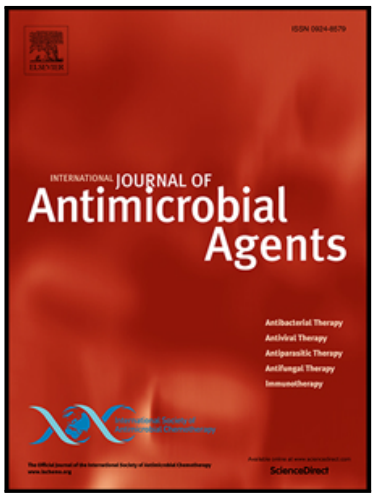

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Serum Trimethylamine-N-oxide Concentrations in People Living with HIV and the Effect of Probiotics Supplementation.

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Keywords: cardiovascular risk, intima media thickness, risk factor, TMAO, HIV, frailty.

Abstract

Background: Cardiovascular disorders show a higher incidence in people living with HIV (PLWH). Traditional and specific risk factors have been described but the role of the gut microbiota-dependent choline metabolite trimethylamine-N-oxide (TMAO) is still unclear.

Methods: A cross-sectional analysis and a longitudinal analysis (with high-dose probiotics supplementation) were performed measuring serum TMAO concentrations through UHPLC-MS/MS. Stable outpatients living with HIV on highly active antiretroviral treatment with no major cardiovascular disease were enrolled. Non parametric tests (bivariate and paired tests) and a multivariate linear regression analysis were used.

Results: In 175 participants serum TMAO concentrations were 165 ng/mL (103-273). An association with age, serum creatinine, number of antiretrovirals, multimorbidity and polypharmacy was observed: at linear logistic regression analysis, multimorbidity was the

only independent predictor of TMAO concentrations. Carotid intima media thickness (IMT) was 0.85 mm (0.71-1.21); we observed a trend towards higher TMAO concentrations in patients with IMT >0.9 mm (p=0.087). In the 25 participants who received probiotic supplementation, TMAO levels did not significant changed after 24 weeks (Wilcoxon paired p 0.220).

Conclusion: Serum TMAO levels in PLWH are associated with multimorbidity, higher cardiovascular risk and subclinical atherosclerosis; no effect of six months of high-dose probiotics supplementation was observed.

Introduction

People living with HIV (PLWH) have a higher cardiovascular (CV) risk then age- and gender- matched controls; this is ascribed to a high prevalence of traditional CV risk factors (including smoking habit, dyslipidemia and hypertension), the use of addiction drugs, the persistence of chronic inflammation and the effect of antiretroviral medications.[1,2] The latter one could be either direct (as it has been shown for abacavir) or indirect (trough the increase in serum lipids or in fat accumulation). Yet traditional risk calculators fail in reliably predicting the incidence of major cardiovascular events (CVEs) in PLWH: therefore several additional biomarkers have been studied in order to carry on tailored preventive interventions.[3]

Ingested phosphatidylcholine (very high in certain foods such as red meat, cheese and eggs) is metabolized in the gut to choline-containing nutrients and, in the large bowel by the intestinal microbiota, to trimethylamine (TMA); the latter is then oxidized to trimethylamine-N-oxide (TMAO) by hepatic flavin-containing monooxygenases. TMAO enhances the accumulation of cholesterol in macrophages and the accumulation of foam cells in artery walls and it has been associated with atherosclerosis, cardiovascular disease and platelet hyper-reactivity in HIV-negative individuals.[4] In PLWH TMAO levels were similar to healthy controls although higher serum concentrations were observed in those receiving combination antiretroviral treatment (cART).[5] Higher TMAO levels have been associated with carotid plaques, endothelial dysfunction and silent cardiac ischemia in HIV-positive subjects.[5,5–9] A recent longitudinal study showed that participants with higher TMAO levels had an increased risk of carotid plaque and that this was partially mediated by biomarkers of monocyte activation and inflammation (sCD14, sCD163).[9] Additionally PLWH with type 2 diabetes despite similar TMAO serum levels showed the highest endothelial dysfunction (and

the effect was associated with TMAO concentrations).[10]

