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ROLE OF THE KYNURENINE PATHWAY IN CARDIOVASCULAR DISEASE RISK IN A COHORT OF PEOPLE LIVING WITH HIV

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1. Introduction

The last UNAIDS report estimates that, worldwide, approximately 39 million people are living with HIV (PLWH), and only 76% of them is taking an antiretroviral treatment (ART)[1]. Since its first appearance, ART has changed the landscape of HIV/AIDS from a death sentence to a chronic and controllable condition, leading to an increased life expectancy which is now close to that of the general population[2].

However, despite the virological and immunological success achieved with always more effective ART drugs, PLWH are burdened by abnormal immune activation and inflammation, characterized by elevated biomarkers such as IL-6, D-dimer, C-reactive protein (CRP), and sCD14[2,3].

Nowadays, clinical data suggest that up to 30% of PLWH only show a modest rise in $CD4^+$ T-cell count[2]. Moreover, chronic immune system activation is a better proxy of disease outcome than plasma viral load (pVL) in PLWH, and levels of inflammation can inform the clinician about the evolution of the infection with the onset of serious non-AIDS events (SNAEs)[2]. SNAEs include neurocognitive disorders, coronary artery disease, chronic liver/kidney dysfunction, metabolic syndrome, osteoporosis, and non-HIV-associated cancers[2]. Another consequence of a modest rise in CD4+ T-cell count is susceptibility to opportunistic infections, a sign of disease progression and poor outcomes[2].

Several mechanisms are being investigated as related to immune activation and inflammation[2]. Among them, the most important is probably the persistence of the HIV reservoir during ART, although gut microbial translocation is considered the spark that ignites the flame[2,3].

In fact, HIV infection causes the loss of integrity of the gut mucosa, allowing microbial products and chemokines to cross the mucosal barrier[3]. These products can stimulate the innate immune system through the pattern recognition receptors such as Toll-like receptors (TLRs), mainly TLR-4, generating a local and systemic proinflammatory state[3]. In addition, after being stimulated by HIV antigens, T cells induce an innate and adaptive immune response[2].

Chronic inflammation, even low-grade one, and immune activation are known risk factors for the development of cardiovascular (CV) diseases (D), which are an increasing burden for PLWH during ART and became the first non-AIDS cause of death in this population[3].

The complexity of this inter-relations between abnormal immune activation, inflammation and comorbidities, and the implications for the quality of life and disease-free time of PLWH, make this a matter of utmost importance. It is fundamental to completely understand the mechanisms by which HIV interacts with the human organism to stop the abnormal immune activation and inflammation and improve the outcomes of non-AIDS-related disorders in PLWH.

1.1. Cardiovascular Disease in PLWH

CVD are the first cause of mortality worldwide, with almost 50% of all adults in the United States having some form of CVD[3–5]. CVD includes four major categories: coronary heart disorders (CHD), cerebrovascular diseases, peripheral artery diseases, and aortic atherosclerosis[3].

Effective ART does not modify CVD risk in PLWH[6,7]. In fact, PLWH have a twofold increased risk of having a CVD compared with the general population, and this risk remains even when adjusting for demographic and clinical confounding variables[3,5–8]. In fact, CVD is now the most common non-AIDS-related cause of mortality in PLWH on ART, with 9-20% of PLWH in developed countries at moderate risk of suffering from a myocardial infarction (MI)[3].

Highest CVD risk in PLWH compared to age-matched HIV-negative individuals is the result of a synergy between **traditional risk factors**, such as *smoking*, *dyslipidemia* and *insulin resistance*, and **HIV-related risk factors**, such as *side effects of ART*, *co-infections*, *chronic low-grade inflammation*, and *persistence of HIV reservoirs*[3,5,8].

1.1.1. Traditional risk factors

1.1.1.1. Lifestyle

PLWH are more likely to smoke and consume alcohol than individuals with no HIV infection[3,6,8]. They are also less likely to quit smoking when prompted, and more affected by hypertension[8]. The excess prevalence of smoking in PLWH is thought to be related to anxiety and other neuropsychiatric disorders pre-existent or related to the diagnosis of HIV infection, stigma, increased risk-taking behaviors, and false perception of smoking risks[3]. Whichever the reason, this excess smoking leads to an even higher risk of MI compared with non-smoking PLWH and negative controls[3].

Lifestyle modifications, such as smoking cessation, regular physical exercise and diet modifications may help reduce the levels of chronic inflammation, improve hypertension, and influence metabolic abnormalities, thus decreasing CVD risk[3,6,8].

Among diet schemes, intermittent fasting is gaining high level of interest because of its multiple potential benefits, such as improvements in glucose metabolism and insulin sensitivity, weight loss, delayed aging, systemic inflammation, neurocognitive and cardiovascular improvements[3].

1.1.2. HIV-related risk factors

Although the prevalence of systolic dysfunction in PLWH on ART seems to be similar to the general population, PLWH are significantly younger (20-30 years) at the onset[6]. Moreover, the prevalence of any diastolic dysfunction is higher in PLWH compared to the general population. This higher prevalence of diastolic dysfunction is related to a different epidemiology of HIV-associated cardiomyopathy since the introduction of ART[6]. Progression of CVD in PLWH has transformed from

a dramatic and acute situation to a progressive and chronic worsening over a long period[6]. Several reasons have been investigated for the persistence of HIV-related CVD despite effective ART.

1.1.2.1. Side effects of antiretroviral treatment

In 2008, the observational cohort Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) observed a 90% increase in the risk of MI in PLWH who were treated with abacavir (ABC)[8,9]. The same study reported about a 49% increased risk of MI in PLWH currently exposed to didanosine (ddI), another nucleoside reverse transcription inhibitor (NRTI)[9]. The result came as a surprise, as ABC was known to be a lipid-friendly drug and did not affect the glucose metabolism[9]. The risk of MI associated to ABC use increased during the first year of treatment, was not affected by the duration of treatment, and did not last after stopping the drug[9]. A follow-up study on a different population found out that PLWH treated with ABC had higher high-sensitivity C Reactive Protein (hs-CRP) and interleukin (IL)-6 levels, thus attributing to ABC a pro-inflammatory effect, not only in genetically predisposed people with HLA-B*5701[9]. In 2016, the D:A:D cohort study revised the use of ABC since the publication of their previous study, noticing that there was a higher chance of stopping ABC treatment in PLWH at high-risk for CVD[10]. They also noticed that, despite stopping ABC treatment in those with high-risk for CVD, the association between ABC use and MI remained[10].

The first generation of protease inhibitors (PIs) were also associated with a high rate of dyslipidemia and high-risk for CVD[6,11]. Dyslipidemia and high CVD risk were also found to be associated to the use of ritonavir-boosted lopinavir (LPV/r)[11]. The use of boosted darunavir (DRV/b) has been related to an increased carotid intima media thickness (CIMT) within the first year of treatment, compared with efavirenz[11]. As for dyslipidemia, it has been highlighted that DRV/b leads to smaller increases in total cholesterol (TC) and triglycerides compared with LPV/r, although the fold-changes in HDL and LDL are similar[11]. Nowadays, major guidelines suggest ART modification to drugs known to have lower CVD risk as a first step in CVD prevention[12].

The introduction of Integrase Strand Transfer Inhibitors (INSTIs) led to a decrease in use of PIs, also because of their reduced side effects on the lipid profile[13]. In fact, switching from a PI-based regimen to a dolutegravir (DTG)-based on leads to a LDL cholesterol decrease of 7.7% after 48 weeks [12]. However, in recent years the RESPOND consortium published several studies highlighting that INSTI drugs seem to be associated with a higher prevalence of CVD and hypertension[13–15].

Recent data suggest that the effects of ART on CVD are small, especially compared to the effects of high HIV viremia and low $CD4^+$ T-cell count[6,8]. Moreover, clinical data suggest that immediate and continuous ART at diagnosis protects against CVD[8].

1.1.2.2. Co-infections

PLWH co-infected with hepatitis C virus (HCV) and cytomegalovirus (CMV), or other human herpesviruses (HHVs) have higher soluble markers of inflammation and T-cell activation, compared with mono-infected PLWH and negative individuals[2,8]. In addition, PLWH coinfected with HCV are

affected by enhanced immune activation, with stronger inflammatory response, platelet activation, and oxidative stress[2].

1.1.2.3. Chronic low-grade inflammation

In chronic inflammatory disorders, the most common cause of death is a major adverse cardiac event (MACE)[4]. In fact, CVD is characterized by a chronic low grade inflammatory state which is crucial to its progression, although this chronic state of inflammation is often underestimate when evaluating the risk of CVD progression[3,4].

Severe inflammation states can increase the risk of MACE by 6.2% when compared to the general population, and treating inflammation may reduce the risk of MACE by ~32%, cardiovascular mortality by ~52%, and all-cause mortality by ~48%[4].

HIV stimulates the immune system and provokes an inflammatory state through the interaction with TLR8 and other TLRs, which, in turn, activate the NLR family pyrin domain containing 3 (NLRP3) inflammasome, a multiprotein platform which activation leads to secretion of pro-inflammatory cytokines and to an inflammatory form of cell-death called "pyroptosis". NLRP3 activation contributes to the inflammation leading to increased CVD risk[3].

ART improves immune dysregulation and inflammation by suppressing viral replication; however, levels of inflammation remain higher than in HIV-negative individuals[6]. Therefore, chronic inflammation and immune activation can explain the increase in CVD risk in PLWH[3,6,8]. Another contributing factor to increased CVD risk in PLWH is immune senescence since senescent cells secrete pro-inflammatory cytokines, worsening the inflammatory state[3]. Moreover, during HIV infection we find decreased levels of antioxidants, together with an increase in the levels of radical oxygen species (ROS)[3]. In addition, pro-inflammatory cytokines such as TNF-a, IL-1, and IL-6 increase levels of the inducible nitric oxide synthase (iNOS) in cardiomyocytes, leading to their apoptosis, especially in PLWH with a low $CD4^+$ T-cell count[6].

Chronic inflammation is not only associated to higher CVD risk, but also with increased mortality after MACE[6,8]. Several studies in PLWH on ART showed that increased levels of IL-6, hs-CPR, sCD163, sCD14, soluble tumor necrosis factor receptor I (sTNFR-I), sTNFR-II, kynurenine-to-tryptophan ratio, and D-dimer levels are independently associated with a higher risk of non-AIDS defining events, including death[3,6–8].

1.1.2.3.1. Intestinal microbial translocation

Gut microbial translocation is thought to be one of the main culprits of the chronic inflammatory state in PLWH[3]. During the first phases of the infection, HIV invades $CCR5^+CD4^+$ T-cells in the gut-associated lymphoid tissues (GALT), destroying the intestinal mucosa barrier[2]. The barrier inefficiency causes intestinal microbes and their metabolites to enter the circulation, stimulating the immune activation and inflammatory response[2]. In fact, lipopolysaccharide (LPS), was found to be significantly increased in PLWH[2]. Moreover, microbial translocation is associated to an increased

proportion of $CD38^+CD8^+$ T-cells, circulating levels of IFN, and immunological failure of ART[2]. In addition, the inflammatory state during the first phases of HIV infection is a vicious cycle which further promotes microbial translocation[2].

High levels of sCD14, a marker of monocyte activation, have been associated with bacterial translocation, a larger size of HIV reservoir, more rapid disease progression, poorer CD4+ T-cell recovery, and a higher burden of morbidity and mortality[12]. As gut microbial translocation is associated to the chronic inflammatory state in PLWH, management of the gut microbiota and its metabolites can be approached as novel therapeutic measures[16].

Talking about CVD, translocation, shedding of the bacterial wall compound, and altered metabolism can worsen the inflammatory state[16]. With regards to altered metabolism, at least four different products have been investigated:

- *Short-chain fatty acids* (SCFAs), generated by anaerobic fermentation of fibers, pivotal for initiating inflammatory signaling[16].
- *Butyrate*, another end metabolite of fermentation of fibers, acts as a down regulator of the inflammation and is a recognized prerequisite to maintain the intestinal barrier[16].
- *LPS* is shed from gut Gram-negative bacteria. Its high levels lead to the worsening of the inflammatory state and compromise cardiac and vascular functions[16].
- *Trimethylamine Oxidase* (TMAO) is another end-stage bacterial metabolite, which levels predict all-cause mortality and CV events[16].

The impacts of inflammatory status induced by gut dysbiosis have been displayed in a wide spectrum of CVDs[16]. Svingen et al showed that gut metabolites, including LPS and indoxyl sulfate, can increase the instability of myocardial substrate and predispose the onset of atrial fibrillation[17]. Tabata et al showed that the pathologic alterations of the gut microbiota are proportional to the NYHA functional class and correlate with the initiation of the inflammatory class[18].

