Serum tryptophan-derived quinolinate and indole-3-acetate are associated with carotid intima-media thickness and its evolution in HIV-infected treated adults

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FOOTNOTES

CONFLICTS OF INTEREST

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ABSTRACT

Background: HIV-infected individuals undergoing effective antiretroviral therapy (ART) present an increased risk of atherosclerotic cardiovascular disease. We identified serum metabolites associated with carotid intima-media-thickness (c-IMT) and its evolution.

Methods: 143 hydrophilic serum metabolites were measured by ultra-performance liquid-chromatography coupled to high-resolution mass-spectrometry in 49 HIV+ART+, 48 HIV+ART-naïve, and 50 HIV-negative age-matched, never-smoking, male triads. Metabolites differentially altered between groups (“features”) were defined as having a Benjamini-Hochberg adjusted p-value <0.05 from a t-test and >0.25 log2 absolute mean fold-change in metabolite levels. c-IMT was measured across 12 sites at inclusion in all individuals and at the carotid-artery (cca) after a median 5.1 years in 32 HIV+ART+ individuals. Difference in c-IMT (cross-sectional analysis) and slope of cca-IMT regression/progression per year (longitudinal analysis) for each log10(area) increase in metabolite level was estimated with linear regression.

Results: Compared to HIV-, metabolite features of HIV+ART+ were increased N6,N6,N6-trimethyl-L-lysine and decreased ferulate and 5-hydroxy-L-tryptophan; while features of HIV+ART-naïve were increased malate, kynurenine, 2-oxoglutarate, and indole-3-acetate and decreased succinate and 5-hydroxy-L-tryptophan. In HIV+ART+ individuals, quinolinate and/or indole-3-acetate were positively associated with c-IMT (p<0.03), cca-IMT (p<0.03) and with cca-IMT progression (p<0.008). These associations were not observed in HIV+ART-naive or HIV-negative individuals. In HIV+ART+ individuals, the metabolites xanthosine and uridine, from nucleotide metabolism, and g-butyrobetaine, from lysine/dietary choline degradation, were also positively or negatively associated with c-IMT and/or cca-IMT (all p<0.01), but not its evolution.

Conclusion: In these highly-selected HIV-positive ART-controlled males, two novel metabolites derived from tryptophan catabolism, indole-3-acetate and quinolinate, were associated with c-IMT and its progression.
Keywords: antiretroviral; carotid intima-media thickness; HIV; metabolomics; tryptophan metabolism; cardiovascular disease.

Key points: This cross-sectional study in antiretroviral (ARV)-treated, HIV-positive individuals found two novel metabolites derived from tryptophan catabolism, indole-3-acetate and quinolinate, were associated with c-IMT and its progression. These associations were not observed in an HIV-negative comparison group, suggesting an ARV/HIV-specific mechanism.
INTRODUCTION

Cardiovascular disease (CVD) has become a major concern in HIV-positive individuals, even for those with well-controlled HIV replication during antiretroviral therapy (ART) [1,2]. Increases in atherosclerotic cardiovascular risk have been reported for HIV-positive individuals, whether based on cardiovascular outcomes or carotid intima-medial thickness (c-IMT), a validated surrogate marker of atherosclerotic vascular disease [3]. Prior studies have linked host factors, such as smoking and illicit drug use, and HIV-related factors, such as known infection duration [4], immune activation [5] and treatment with protease inhibitors [6,7], to this increased risk. Nevertheless, the precise pathophysiological mechanisms remain uncertain to date.

Metabolomics, able to identify numerous circulating metabolites, could provide important information on the processes involved in atherosclerotic cardiovascular risk. Such analyses have been used in past research in which metabolite levels were associated with HIV-serostatus, severity of HIV-infection, low-grade inflammation in HIV-positive individuals with virological suppression, and long-term non-AIDS-related events [8]. Most previous research has focused on the tryptophan (Trp) catabolic pathway and kynurenine/tryptophan ratio (KTR), resulting from indoleamine-pyrrole 2,3-dioxygenase (IDO) activation [9–12], and trimethylamine (TMA) and trimethylamineoxide (TMAO) of the lysine/dietary choline degradation pathway [13,14].

