The microbial metabolite TMAO in association with inflammation and microbial dysregulation in three HIV cohorts at various disease stages

Running head: TMAO, inflammation and dysbiosis in hiv-1

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Introduction

Despite efficient antiretroviral treatment (ART) HIV-1-infected individuals are at increased risk of cardiovascular disease (CVD) ^[1], where chronic immune activation and inflammation secondary to HIV-induced translocation of microbial products and metabolites are potential contributory factors. Recent evidence suggests a linkage between compositional changes of intestinal microbiota and dysregulation of the gut immune barrier function in HIV ^[2-5]. Of note, several studies have observed decreased microbiome diversity in HIV-patients that is directly correlated with increased immune activation ^[3, 6-8]. Trimethylamine-N-Oxide (TMAO) has recently been identified as a microbial metabolite with pro-atherosclerotic properties ^[9, 10]. Elevated levels of TMAO have been associated with increased cardiovascular risk ^[9-14]. TMAO is the primary metabolite of Trimethylamine (TMA) that is produced by gut bacteria and further converted to TMAO by flavin-containing monooxygenase (FMO3) in the liver ^[10, 13, 15, 16]. Diets rich in red meat, eggs and full-fat dairy products provide substrate for TMA conversion and may subsequently increase TMAO levels. Although plasma levels of TMAO are governed by diet, FMO3 enzyme activity and renal clearance, growing evidence suggests that the gut microbial composition is directly linked to circulating TMAO levels in a healthy population ^[10, 15, 17, 18]. Today, there are two main bacterial pathways generating TMA through degradation of dietary substrates in man: the CutC/CutD pathway ^[19, 20] and the Cnt A/B pathway ^[21, 22]. Notably, genetic components for TMA conversion are detected in Firmicutes, Proteobacteria and Actinobacteria, but appears to be absent in Bacteroidetes ^[20, 23]. Considering the potential role of gut microbiota in HIV pathogenesis and the demonstrated effects of TMAO on the atherosclerotic process, we hypothesized that TMAO production

would increase over time as a result of HIV- associated dysbiosis, thus presenting a link between gut microbiota, systemic inflammation and cardiovascular risk in HIV. Exploring this hypothesis we assessed levels of TMAO in two longitudinal cohorts of primary and chronic HIV-1 infected individuals before and after ART initiation in relation to systemic inflammation and microbial composition of the gut. The role of TMAO in relation to cardiovascular co-morbidity was also assessed in a cross-sectional cohort of HIV-1-infected individuals on long-term effective ART.

Methods

Study design

We studied HIV1-infected individuals from two longitudinal studies (before and after ART) ^[8, 24] (**Table 1**) and one cross-sectional study cohort ^[25] in comparison to healthy controls (**Table 2**). Circulating TMAO, markers of systemic inflammation, viral load and cellular immunity were assessed in plasma and whole blood. Additionally, in one cohort (chronic HIV-1) ^[8] the bacterial composition in stool was assessed by high-throughput 16SrRNA sequencing at baseline and after initiation of ART.

Study cohorts

Primary HIV infected cohort (PHI).

Seventeen subjects with primary HIV-1 infection (participants of the Quest study) ^[24, 26] were included with sampling within two weeks of HIV acquisition and 3-4 months after introduction of ART.

Chronic HIV-1 cohort

Twenty-two subjects were included from a study on microbiota and immune status in treatment-naïve chronic HIV-1-patients ^[8] followed for a median of 10 months (IQR 4-15) after ART initiation. Patients were recruited from the HIV outpatient clinic at Karolinska University Hospital, Stockholm, Sweden. Nine sex and age-matched HIV-1-negative individuals were included, consisting of household members and partners of the patients.

Chronic ART cohort

One hundred and one HIV-1 patients on long-term ART (median 9 years, IQR 5-15) were included from a cross-sectional study of vitamin D levels in HIV-1-infected individuals from the HIV outpatient clinic at South General Hospital, Stockholm, Sweden 2012 ^[25]. Six years follow-up data on time to first cardiovascular events (unstable angina, acute

myocardial infarction, transitory ischemic attack and stroke) were registered from medical files. HIV-1-negative healthy controls (n=30) consisting of staff at the Karolinska University Hospital were included from the same study.

