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Gut Microbiota, Plasma Metabolomic Profiles and Carotid Artery Atherosclerosis in HIV Infection

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Disclosures

None.

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Abstract

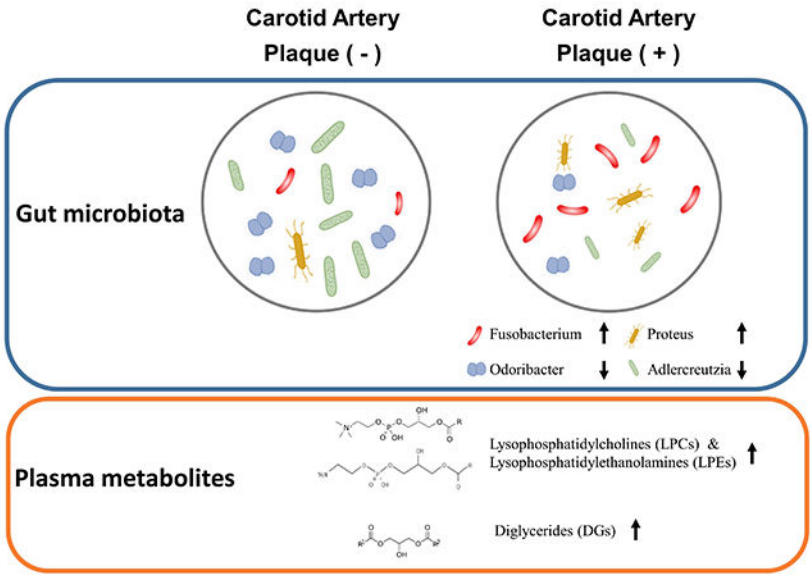
Background: Alterations in gut microbiota and blood metabolomic profiles have been implicated in HIV infection and cardiovascular disease. However, it remains unclear whether alterations in gut microbiota may contribute to disrupted host blood metabolomic profiles in relation to atherosclerosis, especially in the context of HIV infection.

Methods: We analyzed cross-sectional associations between gut microbiota features and carotid artery plaque in 361 women with or at high risk of HIV(67% HIV+), and further integrated plaque-associated microbial features with plasma lipidomic/metabolomic profiles. Furthermore, in 737 women and men, we examined prospective associations of baseline gut bacteria-associated lipidomic and metabolomic profiles with incident carotid artery plaque over 7-year follow-up.

Results: We found two potentially pathogenic bacteria, *Fusobacterium* and *Proteus*, were associated with carotid artery plaque; while the beneficial butyrate producer *Odoribacter* was inversely associated with plaque. *Fusobacterium* and *Proteus* were associated with multiple lipids/metabolites which were clustered into 8 modules in network. A module comprised of 9 lysophosphatidylcholines(LPCs) and lysophosphatidylethanolamines(LPEs) and a module comprised of 9 diglycerides were associated with increased risk of carotid artery plaque(RR[95% CI] = 1.34[1.09, 1.64] and 1.24[1.02, 1.51] per SD increment, respectively). Functional analyses identified bacterial enzymes in lipid metabolism associated with these plasma lipids. In particular, phospholipase A1 and A2 are the key enzymes in the reactions producing LPCs and LPEs.

Conclusion: Among individuals with or at high risk of HIV infection, we identified altered gut microbiota and related functional capacities in the lipid metabolism associated with disrupted plasma lipidomic profiles and carotid artery atherosclerosis.

Graphical Abstract



Keywords

gut microbiota; metabolomics; atherosclerosis; HIV infection

Introduction

Cardiovascular disease (CVD) has become one of the major concerns in people living with human immunodeficiency virus (HIV) infection, since survival has significantly improved because of successful antiretroviral therapy (ART) [1,2]. The complicated interactions among the effects of chronic HIV infection and host-related factors may contribute to excess risk of CVD in people living with HIV [1,3,4], but the underlying mechanisms are not fully understood. Emerging evidence has suggested that gut microbiota might play an important role in the host vascular physiology (e.g., atherosclerosis), and thus may contribute to the development of CVD [5–11]. In addition, alterations in gut microbiota were also observed in people living with HIV infection, and HIV-associated gut microbiota alteration might contribute to disrupted host metabolite profiles in people living with HIV infection [12–14]. However, the role of gut microbiota in the progression of atherosclerosis and CVD remains largely unknown in the context of HIV infection.

Our prior work has demonstrated that HIV infection is associated with new formation of carotid artery plaque, an established subclinical atherosclerosis measure which has been used as a validated surrogate for clinical CVD events[15]. Interestingly, numerous bacteria have been found in human carotid artery plaque tissues, which might be related to oral bacteria that can migrate via the bloodstream [16,17]. A previous study in 15 patients with atherosclerosis identified common bacteria in oral, fecal and carotid artery plaque samples, suggesting linkages of both oral and gut microbiota with atherosclerotic plaques [18] . A recent population-based study of 569 elderly Chinese also found alterations in gut microbiota associated with carotid atherosclerosis measured by carotid intima-media

thickness (CIMT)[6]. However, the association between gut microbiota and carotid artery plaque formation remains unclear, and it is also unknown whether the association differs depending on HIV-infection status. Furthermore, host metabolomic data have not been well-integrated with gut microbiome in previous studies on atherosclerosis and CVD [6,7], though microbiota-related metabolites have been suggested as potential mediators linking gut microbiota and CVD [5,8].

Therefore, in the present study, we aimed to identify gut microbiota features (e.g., overall diversity and individual taxa) associated with carotid artery plaque in women with HIV infection and those without HIV infection from the Women's Interagency HIV Study (WIHS). We also examined associations of plaque-related gut bacteria and microbial functional components with host plasma metabolomic profiles in these women. Moreover, in two HIV cohorts of women (WHIS) and men (Multicenter AIDS Cohort Study, MACS), we further examined the prospective associations of baseline gut bacteria-related metabolite profiles with incident carotid artery plaque over a median 7-year follow-up.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Population

The WIHS is a prospective cohort study of women with or at risk for HIV infection, and details on study design and methods have been described previously[19] . In this study, we included 572 WIHS women whose fecal samples were collected using a home-based self-collection kit [20,21] during 2017–2019. Among these participants, 361 women underwent carotid artery imaging during 2017-2019, and 435 women had metabolomic/lipidomic data on fasting plasma samples collected during 2017–2019, at the closest WIHS core visit to the time of stool sample collection.

In addition, we also included 398 women in the WIHS and 339 men in the MACS with baseline metabolomic/lipidomic data and longitudinal carotid artery imaging data over 7 years[22] . In brief, 737 women and men underwent carotid artery imaging for plaque assessment at a baseline visit (2004-2006) and at a follow-up visit (2011-2013)[23]. After a median follow-up of 7 years, 112 participants developed carotid artery plaques.

