IMPACT OF HIV-RELATED GUT MICROBIOTA ALTERATIONS ON METABOLIC COMORBIDITIES

One sentence summary: HIV-related microbiota alterations were associated with visceral fat accumulation and metabolic comorbidities, in particular in individuals with previous severe immunodeficiency.

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Abstract

Background: We aimed to identify an HIV-related microbiota signature, independent of sexual preferences and demographic confounders, in order to assess a possible impact of the microbiome on metabolic comorbidites.

Methods: Bacterial 16S rRNA analyses were performed on stool samples from 405 HIV-infected and 111 uninfected participants of the Copenhagen Comorbidity in HIV infection (COCOMO) study. Individuals were stratified according to sexual behaviour (men who have sex with men, MSM and non-MSM).

Results: After excluding MSM-associated microbiota traits and adjusting for confounders, we identified an HIV-related microbiota signature, consisting of lower biodiversity, increased relative abundance of the bacterial clades Gammaproteobacteria and Desulfovibrionaceae and decrease in several Clostridia. This microbiota profile was associated with a 2-fold excess risk of metabolic syndrome, driven by increase in Desulfovibrionaceae and decrease in Clostridia *(Butyrivibrio*, *Coprococcus-2*, *Lachnospiraceae UCG-001* and *CAG-56)*. This association was accentuated (5-fold excess risk) in individuals with previous severe immunodeficiency, which also modified the association between HIV-related microbiota signature and visceral adipose tissue (VAT) area (p-interaction 0.012). Accordingly, HIV-related microbiota was associated with 30 cm^2 larger VAT in individuals with history of severe immunodeficiency, but not in those without.

Conclusion: The HIV-related microbiota was associated with increased risk of metabolic syndrome and VAT accumulation, particularly in individuals with previous severe immunodeficiency, driven by increased Desulfovibrionaceae and lower abundance of several Clostridia. Our findings suggest a

potential interplay between HIV-related microbiota, immune dysfunction and metabolic comorbidities. Interventions targeting the gut microbiome may be warranted to reduce cardiovascular risk, particularly in individuals with previous immunodeficiency.

Introduction

People living with HIV (PLWH) have reduced life expectancy [1], partly due to cardiovascular disease and associated metabolic disorders [2]. Accordingly, PLWH have increased risk of developing abdominal obesity and metabolic syndrome, even in individuals well-treated with combination antiretroviral therapy (cART), and independently of demographic and lifestyle factors [3].

While the gut microbiota has been suggested to contribute to cardiometabolic disorders in the uninfected population [4], the determinants of this association are unclear. Disease-associated alterations in microbiota composition, microbial metabolites, disruption of the gut barrier and microbial toxins such as lipopolysaccharides (LPS) have all been associated with the development of metabolic disorders, as reviewed in [4].

The potential association between HIV infection and gut microbiota alterations has been investigated in several studies, with conflicting results [5–11]. Both reduced and increased microbiota diversity have been reported in PLWH, as presented in [12]. A shift from a *Bacteroides*-enriched to a *Prevotella*-enriched phenotype was reported in several studies [5,13], but has later been linked to sexual practice, particularly men who have sex with men (MSM) [13]. In subsequent studies controlling for MSM, other microbiota traits have been associated with HIV, in particular increased proteobacteria and reduced clostridia (reviewed in [14]). Nevertheless, adequately powered studies with matched control populations and control of confounders including sexual behaviour are of utmost importance in order to robustly identify an HIV-related microbiota profile and its possible clinical consequences.

In the present study we aimed to: i) establish an HIV-related microbiota profile, independent of sexual behaviour and other relevant confounders; ii) investigate possible associations between the

82 HIV-related microbiota profile and metabolic comorbidities in a large population from the
83 Copenhagen Comorbidity in HIV infection (COCOMO) study.

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Methods

Study population

The COCOMO study is a longitudinal study aiming to assess the burden of non-AIDS comorbidities in PLWH. All PLWH >18 years old were invited to participate in connection with regular outpatient visits at Department of Infectious Diseases Rigshospitalet and Hvidovre Hospital. In total, 1099 individuals were included in the COCOMO study, constituting approximately 40% of PLWH in the Copenhagen area. Procedures for recruitment and data collection have been described elsewhere [15]. All COCOMO participants were invited to participate in the present study and 405 individuals with stool samples available were included. In addition, 90 uninfected controls from the general population were recruited in the COCOMO study. Of these, 68 individuals with stool samples available were included in the present study. Individuals from the general population were recruited in connection with their participation in a regionwide population research study [16].

Finally, 43 uninfected individuals with MSM behaviour with stool samples available were recruited from the Infectious Diseases Department, Rigshospitalet, in connection with initiation or continuation of Pre-Exposure Prophylaxis (PrEP) treatment. Inclusion criteria for PrEP were self-reported MSM-behaviour, negative HIV test and age ≥18 years.

Ethical approval was obtained by the Regional Ethics Committee of Copenhagen (H-15017350). Written informed consent was obtained from all participants.

Clinical assessments

History of antibiotics use three months preceding stool samples collection were uniformly collected using identical questionnaire in all participants.

Clinical data were only available in PLWH participants.

Information about participants´ demographics, smoking, diet and medication were collected using structured questionnaire, and data regarding HIV-related factors were obtained from review of medical charts [15].

All clinical examinations were performed by trained clinic staff as previously described [15]. Non-fasting venous blood was collected and analyzed for HDL-C, triglycerides, and glucose at Herlev Hospital, Copenhagen [3,15].

