptoms from their carotid stenoses were recruited during investigation of coronary or peripheral artery disease or hypercholesterolemia. The diagnosis of coronary artery disease (CAD) was based on findings on coronary angiograms, i.e., angiographically documented obstruction (≥50%) of at least one main coronary artery. Hypertension and dyslipidemia were defined as taking antihypertensive medication or on statin therapy, respectively. For comparison blood samples were collected from the 62 healthy control subjects who were recruited from the same area of Norway as the patients. All controls were apparently healthy individuals assessed by patient history and clinical examination, and all had CRP levels <10 mg/L. The study protocol was approved by the Regional Committee for Medical and Research Ethics. All study participants signed written, informed consent.

2.2. Carotid ultrasound

Colour duplex ultrasound was performed with a General Electric Vivid 7 (General Electric, Horten, Norway) using a M12L probe (14 MHz) on both carotid arteries. The degree of stenosis was based on velocities according to consensus criteria of the Society of Radiologists in Ultrasound [16].

2.3. Blood sampling protocol

Venepuncture of a forearm vein was performed with patient in a non-fasting state before lunch at the day of study inclusion. Blood was drawn into pyrogen-free tubes without any additives and allowed to clot at room temperature (within 1 h) before centrifugation (2500 *g* for 20 min). Serum samples were stored at −80 °C and thawed <3 times.

2.4. Measurement of Carnitine, γBB, TML and TMAO

Serum levels of Carnitine, γBB, TML and TMAO were quantified by high performance liquid chromatography as described [17].

2.5. Statistical analysis

Continuous variables were compared with the Mann-Whitney *U* test (two groups) or Kruskal-Wallis comparison test when three groups were compared. Student's *t*-test was used for comparison of normally distributed data. The Chi square test was used for analyzing categorical data. Spearman correlation was performed to investigate circulating levels of carnitine-related metabolites in relation to clinical characteristics in patients with atherosclerosis. Kaplan-Meier curves and log rank test were performed to analyze survival. The importance of the metabolites in relation to outcome was investigated by multivariable Cox regression including age and eGFR. Variables were log transformed and expressed per SD for regression. Probability values (2-sided) were considered significant at $P < 0.05$. All calculations were performed with SPSS for Windows statistical software (Version 21.0; SPSS Inc, Chicago, IL).