An expanded description of study populations, microbiome sequencing, metabolomic profiling, assessments of carotid artery plaque and HIV variables, and statistical analyses is provided in Supplemental Methods. The study was reviewed and approved by the institutional review boards at all participating institutions. All participants provided written informed consent.

Microbiome sequencing

The 16S rRNA V4 region sequencing was performed on DNA extracted from fecal samples using MiSeq platform(Illumina, San Diego, CA). The Microbiome bioinformatics analyses, including taxonomic assignment, α- and β-diversity measurements, were performed using

QIIME2 pipeline[24]. The functional components of the microbial community were inferred using PICRUSt2[25]. 84 bacterial genera, which's average relative abundance ≥0.01% and present in >10% of the study population, were included in the analyses.

Metabolomic/lipidomic profiling

Plasma metabolomic/lipidomic profiling was performed using liquid chromatography-tandem mass spectrometry(LC-MS) at the Broad Institute Metabolomics Platform (Cambridge, Massachusetts), as previously described[22] . We included 211 lipids and 167 polar metabolites in the current analysis, and all metabolites had coefficient variation <30% and missing rate <20%.

Carotid Artery Plaque Ascertainment

High-resolution B-mode carotid artery ultrasound was used to image 8 locations in the right carotid artery of participants: the near and far walls of the common carotid artery, carotid bifurcation, internal and external carotid artery[26].Focal plaque measures were obtained at a centralized reading center (University of Southern California). We defined a focal plaque as an area with localized intima-media thickness of >1.5 mm in any of the 8 imaged carotid artery locations[27].

Conventional CVD risk factors included body mass index(BMI), waist to hip ratio, systolic blood pressure(SBP), diastolic blood pressure(DBP), triglycerides, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, fasting glucose, Hemoglobin A1c, anti-cholesterol and anti-hypertensive medication[28]. Assessments of HIV infection and other variables are described in Supplemental Methods.

Statistical analysis

Gut microbiota and prevalent carotid artery plaque.—We first examined cross-sectional associations of gut microbiota features with carotid artery plaque among 361 women with corresponding data available. LefSe[29] was used to identify gut bacterial genera associated with prevalent carotid artery plaque. We then used logistic regression models and linear regression models to examine the multivariate-adjusted associations, adjusting for age, race, study site, antibiotics use, income, education, BMI, current smoking status, marijuana use and HIV status. CSS normalization was conducted for the genus level abundance of taxonomic units before the analyses. In addition, we also applied Analysis of Composition of Microbiomes (ANCOM2)[30], adjusting for aforementioned covariates. We controlled the false discovery rate(FDR) at 10%.

Gut microbiota and plasma lipidomic and metabolomic profiles .—We then examined cross-sectional associations of plaque-associated genera with plasma lipidomic and metabolomic profiles among 435women. Spearman correlation was employed to estimate correlation coefficients of identified genera with lipidomic and polar metabolomic profiles. Network analysis was conducted using a weighted correlation network analysis implemented in the WGCNA R package [31].

Baseline lipidomic/metablomic profiles and incident carotid artery plaque.— We examined the prospective associations of bacteria-associated lipidomic/metablomic profiles with incident carotid artery plaque over 7 years among 737 women and men free of carotid artery plaque at baseline. Poisson regression models were used to estimate risk ratios(RRs) and 95% confident interval(95% CI) of incident carotid artery plaque per SD increment of metabolite/lipid module scores and individual lipids and polar metabolites, adjusting for age, sex, race, study site, education, smoking status, ART use, CD4 counts, HIV viral load, crack cocaine use, injected drug use, and hepatitis C virus infection.

Gut microbiota functional components and plasma lipidomic and metabolomic profiles.—Centered-log-ratio transformation was applied to the PICRUSt2 predicted enzymes. Linear regression models were applied to examine associations of enzymes with metabolite modules, after controlling for the aforementioned covariates. An enrichment test was performed for the 1486 annotated enzymes at EC level III enzyme category, with FDR q <0.10 as cut off. Associations among the functional enzymes, plaque related genera, and metabolite modules were examined using linear regression models with multivariable adjustment for the aforementioned covariates.

The Benjamini-Hochberg false discovery rate (FDR) method was used for the multiple testing correction. Statistical analyses were performed using R 4.0.1. unless otherwise stated. Detailed methods for all the Omics data and statistical analyses are described in Supplemental Methods.

Results

Gut Microbiome and Carotid Artery Plaque

Table S1 shows characteristics of the 361 participants (264 without plaque and 97 with plaque), who were included in the analysis of the association between gut microbiome and carotid artery plaque. As expected, compared with participants without plaque, participants with plaque were older and more likely to have higher levels of CVD risk factors. The demographic and socioeconomic characteristics among participants with and without HIV infection were generally similar and comparable, consistent with the WIHS study design (data not shown). The majority of HIV-infected individuals reported of ART use (93% and 94% respectively, in the participants with/without plaque group), while a large proportion of HIV-infected individuals had undetectable HIV-1 viral load (72% and 78 % respectively).

We first examined associations of gut bacterial community α-diversity and β-diversity with carotid artery plaque. Three α-diversity indices (Shannon index, Chao-1 Index and Simpson's Index) were not significantly different between participants with and without plaque (all P>0.05, Figure S1A and S1B). We did not find significant association between β-diversity measured by Bray-Curtis dissimilarity and plaque status (Figure S1C; R^2 <0.1, P >0.05, PERMANOVA analysis).

In taxonomy analyses, the LefSe indicated that carotid artery plaque was associated with enriched *Fusobacterium* and *Proteus*; and depleted *Odoribacter* and *Adlercreutzia* (All LDA score >3, Figure 1A). We then focused on these four plaque-associated genera in

the subsequent regression analyses. After multivariate adjustment, there were significant differences in the CSS transformed abundance of *Fusobacterium*, *Proteus*, *Odoribacter* and Adlercreutzia between women with and without plaque (Figure 1B). Higher levels of *Fusobacterium* and *Proteus* were associated with elevated odds of carotid artery plaque (OR [95% CI]=1.62 [1.22, 2.15] and 1.28 [1.01, 1.62], respectively, per SD increment in the CSS transformed abundance) while higher levels of *Odoribacter* was associated with lower odds of plaque (OR[95% CI]=0.70[0.53,0.91]) (Figure 1C). A Marginal significant association between *Adlercreutzia* and carotid artery plaque was observed in the multivariate linear regression (P=0.086) and the logistic regression models (P= 0.093). In addition, we also examined the associations between carotid artery plaque and all bacterial genera using ANCOM2, after multivariate adjustment. Consistently, *Fusobacterium* was significantly enriched (detection level 0.7) while *Odoribacter* was significantly depleted (detection level 0.6), in participants with carotid artery plaque compared to participants without plaque. We identified that *Fusobacterium nucleatum* was the most abundant species of Fusobacterium genus (accounting for 95.3%), while for other plaque-associated genera, the amplicon sequence variants (ASVs) were not classified into known species.

