

The carnitine-butyrobetaine-TMAO pathway after cardiac transplant

Impact on cardiac allograft vasculopathy and acute rejection

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Abstract

Background. Alterations in the partly microbiota-dependent carnitine-butyrobetaine (γ BB)-trimethylamine-N-oxide (TMAO) pathway have been linked to the progression of heart failure and atherosclerotic disease. We evaluated if circulating γ BB, TMAO and their common precursors carnitine and trimethyllysine (TML) were dysregulated after heart transplantation and associated with development of cardiac allograft vasculopathy (CAV) and acute rejection. **Methods.** We measured these metabolites in plasma from heart transplant recipients with everolimus (EVR)-based (n=32) and standard cyclosporine-based immunosuppression (n=30), at different time points and accompanied by assessment of CAV by intravascular ultrasound. **Results.** i) Baseline levels of carnitine, TMAO and TML were elevated in heart transplant recipients compared to controls, and TML remained elevated throughout the observation period, ii) The microbiota dependent metabolite γ BB increased steadily during three years follow-up, with a similar decrease in its endogenous precursor TML, iii) The increase in γ BB and change in TML was associated with change in total atheroma volume from baseline to three years, iv) Increase in γ BB and carnitine levels from baseline to 1 year was associated with increased frequency of acute rejection within the first year after heart transplant. **Conclusion.** Our study reveals alterations of the carnitine- γ BB-TMAO pathway after heart transplant, with increasing levels of γ BB being associated with acute rejection and increase in total atheroma volume during three years follow-up. Future studies should clarify whether interactions between dietary factors, immunosuppressive drugs and the gut microbiota could influence acute rejection and CAV development, in order to delineate mechanisms and potential novel treatment targets.

Introduction

Clinical outcome after heart transplantation (HTx) has improved substantially the last decades, but long-term survival is still reduced mainly due to development of cardiac allograft vasculopathy (CAV) ¹. Whereas both immunological and non-immunological mechanisms (e.g., alloimmune responses, non-specific insults including ischemia-reperfusion injury, viral infections, and metabolic disorders) are thought to contribute, the mechanisms leading to CAV are not fully clarified. Since treatment of CAV is limited, identification and prevention of potential triggering and modifiable factors is of clinical major importance ^{1, 2}.

The gut microbiota, comprising the trillions of bugs inhabiting the gastrointestinal tract, may be considered a complex bioreactor with several metabolic and immunological roles. Increasing evidence has linked functions and alterations of the gut microbiota to cardiometabolic diseases such as atherosclerosis, diabetes and obesity³. The gut microbes produce a large range of metabolites which act not only in the gut, but also systemically, and the metabolite trimethyl amine-N-oxide (TMAO) was recently introduced as the first potentially direct link between the gut microbiota and atherothrombotic disease.

Trimethylamine (TMA) is produced by the gut microbiota from nutrients containing L-carnitine, choline and phosphatidylcholine, and is subsequently oxidized by hepatic flavin-containing monooxygenases to TMAO. Elevated plasma levels of TMAO have in several independent cohorts been shown to predict incident myocardial infarction, stroke and all-cause mortality³⁻⁵, as well as poor clinical outcome in heart failure ^{6, 7}. Plasma levels of TMAO may be confounded by liver and kidney function,⁸ and in a recent work, we found γ -butyrobetaine (γ BB), a microbiota-dependent intermediate metabolite in the pathway from carnitine to TMAO⁹, and its endogenous precursor trimethyl lysine (TML), to be even more predictive of cardiovascular mortality than TMAO ¹⁰.

Clinically, CAV resembles coronary heart disease, but is pathologically distinct from the usual coronary atherosclerosis and may affect any graft vessel, with concentric, progressive thickening along the whole length of the vessels that ultimately leads to ischemia, myocardial failure and death. Although the gut microbiota and its products have been implicated in the pathogenesis of atherosclerosis, there is no data on the role of TMAO and related gut microbiota-derived metabolites in CAV. As transplanted patients have a suppressed immune system, the gut microbiota and its metabolites could be of particular relevance for development of CAV.