Gut microbiota has a multitude of metabolic functions, such as the synthesis of vitamins, fermentation of indigestible dietary fibers to short-chain fatty acids (SCFA) and bile acid metabolism[19]. It is thought that intestinal bacteria can preserve cardiovascular health, and to alleviate and rehabilitate CVDs related to metabolic disorders, such as dyslipidemia, hyperglycemia, obesity and hypertension[19].

1.1.2.3.2. Depletion of regulatory T (Treg) cells

During chronic HIV infection, $CD4^+CD25^+$ Treg cells distribution shift from the peripheral blood to lymph nodes and mucosal lymphoid tissues[2]. Treg cells can inhibit T cell activation and proliferation, their susceptibility to HIV infection causes a functional deficit during this infection[2]. Although impaired, they still control immune activation by reducing IL-2 production[2].

1.1.2.3.3. Toll-Like Receptors (TLRs) and Nuclear Factor kappa B (NF-kB) activation

TLRs can recognize multiple pathogen associated molecular patterns (PAMPs), activating pathways which release abundant inflammatory cytokines[2]. Activation of these pathways can also determine organic damage and dysfunctions[2]. HIV tat protein stimulates astrocytes to upregulate the expression of MCP-1, IL-8, and CXCL10 through NF-kB signaling[2]. This hyperactivation of the NF-kB signaling also inhibits the excitatory amino acid transporter 2 (EAAT-2) in astrocyte plasma membranes, increasing levels of extracellular glutamate, especially during HIV-associated Neurologic Disorders (HAND)[2].

1.1.2.4. Persistence of HIV reservoirs

Despite suppressing viral replication and causing immune reconstitution, combined ART cannot eliminate integrated HIV within the reservoir[2,6]. This is the main hurdle to HIV cure and the main reason for chronic immune activation in PLWH[2]. The expression of HIV reservoir, with production of viral particles, stimulates lymphocyte and macrophage activation through different TLRs and inflammatory pathways[2]. Therefore, PLWH are at an increased risk of developing non-AIDS related comorbidities, such as CVDs, and this risk might be proportional to HIV reservoir size[2]. Recent studies have shown that treatment with INSTI might reduce the reservoir size, decrease the number of infected $CD4^+$ T-cells and activated $CD38^+CD8^+$ T-cells[2].

1.1.3. Endothelial dysfunction

Endothelial dysfunction, caused by oxidized LDLs, is associated with traditional risk factors for atherosclerosis, and represents the final pathway of multiple vascular insults[3]. Increased endothelial activation, together with increased inflammation, and increased CIMT lead to diastolic dysfunction and vascular stiffness, which remain unchanged despite the use of ART and viral suppression[6].

1.1.4. Atherosclerosis

Atherosclerosis occurs when inflammatory plaques form inside blood vessels. Monocytes are central in atherogenesis in PLWH for their increased ability to form foam cells associated with hyperactivation[7]. Together with thrombosis, atherosclerosis plays a central role in the development of vascular diseases, including MI[8]. There are two types of MI:

- *Type I MI*: it occurs when lipid-rich, inflammatory plaque breaks or erodes, causing the formation of a thrombus and a vessel occlusion[8].
- *Type II MI*: myocardial oxygen supply is too low compared to demands[8].

PLWH are more at risk for type II MIs than for type I[8]. Even when virologically suppressed thanks to ART, PLWH are more at risk of progression of subclinical atherosclerosis compared with HIV-negative individuals, due to a persistent inflammatory status[12,16]. The NEAT022 study showed that, in PLWH with high CV risk, switching to DTG significantly improved lipid profile and reduced sCD14 and adiponectin levels after 48 weeks[12].

Effectiveness of statins in decreasing CV event rates in individuals at high risk of CVD has led several authors to think that their use might show some benefit even in virologically suppressed PLWH with moderate CV risk[7]. Several attempts were made with rosuvastatin and pravastatin, with no success except for the improvement of plasma lipid profiles, before the REPRIEVE trial, which tested the use of pitavastatin in PLWH with moderate CV risk[7,20–22]. REPRIEVE trial results showed that an oral daily treatment with 4 mg pitavastatin calcium was able to decrease the risk of MACE in PLWH with low-to-moderate risk of CVD after a median follow-up period of 5.1 years[22]. A sub-study of the REPRIEVE trial also highlighted a significant decrease in LpPLA2 levels and oxidized LDLs (oxLDL) cholesterol[20]. However, no significant difference between pitavastatin and placebo was highlighted regarding their effects on sCD14, sCD163, IL-6, Il-1b, IL-18, and IL-10 plasma levels[20].

1.1.5. HIV-associated cardiomyopathy

Myocarditis was a frequent complication of HIV infection before the widespread use of ART, especially in PLWH with CD4+ T-cell counts ≤ 400 cells/µL with opportunistic infections, such as CMV, EBV, *Toxoplasma gondii*, *Cryptococcus neoformans* and *Mycobacterium avium* complex[6,8]. Although symptomatic myocarditis has nowadays decreased, several studies using cardiac magnetic resonance imaging (CMRI) have shown that even PLWH on ART have increased rates of subclinical myocardial inflammation, interstitial fibrosis, lower ejection fraction, increased left ventricular mass, and increased frequency of subclinical myocardial edema leading to increased rates of cardiac dysfunction[6].

Moreover, in PLWH post-MI complications are more frequent and serious, with worse cardiac scarring and increased rates of heart failure[6]. In addition, HIV-associated cardiomyopathy is characterized by worse outcomes than those seen with other causes of cardiomyopathy[6].

1.2. Kynurenine pathway

Tryptophan (Trp) is an essential amino acid which has been recently found to have a critical role in CVD development[23]. More than 95% of Trp is metabolized through the kynurenine pathway (KP) under physiological conditions. The first and rate limiting steps of the KP are catalyzed by tryptophan 2,3-dioxygenase (TDO), indolamine 2,3-dioxygenase 1 (IDO1) and IDO2 and consists of production of Kynurenine (Kyn) from Trp[24]. Ninety-eight per cent (98%) of KP is intra-hepatic under physiological conditions and catalyzed by TDO[25].

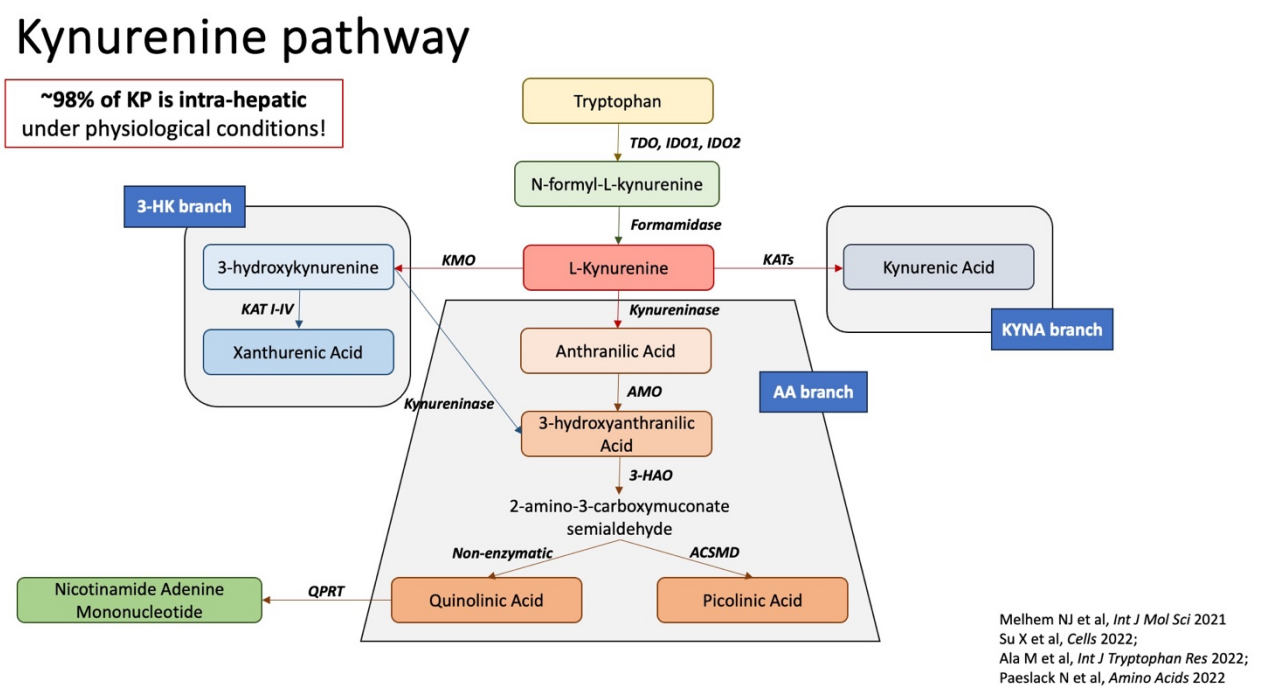

Figure 1. Kynurenine Pathway.

However, under inflammatory conditions, the activation of JAK/STAT pathways by IFNg, increases IDO1 activity, including outside the liver[24,26]. Under these conditions, IDO1 activity can exceed TDO activity, resulting in accumulation of extra-hepatic KP metabolites[27].

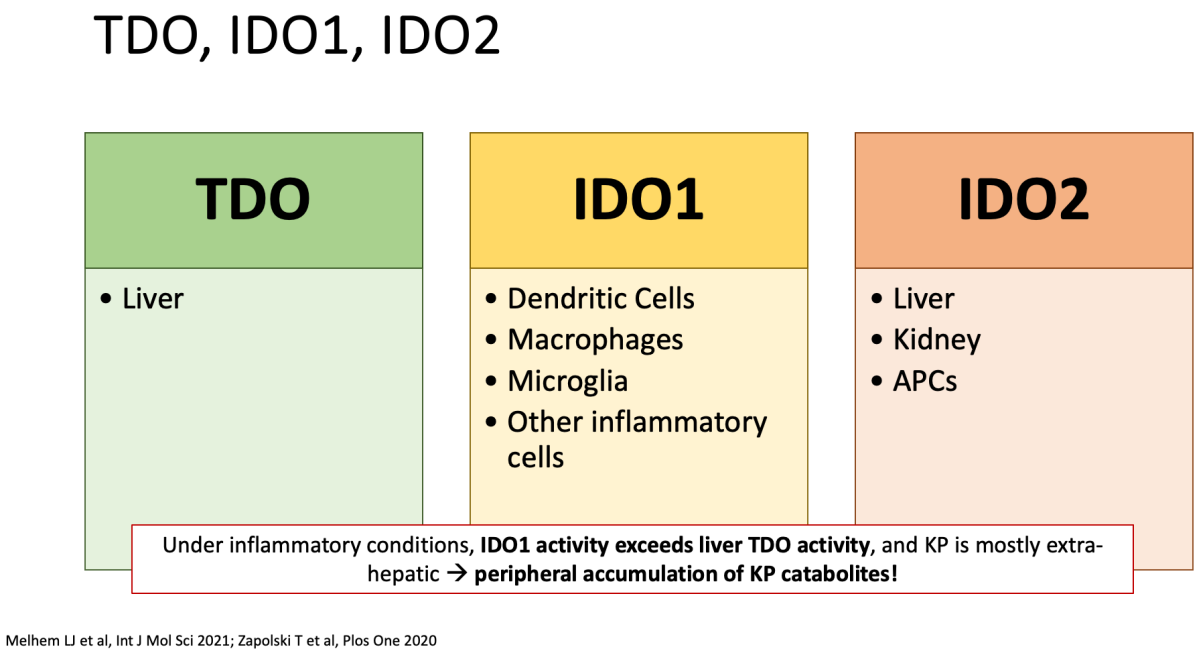

Figure 2 – KP enzymes

KP influence over inflammation, immune cell functions, and its correlation with CVD development has not been fully clarified[28]. It has been established that lower Trp levels and higher

Kynurenic acid (KynA) levels, with a higher KynA/Trp ratio are associated with an increased rate of CVD, even in people with low CVD risk[26].

Several studies have explored the KP pathway and CVD risk in PWH. In 2018, Qi et al[29] measured Trp and KynA in 520 PWH and 217 HIV negative individuals and showed that PWH had lower levels of Trp and higher KynA/Trp ratios. Moreover, they observed that $CD4^+$ T-cell count positively correlated with Trp levels and inversely correlated with KynA/Trp ratio[29]. In addition, Trp was found to be inversely correlated with the risk of carotid artery plaque development, while KynA and KynA/Trp ratio were positively correlated to it[29].