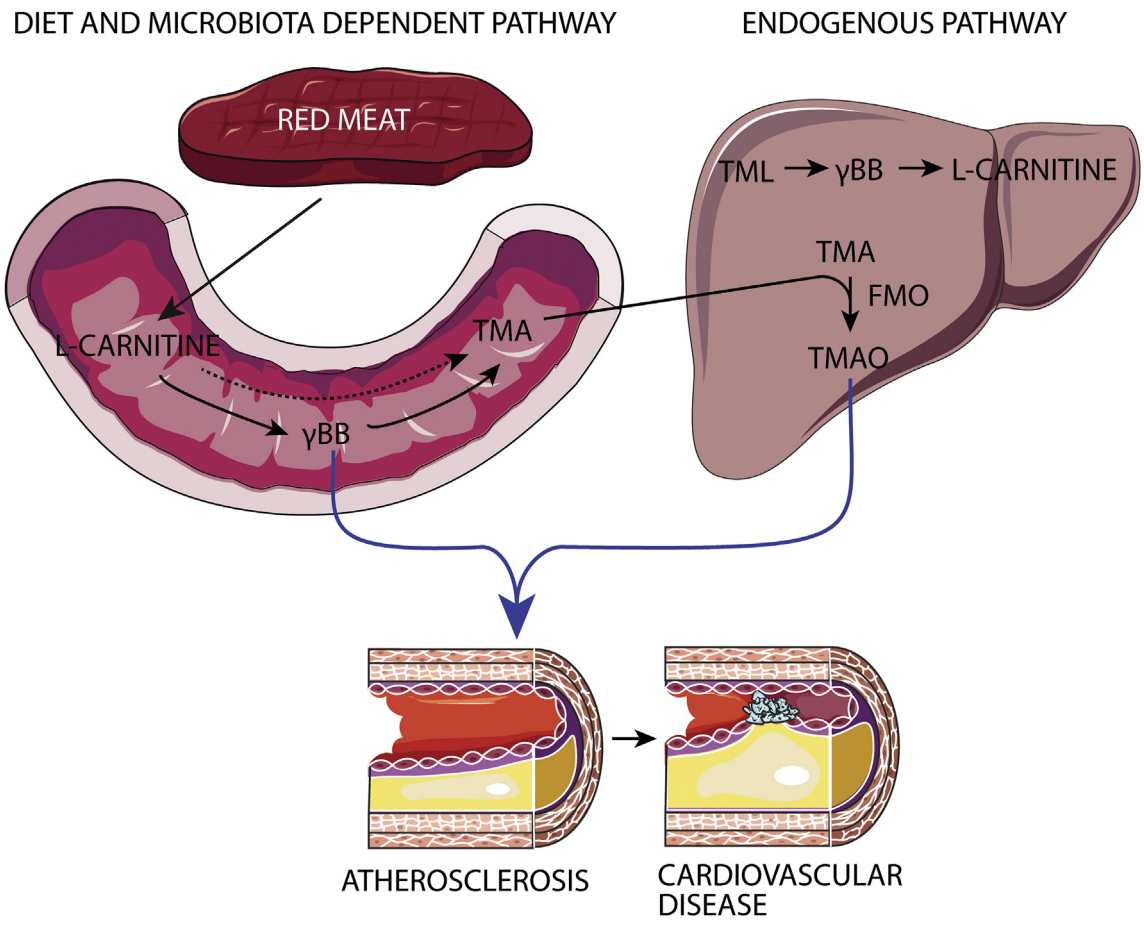

Fig. 1. Dietary L-carnitine is metabolized into γ- butyrobetaine (γBB) that is further converted into trimethylamine (TMA). TMA is absorbed and further metabolized endogenously by the liver, where it is oxidized by flavin mono-oxygenases (FMO) to form TMAO. In our proposed model it may be possible that γBB in addition to exerting pro-atherogenic effects through TMAO, could enhance atherosclerosis directly.

3. Results

3.1. Baseline characteristics of patients and controls

There were no significant differences in gender and age between patients and controls (Table 1). Seventeen percent of the patients had diabetes, 43% had CAD, 86% were on statin therapy, 66% on anti-hypertensive therapy, 87% on anti-platelet medication, and 11% on anti-coagulation therapy (Table 1). As expected, the patient group had significantly higher blood levels of CRP and HbA1c, and lower levels of HDL cholesterol (Table 1). In contrast, LDL cholesterol levels were higher in the control group, probably reflecting the frequent use of statins in the patient group (Table 1).

3.2. Circulating levels of Carnitine-related metabolites in patients with carotid atherosclerosis and healthy controls

Patients with carotid artery atherosclerosis (n = 264) had significantly higher serum levels of Carnitine and γBB, but not of TMAO and TML as compared with controls (n = 62) (Table 1). When analyzing the study group as a whole (n = 326), all these four Carnitine-related metabolites were higher in men than in women (Supplemental data, Table S1), with the same pattern in patients and controls (data not shown).

3.3. Circulating levels of Carnitine-related metabolites in relation to clinical characteristics in patients with carotid atherosclerosis

In general, the Carnitine metabolites were poorly correlated with clinical characteristics in patients (Table 2). However, Carnitine was negatively correlated with CRP, and TML was negatively correlated with HDL cholesterol and positively correlated to presence of CAD in addition to carotid atherosclerosis (Table 2). Moreover, patients with CAD had significantly higher levels of TML, but not of Carnitine, γBB and TMAO, than those without CAD (Table 2). Also, patients with eGFR <60 mL/min/1.73 m^2 (n = 26) had markedly higher levels of TML, γBB and TMAO, but not of Carnitine, than patients with eGFR <60 mL/min/1.73 m^2 (n = 238) (Table 2). In contrast, the presence of hypertension and diabetes did not significantly influence the levels of any of the Carnitine-related metabolites (Table 2). Finally, 42% of the patients (n = 110) were classified as symptomatic and 58% (n = 154) as asymptomatic, according to presence or absence of relevant symptoms within the preceding 2 months. We found, however, no association between serum levels of Carnitine, γBB, TMAO or TML and symptomatology (Table 2).

When analyzing the impact of medication on levels of Carnitine metabolites, we found that except for a higher level of Carnitine in those not taking anti-platelet medication, the use of various medications had no significant influence on these metabolites (Table 2). The lack of correlations between statins and anti-coagulation therapy and levels of the Carnitine metabolites, does, however,

Table 1
Patient demographics and comparison of serum levels of metabolites in patients and controls.

