Altered plasma metabolites and inflammatory networks in HIV-1 infected patients with different immunological responses after long-term antiretroviral therapy

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Research Article

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Abstract

Background: Chronic metabolic changes relevant to human immunodeficiency virus type 1 (HIV-1) infection and in response to antiretroviral therapy (ART) remain undetermined. Moreover, links between metabolic dysfunction caused by HIV and immunological inflammation in long-term treated individuals have been poorly studied.

Methods: Untargeted metabolomics and inflammatory cytokine levels were assessed in 50 HIV-infected individuals including 25 immunological responders (IRs) and 25 non-responders (INRs) before and after ART. The IRs and INRs were matched by age, gender, baseline viral load and baseline CD4+T cell counts. Another 25 age-matched uninfected healthy individuals were also included as controls.

Results: Among the 770 plasma compounds detected in the current study, significant changes were identified in lipids, nucleotides, and biogenic amino acids between HIV-infected patients and healthy controls. Principal Component Analysis (PCA) and Random Forest (RF) model suggested that levels of selected metabolites could differentiate HIV infected patients clearly from healthy controls. However, only three metabolites including maltotetraose, N,N-dimethyl-5-aminovalerate and decadienedioic acid (C10:2-DC) were different between IRs and INRs following long-term ART. The pathway enrichment analysis results revealed that disturbances in pyrimidine metabolism, sphingolipid metabolism and purine metabolism after HIV infection and these changes did not recover to normal levels in healthy controls even with suppressive ART. Correlation analysis of the metabolism-immune network indicated that interleukin (IL)-10, D-dimer, vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1) and TNF-RII were positively correlated with most of the significantly changed lipid and amino acid metabolites, but negatively correlated with metabolites in nucleotide metabolism.

Conclusions: Significant changes of many metabolites were observed in HIV-infected individuals before and after ART, regardless of their immunological recovery status. The disturbed metabolic profiles of lipids and nucleotides in HIV infection did not recover to the normal levels even after long-term ART. These changes are correlated with modified cytokines and biomarkers of chronic non-AIDS events, warranting try out of interventions other than ART.

Introduction

Although the introduction of antiretroviral therapy (ART) has achieved viral suppression and subsequent immune reconstitution in most people living with HIV-1 (PLWH), increased risks of chronic AIDS-related illnesses associated with aging are still challenging in this population(1). Nevertheless, 10–30% PLWH suffer from poor immune recovery despite prolonged successful suppressive ART, which further speeds up the disease progression and contributes to increased mortality, referred to as “immune non-responders” (INRs)(1, 2). Growing evidence has revealed that HIV infection could induce various metabolic changes including impaired glucose metabolism(3, 4), hypertriglyceridemia(3), and down-regulation of sphingomyelin metabolism(4). For example, the transition of resting T cells depends on the catabolic metabolism of glucose, the displacements of amino acids and fatty acids in oxidative
phosphorylation(5). These changes are related to the effects of HIV infection on the proliferation and functioning of immune cells. For example, the transition of resting T cells depends on the catabolic metabolism of glucose and the displacements of amino acids and fatty acids in oxidative phosphorylation; susceptibility of CD4 T cells to HIV-1 causes increased glycolysis and oxidative phosphorylation (OXPHOS) independent of the activation phenotype(5). However, current results of HIV-caused metabolic changes are not consistent, and metabolic changes in HIV patients after long-term ART are unclear.

Metabolomics has been widely applied to screening potential biomarkers and related disease mechanisms, which has been applied to liver disease(6), cardiovascular disease (7), cancer(8) and HIV infection(4, 5, 9, 10). Several metabolomics studies of HIV infection suggest that ART could only partially recover the disturbed metabolic changes due to HIV infection (9, 11). Some antiretroviral drugs may even cause additional metabolic dysfunction (5, 12), such as hyperactive glycolysis (11), enhanced mitochondrial toxicity (11), abnormal amino acid catabolism (9, 10, 13), and imbalances in phospholipid and sphingolipid metabolism (9, 10). While two studies have reported the effects of long-term ART on HIV patients’ metabolism (10, 14), little has been explored regarding metabolic changes between INRs and IRs of ART. Higher accumulation of plasma acylcarnitine(15) and persistent decline of sphingosine-1-phosphate phosphatase 1 activity have been observed in INRs (4, 15), while more high density lipoprotein (HDL) particles, HDL cholesterol and microbial translocation have been reported in IRs (14) (16). However, the available study results regarding IRs and INRs were not consistent.

Therefore, we plan to comprehensively assess the immune-metabolic adaptations occurring with HIV infection and the potential response to long-term ART. The plasma metabolomes were studied in HIV-infected individuals before and following long-term ART according to their varied immune responses and compared with HIV-seronegative people. The study results provide clues for developing improved treatment strategies for the well-being of HIV patients.