The same results were confirmed by a similar, although probably underpowered, study by Hoel et al[30]. Comparing four groups, based on HIV/type 2 diabetes (T2D) status, they observed that PWH had higher Kyn/Trp ratio compared to HIV negative individuals[30].

In 2019, Boyd et al[31] published the results of a study on 150 never-smoker men with no-known risk of CVD and/or no previous CVD. They undertook more extensive analysis of the KP pathway and showed that xanthurenic acid (XA) and quinolinic acid (QuinA) were associated with higher carotid intima media thickness (c-IMT) in PWH on ART[31].

At CROI 2023, two groups shared their results on Trp/KP in PWH. The first group, led by Bayón-Gil (CROI 2023, poster 246), highlighted a high correlation between IFNg activation and upregulation of KP in viremic progressor PWH, while a preservation of Trp metabolism characterized viremic non-progressors. The second group, led by Luo (CROI 2023, poster 668), confirmed Qi et al[29] results, focusing on women with HIV included in that study.

2. Aims of the study

Several knowledge gaps remain about KP and the genesis of CVD in PWH. Especially, the need to prevent the progress to an acute major cardiovascular event of a chronic CVD, requires a deep knowledge of the mechanisms underlying the onset of CVD.

Our group has already shown that there is a correlation between the expression of different genes and production of different cytokines and inflammatory factors between different categories of people at risk, and that these cytokines and inflammatory factors allow to identify clusters of people at risk[32–35]. However, more data are required to understand which mechanisms lead to the production to these different clusters of cytokines and inflammatory factors. It is known that KP influences inflammation both in an anti-inflammatory and a pro-inflammatory way, it will be important to clarify how KP metabolites associates with the clusters.

2.1. Hypothesis

We hypothesize that PLWH belonging to inflammatory clusters will have different levels of KP catabolites compared to PLWH assigned to a non-inflamed cluster.

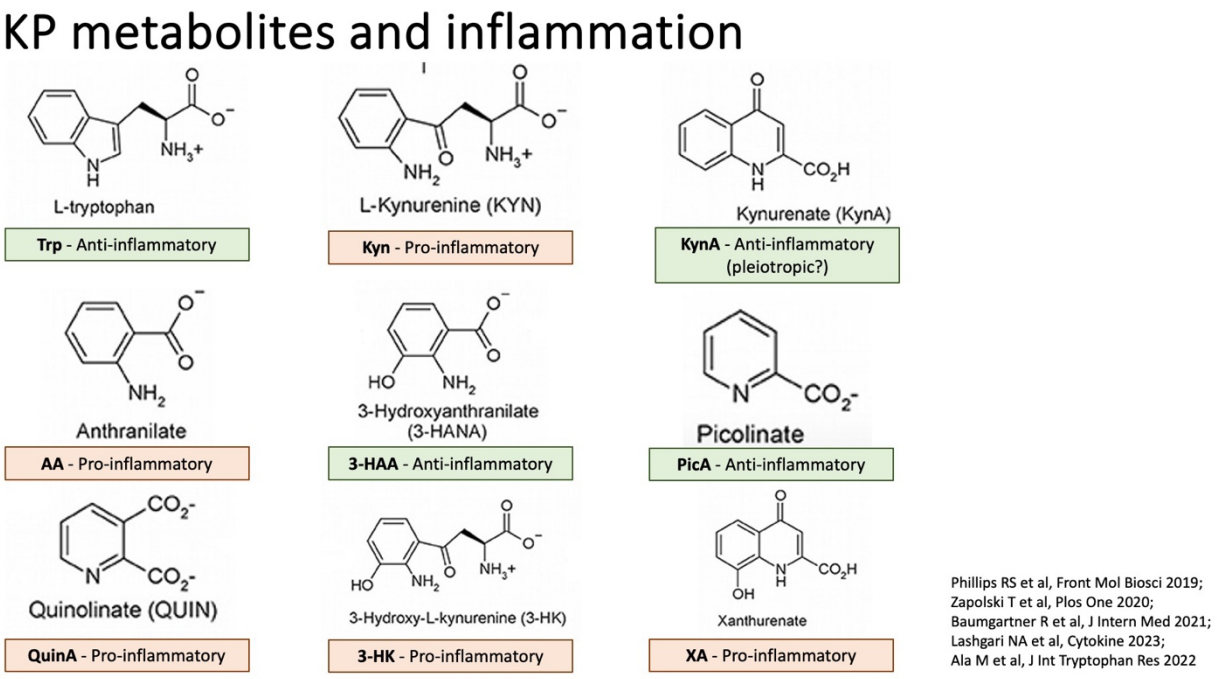

Figure 3 – Hypothetic pro- and anti-inflammatory role of each metabolite of the kynurenine pathway according to literature research.

If our hypothesis will be proven true, we will be a step closer to understanding the genesis of CVDs in PLWH and finding a therapeutic strategy around it.

2.2. Primary objective

- To understand which Trp metabolites of the KP correlate with membership of an inflammatory cluster associated with an increased CVD risk in PLWH.

2.3. Secondary objectives

- To study correlations between KP catabolites and immunological profiles of PLWH
- To study correlations between KP catabolites and inflammatory profiles of PLWH
- To study correlation between KP catabolites and clinical characteristics of PLWH

3. Patients and Methods

3.1. All-Ireland Infectious Diseases cohort

Established in 2019, the Center for Experimental Pathogen Host Research (CEPHR) of the University College Dublin (UCD) unified several research groups focusing on infectious diseases related disorders. At CEPHR, research is mainly focused on all aspects of chronic viral infections, such as diagnostics, upcoming pathogen discovery, HIV infection, viral hepatitis infections, long-term consequences of chronic viral infections and derived persistent inflammation.

CEPHR is the headquarter of several study cohorts. The biggest one is the All-Ireland Infectious Diseases (AIID) cohort, enrolling patients affected by infectious diseases, including HIV, nosocomial infections, and COVID-19, at eleven clinical centers across Ireland. AIID is a multicenter, prospective, longitudinal, observational cohort enrolling consecutive adult subjects (≥ 18 years old) attending hospital services for management of infection.

Enrolled subjects provide written informed consent for use of routine clinical and laboratory data for research. In addition, they sign a written consent to collection and biobanking of biological samples, including respiratory samples such as nasopharyngeal swabs. The AIID Cohort is approved by local institutional review boards. Data and samples within the AIID Cohort are accessed through standardized Data Access Guidelines and all Data Access Requests are approved by the local Ethics Committee.

3.2. Previous studies

Several lines of research about CVD in PLWH have been carried out at CEPHR, and the most important results are represented by these findings:

- ***PLWH have immune activation that contributes to CVD*** – In PLWH immune activation is not turned off by long-term ART, and immune disorders are linked to an increased risk of CVD.

  McGettrick PMC, Mallon PWG. HIV and cardiovascular disease: defining the unmeasured risk. *Lancet HIV* 2018; **5**: e267–9[33].

  McGettrick P, Mallon PWG, Sabin CA. Cardiovascular disease in HIV patients: recent advances in predicting and managing risk. *Expert Rev Anti-Infect Ther* 2020; **18**: 677–88[34].

  McGettrick P, Mallon PWG. Biomarkers to predict cardiovascular disease in people living with HIV. *Curr Opin Infect Dis* 2022; **35**: 15–20[32].

  McGettrick P, Tinago W, O'Brien J, *et al.* Distinct Inflammatory Phenotypes Are Associated With Subclinical and Clinical Cardiovascular Disease in People With Human Immunodeficiency Virus. *J Infect Dis* 2024; jiae007[35].

- ***Monocyte/macrophages in PLWH hold too much cholesterol*** – Monocyte/macrophages contribute to atherosclerosis embedding cholesterol in the wall of blood vessels. In PLWH these cells have an abnormal gene signature, which can be used as a target for treatment, suggesting that they hold too much cholesterol.
- ***ART can modify the risk of CVD*** – Some antiretroviral drugs can alter the function of platelets, increasing the risk of CVD.

3.3. PLWH's selection

Based on previous studies[35] conducted by our group, we identified within the UCD-ID cohort, a subset of the AIID which includes 277 individuals, 3 clusters:

- *Cluster 1* (148 individuals) – PLWH belonging to this group are characterized by overall lower inflammation.
- *Cluster 2* (100 individuals) – PLWH belonging to this group show higher markers of monocyte activation (sCD14, sCD163), systemic inflammation (hs-CRP, TNFR-1 and -2), and vascular endothelial function (P-selectin, E-selectin, sICAM-1, sVCAM-1).
- *Cluster 3* (29 individuals) – PLWH belonging to this group show higher markers of T-cell differentiation and regulation (IFN- IFN-γ, IL-2, IL-4, IL-12, IL-10, TNF-α, IL-6, IL-1β).

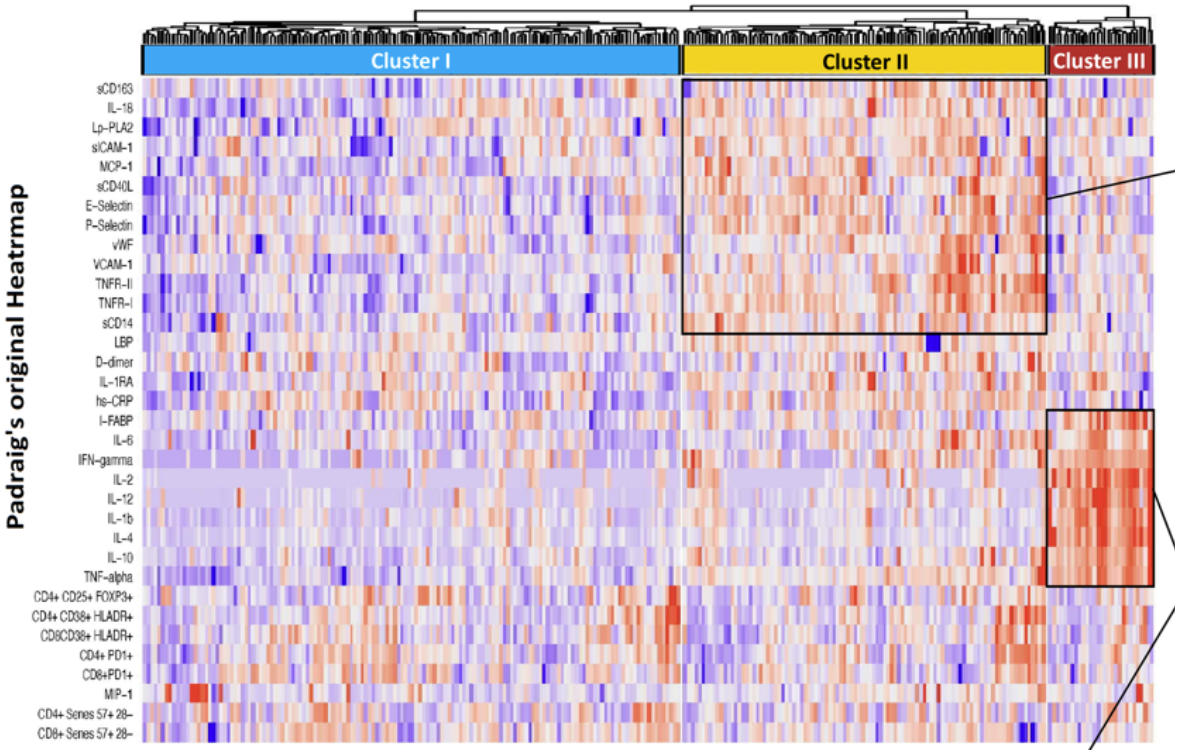

Figure 4. Clusters of PLWH by expression of inflammation markers

We carried out this study as a proof-of-concept one. Therefore, we did not include all the PLWH of the cohort in the analysis, and we chose to match 1:1:1, by sex, ethnicity, and age the 29 PLWH included in cluster 3 with 29 from cluster 1 and 29 from cluster 2.

We performed the matching by a propensity score with the nearest neighbor method, combined with a “distance to the centroid” method by K-means.