As an external control, 23 sex and age-matched HIV-1 negative individuals were selected from a previously presented population-based cohort in the Stockholm region of Sweden ^[27].

Ethics:

All participants provided written informed consent and the study was performed in accordance with the Declaration of Helsinki and with regulations from Karolinska Institutet. The Regional Ethical Committee, Stockholm, Sweden reviewed and approved the study protocols and amendments (Karolinska Institutet: Dnr 98-015, 2009-1485-31-3, 2011-1383-31-3 and 244-001),

Plasma HIV-1 RNA quantification and CD4 counts

Analyses of CD4 counts and plasma HIV-1 RNA load were performed at the Karolinska University Laboratory, Stockholm, Sweden with flow cytometry and Cobas Amplicor (Roche Molecular Systems Inc., Branchburg, New Jersey, USA), respectively.

TMAO

Analysis of plasma levels of TMAO was performed by liquid chromatography/tandem mass spectrometry (LC/MS/MS) at Swedish Metabolomics Centre, Umeå, Sweden utilizing a protocol previously described ^[27] (PHI, chronic ART with controls and sex and age-matched controls), and in the laboratory of Bevital AS (www.bevital.no) (Chronic HIV-1 cohort with controls).

113 Soluble inflammation markers

114 Limulus Amebocyte Lysate (LAL) (Lonza; Basel, Switzerland) and Human sCD14
 115 Quantikine ELISA (R&D, Minneapolis, Minnesota, USA) were used to measure plasma
 116 lipopolysaccharide (LPS) and soluble CD14 (sCD14), as described [8, 25]

117 LPS binding protein (LBP) (Hycult Biotech, Uden, The Netherlands), and D-Dimer
 118 (Technoclone, Vienna, Austria) were determined in plasma samples by ELISA according to
 119 the manufacturers' instructions.

120 Microbiota analysis.

121 Stool samples were collected and stored at -80 °C until further use. Microbiota composition
 122 was determined by 16S rRNA sequencing targeting V3–V4 region of 16S gene using
 123 Illumina MiSeq and analysed using the established bioinformatics pipeline as described
 124 previously [8]. A post hoc analysis was performed with a special focus on gut bacteria with
 125 the propensity to produce TMA (defined by in-vitro studies and/or genomic presence of the
 126 TMA-producing enzymes CutC/D and Cnt A/B)^[19, 20, 22, 23, 28, 29] (**Table 3**).

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128 Statistical analyses

129 Data were expressed as median (10th-90th percentile or range) or *n* (percentage) as
 130 appropriate. Univariate comparisons between groups were performed using Mann Whitney
 131 U test for continuous variables. Wilcoxon matched-pairs signed rank test was used to
 132 evaluate differences between time-points. Spearman correlation was applied to explore
 133 associations between continuous variables.

Determinants of TMAO (baseline, follow-up and delta values) in the PHI and chronic HIV-1 cohorts were assessed by use of repeated measure ANOVA (individually controlling for male gender, Caucasian origin, MSM and PI use) and simple linear regression (age, CD4 count, CD4/CD8 ratio, viral load and inflammatory markers). Multiple linear regression was used to assess determinants of TMAO in the chronic ART-cohort (adjusting for age, MSM, eGFR, years with HIV, ART years, and years with undetectable viral load). Cox proportional hazard regression was used to assess if baseline TMAO predicted risk of cardiovascular events.

$P < 0.05$ was considered as statistically significant. Statistical analyses were performed using statistical software SSPS, version 23 (IBM, USA).

Results

Patient characteristics and demographics

The median age was similar in the PHI and the chronic HIV-1 cohort with healthy controls (**Table 1**). The PHI cohort was dominated by men who have sex with men (MSM) of Caucasian origin, whereas the chronic HIV-1 cohort had a more heterogeneous distribution of gender, ethnic origin and sexual orientation (**Table 1**). All patients in the PHI cohort were started on protease inhibitors (PI). In the chronic HIV-1 cohort 65% were started on a PI and 35% on a NNRTI based regimen (**Table 1**). Subjects in the chronic ART cohort were significantly older than their healthy controls ($p=0.001$) and were predominantly MSM of Caucasian origin (**Table 2**). Twenty-four percent of the HIV-1 infected individuals were on a PI and 54% on a NNRTI containing regimen (**Table 2**).