Stool sample collection and processing

At study inclusion, participants were instructed to collect stool samples using a standardized sampling device and Stool Collection Tubes with DNA Stabilizer (Stratec Molecular GmbH, Germany). Samples were frozen at -80°C upon arrival and eventually shipped on dry ice to Oslo for microbiota analyses. Stool DNA was extracted using the PSPSpin Stool DNA-Plus Kit (Stratec Molecular GmbH, Germany) following the manufacturer's protocol, slightly modified by adding a bead-beating step, as described in [17].

Library preparation and sequencing

DNA libraries were prepared as described in [18]. Briefly, libraries were generated from PCR amplicons targeting the hypervariable regions V3 and V4 of the 16S rRNA gene, and using dual-indexed universal primers 319F (forward) and 806R (reverse) along with Phusion High-Fidelity PCR Master mix m/HF buffer (Thermo Fisher Scientific, USA). Cleaning and normalization of PCR products were performed using the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, USA). Quality control and quantification of pooled libraries were performed using Agilent Bioanalyzer

(Agilent Technologies, USA) and Kapa Library Quantification Kit (Kapa Biosystems, London, UK). Sequencing was performed at the Norwegian Sequencing Centre (Oslo, Norway), applying the Illumina MiSeq platform and v3 kit (Illumina, San Diego, CA, USA), allowing for 300bp paired-end reads.

Bioinformatics

Paired-end reads were filtered for Illumina Universal Adapters and PhiX, demultiplexed, quality trimmed and merged using bbduk 38.25, je 1.2, cutadapt 1.18 and bbmerge. Denoising to ASVs (amplicon sequence variants), taxonomic classification and filtering of contaminants and rare ASVs were done with QIIME2 version 2018.8. Alpha diversity and all further analyses were performed on a rarefied (subsampled) dataset with an ASV count of 6247 per sample. Further details are given in Supplementary methods.

Soluble CD14 and LPS binding protein (LBP) measurements

Plasma levels of soluble CD14 and LBP were measured with enzyme-linked immunosorbent assay (ELISA), as described in Supplementary methods.

Visceral and subcutaneous adipose tissue

Visceral (VAT) and subcutaneous (SAT) adipose tissue area were measured at the level of the 4^{th} lumbar vertebra using 320-multidetector scanner (Aquilion OneViSION Edition, Canon, Japan) in a single rotation (275ms). A detailed description of the scanning protocol is reported in [19].

Statistical analyses

Continuous variables were reported as mean and standard deviation (SD) and categorical variables as frequency and percentage (%). Different groups were compared with t-tests or Mann Whitney U test for continuous data with normal or non-normal distribution, respectively, and chi square/Fisher's tests for categorical data. To identify HIV-related alterations, we compared microbial relative abundances in PLWH with MSM behaviour vs uninfected individuals with MSM behaviour and PLWH without MSM behaviour vs uninfected individuals from the general population, respectively. Differences reproduced in both these comparisons were identified as "HIV-related". P-values were adjusted for False Discovery Rate (FDR) using the Benjamini-Hochberg method for the cohort-comparisons of interest, and taxa with a subsequent q-value<0.05 were used for the HIV-related index.

An HIV-related microbiota index was calculated as previously described [20]. The following formula was used: Log_e((*sum of the relative abundances of clades upregulated in HIV infection*)/(*sum of the relative abundances of clades reduced in HIV infection*)). High HIV-related microbiota index was defined as the highest quartile (≥ 1.145) in the whole study population. The robustness of the HIV-related microbiota index was tested in regression models adjusting for MSM behaviour, age, sex, and antibiotic use 3 months prior to stool sample collection.

Correlations of sCD14, LBP and Shannon diversity index with the HIV-related microbiota index, were tested using Spearman´s test.

Associations between the HIV-related microbiota index and pre-defined outcomes were tested using logistic *a priori* defined regression models adjusted for age, sex, geographical origin

(Scandinavian, other EU, Middle-East and Indian sub-continent, other), smoking, BMI, self-reported physical activity (inactive, moderately inactive, moderately active, very active), and MSM behaviour.

Within the HIV-related microbiota index, bacteria that were more/less prevalent in individuals with metabolic syndrome were identified using Wilcoxon Rank sum test.

Associations between the HIV-related microbiota index, metabolic syndrome, VAT and SAT were also stratified according to previous immunodeficiency: presence of previous AIDS defining events (n = 75), CD4 nadir strata (<50 cells/µl, n = 64; 50-199 cells/µl, n = 98; and ≥200 cells/µl, n = 234), and any previous severe immunodeficiency (history of AIDS and/or CD4 nadir < 50 cells/µl, n = 105).

In sensitivity analyses the association of the HIV-related microbiota index and metabolic syndrome was assessed after further adjustment for dietary habits, collected using structured questionnaires regarding weekly consumption of beef, poultry, vegetables, fruit, and fat used in food preparation.

All statistical analyses were performed using R statistical software version 3.4.1.

Outcome definitions

Metabolic syndrome was defined as three or more of the following:(1) waist circumference ≥ 94cm in men and ≥ 80cm in women, (2)SBP ≥ 130mmHg and/or DBP ≥85mmHg and/or antihypertensive treatment, (3)non-fasting plasma triglyceride ≥ 1.693mmol/l, (4)HDL ≤ 1.036mmol/l in men and ≤ 1.295mmol/l in women, (5)self-reported diabetes and/or antidiabetic treatment and/or plasma glucose ≥11.1mmol/l, as previously reported [3].