Gut microbiota changes have been reported after allogenic liver transplantation, kidney transplantation and hematopoietic stem cell transplantation, and these changes have been potentially linked to complications including acute rejection^{11, 12}, where direct immunological mechanisms and interference with immunosuppressive drugs could be at play¹³. If such mechanisms are also operating after HTx are however not known

We hypothesized that the microbiota dependent pro-atherogenic metabolites TMAO and γ BB, as well as their dietary and endogenous precursors carnitine and TML would be elevated after cardiac HTx and associated with development of CAV as well as acute rejection during the first year of follow-up. We tested our hypothesis by measuring these metabolites in plasma from HTx recipients with everolimus (EVR)-based and standard cyclosporine-based immunosuppression.

Material and methods

Study design, population and conduct

The SCHEDULE trial involved 115 participants. Biobanking was performed in a pre-specified subgroup subset of these patients of which n=62 had repeated measurements and were included in the present study¹⁴. The SCHEDULE (SCandinavian HEart transplant everolimus De novo stUdy with earLy calcineurin inhibitor avoidanceE) trial was a prospective, multicenter, randomized, controlled, open-label trial undertaken at five HTx centers in Scandinavia, in which adult *de novo* HTx recipients (n=115) were randomized in a 1:1 ratio to (i) low-dose EVR, low-dose cyclosporine (CyA), mycophenolate mofetil (MMF) and corticosteroids (CS) with withdrawal of CyA and step up to full dose EVR after 7-11 weeks *or* (ii) conventional treatment with CyA, MMF and CS (Figure 1). A detailed description of the SCHEDULE trial has been reported previously¹⁴. For comparison of carnitine, γ BB, TML and TMAO levels, we also included 76 healthy individuals¹⁰. The study was conducted in compliance with Good Clinical Practice and in accordance with the 2000 Declaration of Helsinki and Declaration of Istanbul 2008. The appropriate ethics committee for each center approved the study and it was registered with clinicalTrials.gov (NCT01266148). Written informed consent was obtained from all study participants prior to inclusions in the study.

Measurement of TMAO, γ BB, carnitine and TML

Blood samples were available from three time points: 7 weeks (before CyA withdrawal), 12 months and 3 years post Tx. Serum levels of TMAO, γ BB, carnitine and TML were quantified by stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described^{7, 10}.

Treatment

Induction therapy with antithymocyte globulin (ATG) was initiated within 12 hours of HTx for all included patients and continued for up to 5 days. In the EVR group, EVR was initiated at a dose of 0.75 mg twice daily no later than the fifth postoperative day. Everolimus target trough levels prior to week 7-11 was 3-6 ng/mL, and after calcineurin inhibitor (CNI) withdrawal 6-10 ng/mL. The target trough level of CyA in the EVR group was 75-175 ng/mL until week 7-11. CyA elimination took place at week 7 unless ongoing rejection was present at that time, in which case discontinuation was allowed to be postponed up to week 11. The target dose of MMF was 1500-2000 mg/day until week 7-11, with a target dose of 1000 mg/day (minimum 750 mg/day) at the time of CyA withdrawal and onwards. In the CNI group, the target CyA trough level was 150-350 ng/mL during month 1-3, 100-250 ng/mL during month 4-6 and 60-200 ng/mL onwards. The target MMF dose was 2000-3000 mg/day. All patients received CS at a minimum dose of 0.1 mg/kg during month 1-3 and 0.05-0.1 mg/kg at month 4-12. Hereafter, CS could be discontinued at the discretion of the investigator¹⁴.

All patients were on statin treatment throughout the study period, unless statin-related side effects precluded their use. The decision to initiate antihypertensive treatment was left to the discretion of the treating physician. Cytomegalovirus (CMV) antibody-negative patients receiving a graft from a CMV-positive donor were treated with oral valganciclovir for at least three months. Furthermore, all of our transplant recipients were prescribed low-dose trimethoprim sulfa for the first six months after transplantation. Altogether, 32 % of the patients had one or more bacterial or fungal infections, which was treated by additional antibiotics.