TMAO levels were decreased in treatment-naïve HIV-1-individuals and increased to normal levels with treatment

Levels of TMAO were lower in untreated HIV-individuals and increased significantly after ART, both in primary infected (median 1.28 vs 2.30 $\mu\text{M/L}$, $p=0.040$) and chronic HIV-infected individuals (median 2.09 vs 4.47 $\mu\text{M/L}$, $p<0.001$) (**Fig 1a**). Despite increased TMAO levels at follow-up in the chronic HIV-1 cohort, levels did not differ significantly from the corresponding healthy controls ($p=0.075$) (**Fig 1a**).

Notably, chronic HIV-1-individuals with longstanding ART had similar levels as their healthy controls ($p=0.89$). To account for the difference in age between the chronic ART cohort and their controls we introduced an age and sex-matched, HIV-1 negative control group (**Table 2**). Compared to the age and sex-matched controls, chronic ART-individuals had significantly lower TMAO levels ($p=0.008$) and higher eGFR ($p<0.001$) (**Fig 1a**).

However, a one-way ANCOVA conducted to compare TMAO levels found no significant difference in mean TMAO levels ($F(1, 106) = 0.563, p = 0.455$) between the two cohorts when controlling for eGFR.

Plasma TMAO and correlations with bacterial composition in stool

Spearman correlation analysis between bacterial taxa and TMAO levels revealed several interesting findings at different taxonomical levels. Thus, at baseline we found that TMAO levels were inversely correlated with Bacteroidetes (Rho: -0.62, $p = 0.002$), and positively correlated with Firmicutes (Rho: 0.65, $p = 0.001$). Additionally, baseline plasma TMAO levels were inversely correlated with the genera *Alistipes* (Rho: -0.47, $p = 0.029$), *Bacteroides* (Rho: -0.58, $p = 0.004$), *Odoribacter* (Rho: -0.60, $p = 0.003$), *Parabacteroidetes* (Rho: 0.52, $p = 0.014$), and positively correlated with *Prevotella* (Rho 0.44, $p = 0.039$); all members of the Bacteroidetes phyla. Furthermore, the genus *Sutarella*, a member of the Proteobacteria phylum was inversely correlated (Rho: -0.64, $p = 0.001$), (**Fig 1b**). However, at follow-up these associations were no longer present and only a positive association between TMAO and *Dialister* (Rho: 0.64, $p = 0.007$), member of the Firmicutes phylum, was observed (**Fig 1b**).

Relative abundance of TMA producing bacteria did not change with ART initiation and did not correlate with circulating TMAO or systemic inflammation.

Since plasma levels of TMAO are partly regulated by TMA producing gut-bacteria, we further assessed the relative abundance of bacteria with the propensity to produce TMA before and after initiation of ART at genus level (**Table 3**). The relative abundance of TMA producing bacteria did not significantly increase after ART, but remained similar to

controls (data not shown). Moreover, changes in the relative abundance of TMA producing bacteria over time (Δ TMA=follow-up – baseline levels) did not correlate with the observed changes in circulating TMAO nor with changes in circulating sCD14, LPS, LBP, and D-dimer (data not shown).

Baseline CD4/CD8 ratio, but not viremia, inflammation or use of ART, predicted increased levels of TMAO at follow-up

To assess if immune status, HIV viremia and inflammation predicted TMAO levels at baseline and follow-up we assessed the individual cohorts by repeated measure ANOVA and linear regression analysis.

In the PHI and chronic HIV-1 cohorts, TMAO levels did not associate with male gender, Caucasian origin or MSM status. Age, viral load (VL), CD4 count or CD4/CD8 ratio did not predict TMAO levels in either cohort, nor did they associate with change in TMAO levels (Δ TMAO: follow-up–baseline) with one exception: In the chronic HIV-1 cohort we found that higher baseline CD4/CD8 ratio predicted increased Δ TMAO ($B=11.92$, $p=0.016$). None of the inflammatory markers (sCD14, LPS, LBP, D-dimer) predicted TMAO levels or Δ TMAO in the chronic HIV-1 cohort. Moreover, in the chronic HIV-1 cohort, neither use of PI nor NNRTI predicted follow-up TMAO levels, or Δ TMAO.