Results

HIV infection and MSM status have opposing effects on gut microbiota diversity

Demographic characteristics of the populations and HIV-specific factors are depicted in Table 1. Microbial diversity (Shannon index) was lower in PLWH MSM compared to uninfected MSM ($p<0.01$), and PLWH non-MSM had lower Shannon diversity than uninfected controls from the general population ($p<0.001$, Fig. 1). In contrast, among PLWH, Shannon diversity was higher in MSM than non-MSM ($p<0.001$). Accordingly, in regression analysis, HIV infection was associated with lower diversity (adjusted β -0.51 [-0.67; -0.34], $p<0.001$) and MSM behaviour with higher diversity (adjusted β 0.19 [0.02;0.37], $p=0.039$) after adjusting for demographic confounders and antibiotic use three months preceding stool sample collection. These results were consistent when considering observed number of operational taxonomic units (OTUs) and chao1 diversity index (Supplementary Table 1).

HIV-related microbiota alterations

Differences in relative abundance of bacterial taxa between the groups are depicted in Supplementary Figure 1 and Supplementary results 1 and 2. Increased relative abundance of the bacterial class Gammaproteobacteria, the family Desulfovibrionaceae, as well as the genera *Eisenbergiella*, *Oscillibacter* and a concurrent reduction in numerous Clostridia, including *Ruminococcaceae UCG-003*, *Romboutsia* and several genera of the family Lachnospiraceae (*CAG-56*, *Butyrivibrio*, *Coprococcus-2*, *Lachnospiraceae UCG-001*, *Lachnospiraceae UCG-004*, *and GCA-900066575*), were found when comparing PLWH MSM vs uninfected MSM and PLWH non-MSM vs uninfected individuals from the general population, respectively (all $p_{FDR} < 0.05$, Fig. 2A), and were used to define a gut microbiota index in HIV (Fig. 2B and Fig. 3). Adjustment for age, sex, MSM

behaviour, and antibiotic use three months preceding stool sample collection did not affect the association between HIV and the microbiota index (Supplementary Table 2).

HIV-related microbiota index is associated with previous immunodeficiency, markers of microbial translocation and reduced microbiota diversity

CD4 nadir <200 cells/µl was independently associated with higher HIV-related microbiota index (adjusted β 0.32 [0.04; 0.61]). However, no associations between the microbiota index and duration of cART, prior exposure to thymidine analogues (TA)/didanosine (ddI) or current CD4 T-cells were found. The microbiota index was positively correlated with markers of microbial translocation (soluble CD14, sCD14: Spearman´s rho 0.16, p<0.001; LPS-binding protein, LBP: rho 0.10, p=0.036), and negatively correlated with Shannon diversity index (rho -0.43, p<0.001).

Association of gut microbiota diversity and HIV microbiota index with metabolic syndrome

One-unit increase in Shannon diversity was associated with 28% reduced risk of metabolic syndrome both before (OR 0.72 [0.54; 0.94]) and after adjusting for confounders (adjusted OR (aOR) 0.72 [0.51; 1.00]).

Elevated HIV-related microbiota index was associated with excess risk of metabolic syndrome both before (OR 1.77 [1.11; 2.83]) and after (aOR 1.97 [1.12; 3.46]) adjusting for confounders. This association was consistent after further adjusting the model for dietary habits (Supplementary Table 3), prior exposure to TA and/or ddI (aOR 2.05 [1.16; 3.63]) and duration of cART (aOR 1.99 [1.11; 3.58]).

This association was mainly driven by excess risk of hypertriglyceridemia (aOR 2.10 [1.26; 3.50]), diabetes (aOR 3.23 [1.08; 9.64]) and hypertension (aOR 1.60 [0.96; 2.66]). No association between

228 elevated HIV-related microbiota index and waist circumference (aOR 1.12 [0.60; 2.11]) or low HDL
229 was found (aOR 1.24 [0.75; 2.05]).

230 **Potential interplay between HIV-related microbiota alterations and immune dysfunction on the**
231 **risk of metabolic comorbidities**

232 Within the bacteria included in the HIV-related microbiota index, increase in Desulfovibrionaceae
233 and reduction in several Clostridia (*Lachnospiraceae UCG001*, *Coprococcus 2*, *CAG56*, and
234 *Butyrivibrio*) characterized individuals with metabolic syndrome (all p-values < 0.05). A similar
235 microbiota profile (outgrowth of *Desulfovibrio* and reduction in Clostridia) was recently reported to
236 trigger the metabolic syndrome and fat accumulation in immunodeficient mice [21].

237 We therefore stratified our findings for low CD4 nadir and/or history of AIDS, finding an accentuated
238 association (5-fold excess risk) between HIV-related microbiota index and metabolic syndrome in
239 individuals with previous severe immunodeficiency (Figure 4).

240 Finally, history of immunodeficiency significantly modified the association between the HIV-related
241 microbiota index and VAT (p-interaction 0.013). Accordingly, the presence of elevated HIV-related
242 microbiota index was associated with 30.8 cm^2 [3.1; 58.5] larger VAT area in individuals with
243 previous immunodeficiency, but not in those without (adjusted β -4.2 cm^2 [-20.0; 11.7] (Table 2).
244 The HIV-related microbiota index was not associated with SAT area (Table 2).

245

Discussion

In the present study, we identified HIV-associated gut microbiota alterations independent of sexual behaviour and other confounders and investigated their potential association with metabolic comorbidities. Whereas HIV infection was associated with reduced gut diversity, MSM status was associated with higher diversity, as previously reported [22,23]. After filtering out the MSM-signal, we identified an HIV-related microbiota index consisting of increased abundance of Gammaproteobacteria and Desulfovibrionaceae as well as lower abundance of several Clostridia. The HIV-related microbiota index was associated with increased risk of metabolic syndrome and VAT accumulation, particularly in individuals with previous severe immunodeficiency, suggesting a possible microbial link between previous immunodeficiency and metabolic comorbidities.