Intravascular ultrasound

The pre-defined efficacy variables were changes from baseline in maximal intimal thickness, percent atheroma volume, and total atheroma volume as measured by intravascular ultrasound (IVUS) ¹⁵. These variables are quantitative, surrogate markers for the extent of CAV.

Statistical analysis

Continuous demographics between the treatment groups were compared by unpaired T-test or Mann Whitney U test depending on distribution. Proportions were analyzed with the chi-square test and correlations were calculated using Spearman's rank correlation coefficient. Differences in metabolites between controls and HTx patients were analyzed by univariate regression with metabolites as dependent (log transformed if necessary), group (i.e. control or HTx patients) as fixed and age, sex and creatinine as covariates. For comparing treatment effects, univariate repeated-measures ANOVA was performed *á priori* and paired (paired T-test) or unpaired (univariate regression adjusting for baseline levels) tests *á posteriori* at individual time-points when appropriate (ANOVA significant). Data from the treatment study are given as estimated means with 95% CI. The association between changes in carnitine-related metabolites and changes in total atheroma volume and acute rejection were analyzed by linear and logistic regression, respectively. P values are two-sided and considered significant when <0.05 .

Results

Baseline characteristic and association with Carnitine-related metabolites

Baseline characteristics of the patients (n=62) are presented in Table 1. Mean (\pm SD) age was 49.8 ± 13.7 years and 26% were female. Median eGFR was 64 ± 15 mL/min/1.73m² and creatinine 104 ± 34 μ mol/L. Treatment groups did not differ significantly. As shown in Supplemental Table 1, there were few associations between plasma levels of carnitine-related metabolites and patient characteristics, although all metabolites correlated with creatinine, and TMAO correlated with age and eGFR as previously reported in patients with heart failure⁷. Also TML correlated with eGFR at baseline.

Circulating levels of Carnitine-related metabolites in HTx patients and healthy controls

At baseline HTx patients (n=62) had significantly higher plasma levels of carnitine, TMAO and TML, but not γ BB, compared with healthy controls (n=76) (Figure 1). Controls were older (mean age of 66.5 ± 7.1 vs 50.5 ± 13.7 , $p<0.001$), more often female (51% vs 24%, $p<0.001$) and had lower creatinine levels (73 ± 14 vs. 104.1 ± 33.2 , $p<0.001$) but similar BMI (25.2 ± 3.1 vs 24.6 ± 3.6 , $p=0.35$) to HTx patients. These factors were adjusted for when comparing patients with controls (creatinine was preferred over GFR since the formulae includes age and sex).

Changes over time and effect of treatment on carnitine-related metabolites

The microbiota-dependent metabolite TMAO decreased from elevated levels at baseline to levels comparable with controls at 1 year, and for the EVR group remained at normal levels also at 3 years. An opposite pattern was seen for the microbiota-dependent intermediate γ BB, with levels comparable to controls at baseline, with a steady increase in both treatment groups over time, reaching significantly higher levels than at baseline at 3 years in the EVR

group. Conversely, TML, an endogenous precursor of γ BB, decreased in both groups at 1 and 3 years, although still elevated compared to healthy controls. Finally, carnitine levels remained consistently elevated in HTx patients during follow-up with only minor fluctuations. However, no significant differences between the EVR and the CyA arm were observed for any of the carnitine-related metabolites (Figure 2). The absolute levels in controls and HTx patients during follow-up are given in Supplemental Table 2.

The changes in carnitine, γ BB and TML from baseline to 1 year and 3 years were not significantly associated with the increase in eGFR observed at these time-points (r 's between 0.02 and 0.23). However, the decline in TMAO was modestly associated with the increase in eGFR at 1 year ($r=-0.26$, $p=0.041$) and 3 years ($r=-0.25$, $p=0.055$) in the HTx population as a whole.

Associations between change in circulating levels of carnitine-related metabolites and total atheroma volume

No associations between baseline levels or change in circulating levels of carnitine-related metabolites and CAV as reflected by maximal intimal thickness, percent atheroma volume or total atheroma volume were detected (Supplementary Table 1 for correlations at baseline). However, changes in total atheroma volume correlated with increase in γ BB ($r=0.41$, $p=0.002$) and change in TML ($r=0.30$, $p=0.029$) from baseline to 3 years. Relevant covariates are given in Supplemental Table 3. When adjusting for age, sex, treatment, β -blocker and changes in LDL cholesterol in a basic linear regression model, the associations between carnitine-related metabolites and total atheroma volume were not markedly affected, nor when adjusting further for statins, ACE inhibitor/angiotensin receptor blocker, total cholesterol, number of infections, ASAT and ASAT (Supplemental Figure 2).