	Patients, (n = 264)	Controls, (n = 62)	P
Age	67.6 ± 8.4	68.0 ± 5.9	0.732
Sex, male*	68.6 (181)	58.1 (36)	0.115
Diabetes*	17 (45)	–	
Coronary artery disease*	43.2 (114)	–	
Anti-hypertensive therapy*	66.3 (175)	–	
Statin therapy*	85.6 (226)	–	
Anti-platelet medication*	87.1 (230)	–	
Anti-coagulation therapy*	11.4 (30)	–	
Symptomatic	41.0 (110)	–	
Leukocytes, 10^9/L	7.5 ± 2.4	5.7 ± 1.4	<0.001
CRP, mg/L	2.8 (0.6–90.0), 4.9	1.2 (0.5–9.90), 1.5	<0.001
Total C, mmol/L	4.3 ± 1.0	6.0 ± 0.9	<0.001
LDL C, mmol/L	2.5 ± 0.9	3.8 ± 0.8	<0.001
HDL C, mmol/L	1.3 ± 0.4	1.7 ± 0.5	<0.001
Creatinine, μmol/L	81.7 ± 32	74.3 ± 13.4	0.071
HbA1c, %	6.1 ± 1.2	5.5 ± 0.5	<0.001
Carnitine, μmol/L	42.8 (12.9–69.0), 11.4	40.4 (26.2–51.8), 9.1	0.001
γBB, μM	1.08 (0.06–2.28), 0.40	1.00 (0.62–1.65), 0.28	0.024
TMAO, μM	9.77 (0.4–161.7), 161	5.8 (1.3–36.8), 3.9	0.773
TML, μM	0.64 (0.29–2.80), 0.22	0.54 (0.30–2.56), 0.24	0.211

The values are given as mean (SD) or *percentage (number). The values for the metabolites and for CRP are given as median (range), interquartile range. CRP = high sensitivity C-reactive protein, C = cholesterol, γ BB = γ-butyrobetaine, TMAO = trimethylamine-N-oxide, TML = trimethyllysine.

Table 2
Circulating levels of Carnitine-related metabolites in relation to clinical characteristics and medication-class in patients with atherosclerosis (n = 264).

	Carnitine	TMAO	γBB	TML
Symptomatic lesion	0.03	0.06	−0.01	0.02
Leukocytes	−0.04	0.04	0.01	0.06
Cholesterol	0.11	−0.01	0.03	−0.01
LDL cholesterol	−0.07	0.002	0.10	0.03
HDL cholesterol	−0.07	−0.12	−0.12	−0.26**
CRP	−0.16*	0.12	0.04	0.08
HaA1c	−0.02	0.09	−0.01	0.06
eGFR >60 Yes/No (p)	43.3/43.1 (0.60)	4.9/13.7 **(<0.001)**	1.0/1.3 **(<0.001)**	0.56/0.91 **(<0.001)**
Diabetes Yes/No (p)	41.9/43.6 (0.19)	5.0/5.3 (0.67)	1.0/1.0 (0.07)	0.55/0.59 (0.34)
Hypertension Yes/No (p)	43.8/42.1 (0.27)	5.2/5.4 (0.52)	1.0/1.1 (0.33)	0.6/0.6 (0.56)
CAD Yes/No (p)	44.3/42.7 (0.37)	5.2/5.4 (0.97)	1.1/1.0 (0.16)	0.6/0.6 **(0.02)**
Statin therapy Yes/No (p)	41.2/42.1 (0.42)	5.3/4.9 (0.67)	1.0/1.0 (1.00)	0.6/0.6 (0.67)
Anti-platelets Yes/No (p)	42.2/46.1 **(0.01)**	5.4/5.0 (0.22)	1.1/1.1 (0.83)	0.6/0.6 (0.83)
Anti-coagulation Yes/No (p)	44.2/43.3 (0.79)	5.9/5.0 (0.29)	1.1 (1.0 (0.16)	0.6/0.6 (0.54)

CRP = high sensitivity C-reactive protein, BB = γ-butyrobetaine, TMAO = trimethylamine-N-oxide, TML = trimethyllysine. For dichotomous variables (eGFR < 60 yes/no, diabetes, hypertension and CAD) and medication-class (statin therapy, anti-platelet medication or anti-coagulation therapy), data are given as median for the actual metabolites (μM) for Yes/No (p-values) (Mann-Whitney *U* test comparison). Twenty-six of the patients had eGFR (mL/min/1.73 m^2) <60 and 238 patients had >60. Numbers of patients with diabetes, hypertension, CAD and on different treatment modalities are shown in Table 1. For the other analyses, correlation coefficients (Spearman correlation) are shown). Statistical significant associations are marked in bold. **p < 0.001 and *p < 0.05.

not exclude any interactions as statins were used by nearly all individuals (86% of patients) and anti-coagulation therapy was taken by only a minority of the patients (11.4%).