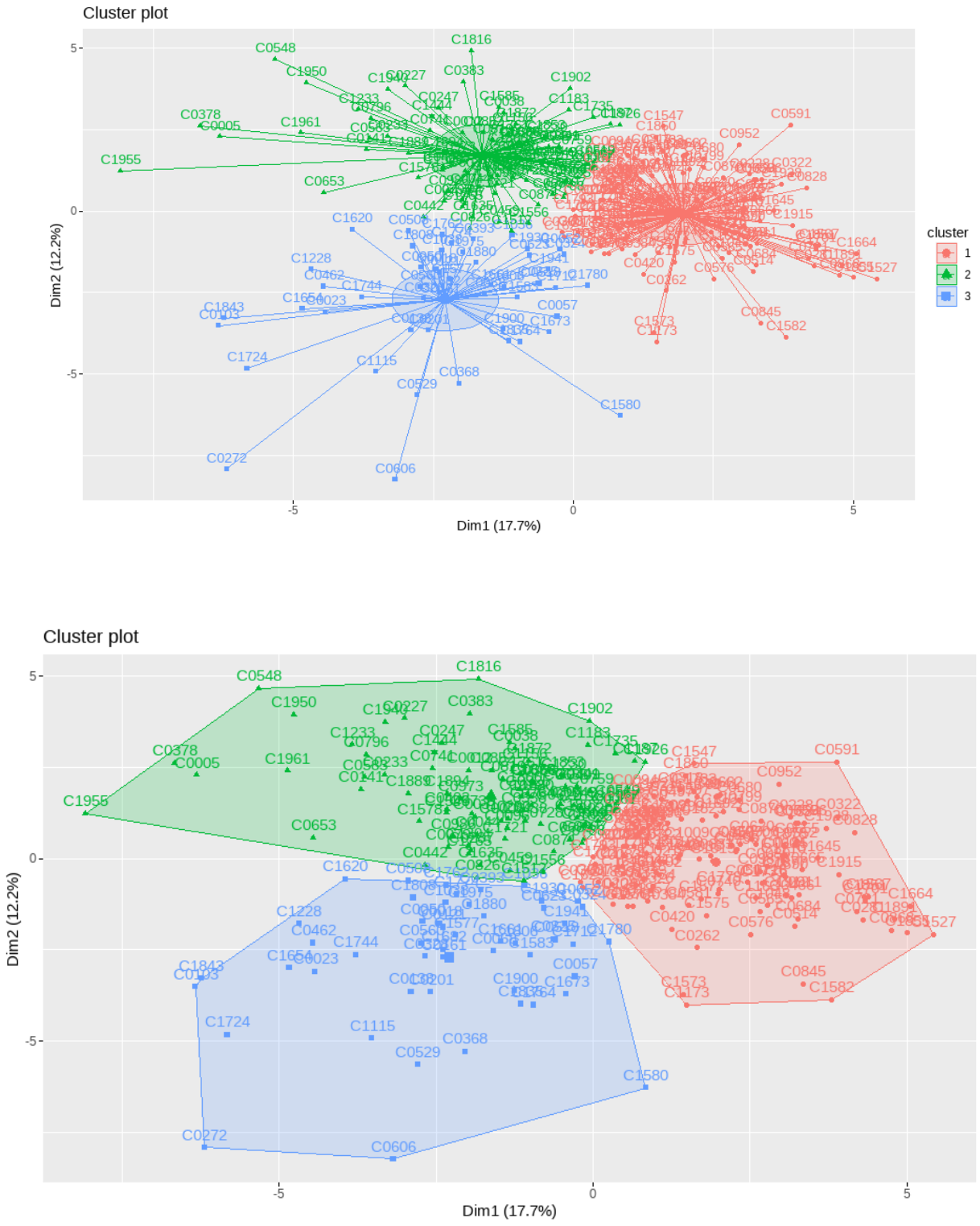

Figure 5 – Cluster definition by a combination of propensity score and distance to centroid methods

Clusters identified by these method were well defined and very stable. Stability of clusters was tested by using a Jaccard Index, a stability assessment method repeated 100 times, which did not

dissolve the clusters. By using this method, we obtained a 0.9 score out of 1.0 for the three clusters. Everything over 0.8 should be considered stable.

We therefore obtained the 29 best matched PLWH for each cluster.

3.4. Metabolite analysis

Metabolite analysis was carried out at an outsource facility, Metabolon Inc. Methods here described were included in the results package.

Sample Accessioning: Following receipt, samples were inventoried and immediately stored at -80°C. Each sample received was accessioned into the Metabolon LIMS system and was assigned by the LIMS a unique identifier that was associated with the original source identifier only. This identifier was used to track all sample handling, tasks, results, etc. The samples (and all derived aliquots) were tracked by the LIMS system. All portions of any sample were automatically assigned their own unique identifiers by the LIMS when a new task was created; the relationship of these samples was also tracked. All samples were maintained at -80°C until processed.

Sample Preparation: Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into multiple fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, while the remaining fractions were reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

QA/QC: Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample, allowed instrument performance monitoring and aided chromatographic alignment. Tables 1 and 2 describe these QC samples and standards. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples

were randomized across the platform run with QC samples spaced evenly among the injections, as outlined in Figure 6 below.

Type	Description	Purpose
MTRX	Large pool of human plasma maintained by Metabolon that has been characterized extensively.	Assure that all aspects of the Metabolon process are operating within specifications.
CMTRX	Pool created by taking a small aliquot from every customer sample.	Assess the effect of a non-plasma matrix on the Metabolon process and distinguish biological variability from process variability.
PRCS	Aliquot of ultra-pure water	Process Blank used to assess the contribution to compound signals from the process.

Table: Description of Metabolon QC Samples

Type	Description	Purpose
RS	Recovery Standard	Assess variability and verify performance of extraction and instrumentation.
IS	Internal Standard	Assess variability and performance of instrument.

Table. Metabolon QC Standards.

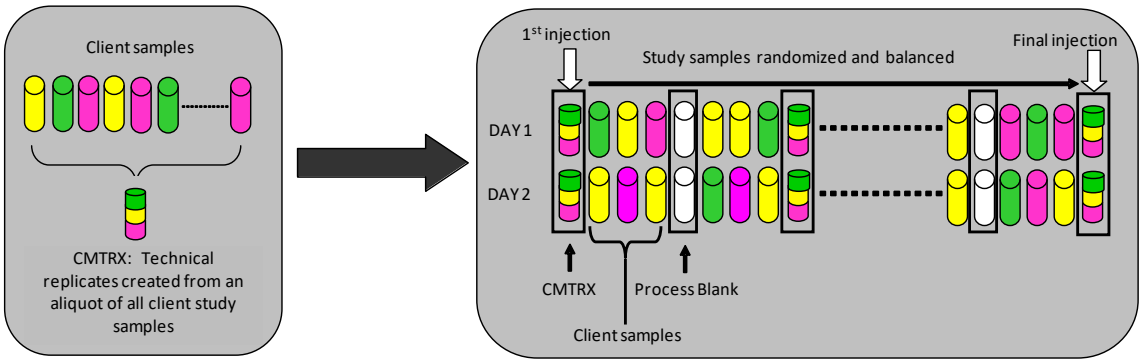

Figure 6. Preparation of client-specific technical replicates. A small aliquot of each client sample (colored cylinders) is pooled to create a CMTRX technical replicate sample (multi-colored cylinder), which is then injected periodically throughout the platform run. Variability among consistently detected biochemicals can be used to calculate an estimate of overall process and platform variability.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS): All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution (PMID: 32445384). The dried sample extract were then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds (PosEarly). In this method, the extract was gradient eluted from a C18 column (Waters

UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions; however, it was chromatographically optimized for more hydrophobic compounds (PosLate). In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column (Neg). The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8 (HILIC). The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slightly between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below.

Bioinformatics: The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

LIMS: The purpose of the Metabolon LIMS system was to enable fully auditable laboratory automation through a secure, easy to use, and highly specialized system. The scope of the Metabolon LIMS system encompasses sample accessioning, sample preparation and instrumental analysis and reporting and advanced data analysis. All of the subsequent software systems are grounded in the LIMS data structures. It has been modified to leverage and interface with the in-house information extraction and data visualization systems, as well as third party instrumentation and data analysis software.

Data Extraction and Compound Identification: Raw data was extracted, peak-identified and QC processed using a combination of Metabolon developed software services (applications). Each of these services perform a specific task independently, and they communicate/coordinate with each other using industry-standard protocols. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (m/z), and fragmentation data on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the

ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between molecules based on one of these factors, the use of all three data points is utilized to distinguish and differentiate biochemicals. More than 5,400 commercially available purified or in-house synthesized standard compounds have been acquired and analyzed on all platforms for determination of their analytical characteristics. An additional 7000 mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis. Metabolon continuously adds biologically relevant compounds to its chemical library to further enhance its level of Tier 1 metabolite identifications.

Compound Quality Control: A variety of curation procedures were carried out to ensure that a high-quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove or correct those representing system artifacts, mis-assignments, mis-integration, and background noise. Metabolon data analysts use proprietary visualization and interpretation software to confirm the consistency of peak identification and integration among the various samples.

Metabolite Quantification and Data Normalization: Peaks were quantified using area-under-the-curve. For studies spanning multiple days, a data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately (termed the "block correction", Figure 7). For studies that did not require more than one day of analysis, no normalization is necessary, other than for purposes of data visualization. In certain instances, biochemical data may have been normalized to an additional factor (e.g., cell counts, total protein as determined by Bradford assay, osmolality, etc.) to account for differences in metabolite levels due to differences in the amount of material present in each sample.

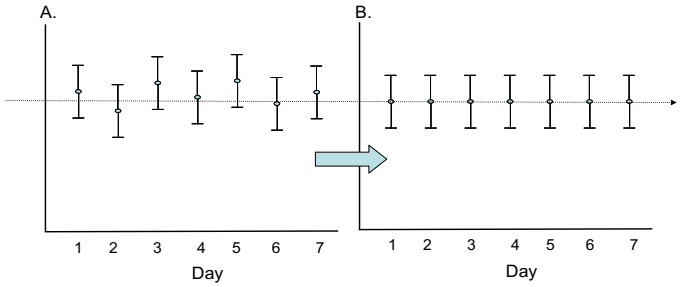

Figure 7. Visualization of data normalization steps for a multiday platform run.

3.5. Statistical analysis

Categorical variables were summarized as count (percentage), while continue variables were described as median (interquartile range, IQR).

To define the distribution for continue variables, we used the Shapiro-Wilk test. Parametric tests, such as t-test and analysis of variance (ANOVA), were used to compare normally distributed variables, while non-parametric tests, such as Mann-Whitney and Kruskall-Wallis tests, were used to compare non-normally distributed variables. For those parameters resulting statistically different, we performed post-hoc analyses with Dwass-Steel-Critchlow-Fligner (DSCF) pairwise comparison method, to reveal specific differences between groups.

Correlation analysis was performed by calculating Pearsons r correlation coefficients and computing them in a matrix. Missing values are managed by deleting them in pairs, instead of deleting all rows of x having any missing variable.

Descriptive statistical analysis was performed with Jamovi version (v.) 2.3.19.0 for MacOS (OpenSource, R language based), while inferential statistics was performed with RStudio v. 2023.12.1+402 (OpenSource, R language based). Graphics were realized with RStudio v. 2023.12.1+402 and GraphPad Prism 10 v. 10.2.1 (GraphPad Software, Boston, MA, US). For inferential statistics, level of significance (p) was set at < 0.05, with a 95% confidence interval (CI).

4. Results

4.1. Characteristics of the PLWH

We included in this study 93 PLWH, 30 female (32.3%). Fifty (53.8%) were Caucasian, 33 (35.5%) were of African origin, while 10 (10.8%) were from South America. Figure 8 shows routes of transmission for the included PLWH.

