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

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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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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).