In line with recent studies, our results suggest that most of the microbiota alterations reported in early HIV cohorts, including enrichment in Prevotellaceae and Erysipelotrichaceae and reduction in Bacteroidaceae is driven by sexual behaviour [13,22]. On the other hand, we found increased abundance of Gammaproteobacteria and Desulfovibrionaceae (both Proteobacteria), and reduction in several Clostridia belonging to the Lachnospiraceae and Ruminococcaceae families to be associated with HIV infection. Our data validate the aggregate findings from several smaller studies (as reviewed in [14]), and in total are consistent with the presence of an HIV-related gut microbiota profile across study cohorts, although differences will remain due to HIV-related factors, demographics and geography.

Interestingly, we found that individuals with high HIV-related microbiota index had 2-fold excess risk of metabolic syndrome, independent of confounders, mainly driven by increase in Desulfovibrionaceae and decrease in several Clostridia (*Butyrivibrio*, *Coprococcus-2*,

Lachnospiraceae UCG-001 and *CAG-56*). In a recent study by Petersen et al, outgrowth of *Desulfovibrio* and reduction in several Clostridia (so-called "obesogenic microbiota") was sufficient to trigger fat accumulation and metabolic syndrome in immunodeficient mice, suggesting a close interplay between gut microbiota and immunodeficiency on metabolic risk [21]. Of note, microbiota alterations characterizing PLWH in our study to a large degree resembles this "obesogenic microbiota", further supported in human cohorts of gestational diabetes [24] and obesity [25]. Notably, the association between the HIV-related microbiota index and metabolic syndrome was considerably more pronounced in individuals with previous severe immunodeficiency, thus supporting the findings by Petersen et al. [21] in a human setting.

We observed that the HIV-related microbiota index was associated with larger VAT area but not SAT area. Mechanistic studies have reported translocation of microbial products from the gut to the surrounding VAT, particularly mesenteric adipose tissue, triggering VAT inflammation and metabolic diseases [26,27], and in a previous study from non-infected individuals undergoing bariatric surgery, we detected bacterial DNA in mesenteric adipose tissue along with a strong correlation between circulating LPS and VAT, but not SAT area [28]. While the HIV-related microbiota index was associated with VAT accumulation in individuals with previous severe immunodeficiency, this association was absent in individuals without. In the study by Petersen et al, the "obesogenic microbiota" seems to interfere with regulation of long chain fatty acids and facilitate lipid absorption in mice with impaired cellular immunity [21], and our findings are consistent with an interacting effect of the gut microbiota and cellular immunodeficiency on VAT accumulation and its related metabolic risk [29].

289 A close interplay between history of severe immune dysfunction and gut dysbiosis in HIV infection
290 has recently been proposed by *Guillen et al* [30]. Accordingly, we found the HIV-related microbiota
291 index to be associated with low nadir CD4 count, possibly pointing to long-term effects on gut
292 microbiota composition resulting from impaired mucosal immunology [31], such as loss of Th17 cells
293 and IL-17 production, which has counteracting effects on dysbiosis, VAT inflammation and
294 development of metabolic syndrome [32]. Of note, we found that HIV-related microbiota index
295 correlated weakly yet significantly, with plasma sCD14 and LBP, which could imply the presence of
296 impaired gut barrier despite long-term treatment with cART. The Desulfovibrionaceae family
297 contains sulphur reducing bacteria that produce hydrogen sulphide (H_2S), a compound with toxic
298 effects on the gut epithelium [33]. Conversely, several Clostridia in the Lachnospiraceae and
299 Ruminococcaceae families are known butyrate producers, which is vital for maintaining the gut
300 barrier [34]. Outgrowth of *Desulfovibrio* at the expense Clostridia has been demonstrated in
301 cohousing experiments [21], and our data suggest that imbalance between these bacteria could be
302 a potential therapeutic target in future studies targeting metabolic comorbidities in HIV infection.

303 The present study has some limitations. First, due to cross-sectional design, conclusions about
304 causality cannot be drawn. Second, differences in age and gender may explain part of the findings,
305 although possible confounding was reduced by adjustment in multi-variable regressions. Third, lack
306 of clinical data from uninfected controls prevented us from exploring a possible HIV-specific effect
307 on the observed associations, although the impact of previous immunodeficiency on the HIV-related
308 microbiota index and its relation with metabolic syndrome suggests a potential clinical relevance in
309 PLWH. By filtering out MSM-related microbiota traits and applying FDR-correction, we may have
310 overlooked alterations of potential clinical relevance in HIV-infected MSM. However, given the
311 conflicting results in previous studies, we have chosen a conservative approach to get closer to

defining the core of HIV-related microbiota alterations. Furthermore, information regarding sexual behaviour was not available in uninfected controls from the general population. Finally, due to the low number of viremic individuals, our results may not be translatable to settings characterized by higher prevalence of uncontrolled viral replication.

In conclusion, HIV-related microbiota alterations were associated with increased risk of metabolic syndrome and VAT accumulation, particularly in individuals with previous severe immunodeficiency. Our findings suggest a potential interplay between microbiota alterations, immune dysfunction and metabolic comorbidities. Interventions targeting the gut microbiome may be warranted to reduce cardiovascular risk, particularly in individuals with previous immunodeficiency.