In the chronic ART cohort, multiple regression analysis demonstrated that neither preART-VL, CD4 count, CD4/CD8 ratio, nor baseline CD4 count or CD4/CD8 ratio predicted TMAO, when controlling for age, MSM, eGFR, years with HIV, years of ART, and years with undetectable viral load. Similarly, markers of baseline inflammation (hsCRP, sCD14

and LPS) did not predict TMAO levels. Nor did use of PI or NNRTI have a significant effect on TMAO levels.

Higher TMAO levels did not infer a significantly increased risk for cardiovascular events

During 6 years follow-up, there were 6 registered cardiovascular events in the chronic ART cohort. Although the study was not powered to predict cardiovascular events, the highest tertile of TMAO ($> 4.93 \mu\text{M/L}$) was non-significantly associated with a 2.8-fold risk (HR 2.76; 95% CI 0.29-26.70, $p=0.38$).

Lack of increase in plasma TMAO levels after ART initiation associated with loss of Bacteroidetes and increased LPS levels.

Intrigued by the strong association between baseline CD4/CD8 ratio and increased TMAO levels after ART initiation in the chronic HIV-1 cohort we further assessed the cohort with regards to changes in TMAO-levels.

We found that increased TMAO levels after ART initiation ($\Delta \text{TMAO} > 1 \mu\text{M/L}$), were present in a subgroup of 10 HIV individuals whereas no increase ($\Delta \text{TMAO} < 1 \mu\text{M/L}$) was observed in 11 individuals (**Fig 2a**). Individuals with increased TMAO levels had significantly higher CD4/CD8-ratio at baseline (**Fig 2b**) compared to subjects with no increase. The two groups had a similar age and sex distribution but in the group with increased TMAO-levels more individuals were treated with PI (80%), compared to the group with no increase (50%) ($p=0.210$). Based on the hypothesis that lower CD4/CD8 ratio may reflect a more dysregulated gut microbiota, we compared the microbial composition in individuals with increase versus no increase in ΔTMAO levels in relation to corresponding healthy controls.

We found that treatment-naïve HIV-individuals were similar to controls in relative abundance of Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria. After ART initiation HIV-individuals with increased Δ TMAO remained stable in their composition of gut microbiota, whereas individuals with no increase demonstrated a significant reduction in relative abundance of Bacteroidetes ($p=0.023$) and a corresponding non-significant expansion of Firmicutes and Proteobacteria compared to controls (**Fig 2c**). Moreover, after ART initiation HIV-individuals with no increase had significantly higher levels of LPS compared to individuals with increased Δ TMAO ($p=0.01$) (**Fig 2d**). Of note, LPS levels correlated inversely with relative abundance of Bacteroidetes at follow-up (Rho -0.90, $p=0.037$).

Discussion

Our study explored circulating TMAO-levels in primary and chronic HIV-1 before and after ART and in HIV-1-infected individuals on long-term effective ART. In support of our original hypothesis we found that TMAO levels increased over time with ART. However, regardless of treatment length, TMAO levels in HIV-1-infected individuals remained similar to healthy controls and were not significantly affected by treatment regime, immune status or degree of systemic inflammation. Notably gut dysbiosis was more pronounced in patients without increase in TMAO levels after ART initiation.

The observation that HIV-1 infected individuals had similar TMAO levels as healthy controls has been reported by other studies assessing circulating TMAO and CVD in HIV [30-32]. Contrary to population based studies [9-11, 13] these studies provide no conclusive evidence that elevated TMAO has a strong impact on atherosclerotic burden, prevalent CVD or adverse cardiac events in HIV-infected individuals [30-33]. Similarly, although the study was not powered to detect relation to cardiovascular events, we found no significant risk with increasing TMAO levels ($p=0.38$) in well controlled HIV-1 infected individuals on long-term ART. Furthermore, our observation suggesting that ART initiation increase TMAO levels was previously reported in a nested case-control study by Haissman et al. [31].

Why does TMAO increase after ART?

It could be argued that the effect might be related to dietary changes and the return of appetite after viral control is established through treatment. However, this argument remains purely hypothetical since we lack data on diet and general well-being. Moreover,

fatigue and loss of appetite is more pronounced in advanced HIV disease that is not clinically represented in the two study cohorts at baseline.