In recent years a great interest arouse on the gut microbiome modifications in PLWH both in terms of its alterations and of the consequences of microbiome changes after pre/probiotics supplementation.[11,12] Several studies compared HIV-infected gut microbiota composition to that of HIV-uninfected control sub-jects finding an enrichment of *Erysipelotrichaceae, Enterobacteriaceae, Desulfovibrionaceae, and Fusobacteria and a depletion of Lachnospiraceae, Ruminococceae, Bacteroides, and Rikenellaceae*. However the interaction between HIV and gut microbiome seems to be modulated by lifestyle and behavioural factors (including sexual habits) as well as intestinal wall structural and functional modifications.[13] Some of the aforementioned studies additionally found correlations between some of these perturbations to the gut community and markers of inflammation and HIV disease progression, including kynurenine to tryptophan ratios, IL-6, sCD14, IL-1β and peripheral T cell activation. Recently gut microbiome traits, after adjusting for confounders, have been associated with metabolic syndrome and coronary heart disease in PLWH.[14,15] Followig these observations several studies have studied the consequences of gut microbiome modulation by probiotics supplementation: despite interesting favourable results the majority of the studies were highly heterogenous or lacking power to demonstrate clinically relevant results.[12]

Aim of this study was to investigate whether TMAO serum concentrations were associated with cardiovascular risk (CVR) or carotid intima media thickness and if they changed after probiotics supplementation.

Methods

Two studies were performed. The first one was a cross-sectional analysis of the determinants of serum TMAO concentrations in PLWH. Data were obtained from a cross-sectional study in Torino and from the baseline of the interventional pilot trial in Rome: both protocols included PLWHs receiving cART. The second study was a "probiotic intervention pilot study" described in details elsewhere: baseline and 6 month TMAO serum concentrations were compared.[16] Briefly HIV-positive patients (on cART with plasma HIV RNA <37 copies/mL and CD4+ T lymphocyte above 400/mm^3) Patients received a high concentration lyophilized multi-strain probiotic supplement (*Lactobacillus plantarum* DSM24730, *Streptococcus thermophilus* DSM24731, *Bifido-bacterium breve* DSM24732, *L. paracasei* DSM24733, *L. delbrueckii* subsp. bulgaricus DSM24734, *L. acidophilus* DSM 24735, *B. longum* DSM24736, *B. infantis* DSM24737) twice a day for 6 months. Patients signed a written informed consent and Ethics approval was obtained by each Institution's Ethics

Committee. Serum was obtained after blood centrifugation within 3 months of the clinical visit and stored at -20°C until analysis. In order to quantify TMAO plasma concentrations, 100 µL of plasma were added with 50 µL of Internal Standard working solution and 90 0µL of acetonitrile. After protein precipitation, 5 µL of the supernatants were directly analysed through a validated Hydrophilic Interaction Liquid Chromatography (HILIC) and Tandem Mass Spectrometric (MS/MS) method, with a UHPLC-MS/MS instrument, settled in positive electrospray ionization. Carotid intima media thickness (IMT) was measured by the same operator at 1 cm from carotid bifurcation, averaged on three different measurements and judged abnormal if ≥0.9 mm. Cardiovascular risk scores were calculated according to the Framingham, D:A:D, ASCVD/AHA/ACC and CUORE algorithms. Multimorbidity and polypharmacy were defined as the presence of ≥3 comorbidities and ≥5 co-medications. Data are described using number (percentage) or median (interquartile ranges) and analysed through non-parametric tests; a multivariate linear regression analysis was performed including variables with bivariate p-values <0.05. All analysis were performed through SPSS version 24 for Mac (IBM Inc). 6 month TMAO serum concentrations were compared to baseline values (in the intervention study) through Wilcoxon's test.

Results

In the cross-sectional study we enrolled 175 participants whose demographic and clinical characteristics are shown in Table 1. They were mostly male (79.4%) of European ancestry (79.4%) with a median age of 50 years (44-55). We observed good immunovirological features with the majority patients showing undetectable plasma HIV RNA and CD4 cell count above 500/mm3. Despite a high prevalence of active smokers (42.7%) other CV risk factors were not so common (with 13.2% showing hypertension) thus generating median 10-year CVR scores around 5% (4-8.6% according to the different algorithms). Comorbidities were observed in several participants and MM was present in 12.5%.