Finally, when analyzing the correlations amongst the different Carnitine-related metabolites in the study group as a whole (n = 326), we found that Carnitine was significantly correlated to γBB and TML, but not to TMAO, γBB was significantly correlated to Carnitine, TML and TMAO, and TMAO was significantly correlated to TML and γBB, but not Carnitine (Supplementary Table S2).

3.4. Circulating levels of Carnitine-related metabolites in relation to clinical outcome

After a mean follow-up of 5.3 ± 2.9 years, 51 patients (20%) died of vascular (n = 24) or non-vascular (n = 27) causes. Five patients were missing to follow-up, making the total number of patients available for outcome analysis 259. Vascular mortality was defined as documented cause of death being MI or stroke. Patients who died due to vascular causes had higher levels of γBB, TML and TMAO (these levels remained significantly higher also after adjustment for gender), whereas patients who experienced non-vascular death had higher levels of TMAO, γBB, TML, compared to survivors (Fig. 2A and Supplementary Table S3). As the strongest association of Carnitine metabolites and mortality was seen in relation to vascular death, these associations were further examined. Kaplan-Meier plots revealed a higher risk of vascular death with increasing tertiles of γBB, TML and TMAO (Fig. 2B). Univariate analysis revealed that neither sex (HR [95%]: 1.46 [0.61–3.47], p = 0.39, Wald 0.7), nor CAD (HR [95%]: 1.86 [0.80–4.35], p = 0.151, Wald = 2.1) was significantly associated with vascular mortality and they were therefore not included in the final analysis. eGFR <60 on the other hand (HR 7.96 [3.35–18.92], p < 0.001, Wald = 22.1) together with age (HR 1.15 [1.08–1.23]. p < 0.001, Wald = 15.9) were strong covariates for outcome. Based on these findings, eGFR and age were included in the final outcome analysis as covariates to the Carnitine derivate. Unadjusted Cox proportional hazard regression models (Fig. 2C) revealed significant associations between log transformed (per SD) γBB, TMAO and TML, and vascular

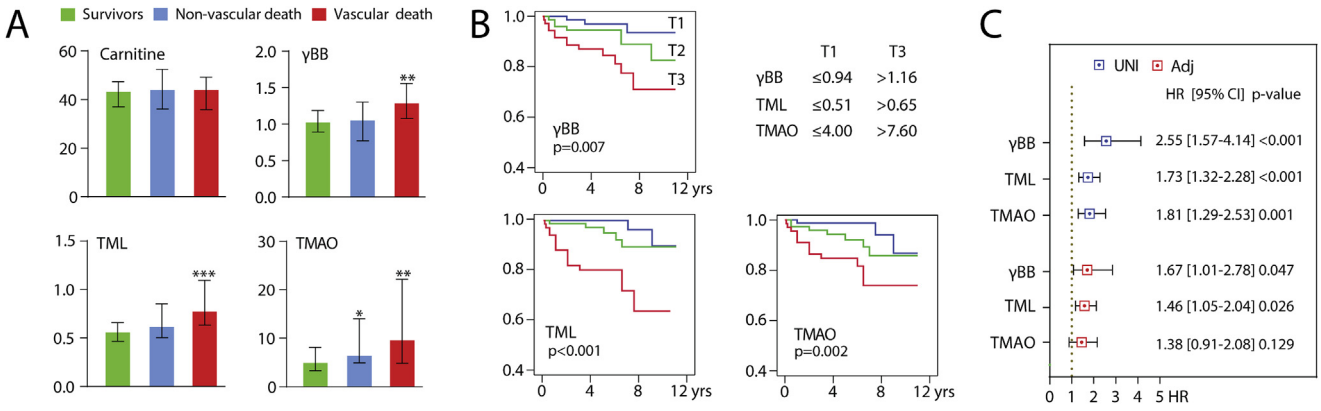

Fig. 2. **(A)** Association between serum levels of free L-carnitine, γ-butyrobetaine (γ BB), trimethylamine-N-oxide (TMAO) and trimethyllysine (TML) (all μmol/L) in patients with carotid atherosclerosis (n = 259) according to mortality status (survivors n = 208, vascular death n = 24 and non-vascular death n = 27). ***p < 0.001, **p < 0.01 and *p < 0.05 versus survivors. **(B)** Kaplan-Meier plots showing the cumulative incidence of vascular death during long-term follow-up (mean years of follow-up 5.3 ± 2.9 [SD], range 1–11), according to dichotomized γBB, TML and TMAO levels. **(C)** Cox-regression model showing univariate (blue) hazard ratios (HR) for γBB, TML and TMAO as well as multivariable models adjusting for age and eGFR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

death. These associations were moderated when adjusting for age and eGFR, however, remained significant for γBB and TML, but not TMAO.