In the stratified analyses by HIV infection status, we observed consistent LefSe results (Figure 1A), and found an additional carotid artery plaque-associated genera, *Pediococcus*, in the strata of women without HIV infection. However, *Pediococcus* was a minor taxa (the average relative abundance was only 0.01%), and the association was not significant in the linear regression model after multivariate adjustment. For the four plaque-associated genera, no effect modifications by HIV infection were observed (All P interaction >0.05, Table S2).

We then examined associations of these 4 plaque-associated genera with conventional CVD risk factors (e.g., obesity measures, blood pressure, glycemic traits, and blood lipids). We did not find many significant correlations between these plaque-associated genera and conventional CVD risk factors, except for a moderate positive correlation between *Fusobacterium* and DBP (r=0.26, P=0.001), a moderate inverse correlation between *Odoribacter* and SBP (r= −0.23, P=0.009), and a moderate inverse correlation between *Adlercreutzia* and HbA1c (r=−0.16, P=0.047) (Figure S2). After further adjustment for conventional CVD risk factors in the regression models (Figure S3 and S4A), the association between Fusobacterium and carotid artery plaque was attenuated but remained significant (Fusobacterium, OR=1.47 [95% CI, 1.07, 2.03] per SD increment in the CSS transformed abundance, P=0.018), and the association between Odoribacter and plaque became marginally significant (Odoribacter, OR=0.76 [95% CI, 0.56, 1.02], P=0.073). Our further analysis indicated that the attenuation might be driven by further adjustment for blood pressure (Figure S4B), which is in line with the observed moderate correlations of *Fusobacterium* and *Odoribacter* with blood pressure.

Since medication use has been reported to influence gut microbiota composition[32], we also examined associations of antihypertensive medication use, anti-diabetes medication use and lipid-lowering medication use with the identified four gut microbial genera. We did not observe any significant differences in these four genera between users and non-users of these medications (all P > 0.05, Figure S5A, S5B, and S5C). In the stratified analyses by HIV status, we observed consistent results (all P >0.05). The observed associations between

these four microbial genera and plaque did not change materially after further adjusted for medication use in logistic regression models (Figure S6).

In addition, we also examined associations of these 4 plaque-associated genera with HIV-related factors (e.g., CD4 count, HIV viral load and ART use). We found an increasing trend of *Proteus* abundance across women without HIV, with aviremic HIV (undetectable viral load ≤20 copies/mL), and viremic HIV (viral load >20 copies/mL) (P=0.037, Figure S7).

Carotid Artery Plaque-associated Gut Microbial Genera and Host Plasma Metabolite Profiles

We next examined the cross-sectional associations between carotid artery plaque-associated gut microbial genera and host plasma lipidomic/metabolomic profiles among 435 participants who had both gut microbiome and host plasma metabolome data available. *Fusobacterium* and *Proteus*, which were positively associated with plaque, showed significant correlations with overall lipidomic and metabolomic profiles measured by top principal component scores, while *Odoribacter* and *Adlercreutzia*, which were inversely associated with plaque, were not correlated with overall lipidomic or metabolomic profiles (Figure S8). Consistent results were observed for the correlations between individual plasma metabolites and plaque-associated gut microbial genera. As shown in Figure 2A and 2B, *Fusobacterium* was significantly correlated with 55 lipids (out of total 211 lipids) and 78 polar metabolites (out of total 167 polar metabolites). *Proteus* was correlated with 17 lipids and 43 polar metabolites. There were 17 lipids and 39 polar metabolites correlated with both genera. *Odoribacter* and *Adlercreutzia* were not significantly correlated with any of lipids or polar metabolites.

We then performed network analysis based on these lipids and metabolites which were significantly associated with *Fusobacterium* and/or *Proteus*. Among these 55 microbiota-associated lipids, 41 lipids were clustered into 4 modules, including a module comprised of 9 lysophosphatidylcholines and lysophosphatidylethanolamines (LPC & LPE module), a module of 9diglycerides (DG module), a module comprised of 13 Sphingomyelins and phosphatidylcholines (SM & PC module), and a module comprised of 10 phosphatidylcholines plasmalogen (PC-P module) (Table S3A). Among 82 microbiota-associated polar metabolites, 55 metabolites were clustered into 4 modules, including a module comprised of 5 acylcarnitines (CARs), a module of 7 amino acids, a module of 29 metabolites which mostly have Methyl and Acetyl groups, and a module of 14 mixed metabolites which belong to different pathways (Table S3B). As expected, *Fusobacterium* and *Proteus* showed significant correlations with these 8 lipid and metabolite modules, while *Odoribacter* and *Adlercreutzia* showed few correlations with these modules (Figure 2C). The network plots (Figure 3A and 3B) illustrate the correlations of *Fusobacterium* and *Proteus* with each of lipids and polar metabolites in these 8 modules.

We also examined the correlations of Fusobacterium and Proteus with plasma lipids/polar metabolites stratified by HIV status and the correlation coefficients were generally consistent between women with and without HIV infection (Figure S9).

Gut microbiota-associated Host Plasma Metabolite profiles and Risk of Carotid Artery Plaque

We further examined the associations between microbiota-associated host plasma metabolite profiles (modules) and risk of incident carotid artery plaque using a prospective dataset of women and men with and without HIV infection in the WIHS and MACS[22] .

Among 737 participants, 112 participants developed carotid artery plaques over a median 7-year follow-up. The 8 lipid and metabolite modules were constructed using the baseline lipidomic and metabolomic data in this study sample and then linked with incident plaque. Consistent with the positive associations of *Fusobacterium* and *Proteus* with prevalent carotid artery plaque, the LPC &LPE module and the DG module, which were positively correlated with *Fusobacterium* and *Proteus,* also showed positive associations with incident plaque (RR=1.36 [95% CI, 1.09, 1.69] and 1.30 [1.06, 1.60], respectively, per SD increment in the module score; both FDR P< 0.05), after adjusting for demographic and behavioral variables (Model 1) (Table 1). These associations did not change materially after further adjustment for HIV-related variables and clinical variables (Model 2). In addition, most of the individual lipids and metabolites within the LPC &LPE module and the DG module were also significantly associated with risk carotid artery plaque (Table S4). After further adjustment for conventional CVD risk factors including blood pressure, total cholesterol, high-density lipoprotein cholesterol, BMI, antihypertensive medication use, and lipid-lowering medication use in the model, the associations between carotid artery plaque and metabolite modules were attenuated but remained significant (LPC &LPE module, RR=1.28 [95% CI, 1.00, 1.64] per SD increment in the module score, P=0.047; DG module, RR=1.25 [1.01, 1.57], P= 0.045).

Associations between these metabolite modules and risk of carotid artery plaque were consistent between those with and without HIV infection, and no effect modifications by HIV infection were observed (all P for interaction > 0.05, Table 1). Results were generally consistent between women and men, except for the PC-P module. The PC-P module was marginally associated with increased risk of carotid artery plaque in women, but not in men (P for interaction =0.028 and 0.066 in Model 1 and Model 2, respectively; Table S5).