Notwithstanding the insights gained from these previous studies, their major limitation is that only specific metabolites of distinct pathways were analyzed. To understand the broader scope of potentially involved metabolites, we analyzed 143 hydrophilic metabolites, including all amino acids and metabolic derivatives, and their association with HIV-infection and c-IMT. Using data from a previous study in closely matched, never-smoking, male adults who were HIV-positive.
ART-controlled, HIV-positive ART-naïve, or HIV-negative, we took advantage of the minimal confounding bias offered from these triads to evaluate the effect of HIV-infection and treatment on metabolite levels. We then focused on HIV-positive ART-treated patients to establish the relationship between metabolite levels and c-IMT. Their correlation with markers of inflammation and HIV-infection severity was also assessed.

METHODS

Study participants and samples

Participants were selected from the Collaboration on HIV, Inflammation and Cardiovascular disease (ANRS-CHIC) study [4]. Briefly, 150 never-smoker men were enrolled in three groups: 50 HIV-1-infected patients >35 years old, taking ART for ≥4 years, and with HIV-1 RNA <400 copies/ml (HIV+ART+); 50 individually age-matched (±5 years) patients, HIV-positive for ≥2 years, who were naïve to ART (HIV+ART-); and 50 HIV-negative patients individually age-matched (±5 years) to the index HIV-positive, treated patient (HIV-). Non-inclusion criteria were current/former smokers; history of coronary heart disease, stroke, angina or myocardial infarction; active/chronic viral hepatitis infection; undergoing systemic chemotherapy or steroids. All participants gave their written informed consent and the protocol was approved by the Hotel-Dieu Ethics Committee.

For this study, we included participants with available serum samples. Individuals with severe renal dysfunction (estimated creatinine clearance <30 mL/min) were excluded due to the strong effect of kidney function on metabolite concentrations [15].
Carotid intima-media thickness measurements

c-IMT was calculated as a composite measure (12-site mean) of the maximal common carotid artery (cca)-IMT, bifurcation IMT, and internal carotid artery IMT bilaterally, outside of plaque, and are reported in mm [4]. c-IMT measurements were performed offline with quality-IMT automatic measurement software. A subgroup of patients in the HIV+ART+ group also had a second measurement of cca-IMT a median 5.1 years (IQR=4.8-5.3) from participating in the cross-sectional study.

Laboratory measurements

Individuals were fasting (12 h) at visit. Blood samples were retrieved and processed at a single center and stored at -80°C until use. Interleukin (IL)-6, IL-10 (Bender Medsystems, Burlingame, CA, USA), resistin, and soluble(s)CD14 (R&D Systems, Minneapolis, MN, USA) levels were analyzed using an enzyme-linked immunosorbent assay. Serum ultra-sensitive C-reactive protein (us-CRP) was measured by immonephelometry on an IMMAGE analyzer (Beckman-Coulter, Miami, FL, USA). Plasma D-dimer was measured by enzyme linked fluorescent assay on a VIDAS analyzer (Biomérieux, Marcy-l’Etoile, France). Inducible protein (IP)-10, fractalkine, MIG, monocyte chemoattractant protein (MCP)-1, E-selectin, and tumor necrosis factor (TNF)-α were quantified from plasma using the BD™ Cytometric Bead Array system (BD, Franklin Lakes, NJ, USA).

Immunological function

Cell samples were available on a randomly-selected subset of 30 HIV+ART+ patients [16]. Activated CD8+ and CD4+ memory T-cells (CCR7-CD27-CD45RA+/-, defined by the
expression of CD38 and Ki67 markers) and immunosenescence (defined by the expression of
CD57 on memory T-cells) were analyzed on a LSR2 flow cytometer (Becton Dickinson, Franklin
Lakes, New Jersey, USA) with appropriate isotype controls and color compensation.

**Ultra-performance-liquid-chromatography coupled to a high resolution-mass-
spectrometry (UPLC-HRMS) metabolomics**

Reference compounds and 13C and 15N stable isotope-labeled mix of amino acids were
purchased from Sigma-Aldrich (Saint Quentin Fallavier, France) as well as LC-MS grade
solvents, acetonitrile and formic acid. Deionized water was obtained from a Milli-Q Elix system
fitted with a LC-PaK and a MilliPak filter at 0.22µm (Merck Millipore, Guyancourt, France).