List of supplementary materials

Supplementary methods – Gut microbiota analyses (bioinformatics), soluble CD14 and Lipopolysaccharide binding protein analyses

Supplementary Table 1. Association of HIV infection and MSM-behaviour with observed bacterial species, Chao1 and Simpson diversity index after adjusting for confounders.

Supplementary Table 2. Association of HIV infection with the HIV-related microbiota index after adjusting for potential confounders

Supplementary Table 3. Association of high HIV-related microbiota index with metabolic syndrome after adjustment for dietary habits

Supplementary Figure 1. Matrix of phylogenetic trees representing differences in bacterial relative abundance between the groups. Green and brown colours represent higher and lower relative abundance in the green term of the comparison, respectively. Only statically significant (p-value < 0.05 after Benjamini-Hochberg multiple comparison correction) are shown.

Supplementary results 1 and 2. Raw results regarding gut microbiota comparison between the four study groups.

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Authors Contribution

MG, BV, JRH, SDN, MT conceived and designed the study. MG, ND, and NK participated in collecting and storing samples from study participants. BV performed DNA extraction from stool samples and established the library preparation protocol. AG extracted DNA and generated the libraries. KH performed the bioinformatic processing and QC. SHH and MG were the primary statistical analysts, with contributions from MT, BV, and JRH. MG, BV, JRH and MT compiled the first draft of the study manuscript and all authors contributed to subsequent revisions. All authors read and approved the final manuscript.

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Competing interests

MG: no competing interests; BV: no competing interests; SHH: no competing interests; KH: no competing interests; ND: no competing interests; NK: no competing interests; BL: no competing interests; AML: Travelling grants from Gilead and GSK; JG: Honoraria for consulting and presenting paid to his institution from Gilead, Abbvie, ViiV, BMS, MSD, Janssen, and Medivir; JL: no competing interests; JRH: advisory boards for Orkla Health and Novartis, research support from Biogen; SDN: Unrestricted research grants from Novo Nordisk Foundation, Lundbeck Foundation, Augustinus

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Data availability

All data associated with this study are available in the main text or the supplementary materials.

Ethics committee approval

Ethics approval was obtained by the Regional Ethics Committee of Copenhagen (COCOMO: H-15017350). Written informed consent was obtained from all participants.

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Tables

Table 1. Demographic table

	PLWH MSM n = 281	**PLWH non-MSM** n = 124	**General population** n = 68	**Uninfected MSM** n = 43	***p***
Age, years, mean (SD)	53.2 (11.3)	53.4 (10.4)	60.6 (9.1)	40.1 (9.6)	*< 0.001*
Sex, male n (%)	281 (100)	60 (48.4)	43 (66.2)	43 (100)	*< 0.001*
Smoking status, n (%)					-
Current smoker	78 (27.6)	24 (18.8)	-	-	
Ex-smoker	114 (40.3)	46 (35.9)	-	-	
Never smoker	88 (31.1)	49 (38.3)	-	-	
Origin, n (%)					-
Other	22 (7.9)	34 (27.6)	-	-	
Other EU	37 (13.5)	12 (9.8)	-	-	
Scandinavian	216 (78.5)	77 (62.6)	-	-	
Metabolic syndrome, yes, n (%)	98 (38.9)	33 (32.7)	-	-	-
Lipid lowering therapy, yes, n (%)	46 (17.0)	15 (13.3)	-	-	-
Antidiabetic therapy, yes, n (%)	12 (4.5)	6 (5.4)	-	-	-
Abdominal obesity, yes, n (%)	176 (63.1)	84 (68.9)			-
Physical activity, n (%)					-
Very inactive	13 (4.8)	10 (9.0)	-	-	
Moderately inactive	106 (39.0)	32 (28.8)	-	-	
Moderately active	127 (46.7)	58 (52.3)	-	-	
Very active	26 (9.6)	11 (9.9)	-	-	
Current CD4, mean (SD)	719.9 (281.5)	711.0 (273.8)	-	-	-
Current CD4 < 500, yes, n (%)	57 (20.3)	32 (26.0)	-	-	-
AIDS defining event, yes, n (%)	47 (16.7)	28 (22.6)	-	-	-
cART duration, years, mean (SD)	15.7 (9.3)	15.9 (9.0)	-	-	-
Prior TA and/or ddI, yes, n (%)	163 (58.0)	76 (61.3)	-	-	-
CD4 nadir < 200 cells, yes, n (%)	109 (39.8)	53 (43.4)	-	-	-
Viral load < 50 copies, yes, n (%)	270 (96.1)	116 (94.3)	-	-	-
Receiving cART, yes, n (%)	275 (98.2)	122 (99.2)	-	-	-
Antibiotic use*, yes, n (%)	64 (22.8)	18 (14.5)	5 (7.4)	16 (38.1)	*< 0.001*
PrEP, yes, n (%)	-	-	-	18 (41.8)	-

Abbreviations: MSM, male to male sex; PLWH, people living with HIV; cART, combination antiretroviral therapy; TA, thymidine analogues; ddI, didanosine; p, p-value; PrEP, pre-exposure prophylaxis
*Antibiotic use in the three months prior to stool samples collection

Table 2. Association of HIV-related microbiota index with visceral and subcutaneous adipose tissue stratified according to history of severe immunodeficiency