Median serum TMAO concentrations were 165 ng/mL (103-273). They were higher in older individuals (rho=0.164, p=0.031), in participants with higher serum creatinine (rho=0.181, p=0.030), in non-smokers (205 vs. 153 ng/mL, p=0.007), in those receiving <3 antiretrovirals (244 vs. 157 ng/mL, p=0.009) and >5 co-medications (250 vs. 144 ng/mL, p=0.004) and in those with multimorbidity (rho=0.343, p=0.002). No significant association was observed with antiretroviral or concomitant medications, with the exception of higher TMAO levels in patients receiving acetyl-salicylic acid (n=6; 299 vs. 146 ng/mL, p=0.004). A bivariate

correlation was observed between CVR scores and TMAO serum levels (with the exception of the ASCVD algorithm and with the highest correlation with the D:A:D score): patients presenting higher D:A:D risk score strata had higher TMAO concentrations (143 vs. 144 vs. 265 ng/mL, p=0.018). Less drug regimens were more commonly used in patients with high CVR (28.5 vs. 10.1% in the low CVR strata) thus potentially explaining the effect observed at univariate analysis. At linear logistic regression (adjusting for age) multimorbidity (presenting ≥3 comorbidities) was independently associated with higher TMAO concentrations (p=0.015) (Fig. 1 left).

IMT has been measured in 84 participants and it was 0.85 mm (0.71-1.21); 39 participants (46.4%) had IMT measurements above the limit of 0.9 mm or carotid plaques. A trend towards higher TMAO concentrations in patients with abnormally high IMT values was observed (166 vs. 129 ng/mL, p=0.087) (Figure 1 right).

In the prospective probiotic supplementation study in Rome 25 participants were enrolled. They were mostly male (18, 72%) and with a median age of 47 years (42-52); 21 (84%) had a plasma HIV RNA <50 copies/mL and median CD4 cell count was 580 (521-716). Clinical and therapeutic characteristics did not differ from the whole cohort (data not shown). TMAO levels at baseline [95 ng/mL (57-180)] and 24 weeks later [166 ng/mL (90-259)] did not significant differ (Wilcoxon paired p 0.220) (Figure 2).

Discussion

In this cohort of PLWH under cART we measured TMAO serum concentrations and report their association with several clinical and therapeutic features; we also report no effect of 6-month high-dose probiotic supplementation on TMAO serum concentrations.

TMAO serum levels here observed are similar to those observed in other studies; the only exception is the study by Srinivasa et al. where serum TMAO concentrations were low (0.7 μM vs. 1.2-5.7 μM in the others) and not associated to study outcomes.[6] We observed higher TMAO concentrations in patients with higher risk of cardiovascular complications, presenting with multimorbidity and polypharmacy. The use of several co-medications has been associated with unfavourable outcomes in elderly HIV-negative individuals and increasing data have being reported in ageing HIV-positive individuals.[17] In our cohort the association between MM and high TMAO levels was significant even after adjusting for age, thus suggesting that it may represent a marker of individual frailty or that nutrition may be a relevant factor for both features.

In this setting the relative contribution of high TMAO levels need to be demonstrated: however being a key factor in atherosclerosis and, less certainly, in renal damage, it may be take into account when planning preventive intervention in high risk patients. The trend towards higher carotid intima media thickness in patients with higher TMAO is in line with previous works in PLWHs: the recent report of longitudinal increase in carotid atherosclerosis in patients with high serum TMAO levels suggest its use as a surrogate biomarker in order to plan tailored interventions. The lack of association with single comorbidities, antiretroviral drugs or comedications support the complex mechanisms in TMAO formation that include diet, gut microbiome and hepatic oxidation.

Two unexpected associations were described. The first one was the observation of higher TMAO levels in patients receiving less than three antiretroviral drugs. This was largely driven by a higher CVR in these individuals that were treated with two-drug regimens in order to avoid NRTIs associated with significant side effects (such as tenofovir disoproxil fumarate and abacavir). The second one, the observation of lower TMAO levels in HIV-positive smokers is, to the best of our knowledge, without a clear explanation. Yet smoke has been associated with changes in intestinal microbiome and local immunity that may affect TMAO formation.[18] Finally we observed no significant change in TMAO levels with six months of high-dose probiotics supplementation; despite the small sample size this suggests that dietary interventions may be more effective in contemporarily affecting TMAO and other pathways supporting atherosclerosis.[19] In HIV-negative participants a higher adherence to a Mediterranean diet (and a higher consumption of plant foodstuffs) was associated with beneficial microbiome-related metabolomic profiles with lower serum and urinary TMAO levels.[20] Yet beneficial immunological, inflammatory and neurocognitive changes were observed with the high dose probiotic supplementation in this cohort thus suggesting an effect on different gut microbes or metabolic pathways.[16,21]

In conclusion, TMAO serum levels in PLWH were associated with multimorbidity, higher cardiovascular risk and subclinical atherosclerosis and were not affected by six months of high-dose probiotics supplementation.