Associations between carnitine-related metabolites and acute rejection

In the current subpopulation of the Schedule trial 59 % experienced one or more acute rejections within the first year, of which 34 % were grade 1 and 25 % were grade 2. ROC analysis revealed that an increase in levels of carnitine ($p=0.013$) and γ BB ($p=0.025$), but not TMAO and TML, from baseline to 1 year was associated with increased frequency of acute rejection in this period (Figure 3A). This was driven by a larger increase of these metabolites in patients with Grade 2 rejection (Figure 3B). While having an infection was not associated with acute rejection at 1 year, the number of infections was (Supplemental Table 3), and therefore included in the basic multivariable adjustment model. Expressing change as standardized change (i.e. change/SD) revealed that a 1SD increase in carnitine (OR 2.15 [1.16 – 4.00] $p=0.015$) and γ BB (OR 2.06 [1.11 – 3.81] $p=0.021$) was associated with around 2 times higher likelihood of acute rejection within 1 year. Adjusting for treatment, age, CMV mismatch and number of infections even enhanced this association for carnitine (OR 2.39 [1.15 – 4.96] $p=0.020$) and γ BB (OR 2.25 [1.12 – 4.53] $p=0.023$), whereas further adjustment did not markedly alter these associations (Supplemental Figure 2).

Discussion

In this substudy of the SCHEDULE trial, we explored the relationship between the microbiota dependent metabolites γ BB and TMAO, as well as their dietary and endogenous precursors, and the development of CAV and rejection, two important complications after HTx. The main results can be summarized as follows: i) Circulating levels of the carnitine-related metabolites carnitine, TMAO and TML were all elevated in HTx patients at baseline compared to controls, and for TML, this elevation was seen throughout the observation period, ii) The microbiota dependent metabolite γ BB increased steadily during three years follow-up, with a similar decrease in its endogenous precursor TML, iii) The increase in γ BB and change in TML were associated with change in total atheroma volume, but not with any other measures of CAV, from baseline to three years, iv) Increase in γ BB and carnitine levels from baseline to 1 year was associated with increased frequency of acute rejection within the first year after HTx.

Several mechanisms may contribute to the changes in carnitine-related metabolites revealed in the present study. As γ BB is converted from carnitine by a microbiota-dependent mechanism⁹, changes in gut microbiota over time could explain the steady increase in γ BB levels during 3 years follow up. The changes in gut microbiota composition after HTx are not known, but it was recently reported from a mouse model that immunosuppressive therapy including MMF, CS and EVR altered the gut microbiota with decreased Bacteroidetes, increased Firmicutes and overgrowth of opportunistic pathogens in feces¹⁶. However, herein we found that TMAO, which is also produced by microbiota-dependent conversion from carnitine and other dietary precursors¹⁷, followed an opposite pattern, with decreased concentration after 1 year follow-up to levels similar to controls. Notably, TMAO is oxidized from trimethylamine (TMA) in the liver and TMAO levels are affected by glycemic control and kidney function⁸. Plasma levels of TMAO were inversely correlated with kidney function

both at baseline and during follow-up. Hence, increased renal clearance of TMAO, altered liver function, as well as interaction between hepatic oxidation of TMAO and hepatic metabolism of drugs may all have contributed to alterations in TMAO levels in the same time period.