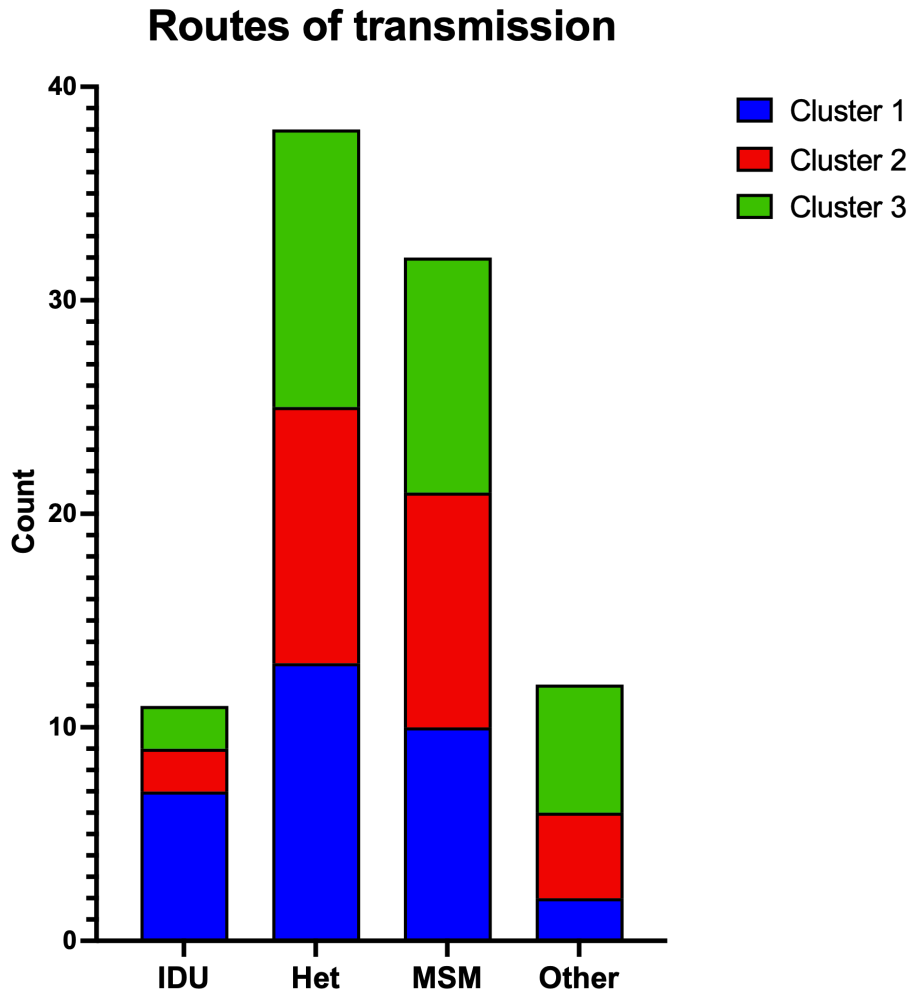

Figure 8. Routes of transmission for the included PLWH. The most frequently reported route of transmission was having a heterosexual intercourse (n = 38, 40.9%), while the second most reported was having a homosexual intercourse (n = 32, 34.4%). The graph also shows the distribution of the routes of transmission within groups. IDUs were mostly represented in Cluster A, while they are present with a lower prevalence in Cluster B and Cluster C. **Abbreviations:** IDU, injection drug users; Het, heterosexual intercourse; MSM, men who have sex with men; PLWH, people living with HIV.

Overall, their median age was 45.0 years old (IQR 40.0-70.0), they had been diagnosed with HIV infection for a median time of 10.5 years (IQR 4.75-16.0). At the time of sampling, median $CD4^+$ T-cell count was 616 cells/µL (IQR 456-797), median $CD4^+/CD8^+$ ratio was 0.83 (IQR 0.60-1.15). Median total cholesterol levels were 4.9 mmol/L (IQR 4.17-5.80), with median HDL 1.21 mmol/L (IQR 0.96-1.38), and median LDL 3.1 mmol/L (IQR 2.5-3.8).

We also stratified these parameters by cluster, obtaining the results summarized in table 1.

Table 1. Characteristics of the patients included in the study at sample collection, by cluster. All data are expressed as median (IQR)

	Cluster 1 (n = 32)	Cluster 2 (n = 29)	Cluster 3 (n = 32)	*p* value
Age (yrs)	45.0 (39.7-49.5)	45.0 (39.0-50.8)	45.0 (40.0-50.0)	0.917
Time from diagnosis (yrs)	11.0 (5.0-16.0)	8.0 (5.0-13.0)	11.0 (4.0-16.0)	0.740
$CD4^+$ T-cell count (cell/µL)	510 (369-712)	686 (554-782)	669 (553-934)	**0.023**
$CD4^+$ T-cell percentage (%)	27.5 (22.0-33.5)	35.0 (29.0-42.0)	34.0 (30.0-41.0)	**0.002**
$CD4^+/CD8^+$ ratio	0.69 (0.53-0.84)	0.88 (0.67-1.20)	1.04 (0.65-1.21)	**0.007**
Total cholesterol (mmol/L)	5.30 (4.60-5.95)	5.00 (4.20-5.50)	4.40 (3.90-5.25)	**0.048**
HDL (mmol/L)	1.14 (0.93-1.30)	1.23 (1.00-1.34)	1.27 (1.00-1.47)	0.241
LDL (mmoL/L)	3.60 (2.90-3.95)	3.10 (2.60-3.60)	2.65 (2.37-3.35)	**0.013**

Abbreviations: n, number; yrs, years; HDL, high-density lipoproteins; LDL, low-density lipoproteins

Post-hoc analysis revealed that $CD4^+$ T-cell count was significantly higher in Cluster 2 than in Cluster 1 ($p = 0.048$), but the difference was not statistically significant when comparing Cluster 1 with Cluster 3 ($p = 0.058$) and Cluster 2 with Cluster 3 ($p = 0.978$).

$CD4^+$ T-cell percentage was significantly lower in Cluster 1 than Cluster 2 ($p = 0.005$) and Cluster 3 ($p = 0.009$), but there were no differences between Cluster 2 and Cluster 3 ($p = 0.901$).

$CD4^+/CD8^+$ ratio was significantly lower in Cluster 1 than in Cluster 2 ($p = 0.021$), and Cluster 3 ($p = 0.016$); however, there were not statistically significant differences between Cluster 2 and Cluster 3 ($p = 0.919$).

Total cholesterol was significantly lower in Cluster 3 than in Cluster 1 ($p = 0.039$), but no statistically significant differences were highlighted when comparing Cluster 1 with Cluster 2 ($p = 0.454$), or Cluster 2 with Cluster 3 ($p = 0.420$).

LDL levels were significantly higher in Cluster 1 than in Cluster 3 ($p = 0.010$), but they were not significantly different when comparing Cluster 1 with Cluster 2 ($p = 0.423$), or Cluster 2 with Cluster 3 ($p = 0.214$).

4.1.1. Traditional risk factors

Among the traditional risk factors, we considered active smoking, alcohol use, drug addiction, history of dyslipidemia, family history of CVD, current diabetes, personal history of CVD, history of hypertension.

Table 2 summarizes the prevalence of traditional risk factors in each cluster. Hypertension rate was significantly different in the three clusters. Post-hoc analysis revealed that hypertension prevalence was significantly higher in cluster 3 compared with cluster 2 ($p = 0.024$), but not in cluster 3 compared with cluster 1 ($p = 0.235$) or in cluster 1 compared with cluster 2 ($p = 0.426$).

Table 2. Traditional risk factors by cluster. All data are expressed in count (%). Percentages are column percentages.

	Total (n = 93)	Cluster 1 (n = 32)	Cluster 2 (n = 29)	Cluster 3 (n = 32)	*p* value
Current smoke	20 (21.5)	5 (15.6)	5 (17.2)	10 (31.2)	0.259
Alcohol use	55 (59.1)	17 (53.1)	12 (41.4)	16 (50.0)	0.925
Drug addiction	12 (12.9)	3 (9.4)	4 (13.8)	5 (15.6)	0.632
History of dyslipidemia	21 (22.6)	9 (28.1)	4 (13.8)	8 (25.0)	0.377
Statin use	19 (20.4)	10 (31.2)	3 (10.3)	6 (18.7)	0.256
Family history of CVD	15 (16.1)	7 (21.8)	4 (13.8)	4 (12.5)	0.427
Personal history of CVD	11 (11.8)	2 (6.2)	6 (20.7)	3 (9.4)	0.306
Diabetes	4 (4.3)	1 (3.1)	0 (0.0)	3 (9.4)	0.187
History of hypertension	17 (18.3)	5 (15.6)	2 (6.9)	10 (31.2)	**0.046**

Abbreviations: CVD, cardiovascular disease.

4.1.2. AIDS-related and non-AIDS related comorbidities

PLWH are often burdened by comorbidities, such as AIDS-defining diseases, chronic kidney diseases, liver disorders, cancer, psychiatric disorders, lung diseases, osteoporosis. Table 3 summarizes the prevalence of comorbidities in each cluster. We also considered the presence of one or more comorbidities except dyslipidemia, and the presence of dyslipidemia together with one or more comorbidities.

Chronic liver disorders were significantly different between the three clusters ($p = 0.014$). Post-hoc analysis revealed that they in cluster 1 there was a significantly higher rate than in cluster 2 ($p = 0.013$), but there were no differences between cluster 1 and cluster 3 ($p = 0.107$) or between cluster 2 and cluster 3 ($p = 0.614$).

Table 3. Prevalence of comorbidities by cluster. All data are expressed as count (%). Percentages are column percentages.

	Total (n = 93)	Cluster 1 (n = 32)	Cluster 2 (n = 29)	Cluster 3 (n = 32)	*p* value
AIDS-defining diseases	15 (16.1)	6 (18.7)	4 (13.8)	5 (15.6)	0.837
CKD	7 (7.5)	4 (12.5)	1 (3.4)	2 (6.2)	0.352
Liver disorders	13 (14.0)	9 (28.1)	1 (3.4)	3 (9.4)	**0.014**
Cancer	6 (6.4)	4 (12.5)	2 (6.9)	0 (0.0)	0.132
Psychiatric disorders	8 (8.6)	1 (3.1)	3 (10.3)	4 (12.5)	0.377
Lung diseases	12 (12.9)	4 (12.5)	3 (10.3)	5 (15.6)	0.825
Osteoporosis	5 (5.4)	2 (6.2)	0 (0.0)	3 (9.4)	0.295

Comorbidity	29 (31.2)	12 (37.5)	7 (24.1)	10 (31.2)	0.531
Multimorbidities	29 (31.2)	10 (31.2)	7 (24.1)	12 (37.5)	0.531
Comorbidity + dyslipidemia	7 (7.5)	4 (12.5)	0 (0.0)	3 (9.4)	0.161
Multimorbidities + dyslipidemia	8 (8.6)	2 (6.2)	3 (10.3)	3 (9.4)	0.835

Abbreviations: AIDS, acquired immunodeficiency syndrome; CKD, chronic kidney disease.

4.1.3. History of co-infections

We also collected data about history of Hepatitis B virus (HBV) infection, Hepatitis C virus (HCV) infection, and tuberculosis (TB).

Table 4 summarizes prevalence of history of HBV infection, positive HCV serology, and history of TB in this cohort, also showing the differences, if any, between the three clusters.

Table 4. Prevalence of co-infections by cluster. All data are expressed as count (%). Percentages are column percentages.

	Total (n = 93)	**Cluster 1 (n = 32)**	**Cluster 2 (n = 29)**	**Cluster 3 (n = 32)**	***p* value**
History of HBV infection	9 (9.7)	1 (3.1)	2 (6.9)	6 (18.7)	0.089
Positive HCV serology	14 (15.0)	7 (21.9)	4 (13.8)	3 (9.4)	0.367
History of TB	15 (16.1)	6 (18.7)	3 (10.3)	6 (18.7)	0.594

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; TB, tuberculosis

4.2. Expression of KP metabolites in clusters

We evaluated the different expression of the Trp metabolites along the KP. Figure 9 shows a boxplot representation of the different median levels at which the metabolites are expressed within the three clusters. Tryptophan, kynurenine, serotonin, quinolinate, and nicotinamide seem to be equally expressed between the three clusters. On the other hand, N-acetyl-tryptophan, N-acetyl-kynurenine, kynurenate, xanthurenate, and picolinate appear to be downregulated in cluster 3 compared to cluster 1 and 2.