Gut Microbiome Functional Components and the Potential Mechanism

Since the DG and LPC & LPE lipid modules were associated with both gut bacteria (*Fusobacterium* and *Proteus*) and risk of carotid artery plaque, we then examined microbial functional components to better understand the potential mechanisms and interrelationship between specific plaque-associated bacteria genera and host plasma lipidomic profiles.

The GMB functional profiles were obtained using PICRUSt2, and a total of 1486 known enzymes with specific Enzyme Commission (EC) numbers were annotated and included in the functional analyses. We first examined associations between host plasma DG and LPC & LPE modules and the 1486 known enzymes. After controlling for the demographic, behavioral, clinical variables and HIV specific variables, we identified 337 enzymes associated with DG module and 198 enzymes associated with LPC & LPE module (all FDR P <0.1). We then performed enrichment tests at EC enzyme category level III, and

found that the DG module was associated with the enrichment of enzymes belonging to three categories (i.e., EC 2.3.1 Transferring groups other than aminoacyl groups, EC 3.1.1 Carboxylic-ester hydrolases, and EC 3.2.1 Glycosidases that hydrolyse O-/S-glycosyl compounds ; all FDR P< 0.1, Table S6A), while the LPC &LPE module was associated with the enrichment of enzymes belonging to one specific category (EC 3.1.1 Carboxylic-ester hydrolases; FDR P< 0.1, Table S6B).

We thus focused on the Carboxylic-ester hydrolases category as it was associated with both LPC&LPE and DG lipid modules and many enzymes under this category are involved in the lipid metabolism (e.g., Phospholipase A1 and A2, etc.). In our study, 12 enzymes were detected under the Carboxylic-ester hydrolases category and most of these enzymes showed moderate- to- high correlations with each other (Figure 4). After multivariate adjustment, we found 9 and 8 enzymes out of the 12 Carboxylic-ester hydrolases were positively associated with *Fusobacterium* and *Proteus,* respectively. Consistently, many of these enzymes were also positively associated with the LPC &LPE module and DG module (Figure 4). For example, Phospholipase A1 and A2 (EC 3.1.1.32 and EC 3.1.1.4) are known as representative enzymes in this hydrolases family, which can hydrolyze phospholipids such as phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) and produce LPC and LPE[33,34].

Discussion

To the best of our knowledge, this is the first study to examine the relationship between gut microbiota and subclinical atherosclerosis in the context of HIV infection. Several previous studies have examined the associations of gut microbiota with atherosclerosis and CVD in human populations without HIV infection and yield various results [6,7,9–11]. A case-control study of Chinese in Shanghai reported lower gut microbiome α-diversity in 70 patients with coronary artery disease compared to 98 healthy controls [9] , while one smaller study in Tibetan Chinese found a lower α-diversity Simpson index in 12 patients with non-stenosis coronary heart disease (CHD) but not in those with CHD (n=18), comparted to healthy controls (n=23)[10] . A relatively larger case-control study in Han Chinese did not find significant differences in gut microbiome α-diversity between 218 patients with atherosclerotic cardiovascular disease and 187 healthy controls [7], which is in line with our results. Alterations of various bacterial taxa in CVD groups have also been reported in these case-control studies[7,9,10], but only few results are consistent across studies (e.g., depleted *Roseburia* genus in individuals with CVD) [7,9]. A recent population-based study in 569 elderly Chinese found several gut bacterial genus (e.g., *Faecalicatena* and *Libanicoccus*) associated with subclinical atherosclerosis measured by CIMT[6]. In addition, one study from Sweden (n=25) found that *Collinsella* was enriched in patients with symptomatic carotid atherosclerotic plaques (n=12) whereas *Roseburia* and *Eubacterium* were enriched in healthy controls (n=13) [11] . However, we did not find these previously reported bacteria associated with carotid artery plaque in our study of 361 women with or at high risk of HIV infection. The inconsistency among these studies might be due to various CVD outcomes studied (e.g., clinical CVD vs. subclinical atherosclerosis measured by CIMT or plaque), relatively small sample sizes in most studies, and differences in participant characteristics. Race/ethnicity, geographic location, and environmental and behavior factors may influence the human gut

microbiome and its relationship with human health and disease [35–37]. In particular, HIV infection has been associated with increased risk of carotid artery plaque, and alterations of gut microbiome [12], although this study found little evidence for the potential effect modification of HIV infection on the relationship between gut microbiome and carotid artery plaque.

Fusobacterium is a Gram-negative anaerobic genus commonly residing in oral cavity and gastrointestinal tract [18,38]. Higher level of gut *Fusobacterium nucleatum*, the most abundant species of this genus (accounting for 95.3% of Fusobacterium in our study), has been associated with inflammatory bowel disease [39]and intestinal tumorigenesis [40], and thus considered as a marker for the early gut microbial dysbiosis [38] . Although the association between gut *Fusobacterium* and CVD has not been reported before, *Fusobacterium nucleatum* was detected in human carotid artery plaque tissues [16–18] . Our findings provide new evidence supporting that both gut and oral *Fusobacterium* might be potential sources of *Fusobacterium* in carotid artery plaque [18]. We also found that gut *Fusobacterium* was associated with multiple plasma lipids and metabolites, especially higher levels of LPCs, LPEs and DGs which were further found to be associated with increased risk of carotid artery plaque. Interestingly, our microbial functional analysis identified several microbial enzymes, which are involved in the lipid metabolism, associated with these lipid profiles as well as gut *Fusobacterium*. In particular, Phospholipase A1 and A2 are the key enzymes in the reactions producing LPCs and LPEs from PCs and PEs, respectively[33,34]. Both LPCs and LPEs are phospholipids in the cell membrane and may play a role in cell signaling and immune modulating [41], and LPCs have been found to induce endothelial cell activation during early atherosclerosis thus may contribute to the development of CVD[42] . Taken together, these results suggest a potential link between higher abundance of gut *Fusobacterium* and related microbial functions in the lipid metabolism and alternations of host plasma lipid profiles associated with atherosclerosis and CVD. However, it should be noted that both host factors (e.g., genetics, dietary intake) and gut microbiota may contribute to host plasma lipid profiles since these lipids are not gut microbiota dependent metabolites. In addition, the mechanisms underlying these associations remains unclear.