	Visceral adipose tissue			Subcutaneous adipose tissue		
	PLWH ***with*** history of severe immunodeficiency *n = 105*	PLWH ***without*** history of severe immunodeficiency *n = 290*		PLWH ***with*** history of severe immunodeficiency *n = 105*	PLWH ***without*** history of severe immunodeficiency *n = 290*	
	Effect size in cm^2 [95% CI]	Effect size in cm^2 [95% CI]	*p-int**	Effect size in cm^2 [95% CI]	Effect size in cm^2 [95% CI]	*p-int**
High HIV-related microbiota index, yes vs no						
Base model	30.8 [3.1; 58.5]	-4.2 [-20.0; 11.7]	0.013	5.3 [-19.2; 29.8]	5.4 [-12.3; 23.1]	0.786
Base model + prior TA/ddI	31.4 [3.5; 59.4]	-3.0 [-18.9; 12.8]	0.016	6.2 [-18.4; 30.8]	4.4 [-13.3; 22.1]	0.725
Base model + duration of cART	30.9 [3.1; 58.7]	-5.6 [-21.6;10.4]	0.015	6.5 [-18.5; 31.4]	3.0 [-14.9; 21.0]	0.736

The effect size in cm^2 of visceral (and subcutaneous) adipose tissue represent the β-coefficient of multivariable linear regression models associated with the presence of High HIV-related microbiota index. History of severe immunodeficiency was defined by at least one of: history of AIDS and CD4 nadir <50 cells/µl. Confounders included in the base model were age, sex, BMI, smoking, origin, MSM behaviour, and physical activity. Abbreviations: TA, thymidine analogues; ddI, didanosine; cART, combination antiretroviral treatment

**p-int*, p-interaction

Figures and Figure legends

Figure 1. Differences in Shannon alpha diversity index between the four study groups

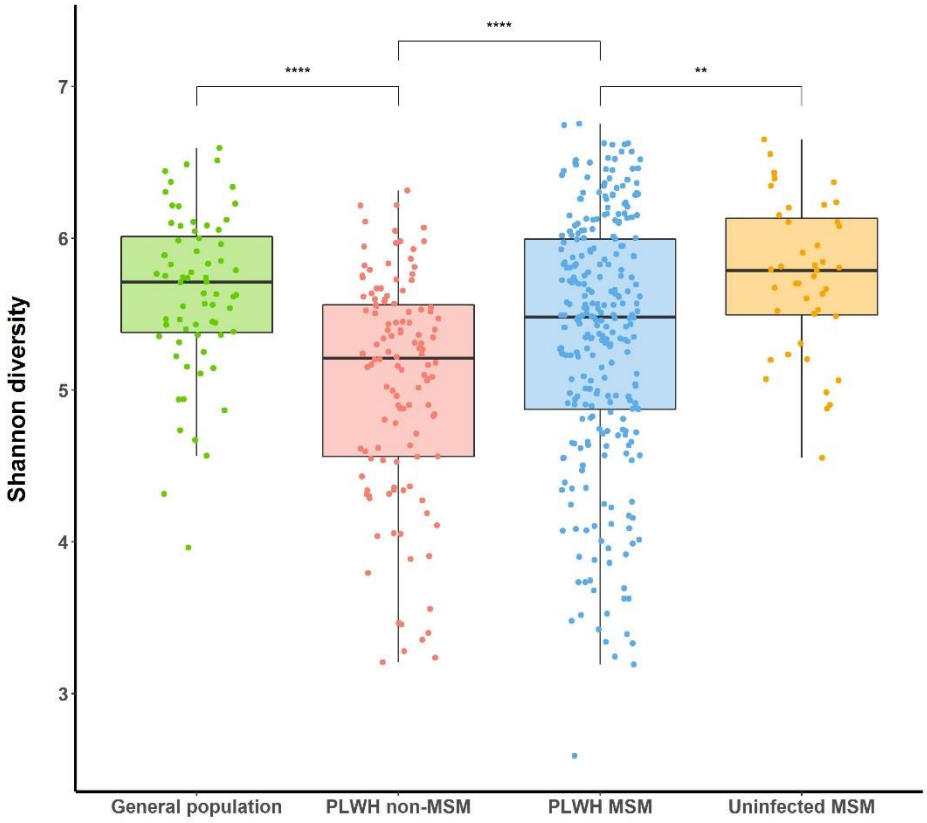
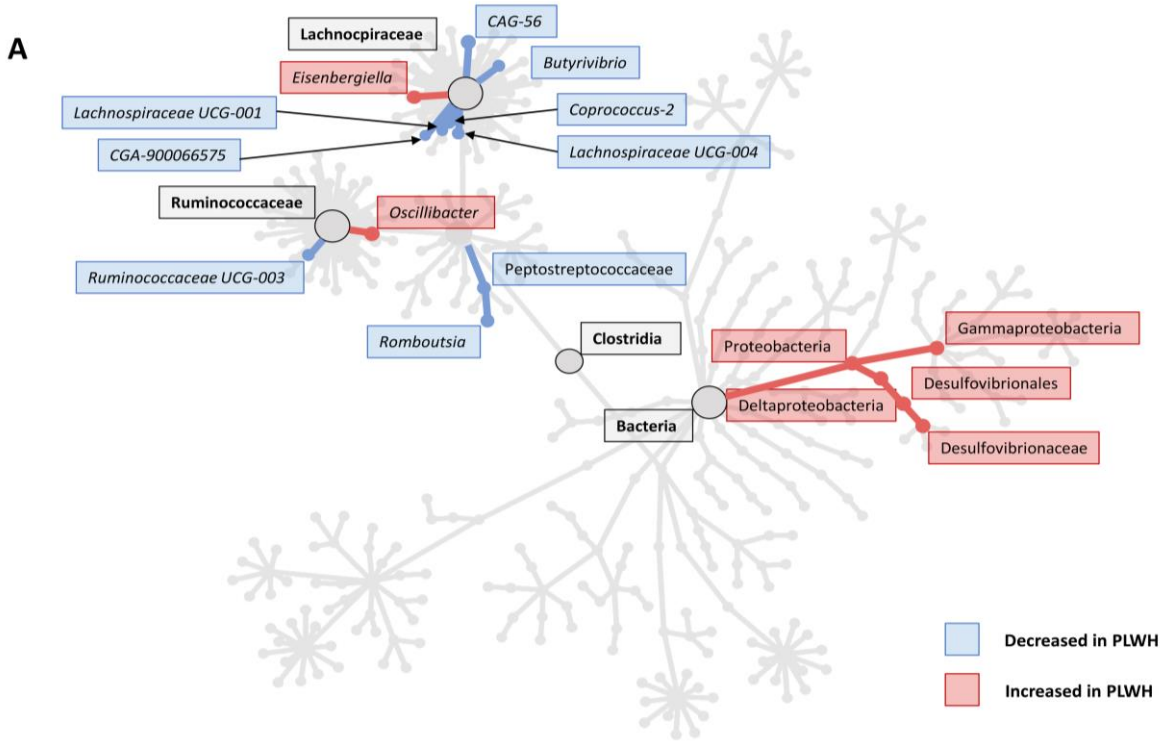