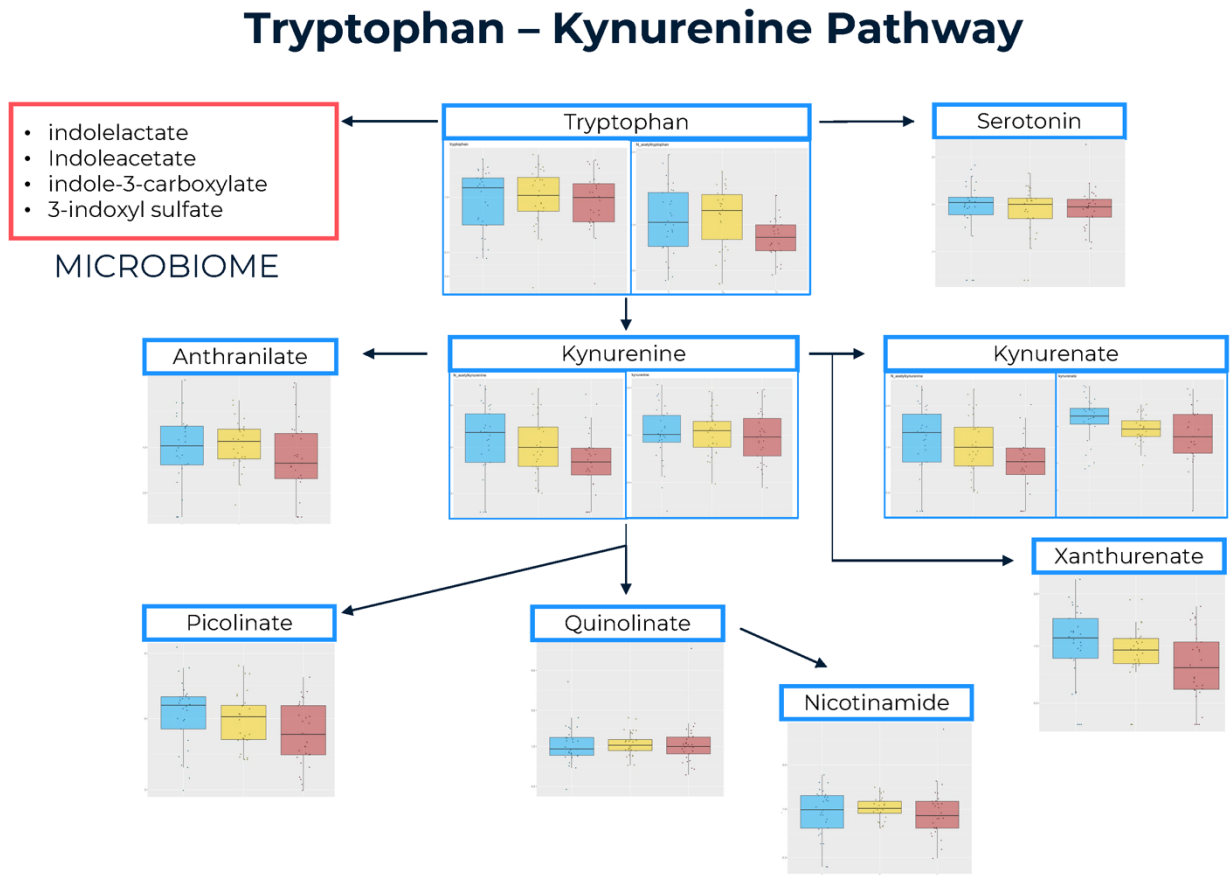

Figure 9. This boxplot graph represents the distribution of the KP metabolites + serotonin in each cluster. It shows that tryptophan, kynurenine, serotonin, quinolinate and nicotinamide are expressed at overlapping levels. On the other hand N-acetyl-tryptophan, N-acetyl-kynurenine, kynurenate, xathurenate, and picolinate are downregulated in cluster 3 compared with cluster 2 and cluster 1, although the distribution within the cluster appears to be quite variable.

We also evaluated the fold change per each Trp metabolite. The unadjusted analysis revealed that several KP metabolites, such as kynurenate, N-acetyl-kynurenine, N-acetyl-tryptophan, picolinate, 8-methoxykynurenate, and xanthurenate were significantly lower in cluster 3 than in cluster 1, confirming what we did see at first observation with boxplots. Kynurenate was significantly downregulated in cluster 2 compared with cluster 1 too. Pooled analysis (cluster 3 vs. clusters 1+2) showed that also anthranilate is downregulated in cluster 3 compared to the other 2 combined clusters, while indolepropionate, a Trp metabolite derived from gut microbiota, is upregulated.

Adjusting the analysis with the Benjamini-Hochberg method these significant differences were not confirmed. Figure 10 shows the fold changes for each metabolite, with *p* values and adjusted *p* values.

Cluster comparisson table									
Biochemical Name	Cluster 3 vs 1			Cluster 2 vs 1			Cluster 3 vs (Cluster 2 & Cluster 1)		
	Fold Chg.	p-value	Adj. p-value	Fold Chg.	p-value	Adj. p-value	Fold Chg.	p-value	Adj. p-value
alanine	1.04	0.828	0.670	1.07	0.305	0.907	1.00	0.825	0.907
anthranilate	0.87	0.145	0.907	1.00	0.852	0.436	0.87	0.049	0.216
indole_3_carboxylate	0.86	0.132	0.675	0.98	0.321	0.414	0.87	0.106	0.369
indoleacetate	1.74	0.484	0.907	1.17	0.840	0.781	1.60	0.420	0.781
indolelactate	0.85	0.263	0.907	0.93	0.768	0.651	0.88	0.219	0.629
indolepropionate	1.39	0.074	0.833	1.23	0.619	0.286	1.25	0.035	0.180
kynurenate	0.77	0.020	0.147	0.79	0.009	0.159	0.86	0.049	0.216
kynurenine	0.95	0.514	0.907	0.97	0.852	0.788	0.97	0.485	0.781
N_acetylkynurenine	0.56	0.009	0.781	0.86	0.437	0.147	0.60	0.006	0.147
N_acetyltryptophan	0.71	0.028	0.844	1.01	0.641	0.159	0.71	0.003	0.147
nicotinamide	1.08	0.504	0.907	1.00	0.803	0.788	1.09	0.233	0.640
nicotinamide_riboside	0.78	0.717	0.950	0.80	0.925	0.907	0.86	0.607	0.833
picolinate	0.70	0.029	0.651	0.87	0.276	0.159	0.75	0.029	0.159
quinolinate	1.30	0.950	0.833	0.90	0.619	0.950	1.37	0.777	0.907
serotonin	1.02	0.327	0.781	0.73	0.428	0.675	1.18	0.580	0.833
tryptophan	1.00	0.828	0.844	1.02	0.652	0.907	0.98	0.592	0.833
tryptophan_betaine	1.45	0.455	0.651	1.38	0.276	0.781	1.22	0.868	0.909
X1_methylnicotinamide	0.86	0.290	0.643	1.12	0.243	0.661	0.81	0.063	0.260
X3_indoxyl_sulfate	0.95	0.950	0.702	1.13	0.351	0.950	0.90	0.531	0.797
X5_hydroxyindole.sulfate	0.92	0.483	0.907	1.12	0.767	0.781	0.87	0.479	0.781
X8_methoxykynurenate	0.57	0.019	0.414	0.64	0.127	0.159	0.70	0.019	0.159
xanthurenate	0.63	0.019	0.314	0.75	0.086	0.159	0.72	0.023	0.159

Figure 10. Cluster comparison table. This table shows the fold changes for each metabolite, when comparing two clusters, or one cluster with the other two combined. Unadjusted p values show several significant differences between cluster 3 and cluster 1. However, these differences disappear when adjusting the analysis with the Benjamini-Hochberg testing

We performed a partial least square discriminant analysis (PLS-DA, figure 11), which allows to clarify that several metabolites of the KP are downregulated in cluster 3 compared with cluster 2 and cluster 1. Among them, those with the highest VIP score are kynurenate and 8-methoxykynurenate, meaning that, in this model, those two variables have the highest importance.

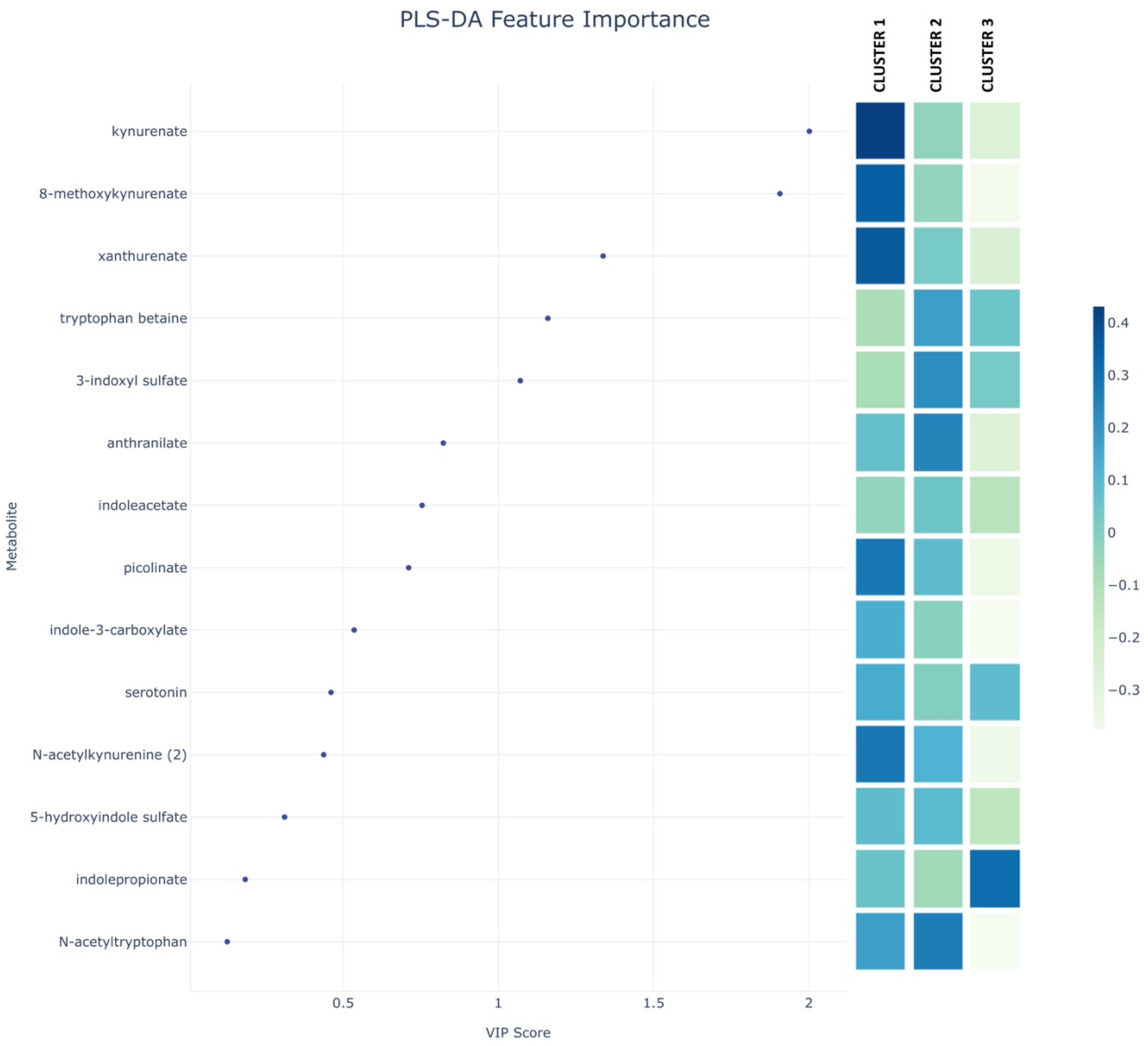

Figure 11. Partial Least Square Determinant Analysis (PLS-DA) confirms that several KP metabolites are downregulated in cluster 3, compared with cluster 2 and cluster 1, while indolepropionate is upregulated in cluster 3 compared with cluster 2 and cluster 1. The VIP score, revealing the importance of each variable in the model, shows that kynurenate and 8-methoxykynurenate have the highest importance in this model.

4.3. Correlation of KP metabolites with markers of inflammation

A secondary aim of this study was to find any correlation between KP metabolites and markers of inflammation in our cohort. To do so, we computed the results of our previous study[35] and normalized measurements of KP metabolites in a table, elaborating a correlation matrix and its relative correlation plot (figure 12). We studied the whole sub-cohort of 93 PLWH, finding out that not differentiating the population by its inflammatory profile, there are several significant correlations between markers of inflammation and KP metabolites. In particular, TNFR-II, IL-1b, and von Willebrand Factor (vWF) are significantly correlated with xanthurenate, TNFR-II and IL-1b are significantly correlated with 8-methoxykynurenate, and ICAM is significantly correlated with N-acetyl-tryptophan and N-acetyl-

kynurenine. On the other hand, P-selectin is inversely correlated with N-formyl-anthranilic acid, and VCAM is inversely correlated with kynurenine.

We also carried out the correlation per cluster. Markers of inflammation such as IL-6 and IL-1b show an intense correlation with KP metabolites in cluster 1, but not in cluster 2 and cluster 3; moreover, VCAM has a strong inverse correlation with KP metabolites in cluster 1, but not in clusters 2 and 3.