As Gram-negative bacteria, members of the *Proteus* genus (e.g., *Proteus mirabilis*, *Proteus vulgaris*) are inflammation inducers [43] and clinically recognized as a cause of urinary-tract infections and bacteremia[44,45]. Similar to *Fusobacterium*, although no previous studies have linked gut *Proteus* with atherosclerosis or CVD, *Proteus* species have been identified in human coronary plaque tissues [46] . A recent experimental study found that the *Proteus mirabilis* may interact with atherosclerotic plaques in human coronary arteries via dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin, and higher abundance of *Proteus mirabilis* was found in the hearts from atherosclerotic mice compared to control mice [47]. These previous results are in line with our findings, and all suggest a potential involvement of *Proteus* in the atherosclerotic plaque formation. Moreover, we found similar but relative weaker associations of gut *Proteus,* compared to gut *Fusobacterium,* with microbial functions in the lipid metabolism and alternations of host plasma lipid profiles (e.g., LPCs, LPEs, and DGs) associated with carotid artery atherosclerosis, though the underlying mechanisms warrants further investigation. Interestingly, our results also suggest that HIV infection and progression might be associated with increased levels of gut Proteus,

but did not observe significant effect modification by HIV on the association between Proteus and plaque. Further studies are needed to elucidate the potential biological link between HIV infection and Proteus and its potential impacts on atherosclerosis and CVD.

Our study also identified two genera, *Odoribacter* and *Adlercreutzia*, inversely associated with carotid artery atherosclerosis. As a butyrate-producing genus, *Odoribacter* in gut has been favorably correlated with SBP in pregnant women [48], potentially related to the production of butyrate which may have beneficial effects on anti-inflammation and cardiometabolic health.[49] Consistently, we also observed an inverse correlation between *Odoribacter* and SBP, a well-known risk factor for atherosclerotic plaques and CVD. Of note, the observed association between *Odoribacter* and plaque was attenuated after further adjustment for blood pressure, suggesting that the beneficial association between *Odoribacter* and carotid artery atherosclerosis might be partially explained by blood pressure.

Adlercreutzia is an obligately anaerobic coccobacillus, and members of this genus (e.g., *Adlercreutzia equolifaciens*) are known to play a role in the breakdown of isoflavones and the production of equol[50]. Few studies has linked gut *Adlercreutzia* with human cardiometabolic health, but equol, which serves as a phytoestrogen, has been shown to have beneficial effects on both vasodilation and nitric oxide metabolism that may have a favorable impact on vascular health[51,52]. Thus, the observed inverse associations of gut *Odoribacter* and *Adlercreutzia* with carotid artery atherosclerosis might be related to some beneficial metabolites produced by these bacteria (e.g., butyrate, equol) [48,51,52] .Unfortunately, butyrate, equol or other related metabolites were not captured by our lipidomics/metabolomics, and we did not find any significant associations of these two bacteria with plasma lipids or metabolites measured in this study. Nevertheless, our data together with previous findings suggest potential cardiometabolic benefits of *Odoribacter, Adlercreutzia* and related bacterial metabolites, and studies are needed to test the potential of these bacteria as probiotics in the prevention of atherosclerosis and CVD.

Our study has several limitations. The associations of gut microbiota with host plasma lipid and metabolite profiles and carotid artery plaque were examined in a cross-sectional dataset of women with or at high risk of HIV. However, using a longitudinal dataset of both women and men with or at high risk of HIV, we found that baseline plasma gut microbiota-related lipid profiles were significantly associated with incident carotid artery plaque over 7 years. Due to the limitation of the 16S sequencing data, our analyses focused on the bacterial genus level, and for some plaque-associated genera, we were unable to assign the ASVs into specific known species. Our lipidomics/metabolomics measured a total of 387 lipid species and polar metabolites in plasma, however, levels of these metabolites were semi-quantified without absolute values. Our methoddid not capture some microbial metabolites, such as short-chain fatty acids which need to be measured using a different method [53]. Targeted methods on the identified lipids and metabolites are warranted to validate our findings and better understand the underlying biological mechanisms. In our metabolic analyses, we did not control for kidney function measures, since some metabolites are excreted via the kidneys. The microbial functional component data should be interpreted with caution, although the inferred functional contents by PICRUSt2 have been shown to be

robust, particularly for the human gut microbiome[54]. Due to the nature of our observational study design, causal inference could not be established without further evidence. In vivo experimental studies are needed, such as fecal microbiota transplantation in gem-free atherosclerosis animal models.

In summary, this study identified alterations in gut microbiota, specifically the enrichment of *Fusobacterium* and *Proteus*, and the depletion of *Odoribacter* and *Adlercreutzia*, were associated with carotid artery atherosclerosis in women with or at high risk of HIV. Notably, higher levels of gut *Fusobacterium and Proteus* were also associated with multiple plasma lipid and metabolite profiles, especially higher levels of LPCs, LPEs and DGs which were associated with progression of carotid artery atherosclerosis over time. We also found several bacterial enzymes in the lipid metabolism (e.g., Phospholipase A1 and A2) associated with plasma lipid metabolite levels (e.g., LPCs and LPEs), suggesting a potential contribution of gut microbiota to host plasma lipid profiles associated with carotid artery atherosclerosis. Our study contributes to the increasing body of gut microbiota studies on atherosclerosis and CVD by extending the relationship among gut microbiota, host lipid/metabolite profiles, and CVD to a significantly under-studied group, people living with HIV infection, with high CVD burden. Our study also supports the concept of a potential therapeutic role of modulating the gut microbiota and related bacterial metabolites in the prevention of atherosclerosis and CVD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

CVD	Cardiovascular disease
HIV	human immunodeficiency virus
ART	antiretroviral therapy
CIMT	carotid intima-media thickness
ASV	amplicon sequence variant
BMI	body mass index
SBP	blood pressure
DBP	diastolic blood pressure
LPC	lysophosphatidylcholines
LPE	lysophosphatidylethanolamines
DG	diglycerides
WIHS	Women’s Interagency HIV Study
MACS	Multicenter AIDS Cohort Study

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Highlights

- In women with or at high risk of HIV, carotid artery plaque is associated with enrichment of gut microbial genera *Fusobacterium* and *Proteus*, and depletion of *Odoribacter* and *Adlercreutzia*.
- Gut *Fusobacterium* and *Proteus* are associated with multiple plasma lipids such as lysophosphatidylcholines (LPCs) and lysophosphatidylethanolamines (LPEs), and diglycerides (DGs).
- The circulating levels of microbial-associated lipidomic LPCs & LPEs and DG modules are prospectively associated with increased risk of carotid artery plaque.
- Several microbial functional enzymes in the lipid metabolism are associated with circulating levels of plasma lipids, as well as gut Fusobacterium and Proteus. In particular, phospholipase A1 and A2 are the key enzymes in the reactions producing LPCs and LPEs from phosphatidylcholines and phosphatidylethanolamines.