Sample preparation and liquid chromatography coupled to high resolution mass spectrometer
(LC-HRMS) experiments were carried out as reported [17,18]. Briefly, 8 volumes of frozen
acetonitrile (-20°C) containing internal standard (labeled mixture of amino acids at 12.5 µg/mL)
were added to 100 µL of serum samples and vortexed. Resulting samples were then sonicated
and incubated at 4°C during 1h for slow protein precipitation. Samples were centrifuged for 20
min at 20.000×g at 4°C. Supernatants were transferred to another series of tubes and then
dried and stored at -80°C prior to LC-MS analyses. Samples were reconstituted in a three-fold
dilution of H2O/ACN (95/05).

UPLC-HRMS experiments were performed using a chromatographic column Discovery HS F5-
PFPP, 5µm, 2.1×150 mm (Sigma, Saint Quentin Fallavier, France) at 35°C in a UPLC® Waters
Acquity (Waters Corp, Saint-Quentin-en-Yvelines, France) and Q-Exactive™ mass
spectrometer (Thermo Fisher Scientific, Illkirch, France).
Statistical analysis

To perform our analysis, we used pathways of the major mapped metabolites from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [19] that have been involved in previous studies on HIV/ART or c-IMT. These metabolic pathways were purine and pyrimidine metabolism, lysine and dietary choline catabolism, tryptophan catabolism, and citrate cycle. All metabolite levels are reported in log\textsubscript{10}-transformed area values (log\textsubscript{10}(area)).

In an initial analysis, we examined metabolite differences between HIV/ART groups. First, we compared the overall variation of metabolite levels, within the four selected pathways, across groups using an $F$-statistic from Wilks' lambda in a multivariate analysis of variance (MANOVA). Second, we identified metabolite features that were differentially altered between HIV/ART groups. Mean metabolite levels were compared between (i) HIV+ART+ versus HIV-, (ii) HIV+ART- versus HIV-, and (iii) HIV+ART+ versus HIV+ART- using an unpaired $t$-test. $P$-values were adjusted using the Benjamini-Hochberg procedure. The log\textsubscript{2} mean fold-change of the log\textsubscript{10}(area) levels between groups was also calculated for each metabolite. We defined metabolite features as those with an adjusted $p$-value $<0.05$ and a $>0.25$ or $<-0.25$ log\textsubscript{2} mean fold-change in log\textsubscript{10}(area). This analysis was carried out using the R package ‘omu’.

In a subsequent analysis, we focused on HIV+ART+ participants and studied the relationship between metabolite levels and c-IMT. We first used univariable analyses to identify candidate metabolites, which were tested in subsequent analyses. First, change in c-IMT for each log\textsubscript{10}(area) increase in metabolite level was estimated with linear regression in univariable analysis. A multivariable analysis model was constructed in which an \textit{a priori} selection of covariables based on prior research [4,15] (age, prior hypertension, diabetes, and creatinine clearance) and metabolites with $p<0.1$ in univariable analysis were included, while metabolites
with $p>0.1$ being removed in backwards-stepwise fashion. Second, the same metabolites associated with c-IMT in univariable analysis were used to model (1) change in cca-IMT for each $\log_{10}(\text{area})$ increase in metabolite level and (2) change in slope of cca-IMT regression/progression per year for each $\log_{10}(\text{area})$ increase in metabolite concentration among those with two cca-IMT measurements. Univariable and multivariable models were constructed as above. Significance for these analyses was determined by a $p$-value <0.05 and no $p$-value adjustments were made in order to avoid adverse reduction in Type II error [31].

Other analyses were conducted to determine the correlation (Spearman rank) between metabolites of the same pathway, as illustrated with correlation networks using Cytoscape v3.6.1 [20], and to determine the correlation between metabolites and markers of inflammation/immunity or HIV severity, as illustrated with heatmaps using the R package ‘gplots’. Significance for these analyses were determined by a $p$-value <0.05.

Statistical analysis was performed using STATA (v12.1, College Station, TX) and R (v3.2.0, Vienna, Austria).

RESULTS

Characteristics of the study population

Of the 150 included males, two without available serum sample and one with severe renal dysfunction in the HIV+ groups were not included.
Demographic, HIV- and cardiovascular-related characteristics are described in Supplementary Table 1. The large majority had a BMI <30kg/m² (97%) and only 6% had ≥1 comorbidity (diabetes, prior hypertension or moderate renal dysfunction). Any use of cannabis, cocaine and/or methamphetamine within <12 months was most common in HIV+ART+ participants (28.6%), followed by HIV+ART- (12.5%) and HIV- (2.0%) participants (p<0.001). In the HIV+ART+ group, HIV-infection was mostly controlled with median ART duration longer than 4 years and 94% under viral suppression (<50 copies/mL).