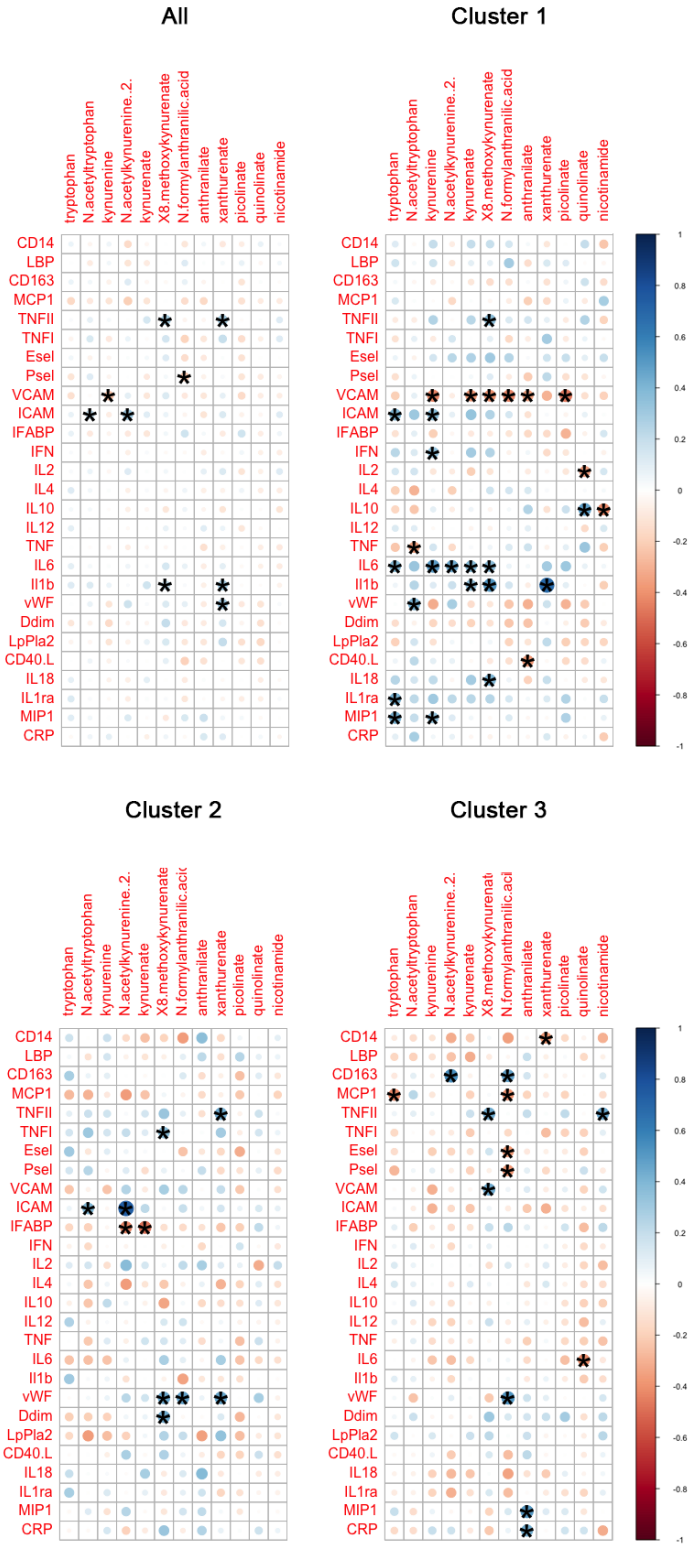

Figure 12. Correlation plot showing the correlations between KP metabolites and markers of inflammation characterizing the clusters. Color intensity and areas of the circles are proportional to the correlation coefficient value.

A blue color means a proportional correlation, while a red color means an inverse correlation. **All.** In the first quadrant, we analyzed the correlations existing when considering the whole cohort. We found out that TNFR-II and IL-1b have proportional correlations with X8-methoxykynurenate and xanthurenate, ICAM has proportional correlations with N-acetyl-tryptophan and N-acetyl-kynurenine, vWF has significant correlation with xanthurenate; however, P-selectin has inverse correlation with N-formyl-anthranilic acid and VCAM shows an inverse correlation with kynurenine. **Cluster 1**. In the second quadrant, we analyzed the correlations existing when considering only PLWH belonging to Cluster 1[35]. We found out that in Cluster 1, markers of inflammation such as IL-6 and IL-1b have strong proportional correlations with KP metabolites. In particular, IL-6 shows significant correlations with tryptophan, N-acetyl-kynurenine, kynurenine, kynurenate, and X8-methoxykynurenate; IL-1b shows strong correlations with kynurenate, X8-methoxy-kynurenate and xanthurenate. On the other hand, VCAM shows strong inverse correlations with kynurenine, kynurenate, x8-methoxy-kynurenate, N-formyl-anthranilic-acid, anthranilate, and picolinate. IL-10 has a proportional correlation with quinolinate and an inverse one with nicotinamide. **Cluster 2.** In the third quadrant we analyzed the correlations existing when considering only PLWH belonging to Cluster 2[35]. We found out that in Cluster 2, TNFR-II is proportionally correlated to xanthurenate, TNFR-I is proportionally correlated to X8-methoxy-kynurenate, ICAM is proportionally correlated to N-acetyl-tryptophan and N-acetyl-kynurenine; vWF is significantly correlated to X8-methoxy-kynurenate, N-formyl-anthranilic acid, and xanthurenate; D-dimer is significantly correlated to X8-methoxy-kynurenate. IFABP is inversely correlated to N-acetyl-kynurenine and kynurenate. **Cluster 3.** In the fourth quadrant we analyzed the correlations existing when only considering PLWH belonging to Cluster 3[35]. We found out that in Cluster 3, sCD163 is correlated to N-acetyl-kynurenine and N-formyl-anthranilic-acid; TNFR-II is significantly correlated to X8-methoxy-kynurenate and nicotinamide; vWF is significantly correlated to N-formyl-anthranilic acid, MIP1 and hs-CRP are significantly correlated to anthranilate. On the other hand, MCP-1 is inversely related to tryptophan and N-formyl-anthranilic acid. E-selectin and P-selectin also are inversely related to N-formyl-anthranilic acid, while IL-6 is inversely correlated to quinolinate. **Abbreviations:** CD14, soluble cluster of differentiation 14; LBP, lipopolysaccharide binding protein; CD163, soluble cluster of differentiation 163; MCP1, monocyte chemoattractant protein 1; TNFII or TNFR-II, tumor necrosis factor receptor II; TNFI or TNFR-I, tumor necrosis factor receptor I; Esel, E-selectin; Psel, P-selectin; VCAM, vascular cellular adhesion molecule; ICAM, intercellular cellular adhesion molecule; IFABP, intestinal fatty acid binding protein; IFN, interferon gamma; IL2, interleukin 2; IL4, interleukin 4; IL10, interleukin 10; IL12, interleukin 12; TNF, tumor necrosis factor alfa; IL6, interleukin 6; IL1b, interleukin 1 beta; vWF, von Willebrand factor; Ddim, D-dimer; LpPla2, lipoprotein-associated phospholipase A2; CD40L, soluble cluster of differentiation 40 ligand; IL18, interleukin 18; IL1ra, interleukin 1 receptor antagonist; MIP1, macrophage inflammatory protein 1; CRP or hs-CRP, high sensitivity C reactive protein.

4.4. Expression of KP metabolites according to history of comorbidities

Another secondary aim we had for this study, was to explore the existence of differences expression of KP metabolites according to history of comorbidities.

As our objective was to find any correlation with cardiovascular diseases, we explored possible differences in expression of KP metabolites between PLWH who had a history of previous CVD and those who had not (figure 13). We found that, except for Cluster 2, all the metabolites have higher concentrations in those who had a history of CVD than in those who had not. Significant differences have been found in cluster 1 for anthranilate ($p = 0.017$) and picolinate ($p = 0.017$); in cluster 2 for

tryptophan (p = 0.029), and in cluster 3 for quinolinate (p = 0.016). All other differences are not statistically significant.

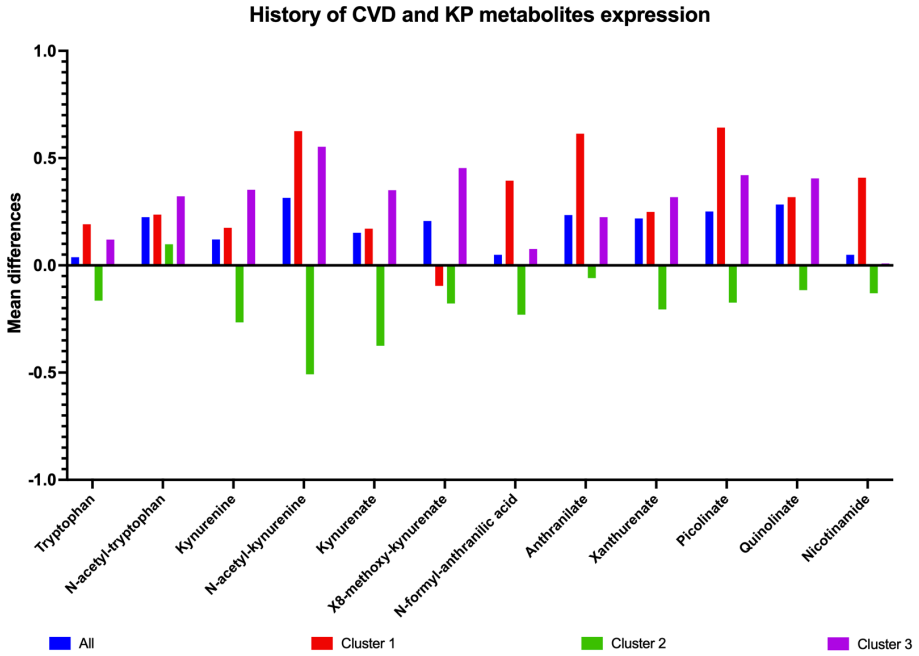

Figure 13. Mean differences in expression of KP metabolites between PLWH who have a history of CVD and those who have not. **Abbreviations:** CVD, cardiovascular disease; KP, kynurenine pathway; PLWH, people living with HIV.

In PLWH with history of hypertension, we highlighted a statistically significant difference only in Cluster 1 for picolinate (p = 0.011). No other statistically significant difference was highlighted, overall or in clusters.

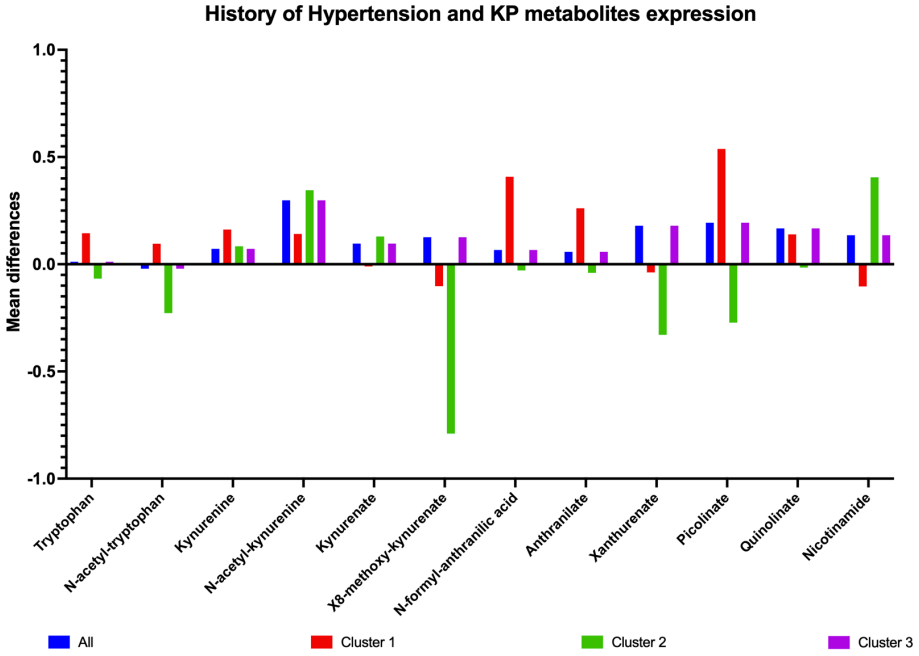

Figure 14. Mean differences in expression of KP metabolites between PLWH who have a history of hypertension and those who have not. **Abbreviations:** KP, kynurenine pathway; PLWH, people living with HIV.

In PLWH with history of dyslipidemia, we did not observe any statistically significant difference in KP metabolite expression either overall or when looking at clusters 1 and 2. In cluster 3, we did observe a statistically significant difference in quinolinate ($p = 0.013$).