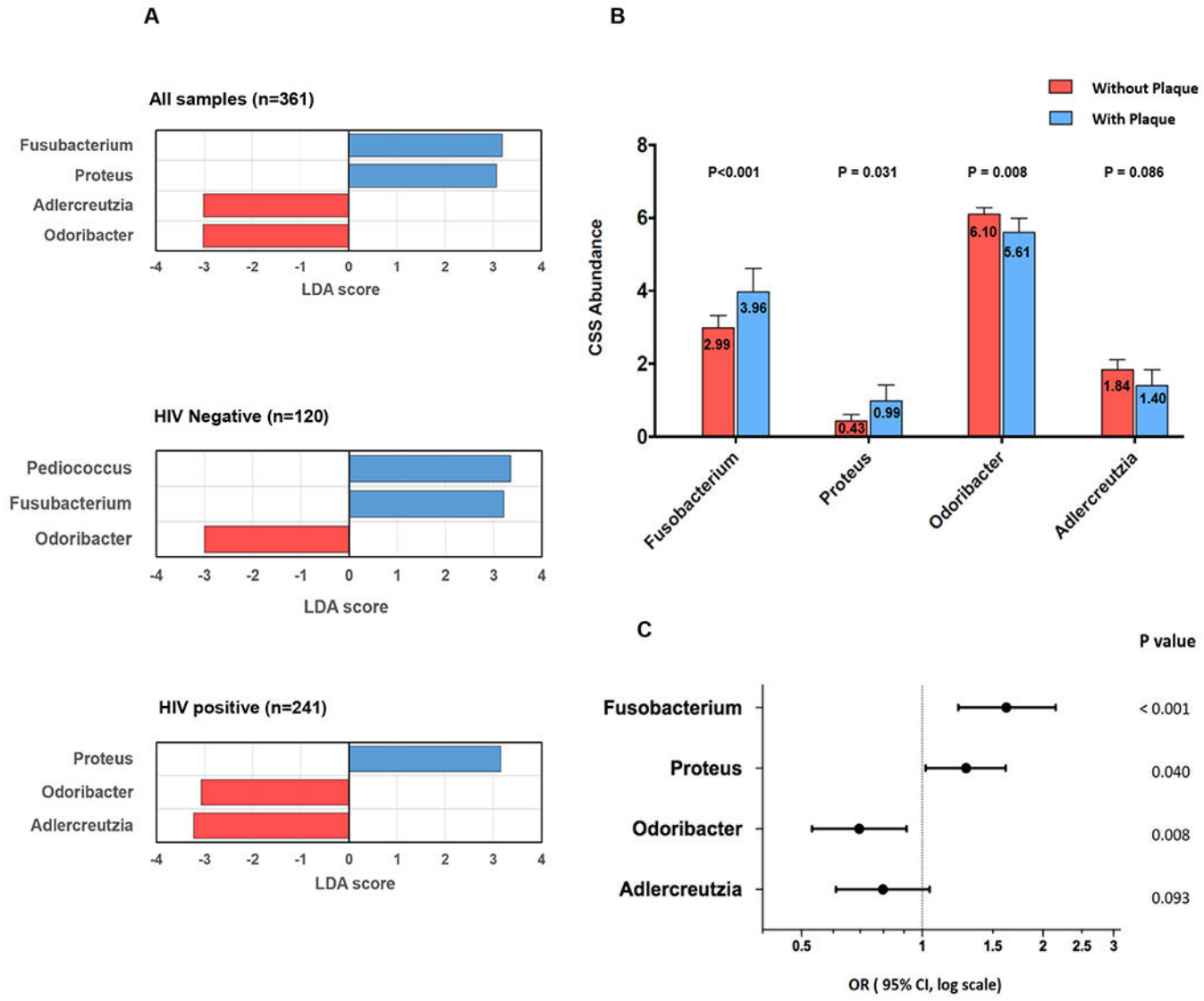

Figure 1. Differentially abundant genera according to carotid artery plaque status.

(A) Taxonomic Linear discriminative analysis (LDA) effect size (LefSe) analysis by carotid artery plaque status, in unstratified samples (n=361), HIV negative samples (n=120), and HIV positive samples(n=241)

(B) Means and 95% CIs of CSS transformed abundance of gut bacterial genera, by carotid artery plaque status. P value were estimated in the linear regression models, adjusting for age, race, study site, antibiotics use, income, education, BMI, current smoking status, marijuana use and HIV status.

(C) Associations between genera and plaque status. Data are odds ratios (ORs) and 95% confidence intervals (CIs) for carotid artery plaque per standard deviation increment of CSS transformed abundance of gut bacterial genera, adjusting for aforementioned covariates.