**Differences in metabolites between HIV/ART groups**

Using MANOVA, significant variation in metabolite levels were observed in the tryptophan metabolism (p<0.001) and citrate cycle (p<0.001) pathways, but not purine/pyrimidine metabolism (p=0.08) or lysine and dietary choline catabolism (p=0.14). Metabolite features were as follows: HIV+ART+ versus HIV-: increased relative levels of N6,N6,N6-trimethyl-L-lysine, and decreased ferulate, and 5-hydroxy-L-tryptophan (Figure 1A); HIV+ART- versus HIV-: increased malate, kynurenine, 2-oxoglutarate, and indole-3-acetate and decreased succinate and 5-hydroxy-L-tryptophan, (Figure 1B); and HIV+ART+ versus HIV+ART-: decreased succinate (Figure 1C).

**Correlation between metabolites of the same pathway according to HIV/ART groups**

In the purine/pyrimidine metabolism pathway (Figure 2A), a strong correlation was observed between inosine and guanosine in HIV+ART+ individuals (rho=0.836, p<0.001), which remained in HIV- individuals (Supplementary Figure 1A). In the lysine and dietary choline catabolism pathway (Figure 2B), strong correlations were observed between pipecolate and carnitine (rho=0.408, p=0.004), N6,N6,N6-trimethyl-L-lysine and lysine (rho=0.322, p=0.02), and
N6,N6,N6-trimethyl-L-lysine and 2-amino adipate (\( \rho=0.391, \ p=0.005 \)) in HIV+ART+ individuals, while these correlations were not apparent in HIV- individuals (Supplementary Figure 1B). In the tryptophan metabolism pathway (Figure 2C), fairly weak correlations were observed between metabolites in HIV+ART+ individuals with the exception of indole-3-acetaldehyde and serotonin (\( \rho=0.989, \ p<0.001 \)) and while this correlation held in HIV- individuals (Supplementary Figure 1C), other correlations emerged. In the citrate cycle pathway (Figure 2D), only citrate and isocitrate were significantly correlated (\( \rho=0.439, \ p=0.002 \)), while this correlation and one between malate and isocitrate emerged in HIV- individuals (Supplementary Figure 1D).

**Metabolites associated with IMT and its progression**

Focusing on HIV+ART+ individuals, we identified four metabolites associated with higher c-IMT levels (xanthosine, uridine, indole-3-acetate, and quinolinate) and two with lower c-IMT (pipocolate and g-butyrobetaine) in univariable analysis (Table 1). All except pipocolate remained significant in multivariable analysis with additional adjustment for age, prior hypertension, diabetes, and creatinine clearance. Metabolites that were not associated with c-IMT in univariable analysis are presented in the Supplementary Table 2.

We used the metabolites associated with c-IMT in univariable analysis as candidates for two subsequent multivariable analyses. First, cca-IMT was used as an end-point and was found to be significantly associated with only g-butyrobetaine and indole-3-acetate (Table 1). Second, when examining the evolution of cca-IMT in the subset of participants with two measures, significantly faster progression of IMT was observed with higher baseline indole-3-acetate and quinolinate levels.
Of note, these associations were not observed in HIV+ART-naïve (data not shown) or HIV-negative participants (Supplementary Table 3).

**Metabolites associated with immune and inflammatory markers**

Of the metabolites associated with c-IMT levels, most were not associated with profiles of high inflammation levels (Figure 3). However, uridine of the purine/pyrimidine metabolism pathway was strongly correlated with several markers of inflammation and cytokines (Figure 3A) and there was a significant correlation between quinolinate of the tryptophan metabolism pathway and TNF-α levels (rho= 0.334, p=0.02) (Figure 3C). Of all the candidate metabolites, g-butyrobetaine was significantly correlated to percent of naïve CD4+CD27+CD45RA+ (rho=0.409 p=0.03), memory CD4+ (rho=-0.469, p=0.009), naïve CD8+CD27+CD45RA+ cells (rho=0.469, p=0.009) and memory CD8+CD57+ senescent cells (rho=0.422, p=0.02) (Supplementary Figure 2). Pipecolate was also significantly correlated with percent of CD4+CD57+ cells (rho=0.378, p=0.04).