Cardiac allograft vasculopathy is a major impediment to long-term survival in heart transplant recipients^{18, 19}. The etiology is likely to be multifactorial. Whereas aspects of the disease are probably caused by low grade inflammation and chronic, antibody-mediated rejection,^{18, 19} classical risk factors for coronary artery disease are thought to contribute^{20, 21}. Although several carnitine-related metabolites including the potential pro-atherogenic metabolite TMAO were elevated in heart transplant recipients at baseline, none of them predicted the development of CAV during the first three years after HTx. However, the increase in γ BB and change in TML correlated with an increase in total atheroma volume over three years. If this reflects any causal relationship is at present unknown, but the association with atheroma volume, and not intimal thickening, could imply that these metabolites are associated with traditional atheromatosis rather than the immune component of CAV. Intimal thickening is an established predictor of all-cause mortality, myocardial infarction, and angiographic abnormalities amongst HTx recipients²², and we found no association between this established CAV parameter and any of the carnitine-derived metabolites.

The increase in γ BB and change in carnitine levels during the first year after HTx were also associated with acute rejection. In addition to microbiota-dependent conversion from carnitine, γ BB is also produced from the endogenous precursor TML. As carnitine levels remained stable whereas TML was reduced, increased conversion from TML to γ BB after HTx is a possible explanation for the increase in γ BB in our study. Whether this conversion is also facilitated by the gut microbiota remains unknown. Microbiota changes

occur after allogenic liver transplantation, kidney transplantation and hematopoietic stem cell transplantation, and these changes may be linked to complications including acute rejection¹¹,¹². If such mechanisms are also operating after HTx is not known, although a recent case report of CAV and severe mixed rejection following fecal microbial transplant for *Chlostridium Difficile* infection in a 3-year-old male with HTx²³ warrants further longitudinal studies to clarify whether different gut microbiota profiles could contribute to acute rejection and/or CAV. Of interest, number of infections was associated with acute rejections at 1 year in our study. Furthermore, the association between temporal changes in metabolites and CAV/rejection could reflect overall changes in microbiota composition rather than specific metabolites being relevant for these complications. Alterations in the gut microbiome could contribute to rejections through several mechanisms, including increased immune activation and/or impaired immune regulation which could have direct effects on the transplant, but also indirect effects through interactions with immunosuppressive drugs¹³. In Figure 4 we have summarized the interaction between carnitine-related metabolites and hypothesized how γ BB could influence the progression of CAV and acute rejection.

Of note, the pathway from carnitine to γ BB is bidirectional, with TML being converted to γ BB and further to carnitine^{10, 17}, and several dietary, pharmacological and metabolic factors could have contributed to the findings from the present study. L-carnitine is a semi-essential nutrient involved in fatty acid oxidation and energy production in the mitochondria. Propionyl-L-carnitine prevents early graft dysfunction in allogenic rat kidney transplantation²⁴, and L-carnitine supplementation has been associated with a significant reduction in all-cause mortality in the setting of acute MI²⁵. Hence, carnitine deficiency at baseline could be an alternative explanation for the association between increasing carnitine levels and acute rejection. However, as carnitine levels were consistently elevated after HTx,

this potential lack of micronutrients would probably be relevant only for a subgroup of patients.

This study has several limitations, including a limited number of patients, and in particular a low number of grade II acute rejections, resulting in wide confidence intervals and increased risk of type 1 statistical error (supplemental figure 3). Furthermore, when the SCHEDULE trial was conducted, the field of quantitative microbiota analysis was in its infancy, and we did not collect stool samples. The evidence linking the suggested changes in the gut microbiome and the changes in the measures of CAV and the risk of acute rejections is therefore circumstantial. Given the lack of statistical power to adjustment for risk factors for CAD with relation to microbiota-related progression of CAV, and the lack of stool samples, this work may open future areas of investigation for more definitive exploration of the gut microbiome after HTx. Of note, the metabolites measured may be influenced by various factors, including gut microbiome (TMAO, γ BB), diet (carnitine, TMAO, γ BB), liver function (TMAO), renal function (probably all metabolites) and a lot of concurrent medications. However, all of these factors have been adjusted for in multivariate models. In the SCHEDULE trial, we used cyclosporine, rather than tacrolimus, in the CNI arm. Tacrolimus is now the preferred CNI worldwide. We cannot rule out that the one CNI differs from the other with regard to its effect on the microbiome and its associated metabolites. However, we are not aware of data supporting this notion.