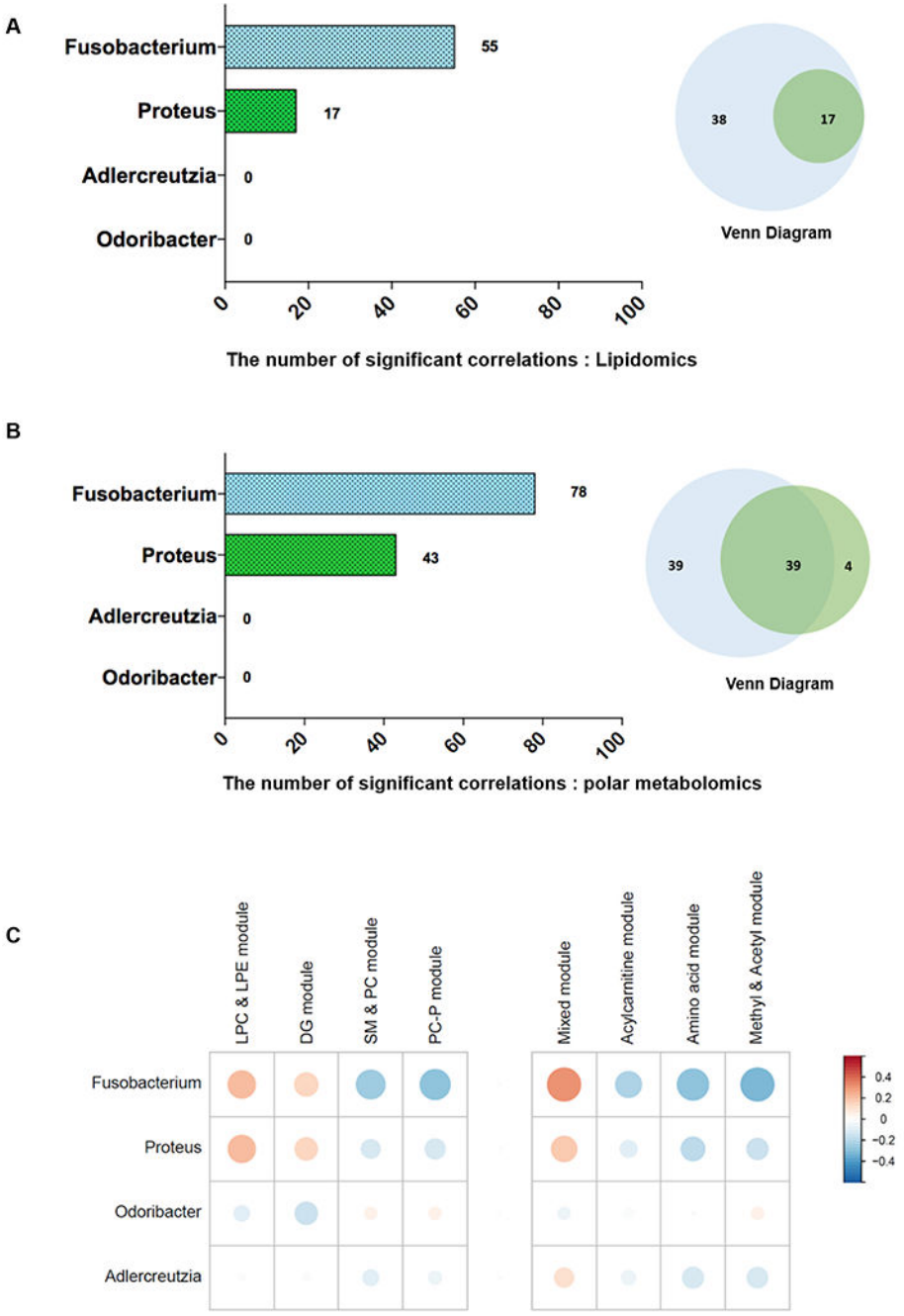

Figure 2. Associations of plaque-associated bacterial genera with host plasma lipidomic and metabolomic profiles.

(A) The number of significant correlations between plaque-associated genera and lipids (FDR $p < 0.05$). The Venn diagrams depict overlaps between the *Fusobacterium/Proteus* correlated lipids.

(B) The number of significant correlations between plaque-associated genera and polar metabolites (FDR $p < 0.05$). The Venn diagrams depict overlaps between the *Fusobacterium/Proteus* correlated polar metabolites.

(C) Correlations between bacterial genera and the 8 modules of lipids and metabolites.

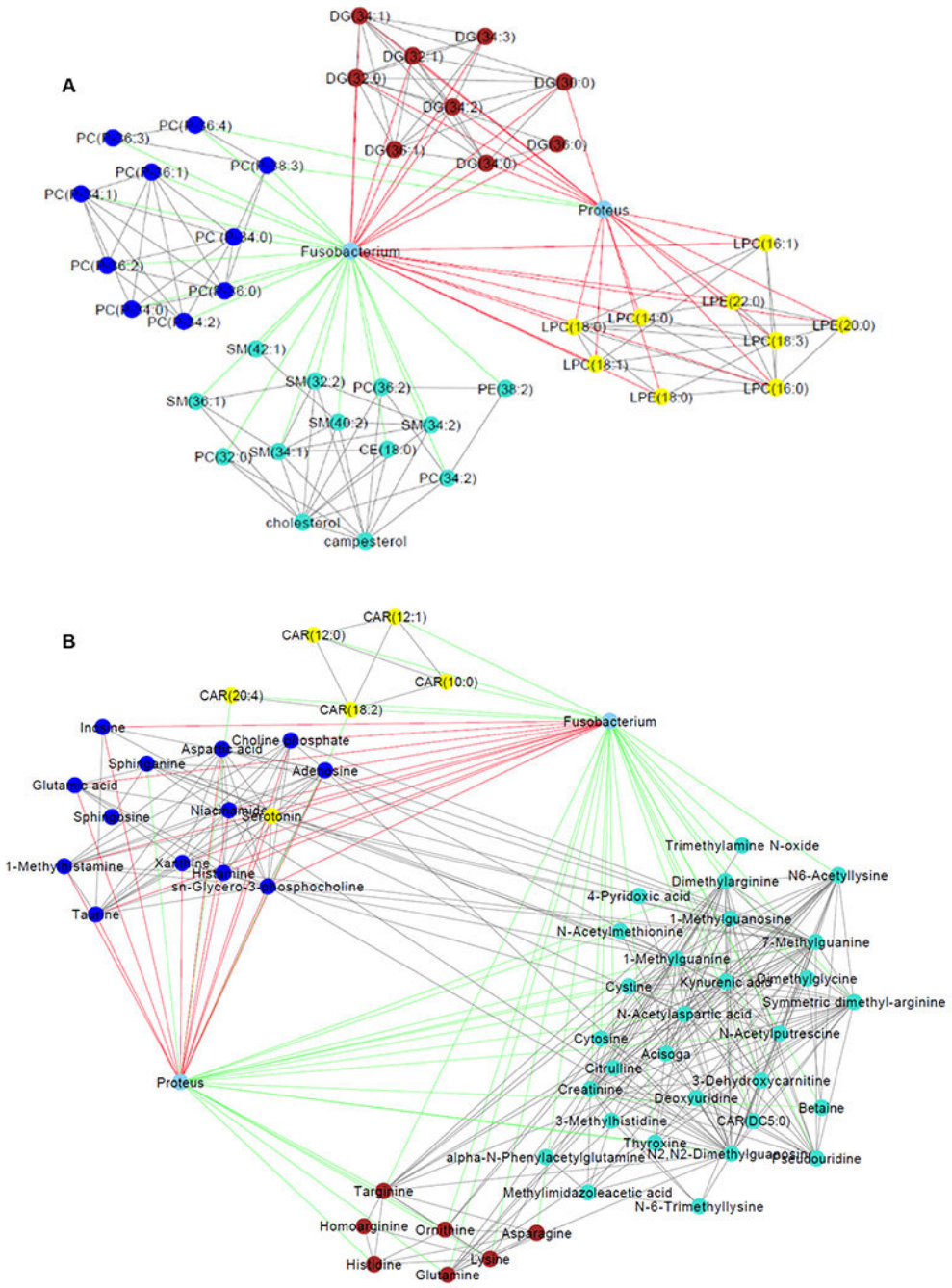

Figure 3. Network analysis of plaque-associated bacterial genera, host plasma lipidomic and metabolomic profiles.

(A) Network plot illustrating the *Fusobacterium* and *Proteus* correlated lipids modules. The color of node reflects the module of each metabolites and the grey edges indicate the weighted correlation coefficients among metabolites. Red/green lines indicate the significant positive/inverse correlations between bacterial genera and metabolites.

(B) Network plot illustrating the *Fusobacterium* and *Proteus* correlated polar metabolites modules.

Abbreviations: LPC, lysophosphatidylcholine ; LPE, lysophosphatidylethanolamine; DG, diglyceride; SM, Sphingomyelin; PC, phosphatidylcholine; PC-P, phosphatidylcholines plasmalogen.