It could also be argued that ART favours a more TMA prone microbiome. However, we could not find evidence supporting an expansion of TMA producing bacteria after ART initiation in chronic HIV-1 infected individuals. Notwithstanding, the data concerning TMA production of gut bacteria are not complete and any such analysis should be interpreted cautiously. Notably, phylogeny appears to be a poor predictor of TMA production [29].

Despite the lack of association between TMA-producers and systemic TMAO-levels after ART we found a positive correlation between plasma TMAO and Firmicutes at baseline and at follow-up and an inverse correlation with members of Bacteroidetes at baseline with the exception of *Prevotella*. Our observations support published data demonstrating a widespread TMA production propensity among members of Proteobacteria and Firmicutes that appears to be missing in Bacteroidetes [19, 20, 22, 23, 29]. Further, several studies have observed a positive association between *Prevotella* and TMAO levels [10, 34, 35], but the underlying mechanisms remain to be elucidated.

Furthermore, although there is no established link between FMO3 and HIV-1 it could be argued that FMO3 activity might be affected by ART. Of note, a recent publication presented evidence that FMO3 was involved in metabolising F18, a new experimental NNRTI [36]. To our knowledge no studies have assessed the role of FMO3 in metabolising PI, or commercially available NNRTI. Whereas Haissman et al. observed that PI, but not

NNRTI, was associated with increased TMAO levels following ART initiation^[31], we found no significant impact of choice of PI or NNRTI on TMAO levels at follow-up (p=0.17) or changes in TMAO levels (p=0.21) in the chronic HIV-cohort. Nor did PI (p=0.99) or NNRTI (p=0.59) predict TMAO levels in the chronic ART cohort. However, although no significant association between PI treatment and TMAO levels was established in the chronic HIV-1 cohort, PI was the dominating ART in the group with increased TMAO levels at follow-up.

Finally, it is possible that the decreased TMAO levels observed in treatment-naïve HIV-1 infected individuals relate to down-regulation of the TMA converting enzyme FMO3 by gut related inflammation, as observed in a model of LPS-induced sepsis in mice^[37]. Indirect support of this hypothesis is provided by a recent clinical study which reported reduced TMAO levels in IBD patients with active disease compared to inactive disease and healthy controls^[38]. Moreover, we found a more pronounced loss of Bacteroidetes and higher levels of LPS in chronic HIV-1 infected individuals who did not increase their TMAO-levels after ART despite viral control. Loss of Bacteroidetes and increase in Proteobacteria has been linked to increased microbial translocation and systemic inflammation in both HIV and inflammatory bowel disease (IBD)^[39, 40]. Finally, chronic HIV-1-infected individuals with no increase in TMAO had lower CD4/CD8 ratios at baseline, suggesting that lack of gain in TMAO might serve as a surrogate-marker for a more dysregulated gut immune barrier function that prevails despite effective ART.

FMO3 downregulation may also be attributed to reduction of the bile acid-activated Farnesoid X receptor (FXR). FXR modulates bile acid homeostasis and inflammation in the

gut^[41-43] and is involved in inducing FMO3 in mice-models^[16, 34, 44]. Importantly, in HIV-patients downregulation of FXR expression has been observed in monocytes and hepatocytes despite functional ART^[45, 46]. However, the link between FXR-expression and TMAO-levels is not yet established in clinical cohorts.

The strength of our study is the consistency in trend observed both in longitudinal and cross-sectional study cohorts representing both primary HIV and individuals with chronic HIV infection at different treatment length and matching healthy controls. Moreover, longitudinal data on microbiome composition provided a unique opportunity to assess its relation to TMAO. The limitations of the study include the relatively low number of patients in each cohort, lack of power to study cardiovascular event rate, lack of information regarding MSM status in the control cohorts and lack of dietary data. However, the healthy controls of the chronic HIV-1 cohort consisted of household members and partners of the patients indicating similar sexual orientation and food intake.

To conclude, although we cannot exclude that elevated TMAO may contribute to cardiovascular risk in general, our data do not support the hypothesis that TMAO is a significant link between gut dysbiosis, inflammation and cardiovascular risk in HIV-1-infected individuals. TMAO levels appear to be disparately regulated by HIV-infection, ART and microbiota, thus limiting its role as a cardiovascular risk marker in HIV-infected individuals.