Of note, four of the five metabolites involved in the citrate cycle were significantly associated with TNF-α and/or D-dimer (Figure 3D).

**Metabolites associated with markers of HIV-infection severity**

As shown in Table 2, none of the metabolites associated with c-IMT in univariable analysis were significantly correlated with CD4+ T-cell count, nadir CD4+, or CD4:CD8 ratio in HIV+ART+ participants. Nevertheless, higher levels of xanthine were significantly correlated with lower CD4+ nadir and higher orotate and L-carnitine levels with higher CD4+ nadir (Table 2). Higher
indolelactate levels were significantly correlated with a lower CD4:CD8 ratio and kynurenate with lower CD4+ T-cell count. All other correlations are provided in Supplementary Table 4.

DISCUSSION

We report that in ART-controlled HIV-positive males but not HIV-positive ART-naïve or HIV-negative controls, c-IMT and its progression were positively associated with metabolites derived from the Trp catabolism pathway, indole-3-acetate and quinolinate. Xanthosine and uridine metabolites derived from purine/pyrimidine metabolism were positively associated with c-IMT while the derivative g-butyrobetaine from the lysine and dietary choline degradation was inversely associated with c-IMT.

Of all pathways studied, metabolites of Trp catabolism were the only ones consistently associated with c-IMT/cca-IMT at the cross-sectional visit as well as cca-IMT progression. Kynurenine and, to a lesser extent, kynurenic acid (KA) have already been identified as correlates with c-IMT and CVD, but these associations were observed mostly in patients with end-stage renal disease [15]. Only few studies have evaluated the association between these metabolites and CVD outcomes in HIV-positive individuals. For instance, a study in 105 ART-naïve HIV-positive from Uganda demonstrated that lower absolute values of KTR six months after ART initiation, specifically among those achieving undetectable HIV viral load, were associated with lower c-IMT levels when measured seven years after treatment initiation [21]. In other cohorts, HIV-positive individuals, compared to HIV-negative, were found to have lower levels of Trp but comparable levels of KA, while higher levels of Trp and lower KA or KA/Trp ratio were associated with a decreased risk of carotid artery plaque formation after a median seven years of follow-up [22]. Although this study importantly stresses the role of Trp catabolites
in increased CVD risk among HIV-positive patients, the relationship with c-IMT was not provided and other classical Trp catabolites, such as kynurenine, were not measured. Furthermore, this previous observation stemmed from study populations with a high prevalence of comorbidities and smokers, thereby increasing the risk of residual confounding bias.

In our study, we observed that Trp and KA were never identified as metabolite features between HIV/ART groups, yet 5-hydroxy-L-tryptophan was a feature with lower levels in HIV-positive individuals, regardless of ART status. We also confirmed the higher levels of kynurenine in HIV+ART- individuals compared to HIV-negative individuals [10,11,23]. None of these metabolites, however, were associated with c-IMT in our study and instead only indoleacetate and quinolinate were positively associated with c-IMT after adjusting on age, hypertension, diabetes, and creatinine clearance. Importantly, this association was not apparent in HIV+ART-naïve and HIV-negative controls, implying that the underlying role of these metabolites in CVD is specific to ART-controlled HIV-infection. It should be mentioned that indole-3-acetate and quinolinate have been associated with CVD and/or c-IMT in patients with end-stage renal disease [24,25]. Since all but two patients in our cohort had normal creatinine clearance levels, any impact of renal function on our results was likely minimal.

How these metabolite imbalances could consequently impact CVD remains debatable. In a previous study, the KTR largely explained the dysbiosis in gut microbiota observed in HIV+ART+ versus HIV-negative individuals [26], while another study found a close link between Trp or KA levels and several taxa of gut microbiota in HIV-positive individuals [27]. Meanwhile, the KTR has been associated with both altered gut microbiota and endothelial dysfunction in the HIV-positive population [28], suggesting some indirect mechanism associated with intestinal flora. We did observe a strong correlation between KTR and kyurenine levels with sCD14, a marker of active microbial translocation but also innate immune activation [29]. However, their
levels were not associated with c-IMT. Systemic inflammation from metabolite imbalances could be another reason for increases in c-IMT, as reported mainly in ART-naïve patients [10]. In our study, kynurenine and KA were strongly associated with D-dimer, while quinolinate was only correlated with TNF-α and no other markers of inflammation. Furthermore, there was no correlation between quinolinate or indole-3-acetate and activated or senescent CD4+/CD8+ cells, which could promote systemic inflammatory responses [30]. These observations overall do not support the influence of low-grade inflammation as an explanation for these findings.