4. Discussion

This study investigated the potential impact of microbiota-associated Carnitine metabolites γBB and TMAO, as well as their common precursors Carnitine and TML, on carotid atherosclerosis and cardiovascular mortality. The major findings were as follows: (i) Patients with carotid atherosclerosis had increased serum levels of Carnitine and γBB, but not TMAO and TML compared with healthy controls. (ii) Higher serum levels of γBB and TML, but not TMAO or Carnitine, were independently associated with cardiovascular death also after adjustment for age and eGFR. Our findings may suggest an association between Carnitine metabolites, and in particular γBB and carotid atherosclerosis, potentially involving a microbiota dependent mechanism.

Whereas previous studies on gut microbiota-dependent metabolites and atherosclerosis have focused on TMAO, this is, to the best of our knowledge, the first study to report an association between γBB and atherosclerosis in humans. Several microbiota-dependent pathways can lead to the Carnitine-related formation of TMA [7,9,14,18,19], which is subsequently converted to TMAO in the liver, and γBB was recently identified as an intermediate in these pathways. Whereas Carnitine may be converted directly to TMA via bacterial enzyme systems in the colon, the conversion of Carnitine to γBB seems to occur in the small intestine [7], and interestingly, γBB is further partly converted to TMA in the colon, suggesting that γBB could mediate some of its effects through TMA and subsequently TMAO [7]. However, in addition to microbiota-dependent metabolism from carnitine, γBB is also produced endogenously from TML [7]. The finding in this current study that both γBB and TML, but not TMAO, were independently associated with cardiovascular mortality in a model including all three metabolites, may support an association between Carnitine related pathways and carotid atherosclerosis, at least partly independent of TMAO formation. This association could potentially include both microbiota-dependent and endogenous pathways, where γBB appears to be the most sensitive marker of Carnitine pathway activity in relation to carotid atherosclerosis.

Previously, an increase of atherosclerotic lesions was reported in animals fed with γBB compared to those fed the same diet supplemented with an antibiotic mixture [7]. This could suggest that γBB may have a proatherogenic effect that requires the presence of microbes, although these findings have been questioned [14]. Indeed, the absence of pro-atherogenic effects from γBB in germ free mice could mean that the potentially pro-atherogenic effect of γBB is indirect and dependent on other factors in the gut microbiota, such as TMAO [20], that again could promote foam cell formation [13]. However, studies from rodents are not necessarily directly applicable in humans [21]. Rodent studies dependent on large bowel bacteria that can be assumed inappropriate for humans due to longer transit time. Additionally, the mice in animal studies have been fed very high doses of Carnitine (the equivalent of 80 half-pound steaks per day) with corresponding blood levels of metabolites therefore likely not representative of normal dietary intake of Carnitine in humans. This highlights the need for separate studies with human subjects. We have previously reported elevated levels of γBB and Carnitine in patients with heart failure [22], and have recently found that γBB and Carnitine, but not TMAO, were closely associated with visceral to subcutaneous adipose tissue ratio in bariatric surgery patients (M Trøseid, unpublished data). Hence, the association between γBB and obesity and clinical atherosclerosis as in the present study could encourage further studies investigating the role of γBB in clinical atherosclerosis.

This study has some limitations. Firstly, although blood samples were taken before lunch, reducing the likelihood of a large meal intake containing substantial amount of Carnitine, the use of non-fasting samples as well as lack of information of vegetarians versus omnivores is a limitation of our analyses. Secondly, the controls were not investigated with carotid ultrasound and we can not exclude the possibility that some of the controls also had carotid atherosclerosis. Additionally, the inclusion of patients with carotid atherosclerotic plaques without accompanying significant carotid stenosis would have been of interest to evaluate the relation of the Carnitine-related metabolites to the degree of atherosclerosis. Finally, given the relatively small event rate in this study, a larger patient population and more long-term follow-up is required to make definite conclusions on the impact of Carnitine-related metabolites on cardiovascular mortality.

In conclusion, our data show that elevated levels of γBB and its precursor TML were increased in patients with carotid atherosclerosis and associated with cardiovascular mortality in these patients. Long-term clinical studies of γBB as a cardiovascular risk marker and its role in the microbiota-dependent carnitine-TMA-TMAO

pathway, as well as mechanistic studies on γBB as a mediator in atherogenesis are warranted.

Conflicts of interest

None.

Acknowledgement

The authors wish to acknowledge SERVIER Medical Art (www.servier.fr) for use of their medical art kits when making the illustration in the article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.01.033.

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