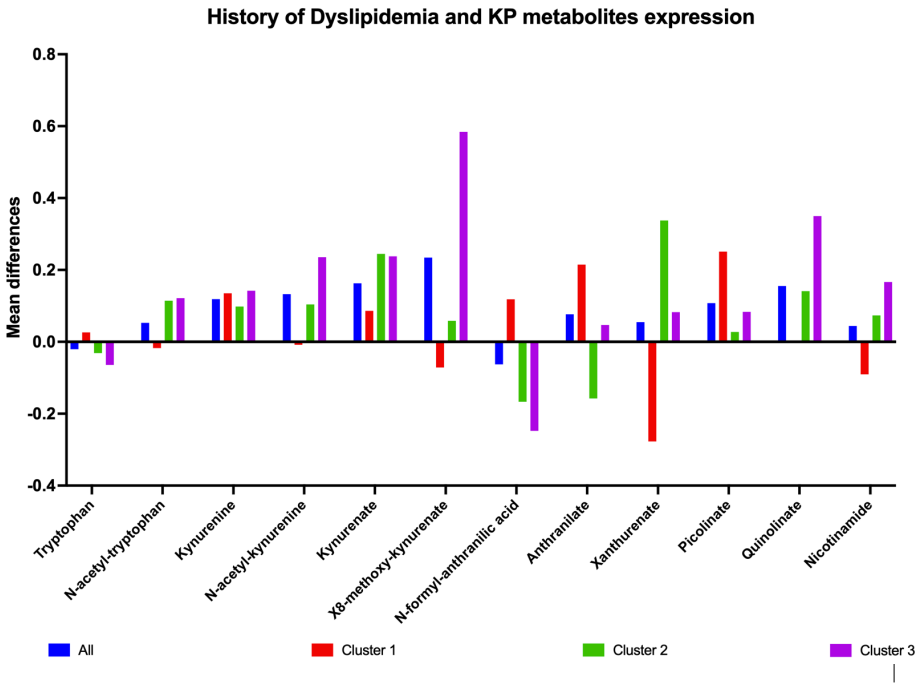

Figure 15. Mean differences in expression of KP metabolites between PLWH who have a history of dyslipidemia and those who have not. **Abbreviations:** KP, kynurenine pathway; PLWH, people living with HIV.

5. Discussion

With this study, we explored the hypothesis that metabolites derived from the kynurenine pathway of degradation of the tryptophan influence CV risk. To do so, we used a known cohort in which inflammatory profiles were already studied and established, and selected age, ethnicity, and sex matched PLWH to deepen our knowledge[35].

5.1. Characteristics of the included PLWH

One third of the PLWH included in this study are female. This fact is important, because it reflects the percentages of women usually included in cohorts and clinical trials, and of PLWH in active follow-up at outpatient clinics throughout the Western world, although the latest UNAIDS estimates report that, worldwide, the percentage of women living with HIV is 53%[1]. Another important information about the population in study is that only around 55% is Caucasian, while around 35% is of African origin and the remaining 10% is of Asian descent. Mixed race in this study is an important feature, as literature shows that race can influence levels of inflammation and microbiota composition, therefore influencing metabolite production[36].

PLWH included in the three different clusters showed significantly different levels of $CD4^+$ T-cell counts and percentage, and of $CD4^+/CD8^+$ ratio. Surprisingly, we found out that $CD4^+$ T-cell counts and percentages, together with $CD4^+/CD8^+$ ratio were higher in PLWH included in clusters 2 and 3, compared with those in cluster 1. This result contrasts with the knowledge we have from literature: higher $CD4^+$ T-cell counts and $CD4^+/CD8^+$ ratio are surrogate markers of lower inflammation[37–40]. However, from our previous work we could observe that cluster 3 is the most inflamed among the three patterns, expressing markers of gut epithelial dysfunction, T-cell differentiation and regulation, and systemic inflammation[35]. Moreover, although it is true that higher $CD4^+$ T-cell counts and $CD4^+/CD8^+$ ratio usually mean lower systemic inflammation, it is necessary to remember that at least seven different subtypes of $CD4^+$ T-cells differentiate from naïve $CD4^+$ T-cells, all of them responding to different cytokine stimulation. In fact, cytokines upregulated in cluster 3 can stimulate differentiation towards all $CD4^+$ T-cell subtypes, determining a de-regulated immune status like the one we can observe during sepsis[41]. Keeping in mind that cluster 3 represent around 10% of the whole AIID cohort, more studies and larger cohorts will be needed to explore the causes leading to this pattern of markers of inflammation.

Also surprisingly, levels of total cholesterol and LDL cholesterol were significantly lower in cluster 3 compared to cluster 1, a result that only partially finds its explanation in statin use, since the highest frequency of use is in cluster 1, although the difference in frequency is not statistically significant when compared to the other clusters.

We accounted for several traditional risk factors, such as current smoking, alcohol use, drug addiction, personal history of dyslipidemia, hypertension, CVD, and diabetes, use of statins and family history of CVD, finding out that our clusters differed only for history of hypertension. In fact, in cluster 3 PLWH were more frequently affected by hypertension compared to cluster 2, even though there were no statistically significant differences when compared to cluster 1. The prevalence of comorbidities was similar in all the three clusters, although the rate of liver disorders was significantly higher in cluster 1 compared with cluster 2, but not with cluster 3.

Traditional risk factors, together with increased inflammation, comorbidities, and HIV infection, increase dramatically the risk of MACE, as they are all parts of a vicious cycle[42–45]. It is known that median age for the onset of hypertension and metabolic disorders, such as diabetes, in the general population is around 55-60 years, and that for PLWH this age is around 10 years lower[46]. Our cohort had a median age of 45.0 years, and one third of the included PLWH suffered from one comorbidity, another third was affected by more than one comorbidity, not accounting for dyslipidemia, around 10% was affected by one comorbidity and dyslipidemia, and another 10% had more than one comorbidity and dyslipidemia. Nowadays, PLWH have the same life expectancy of the general population. Therefore, preventing CVD and MACE is of utmost importance, to reduce mortality and increase morbidity-free time in this population[47].

5.2. Expression of KP metabolites in clusters

From previous works, we know that KP is intra-hepatic under physiological conditions for the 98%, and that the first step is catalyzed mostly by TDO[23–26,48]. However, when there are inflammatory conditions, the activation of JAK/STAT pathways increases the activity of IDO1, leading to an accumulation of KP metabolites outside the liver[23–26,48].

Starting from these premises, we measured KP metabolites in PLWH plasma, hypothesizing a pro- or anti-inflammatory role for each of them. Our primary objective was to understand which Trp metabolite of the KP, if any, correlates with membership of an inflammatory cluster associated with increased CVD risk in PLWH, as previously defined[35]. Comparing the levels of each KP metabolite within the three clusters, we observed that in cluster 3 several metabolites, such as N-acetyl-tryptophan, N-acetyl-kynurenine, kynurenate, xanthurenate, and picolinate appear to be downregulated compared to cluster 1 and 2, although with a high variability. The analysis of the fold-change consented to highlight several significant differences that were not confirmed after adjustment, probably for the small number of PLWH included in the study. Therefore, we could not find a conclusive association of one or more KP metabolites with one or more specific clusters, although we can hypothesize that by increasing the size of each cluster, we could verify the tendency towards downregulation in cluster 3, compared with other clusters, of several KP metabolites.

5.3. Correlation of KP metabolites with markers of inflammation

Previous studies have tried to clarify KP metabolites influence over inflammation and immune cell functions, with little success, although it seems clear that lower Trp levels, together with higher Kynurenic acid levels and KynA/Trp ratio are associated with a higher CVD risk[26,28]. This information was also confirmed in PLWH[29,30].

In our study, we tried to correlate KP metabolite levels with concentrations of markers of inflammation and immune activation in three different clusters which were previously identified and characterized by different patterns of inflammation, with cluster 1 showing a lower expression of markers of inflammation, cluster 2 being the one characterized by higher expression of markers of monocyte activation, systemic inflammation, and vascular endothelial function, and cluster 3 being the one characterized by higher expression of markers of T-cell differentiation and regulation[35].

We found out that KP metabolites show the strongest correlation with markers of inflammation in cluster 1[49]. In fact, in PLWH belonging to cluster 1, KP metabolites show a strong positive correlation with IL-6 and a strong negative correlation with VCAM[49].

IL-6 has a pleiotropic pro- and anti-inflammatory role, depending on the secreting cell[49]. When produced by macrophages, IL-6 has a pro-inflammatory role[49]. It stimulates the production of acute phase proteins and the release of neutrophils from the bone marrow during the acute phase of an infection[49]. On the other hand, it is also produced by myocytes during exercise, with an anti-inflammatory effect, preventing obesity and increasing lipolysis[50]. In PLWH belonging to cluster 1, IL-6 is positively correlated with tryptophan, kynurenine and kynurenate. Although kynurenine has been shown to have a pro-inflammatory role, in this case we could infer that kynurenine increases with Trp as it is the first step of its metabolism through this pathway, towards kynurenate[27,48,51,52]. Both Trp and kynurenate have been shown to have anti-inflammatory properties[27,48,51,52].

VCAM is expressed on the surface of large and small vessels after stimulation of the endothelial cells with cytokines, and it mediates the adhesion of immune cells to the endothelium during inflammatory states[5,53]. In cluster 1, VCAM shows a strong inverse correlation with kynurenine, kynurenate, anthranilic acid and anthranilate, and picolinate. While anthranilic acid has pro-inflammatory properties, it is quickly metabolized in anthranilate, which has anti-inflammatory properties instead [27,48,51,52]. Picolinate has been shown to have anti-inflammatory properties [27,48,51,52].

Taken together, the correlation of KP metabolites with IL-6 and VCAM in cluster 1 support the hypothesis that Trp metabolism through KP is enhanced at low levels of inflammation, with an anti-inflammatory role, as an adjunctive control system. In fact, it must be remembered that although cluster 1 shows a lower expression of markers of inflammation and immune activation compared to cluster 2 and cluster 3, it is still higher than in general population[54,55]. Lower levels of correlation with markers of inflammation and immune activation in clusters 2 and 3 might be explained as a failure to control inflammation.

5.4. Expression of KP metabolites according to history of comorbidities

We found that KP metabolites are expressed in higher concentrations in PLWH who have a history of CVD in cluster 1 and 3, with significant differences found in cluster 1 for anthranilate and picolinate, which have anti-inflammatory properties, and in cluster 3 for quinolinate, which have pro-inflammatory properties[27,48,51,52]. In cluster 2, KP metabolites are expressed in lower concentrations in PLWH who have a history of CVD, with significant differences only found for tryptophan, which have anti-inflammatory properties[27,48,51,52]. Taken together, these data support the conclusions of our previous work that PLWH belonging to cluster 1 have a lower CVD risk compared with clusters 2 and 3[35].

5.5. Limitations of the study

This study has several limitations. First, its cross-sectional design did not allow to study if changes occur over time in KP metabolites concentrations. The small sample size might reduce the power of our conclusions. No information about the virological status of the included PLWH was available. Although viral suppression was an inclusion criterion for this study, no information about the duration of the viral suppression was available, which is a critical data to estimate the influence that the viral reservoir might have on the inflammatory status[56]. We hypothesized that, since all the included PLWH were diagnosed for a median time of 10.5 years at the time of sampling, with a similar time on ART, all PLWH were suppressed for more than one year, decreasing the chances that the immune activation was influenced by active replication[56].

6. Conclusions

Although we could not find any definite correlation between KP metabolites and membership to an inflammatory cluster associated with an increased CVD risk in PLWH, this study provided several insights to the correlation of kynurenine pathway metabolites and markers of inflammation.

First, KP metabolites correlate with IL-6 and VCAM in cluster 1 but not in cluster 2 and 3. The correlations existing with IL-6 and VCAM in cluster 1 support the hypothesis that KP works as an adjunctive inflammation control pathway, especially during low level inflammation. This hypothesis is also supported by the scarce correlation with markers of inflammation and immune correlation when expressed at higher levels in clusters 2 and 3.

Second, KP metabolites correlates with CVD risk. In PLWH who have a history of CVD, KP metabolites with anti-inflammatory properties are expressed at higher levels in cluster 1, while they are expressed at lower levels in cluster 2. Moreover, in cluster 3, KP metabolites with pro-inflammatory properties are expressed at higher levels in PLWH who have a history of CVD.

More studies and larger cohorts will be needed to better explore the correlations existing between KP metabolites, markers of inflammation and immune activation, and CVD.

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