Among metabolites of the lysine, carnitine and dietary choline catabolic pathways, TMAO was not identified as a metabolite feature of HIV+ART+ compared to HIV- individuals and was not associated with c-IMT [31]. This result is in line with previous findings on coronary artery plaque level [32]. Nevertheless, TMAO might not be an adequate predictor of cardiovascular disease in HIV-positive individuals [31]. We did identify another metabolite from this pathway, g-butyrobetaine, which was negatively associated with c-IMT and cca-IMT. Others have shown the proatherogenic properties of g-butyrobetaine [33] and its levels have already been associated with both carotid artery atherosclerosis and cardiovascular mortality in patients with carotid artery stenosis [13]. However, levels of this metabolite could be reflective of lacking synthesis to carnitine, which leads to TMAO production and further vascular damage [34], or imbalances in gut microbiota [33]. G-Butyrobetaine was significantly and positively correlated with naïve T-cells, but also senescent CD8+ cells, suggestive of highly active adaptive immune responses in individuals with higher g-butyrobetaine levels. However, the indirect relationship of these metabolites to immunity is unclear. The mechanisms driving the association between g-butyrobetaine and c-IMT require further study in HIV+ART+ individuals.

Of the metabolites derived from purine/pyrimidine metabolism pathway, higher xanthosine and uridine levels were associated with higher c-IMT in the present study. Such an association has
not been reported previously. Nonetheless, altered levels of metabolites in these pathways, particularly xanthosine and pseudouridine, have been reported in older individuals with high expression of genes linked to inflammasome and in turn associated with their increased risk of all-cause mortality [35]. A recent study has reported that glutamine levels were increased in matched HIV-positive individuals with versus without coronary artery disease [36], but only evaluated 15 amino-acids. Glutamine levels were decreased in HIV+ART+ versus HIV+ART- in our study, yet not associated with c-IMT.

Considering the significant differences in metabolite levels between HIV/ART groups observed in our study population and others [8,23,37,38], it is possible that some metabolites are related to the severity of HIV-infection [8,37]. Accordingly, we observed that carnitine from lysine and dietary choline catabolism and xanthine and orotate from the purine/pyrimidine pathway were correlated with nadir CD4+ T-cell count. Higher levels of metabolites from Trp catabolism pathway were more strongly correlated with lower CD4+ T-cell counts and CD4:CD8 ratio.

HIV-infection has also been previously associated with mitochondrial dysfunction [8,37,38], whereby mitochondrial content within cells increases and results in higher KTR and levels of metabolites from the TCA cycle [38]. Indeed, most metabolites derived from the TCA cycle were identified as metabolite features with higher levels in HIV+ART- participants and some were strongly correlated with several inflammatory markers in HIV+ART+ participants, arguing for mitochondrial dysfunction in the presence of low-grade inflammation during effective ART. Nonetheless, this seemed unrelated to c-IMT.

There are certain limitations of our study. First, analysis was limited to 48-50 participants in each HIV/ART group. The small sample sizes precluded any in depth analysis of other factors, such as specific classes of antiretroviral agents. Second, aside from the reduced confounding
bias of important factors, namely smoking and gender, from the exposure-matched design, our results might not be generalizable to other populations. Third, the strict selection of this population likely reduced variability of metabolite levels, explaining why the criteria for metabolite features included a 0.25-fold log₂ mean change compared to the more commonly used 2-fold change. Nevertheless, the 2-fold cutoff has been criticized as arbitrary and not entirely applicable to metabolomics data [39]. Fourth, HIV+ART+ individuals had a significantly higher proportion with illicit drug use, higher HDL and lower triglyceride levels than HIV-individuals. These differences could have confounded our findings, yet it is unclear how drug use or lipid parameters can influence metabolite concentrations. Finally, analysis on cellular markers of immunity and cca-IMT progression were conducted in a subset of the original study population.