Declarations

Funding: The study was funded through an Investigator initiated research grant received from ViiV healthcare.

Competing Interests: AC, SB and GDP received research grants from ViiV and consultancy fees/speakers' honoraria from Gilead, MSD, Janssen-Cilag and ViiV. All other authors have

no conflict to declare.

Ethical Approval: Yes, Comitato Etico Interaziendale Ospedale Molinette, 7/644/17

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Tables

	number or median	% or IQR
Age: years	50	44-55
Male gender: n (%)	139	79.4%
European ancestry: n (%)	139	79.4%
CD4+ T lymphocyte: Cells/uL	564	389-760
CD4/CD8 ratio:	0.9	0.5-1.1
CD4+ T lymphocyte nadir: Cells/uL	190	85-331
Plasma HIV RNA <50 copies/mL	156	90.7%
Active smokers*	64	42.7%
Hypertension*	20	13.2%
Type 2 Diabetes*	9	5.9%
Dyslipidemia*	13	8.6%
Number of comorbidities:*	1	1-2
Multimorbidity:* n(%)	19	12.5%
10 year Framingham CVR: %	8.6	4.6-17.5
5 year D:A:D CVR: %	3.2	1.2-6.8
10 year ASCVD CVR: %	5.1	2.4-12.2
10 year CUORE CVR: %	4.0	1.6-10
ARV classes: number of participants using		
NRTIs	142	81.1%
NNRTI	50	28.6%
PI	89	50.9%
INSTI	77	44%
R5-inhibitors	7	4%
Two drug regimens	26	14.9%
INSTI + PI	18	10.2%
Lamivudine + PI	4	2.3%
Lamivudine + INSTI	1	0.6%
NNRTI + INSTI	2	1.1%
NNRTI + PI	1	0.6%
Number of comedications	2	1-4
Polypharmacy: n (%)	17	11.2%

*detailed information were available in 151 participants

Table 1. Participants' characteristics. Data are expressed as numbers (percentage) or median values (interquartile ranges). "CVR", cardiovascular risk; "D:A:D", Data collection on Adverse Effects of Anti-HIV Drugs Study; "ASCVD", Atherosclerotic Cardiovascular Disease from the American Heart Association/American College of Cardiology; "ARV", antiretroviral; "NRTIs", nucleos(t)ide reverse transcriptase inhibitors; "NNRTI", non-nucleoside reverse transcriptase inhibitors; "PI", protease inhibitors; "INSTI", integrase strand transfer inhibitors.

Figures

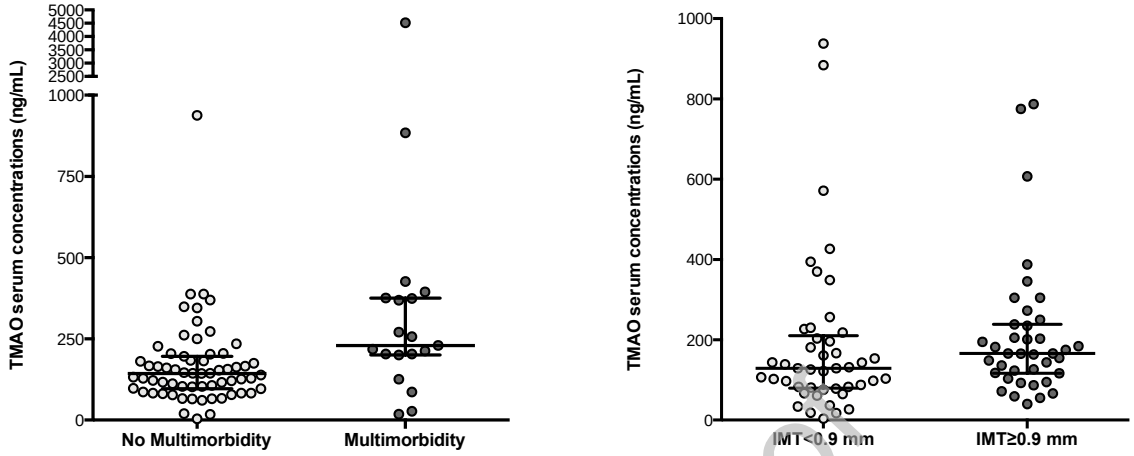

Figure 1. Serum Trimethylamine-N-oxide Concentrations according to the presence/absence of multimorbidity (left) or abnormal carotid intima media thickness (right).