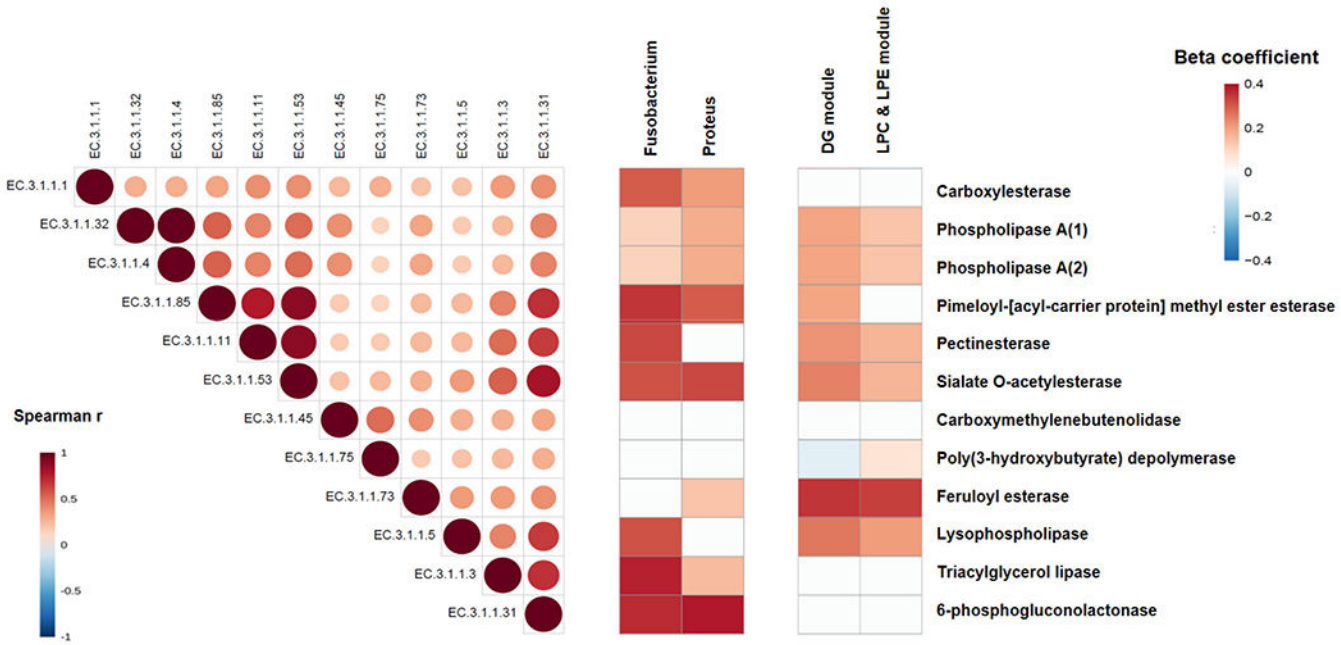

Figure 4. Associations between Gut microbiota functional enzymes, specific bacteria genera, and host metabolomics modules.

The Spearman correlation heatmap include 12 microbial functional enzymes under carboxylic-ester hydrolases category. Associations between the 12 microbial functional enzymes and the plaque-related bacterial genera *Fusobacterium/Proteus,* were estimated by linear regression models after adjustment for age, race, study site, antibiotics use, income, education, BMI, current smoking status, marijuana use and HIV status. Associations between the 12 enzymes and lipidomics DG module and LPC & LPE module were also estimated by linear regression models, adjusting for aforementioned covariates. Abbreviations: LPC, lysophosphatidylcholine ; LPE, lysophosphatidylethanolamine; DG, diglyceride;

Table 1.

Associations of Metabolite Modules with Risk of Carotid Artery Plaque

Module		All (n= 737)			HIV + (n= 520)			HIV - (n= 217)			P for interaction
		RR	95% CI	p value	RR	95% CI	p value	RR	95% CI	p value	
LPC & LPE module	Model 1	1.36	(1.09 - 1.69)	0.007 *	1.31	(1.02 - 1.67)	0.034	1.39	(0.80 - 2.43)	0.244	0.414
	Model 2	1.32	(1.05 - 1.65)	0.018	1.28	(0.99 - 1.66)	0.056	1.45	(0.83 - 2.55)	0.189	0.379
DG module	Model 1	1.30	(1.06 - 1.60)	0.011 *	1.30	(1.03 - 1.65)	0.030	1.12	(0.71 - 1.76)	0.634	0.957
	Model 2	1.29	(1.05 - 1.59)	0.017	1.34	(1.05 - 1.70)	0.016	1.18	(0.74 - 1.88)	0.496	0.943
SM & PC module	Model 1	1.24	(1.02 - 1.52)	0.035	1.17	(0.94 - 1.45)	0.173	1.79	(1.04 - 3.06)	0.034	0.136
	Model 2	1.25	(1.02 - 1.54)	0.029	1.17	(0.93 - 1.47)	0.179	1.89	(1.08 - 3.32)	0.026	0.127
PC-P module	Model 1	1.05	(0.85 - 1.28)	0.673	1.02	(0.81 - 1.30)	0.858	1.31	(0.80 - 2.12)	0.281	0.354
	Model 2	1.08	(0.88 - 1.33)	0.472	1.03	(0.81 - 1.31)	0.826	1.29	(0.78 - 2.12)	0.319	0.415
Acylcarnitine module	Model 1	1.06	(0.86 - 1.30)	0.589	1.15	(0.91 - 1.45)	0.232	0.87	(0.56 - 1.36)	0.536	0.546
	Model 2	1.08	(0.88 - 1.33)	0.462	1.15	(0.91 - 1.44)	0.247	0.91	(0.58 - 1.43)	0.672	0.563
Amino acid module	Model 1	0.83	(0.66 - 1.03)	0.094	0.89	(0.70 - 1.14)	0.357	0.67	(0.39 - 1.15)	0.150	0.987
	Model 2	0.86	(0.69 - 1.08)	0.195	0.90	(0.70 - 1.15)	0.382	0.68	(0.39 - 1.19)	0.179	0.973
Methyl and Acetyl module	Model 1	1.14	(0.92 - 1.42)	0.239	1.11	(0.88 - 1.40)	0.372	1.16	(0.62 - 2.14)	0.646	0.636
	Model 2	1.14	(0.92 - 1.41)	0.245	1.12	(0.89 - 1.42)	0.325	1.16	(0.62 - 2.17)	0.643	0.632
Mixed module	Model 1	1.06	(0.83 - 1.37)	0.631	1.10	(0.82 - 1.48)	0.519	1.04	(0.57 - 1.91)	0.889	0.499
	Model 2	1.05	(0.82 - 1.36)	0.688	1.08	(0.80 - 1.45)	0.610	1.02	(0.55 - 1.90)	0.948	0.574

The data are adjusted risk ratios (RR) and 95% CI of carotid artery plaque per standard deviation increment in metabolite modules (inverse-normal transformed).

Model 1: Adjusted for age, sex, race, study site, education, and Smoking status.

Model 2: Further adjusted for ART use, CD4 counts, HIV viral load, crack cocaine use, injected drug use, hepatitis C virus infection.

* FDR P<0.05. FDR correction was not applied in Model 2 or stratification analyses by HIV infection status.

Abbreviations: LPC, lysophosphatidylcholine ; LPE, lysophosphatidylethanolamine; DG, diglyceride; SM, Sphingomyelin; PC-P, phosphatidylcholines plasmalogen; CAR, acylcarnitines.