In conclusion, our data suggest that disturbances in the Trp, nucleoside and Lys-dietary choline catabolic pathways could be involved in HIV-related atherosclerosis. Given that Trp catabolites TMAO and butyrobetaine are also products of gut microbiota, one attractive explanation would be that altered gut microbiota observed in HIV-infected individuals, occurring during ART-induced viral suppression, could contribute to increased risk of atherosclerosis via alterations in the Trp catabolism and lysine and dietary choline degradation pathways. Further research would be needed to confirm this hypothesis.

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of the manuscript. FI coordinated data collection on metabolite analysis, helped with data interpretation, drafted parts of the manuscript, and provided critical revisions of the manuscript. J-PB and SF were responsible for quantification of inflammatory markers and provided critical revisions of the manuscript. AS, DS, and BA were responsible for quantification of cellular and some inflammatory markers and provided critical revisions of the manuscript. NH and AC (along with FB) measured cardiovascular parameters and provided critical revisions of the manuscript. All authors have approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1. Metabolite features of human immunodeficiency virus (HIV) and antiretroviral therapy (ART) groups

Adjusted \( p \)-values (-log\(_{10}\)) from a \( t \)-test comparing mean metabolite levels in log\(_{10}\)(area) between groups are plotted against their relative log\(_{2}\) mean fold change. Each dot represents a metabolite, while metabolite names are only given for those fulfilling criteria as a metabolite feature: adjusted \( p \)-value <0.05 and log\(_{2}\) mean fold-change >0.25 or <-0.25. Comparisons are between: HIV+ART+ versus HIV- (A), HIV+ART- versus HIV- (B), and HIV+ART+ versus HIV+ART- (C).

Figure 2. Correlation networks between metabolites in HIV-positive patients treated with antiretroviral therapy

Correlation networks between metabolites of the purine/pyrimidine metabolism (A), lysine degradation (B), tryptophan metabolism (C), and citrate cycle (D) pathways. Positive and negative correlations are depicted in blue and red, respectively. Stronger correlations have thicker lines and colored shading. Metabolites are arranged in an edge-weighted, spring embedded layout.

Figure 3. Correlation between metabolites and inflammatory markers in HIV-positive patients treated with antiretroviral therapy
Spearman correlations between metabolites and inflammatory markers are depicted in heatmaps for metabolites involved in purine/pyrimidine metabolism (A), lysine degradation (B), tryptophan metabolism (C), and the citrate cycle (D).
## TABLES

### Table 1. Metabolites associated with carotid intima-media thickness (c-IMT) and common carotid artery (cca)-IMT in HIV-positive patients treated with antiretroviral therapy

| Metabolites associated with c-IMT | Univariable* | Multivariable** |
|----------------------------------|--------------|------------------|
| Total c-IMT cross-sectional† (N=49) | Diff in mm (95%CI) | p | Diff in mm (95%CI) | P |
| **Purine/Pyrimidine metabolism** | | | | |
| Xanthosine | 0.160 (0.005, 0.314) | 0.04 | 0.128 (0.040, 0.216) | 0.005 |
| Uridine | 0.283 (0.069, 0.497) | 0.01 | 0.165 (0.041, 0.289) | 0.01 |
| **Lysine degradation** | | | | |
| Pipecolate | -0.234 (-0.337, -0.130) | <0.001 | | |
| g-Butyrobetaine | -0.344 (-0.577, -0.110) | 0.004 | -0.190 (-0.314, -0.067) | 0.003 |
| **Tryptophan metabolism** | | | | |
| Indole-3-acetate | 0.102 (0.009, 0.194) | 0.03 | 0.056 (0.007, 0.106) | 0.03 |
| Quinolinate | 0.169 (0.011, 0.327) | 0.04 | 0.115 (0.018, 0.211) | 0.02 |

| Metabolites associated with cca-IMT | Univariable* | Multivariable** |
|-----------------------------------|--------------|------------------|
| cca-IMT cross-sectional† (N=49) | Diff in mm (95%CI) | p | Diff in mm (95%CI) | P |
| **Purine/Pyrimidine metabolism** | | | | |
| Xanthosine | 0.108 (-0.032, 0.248) | 0.13 | | |
| Uridine | 0.218 (0.024, 0.413) | 0.03 | | |
| **Lysine degradation** | | | | |
| Pipecolate | -0.193 (-0.288, -0.099) | <0.001 | | |
| g-Butyrobetaine | -0.293 (-0.504, -0.082) | 0.007 | -0.227 (-0.399, -0.054) | 0.01 |
| **Tryptophan metabolism** | | | | |
| Indole-3-acetate | 0.118 (0.035, 0.201) | 0.006 | 0.078 (0.010, 0.145) | 0.03 |
| Quinolinate | 0.099 (-0.046, 0.243) | 0.18 | | |
Table 1 (con’t).