Conclusion

Our study reveals alterations of the carnitine- γ BB-TMAO pathway after HTx, with increasing levels of γ BB being associated with acute rejection and increase in total atheroma volume during three years follow-up. It was very recently shown in a mouse model that gut microbiota could influence MMF toxicity in the gastrointestinal tract²⁶, and in an Editorial

comment, it was underscored that understanding the organized chaos within the gut microbiome may open doors to disease modification as part of personalized medicine²⁷. Future studies should clarify whether interactions between dietary factors, immunosuppressive drugs and the gut microbiota could influence acute rejection and CAV development, in order to delineate mechanisms and potential treatment targets.

Acknowledgements, funding sources and relevant disclosures

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Figure Legends

Figure 1. Circulating levels of Carnitine-related metabolites ($\mu\text{mol/L}$) in HTx patients (n=62) at baseline (i.e. x weeks post HTx) compared to healthy controls (n=76). Data are given as Tukey plots with outliers and P-values are adjusted for age, gender and creatinine levels.

Figure 2. Effect of treatment with Cyclosporine (CyA, n=30) or Everolimus (EVR, n=32) on circulating levels of Carnitine-related metabolites ($\mu\text{mol/L}$) in HTx patients. Data are reported as estimated means from the general linear model procedure adjusting for baseline levels with 95% CI. The grey area represent the 95% CI from healthy controls. †p<0.05 vs. CyA same timepoint. *p<0.05, **p<0.01 vs. baseline. †p<0.05, ††p<0.01, †††p<0.001 comparing controls and the whole HTx population at the indicated time-points adjusting for age, sex and serum creatinine.

Figure 3. Carnitine-related metabolites and acute rejection. Change in circulating levels of carnitine and γBB (both $\mu\text{mol/L}$) the first year according to acute rejection grade shown as Tukey plots with outliers. *p<0.05, **p<0.01 vs. grade 0.

Figure 4. Dietary l-carnitine is metabolized into γ - butyrobetaine (γBB) that is further converted and metabolized to form trimethylamine-N-oxide (TMAO). This pathway is microbiota-dependent, but γBB can also be oxidized from trimethyllysine (TML) endogenously. In our proposed model we suggest that γBB could promote progression of cardiac allograft vasculopathy (CAV) and acute rejection directly through effects on inflammation and activation of endothelial cells. CAV and acute rejection could also have effects on carnitine-related metabolites by interfering with gut microbiota function and other pathways.

Table 1. Baseline clinical and demographic characteristics

	Controls (n=76)	Cyclosporine (n=30)	Everolimus (n=32)	<i>p-value</i>
Recipient characteristics				
Age (years)	66.5 ± 7.1*	49.1 ± 12.9	50.5 ± 14.9	0.69
Female	37 (49)*	8 (27)	8 (25)	0.88
Systolic blood pressure (mmHg)		106.6 ± 17.2	107.9 ± 18.2	0.78
Diastolic blood pressure (mmHg)		67.3 ± 12.0	67.0 ± 13.1	0.79
BMI (kg/m ²)	25.2 ± 3.1	25.5 ± 3.5	23.7 ± 3.6	0.063
Primary reason for heart transplantation,				
Idiopathic cardiomyopathy		22 (73)	22 (69)	
Coronary artery disease		7 (23)	7 (22)	
Other		1 (3)	3 (9)	
Left ventricular assist device		7 (23)	9 (28)	0.67
Medical history				
Diabetes mellitus		6 (20)	3 (9)	0.24
Chronic obstructive pulmonary disease		1 (3)	1 (3)	0.96
Hypertension		3 (10)	5 (16)	0.51
Creatinine	73 ± 14*	102 ± 29	106 ± 39	0.60
eGFR mL/min/1.73m ²		63 ± 14	64 ± 17	0.87
Donor characteristics				
Age (years)		44.4 (12.9)	40.0 ± 14.7	0.21
Female		9 (30)	13 (41)	0.38
Cold ischemia time (min)		197 ± 80	185 ± 79	0.57
Recipient negative /donor positive for cytomegalovirus		7 (23)	4 (13)	0.26

Continuous data are given as mean±SD (standard deviation) while proportions are reported as n and (percent); BMI, body mass index. *p<0.001 vs both transplant groups.

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