Figure 2. Identification of HIV-related microbiota changes



B

$$\text{Log}_e \left[\frac{\text{Desulfovibrionaceae} + \text{Gammaproteobacteria} + \textit{Eisenbergiella} + \textit{Oscillobacter}}{\textit{Lachnospiraceae CAG-56} + \textit{Lachnospiraceae Butyrivibrio} + \textit{Lachnospiraceae Coprococcus-2} + \textit{Lachnospiraceae UCG-001} + \textit{Lachnospiraceae UCG-004} + \textit{Lachnospiraceae GCA-900066575} + \textit{Ruminococcaceae} + \textit{Romboutsia}} \right]$$

HIV-related microbiota index:

Figure 3. Differences in HIV-related microbiota index between the four study groups

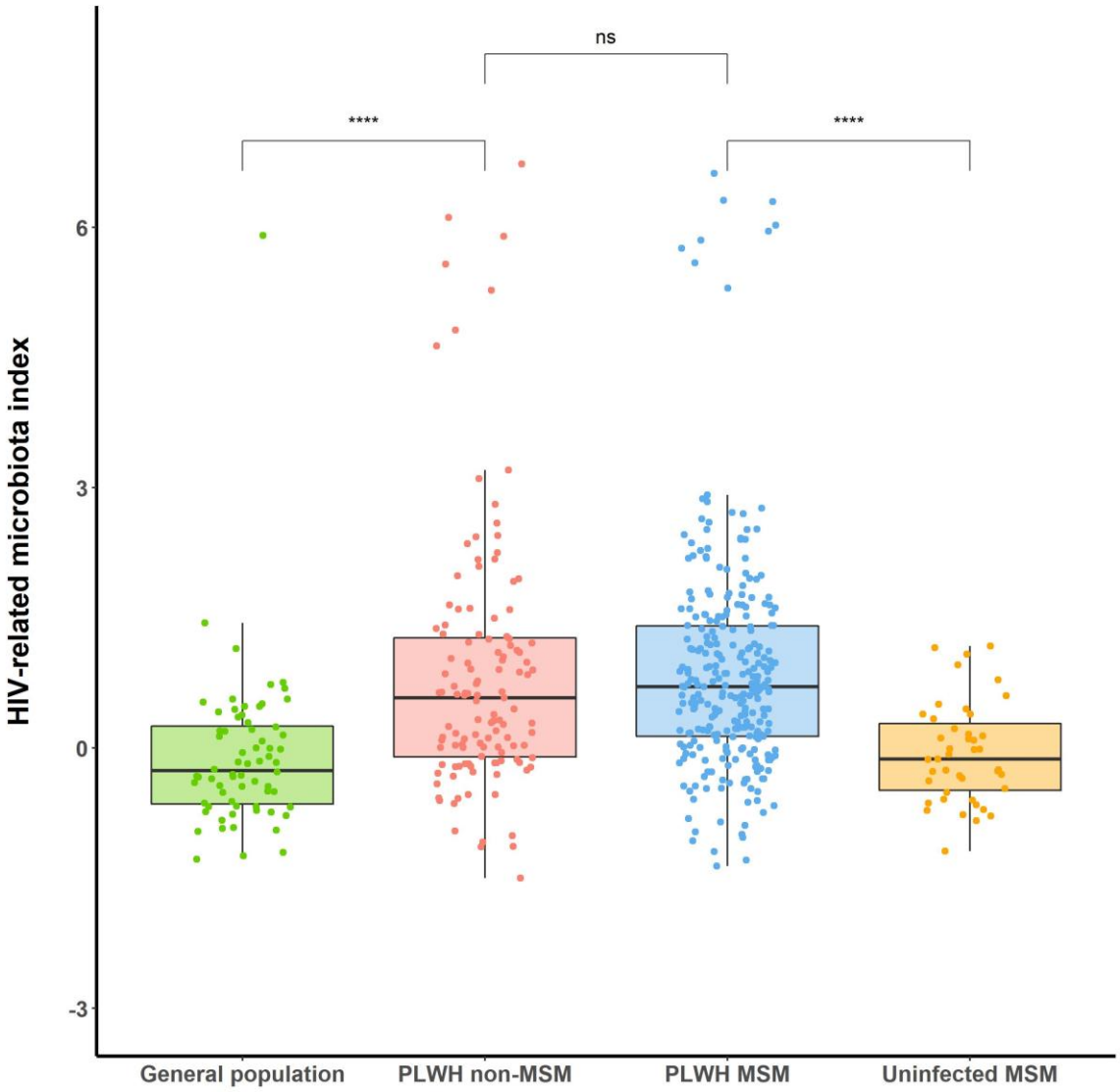


Figure 4. Association of high HIV-related microbiota index with risk of metabolic syndrome before and after stratification according to history of severe immunodeficiency