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356 Analysed the data: C.M, U.N, P.S, M.T, P.N, P.B. Wrote the paper: C.M. Reviewed and

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Fig 1. Distribution of plasma TMAO levels and correlation to bacterial genera in faecal samples.

TMAO levels in primary infected and chronic HIV-1-infected individuals followed from baseline (BL) to follow-up (FU) after 4-10 months ART treatment, compared with HIV-1-infected individuals on long-term ART and HIV-1-negative controls (a). *P* values are generated by Wilcoxon signed-rank (BL-FU) and Mann-Whitney *U* test. Spearman correlation heat map of plasma TMAO and relative abundance of genera in faecal sample of HIV-1-patients at baseline (b). Only 15 patients at follow-up due to missing faecal samples. Only bacterial genera with more than 0.001% abundance present in at least 75% of all patient samples were included. * *P* value <0.01, ** *P* value <0.001.

515 **Fig. 2. Differential change in plasma TMAO levels after ART initiation (Δ TMAO:**
516 **follow-up – baseline <1) (a) in relation to CD4/CD8 ratio at baseline (b), microbial**
517 **composition (c), and LPS levels at follow-up (d).** Number of participants is based on
518 available samples. P values are generated by Wilcoxon signed-rank test (a) and Mann-
519 Whitney *U* test (b, d).

520

521

Figure 1a

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Figure 1b

Figure 2

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Table 1. Patient characteristics in ART-naïve HIV cohorts

	Primary HIV-1 cohort		Chronic HIV-1 cohort		
	HIV	HIV	Controls	P-value	
Participants, <i>n</i>	17	22	9		
Age, years	35 (27-53)*	38 (19-61)*	44 (28-62)*	0.425	
Male gender	15/17 (88)	14/22 (64)	5/9 (56)		
Caucasian origin	16/17 (94)	13/22 (52)	7/9 (78)		
MSM	13/17 (76)	3/15 (20)	NA		
PI initiated	17/17 (100)	13/20 (65)	NA		
NNRTI initiated	0/17 (0)	7/20 (35)	NA		
HIV RNA Log¹⁰ copies/mL	4.77 (4.00-5.59)	5.15 (3.37-6.23)	NA		
CD4, cells/μL	591 (404-993)	365 (239-530)	NA		
CD4/CD8	0.51 (0.14-1.74)	0.38 (0.21-0.75)	NA		
TMAO, μM/L	1.28 (0.99-5.13)	2.09 (0.85-8.27)	3.15 (1.24-7.03)	0.623	
sCD14, μg/mL	NA	1.39 (1.03-1.89)	1.58 (1.08-1.87)	0.588	
LPS, pg/mL	NA	215 (151-306)	285 (253-323)**	0.007	
LBP, ng/mL	NA	6.18 (5.07-8.80)	7.54 (4.89-15.2)	0.185	
D-dimer, ng/mL	NA	68.4 (0.45-684)	42.3 (0.45-146)	0.286	

Data are expressed as median (10th-90th percentile, or range*), or *n* (percentage) as appropriate. ** Data derived from 6 controls due to missing values.

MSM: men that have sex with men, ART: antiretroviral treatment, PI: protease inhibitors, NNRTI: non-nucleoside reverse-transcriptase inhibitors,

Table 2. Patient characteristics in long-term ART treated cohort

	Chronic ART cohort			Age and sex-matched controls	
	HIV	HIV-negative controls	P-value	HIV-negative controls	P-value
Participants, <i>n</i>	101	30		23	
Age, years	51 (30-60)*	42 (25-67)*	<0.001	52 (28-60)*	0.357
Male gender	97/101 (96)	100/100 (100)	0.573	22/23 (96)	>0.999
Caucasian origin	100/100 (100)	100/100 (100)	NA	23/23 (100)	NA
MSM	92/100 (92)	NA	NA	NA	NA
<u>Patient history</u>					
HIV years	15 (4-25)	NA	NA	NA	NA
ART years	9 (3-17)	NA	NA	NA	NA
Pre-ART CD4	222 (31-348)	NA	NA	NA	NA
Pre-ART HIV RNA, Log¹⁰ copies/mL	5.4 (4.5-6.0)	NA	NA	NA	NA
Years undetectable VL (<20 copies/mL)	6 (2-14)	NA	NA	NA	NA
<u>Baseline data</u>					
Use of PI	24/101 (24)	NA	NA	NA	NA
Use of NNRTI	55/101 (54)	NA	NA	NA	NA
HIV RNA copies/mL	< 20 (<20)	NA	NA	NA	NA
CD4, cells/μL	653 (409-1024)	NA	NA	NA	NA
CD4/CD8	0.81 (0.46-1.37)	NA	NA	NA	NA
TMAO, μM/L	3.96 (2.01-12.52))	4.30 (1.36-27.5)	0.893	6.43 (2.81-12.58)	0.010
hsCRP, mg/L	1.20 (0.32-6.10)	0.77 (0.32-2.28)	<0.001	1.10 (0.40-7.18)	0.831
sCD14, μg/mL	1.70 (1.30-2.40)	1.40 (0.96-1.73)	<0.001	NA	NA
LPS, pg/mL	156 (132-183)	152 (132-184)	0.402	NA	NA
eGFR	102 (77-122)	NS	NA	84 (72-107)	<0.001