| Metabolite                          | Diff in mm ∆/year (95%CI) | p   | Diff in mm ∆/year (95%CI) | p   |
|-------------------------------------|---------------------------|-----|---------------------------|-----|
| **Purine/Pyrimidine metabolism**    |                           |     |                           |     |
| Xanthosine                          | 0.062 (-0.161, 0.285)     | 0.6 |                           |     |
| Uridine                             | 0.235 (-0.098, 0.569)     | 0.16|                           |     |
| **Lysine degradation**              |                           |     |                           |     |
| Pipecolate                          | -0.105 (-0.307, 0.098)    | 0.3 |                           |     |
| g-Butyrobetaine                     | -0.189 (-0.560, 0.183)    | 0.3 |                           |     |
| **Tryptophan metabolism**           |                           |     |                           |     |
| Indole-3-acetate                    | 0.148 (0.014, 0.282)      | 0.03| 0.182 (0.053, 0.311)      | 0.008|
| Quinolinate                         | 0.341 (0.083, 0.599)      | 0.01| 0.400 (0.149, 0.651)      | 0.003|

Abbreviations: c-IMT, carotid intima-media thickness; cca, common carotid artery; diff, difference; ∆, change.

Data were obtained from HIV-infected patients treated with antiretroviral therapy.

*Only metabolites with p-values <0.1 in univariable analysis (for the c-IMT cross-sectional analysis) are provided, while all other estimates are provided in Supplementary Table 2. The metabolites in the c-IMT cross-sectional univariable analysis were used as candidates for the cca-IMT cross-sectional and progression analysis.

**Multivariable models were adjusted for age, hypertension, diabetes, and creatinine clearance. The following metabolites were excluded: c-IMT cross-sectional – pipecolate (p=0.899); cca-IMT cross-sectional – uridine (p=0.131), pipecolate (p=0.432); cca-IMT progression – none.

†An evaluation of metabolite levels and c-IMT/cca-IMT was performed at the same moment in the “cross-sectional” studies. “Diff” represents the mm change in IMT for each log_{10}(area) increase in metabolite level.
In a subset of patients with a second cca-IMT measure [occurring a median 5.1 years (IQR=4.8-5.3) after the first measure], metabolite levels at the time of first IMT measure were used to model mm change in cca-IMT per year. “Diff in mm ∆” represents the mm change in slope of IMT regression/progression for each \( \log_{10}(\text{area}) \) increase in metabolite level.
Table 2. Association between metabolites and immunological parameters in HIV-positive patients treated with antiretroviral therapy

|                         | Correlation          |
|-------------------------|----------------------|
|                         | CD4+ T-cell | CD4+ nadir | CD4:CD8 ratio |
| **Purine/Pyrimidine metabolism** |           |           |               |
| Xanthine                | -0.0486     | **-0.2876** | -0.0912       |
| Xanthosine              | 0.1053      | 0.1359     | **0.2084**    |
| Orotate                 | 0.0398      | **0.2889** | -0.0345       |
| Uridine                 | 0.1494      | 0.1091     | 0.2433        |
| **Lysine degradation**  |           |           |               |
| Pipecolate              | 0.0510      | 0.0304     | -0.0441       |
| g-Butyrobetaine         | 0.0844      | 0.0541     | -0.0733       |
| Carnitine               | 0.0848      | **0.2886** | 0.2886        |
| **Tryptophan metabolism** |           |           |               |
| Indole-3-acetate        | 0.0310      | 0.0310     | 0.0310        |
| Kynurenate              | -0.2970     | -0.0900    | -0.0647       |
| Indolelactate           | -0.1000     | -0.0814    | **-0.3054**   |
| Quinolinate             | 0.0992      | -0.0200    | 0.0825        |

Only metabolites with significant Spearman’s correlations (as well as metabolites identified in the c-IMT cross-sectional analysis of Table 1) are provided, while those with non-significant correlations are provided in Supplementary Table 4.

Significant correlations are in bold.
Figure 2
Figure 3