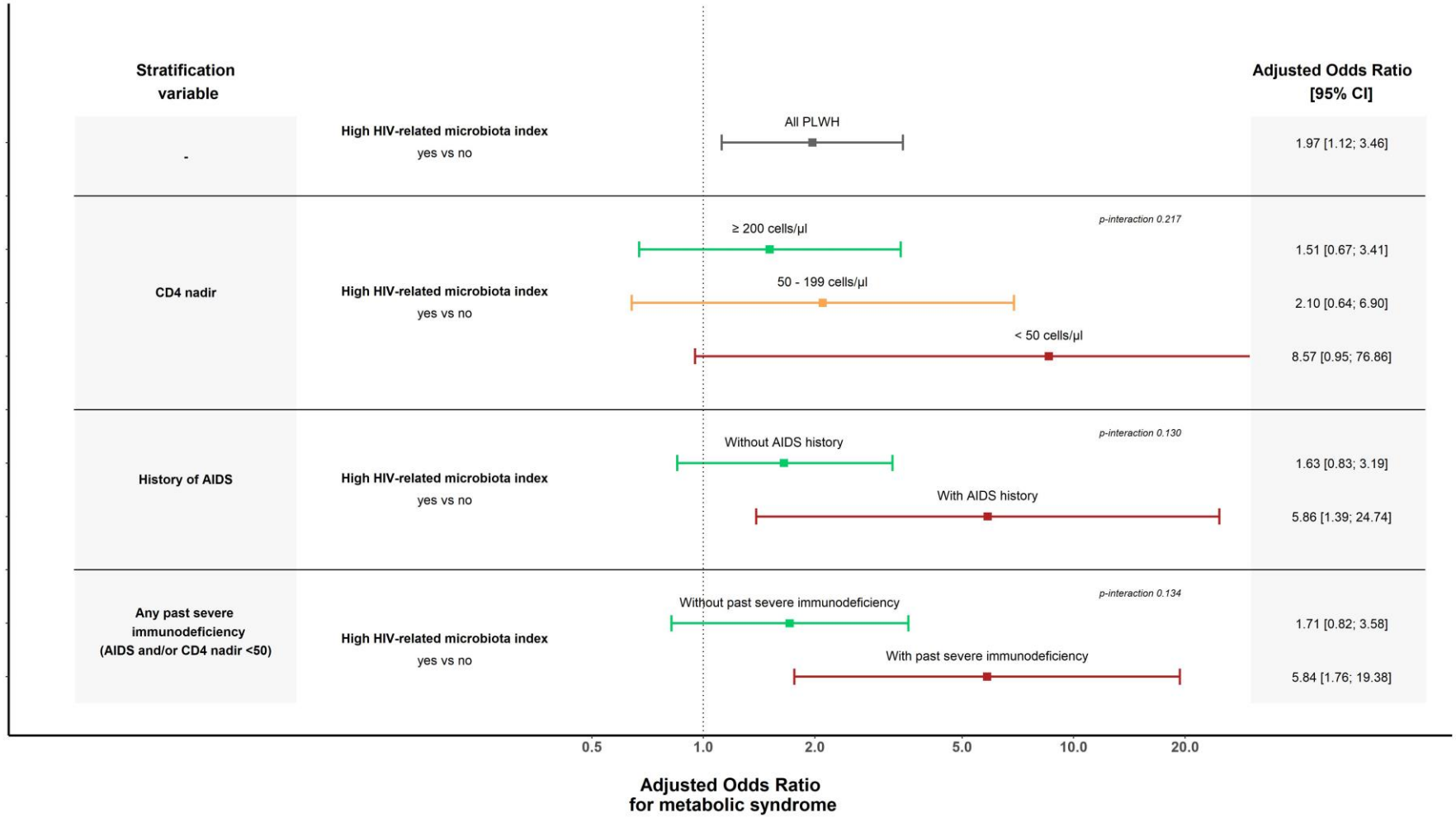


Figure Legends

Fig. 1 Difference in Shannon diversity in the four study groups: uninfected individuals from the general population (green), people living with HIV (PLWH) who are not men who have sex with men (MSM) (red), PLWH MSM (blue), and uninfected individuals who report to be MSM (orange). Levels of significance: *, < 0.05; **, <0.01; ***, < 0.001; ****, <0.0001.

Fig. 2 A. HIV-related gut microbiota alterations. To identify HIV-related gut microbiota alterations, we compared relative abundance differences in PLWH MSM vs uninfected individuals who reported to be MSM and PLWH non-MSM vs uninfected from the general population, respectively. Differences reproduced in both these comparisons were identified as "HIV-related". **B)** Formula used in the HIV-related gut microbiota index computation, as previously presented in [20].

Fig 3. HIV-related microbiota index in individuals from the general population (green), PLWH non-MSM (red), PLWL MSM (blue), and uninfected MSM (orange). Level of significance: ****, < 0.0001; ns, non-significant.

Fig 4. Association of high HIV-related microbiota index (highest quartile) with metabolic syndrome before and after stratification according to CD4 nadir, history of AIDS and any past severe immunodeficiency (AIDS and/or CD4 nadir < 50 cells/µl). Confounders included in the base model were age, sex, BMI, smoking, origin, MSM behaviour, and physical activity.