Data are expressed as median (10th-90th percentile, or range*), or *n* (percentage) as appropriate.

MSM: men that have sex with men, ART: antiretroviral treatment, VL: viral load, PI: protease inhibitors, NNRTI: non-nucleoside reverse-transcriptase inhibitors, eGFR: estimated glomerular filtration rate.

Table 3. Bacteria with the propensity to produce TMA

Phyla	Genus	Reference
Actinobacteria	<i>Mobiluncus</i> [^]	Cruden et al. 1988
	<i>Collinsella</i> ^{*^}	Falany et al. 2015, Rath et al. 2017
	<i>Corynebacterium</i> ^{*^}	Falany et al. 2015
	<i>Olsenella</i> [*]	Craciun et al. 2012 Martinez-del Campo et al. 2015. Rath et al 2017
Firmicutes	<i>Anaerococcus</i> ^{*^}	Craciun et al. 2012, Romano et al. 2015, Falany et al. 2015, Rath et al. 2017
	<i>Bacillus</i> ^{*^}	Craciun et al. 2012, Romano et al. 2015, Falany et al. 2015, Rath et al. 2017
	<i>Clostridium</i> ^{*^}	Craciun et al. 2012, Martinez-del Campo et al. 2015, Romano et al. 2015, Falany et al. 2015, Rath et al. 2017
	<i>Desulfitobacterium</i> [*]	Craciun et al. 2012, Rath et al. 2017
	<i>Enterococcus</i> ^{*^}	Rath et al. 2017
	<i>Eubacterium</i> ^{*^}	Rath et al. 2017
	<i>Staphylococcus</i> [^]	Robinson et al. 1952
	<i>Streptococcus</i> ^{*^}	Rath et al. 2017
Proteobacteria	<i>Acinetobacter</i> ^{*^}	Zhu et al. 2014, Falany et al. 2015, Rath et al. 2017
	<i>Citrobacter</i> [*]	Zhu et al. 2014, Falany et al. 2015, Rath et al. 2017
	<i>Desulfovibrio</i> ^{*^}	Craciun et al. 2012, Rath et al. 2017
	<i>Echerichia</i> ^{*^}	Zhu et al. 2014, Denby et al 2015, Martinez-del Campo et al. 2015, Romero et al. 2015, Falany et al. 2015
	<i>Enterobacter</i> ^{*^}	Craciun et al. 2012, Falany et al. 2015, Rath et al. 2017
	<i>Klebsiella</i> [*]	Zhu et al. 2014, Falany et al. 2015, Rath et al. 2017
	<i>Proteus</i> [*]	Craciun et al. 2012, Martinez-del Campo et al. 2015, Romano et al. 2015, Falany et al. 2015, Rath et al. 2017
	<i>Providencia</i> [*]	Craciun et al. 2012, Romano et al. 2015, Falany et al. 2015, Rath et al. 2017
	<i>Pseudomonas</i> ^{*^}	Robinson et al. 1952, Falany et al. 2015
	<i>Serratia</i> ^{*^}	Rath et al. 2017
	<i>Shigella</i> ^{*^}	Rath et al. 2017
	<i>Yokenella</i> [*]	Craciun et al. 2012, Rath et al. 2017

*CutC/D and/or CntA/B gene pathway present.

[^] Detectable genera present in fecal samples from chronic HIV-1 infected individuals.