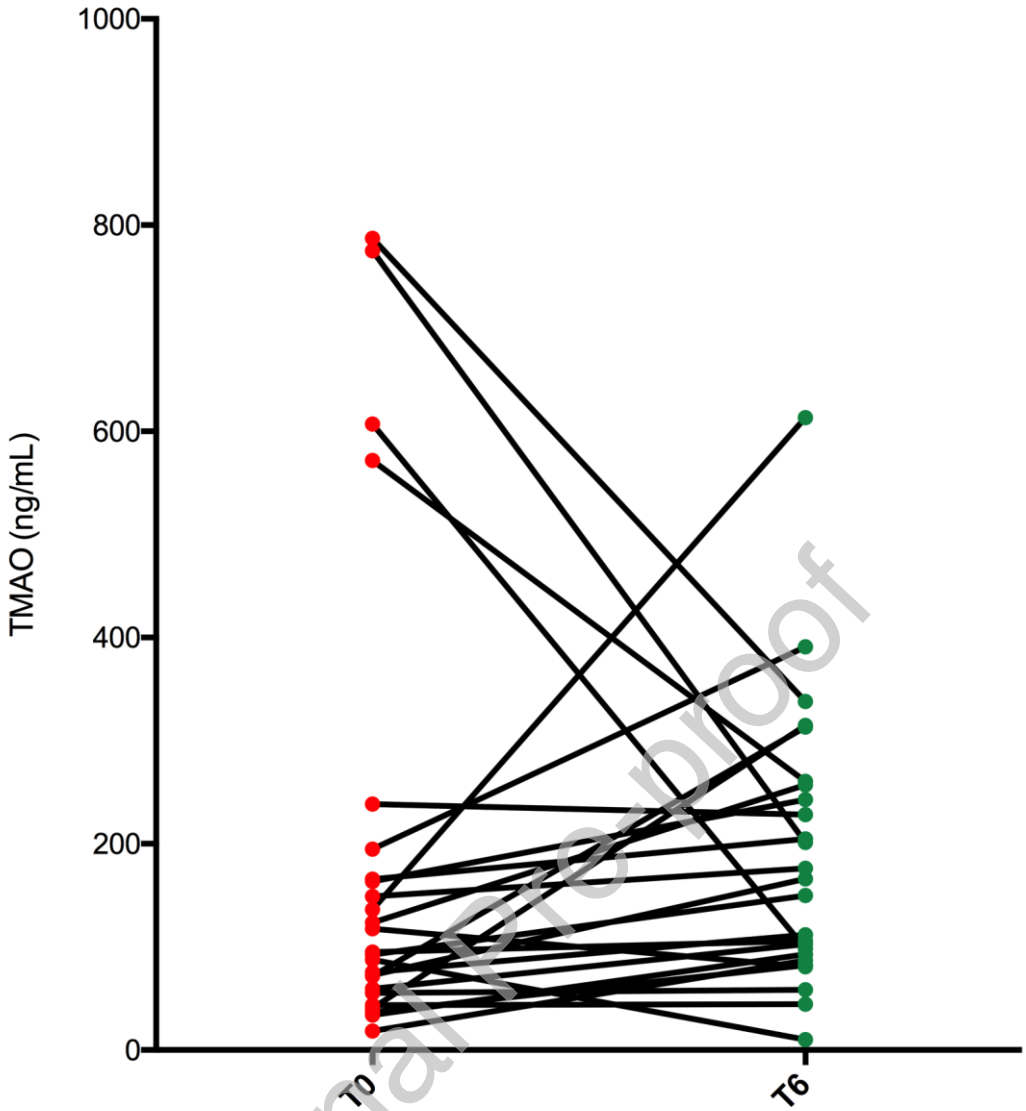

Figure 2. Serum Trimethylamine-N-oxide Concentrations at baseline and six months after probiotics supplementation in 25 participants.