**Materials and methods**

**Study subjects and study design.**

Eligible participants were recruited from the HIV/AIDS outpatient clinic, Peking Union Medical College Hospital (PUMCH), China. All participants have been regularly followed with suppressed viraemia (< 50 copies/ml) for at least three years. Of them, INRs were defined if a patient’s CD4 count remains ≤ 350 cells/µL after long-term ART (n = 25, male 24/25), and IRs were defined as if a patient’s CD4 count > 500 cells/µL (n = 25; male 24/25) (Fig. 1). HIV-seronegative subjects matched by age and gender (n = 25, 23/25 male) to the HIV patients were recruited at the same time as controls. The IRs and INRs were matched by baseline CD4 T cells, viral loads, age and gender, and HIV patients co-infected with HBV or HCV were excluded. Demographics and clinical data of the participants were collected from this mini study. Samples from different time-points were analyzed according to the study protocol. The study was
approved by an independent ethics committee and the institutional review board of PUMCH (Peking Union Medical College Hospital, JS-1431).

**Laboratory testing**

**Untargeted metabolomics**

Untargeted metabolomic analysis was conducted by Calibra Lab at DIAN Diagnostics (Hangzhou, Zhejiang, China) on their CalOmics metabolomics platform. Samples were extracted using methanol in a ratio of 1:4. The mixtures were shaken for 3 minutes and precipitated by centrifugation at 4000 × g, 10 minutes at 20 °C. Four aliquots of 100 µL supernatant were transferred to sample plates and dried under blowing nitrogen, then re-dissolved in reconstitution solutions for sample injection into UPLC-MS/MS systems. The instruments for the four UPLC-MS/MS methods are ACQUITY 2D UPLC (Waters, Milford, MA, USA) plus Q Exactive (QE) hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, USA). QE mass spectrometer was operated at a mass resolution of 35000, the scan range was 70-1000 m/z. In the first UPLC-MS/MS method, QE was operated in positive ESI mode and the UPLC column was C18 reverse-phase (UPLC BEH C18, 2.1x100 mm, 1.7 um; Waters); the mobile solutions used in the gradient elution were water (A) and methanol (B) containing 0.05% PFPA and 0.1% FA. In the second UPLC-MS/MS method, QE was operated in negative ESI mode, and the UPLC column was C18 reverse-phase (UPLC BEH C18, 2.1x100 mm, 1.7 um; Waters), the mobile solutions used in the gradient elution were water (A) and methanol (B) containing 6.5 mM ammonium bicarbonate at pH 8. The third UPLC-MS/MS method had the QE operated in ESI positive mode and the UPLC column was C18 reverse-phase (UPLC BEH C18, 2.1x100 mm, 1.7 um; Waters), the mobile solutions were water (A) and methanol/acetonitrile/water (B) contain 0.05% PFPA and 0.01% FA. In the fourth method, QE was operated in negative ESI mode, the UPLC column was HILIC (UPLC BEH Amide, 2.1x150 mm, 1.7 um; Waters), and the mobile solutions were water (A) and acetonitrile (B) with 10 mM ammonium formate.

**Virological and immunological measurements**

The plasma HIV-1 RNA load was measured using the COBAS Ampliprep/TaqMan 48 real-time RT-PCR Test (Roche, CA, USA) according to the manufacturer’s instructions. Immune profiles of peripheral blood lymphocytes were analyzed by three-color flow cytometry (Epics XL flow cytometry; Beckman Coulter, USA) as previously described (17). The inflammatory cytokine panel was measured by a standardized Luminex assay (Bio-Rad Laboratories, Hercules, CA, USA)) according to the manufacturer’s instructions, and 14 cytokines were measured: interleukin (IL) -1β, IL-2, IL-6, tumor necrosis factor (TNF) -RII, IL-8, IL-10, IL-7, Chemokineligand-10 (CXCL-10), Monocyte chemotactic protein-1 (MCP-1), Vascular Cell Adhesion Molecule-1 (VCAM-1), Intercellular Cell Adhesion Molecule-1 (ICAM-1), D-dimmer, interferon (IFN) -γ, and soluble CD40 ligand (CD40L).

**Statistical analysis**
The statistical analysis and figure drawing were conducted using Metaboanalyst 5.0 software (www.metaboanalyst.ca/), Originpro 2023 software, and R statistical package version 4.0.0 (R Foundation for Statistical Computing, Vienna, Austria). For the descriptive comparisons, categorical variables were compared with the chi-square test and continuous variables with Welch's t-test. For the metabolomics data, raw peak areas were median normalized to adjust for system fluctuation among different run days. The normalized peak areas were log transformed to reduce data distribution bias and to be in an approximate normal distribution (Gaussian distribution). Missing values in the peak matrix (under detection limit) were imputed with the minimum detection value of a metabolite in all samples.

Unsupervised Principal Component Analysis (PCA) and supervised Partial Least Square Discrimination Analysis (PLS-DA) were conducted to visualize sample clustering. Based on their variable importance in the projection (VIP) values in the first principal component (PC) of PLS-DA analysis, metabolites with VIP > 1.0 and p < 0.05 in the student t-test and volcano plots were selected as the differentially expressed metabolites. After the Benjamin–Hochberg procedure was applied to control false discovery rate (FDR) due to multiple testing, metabolites with log₂ (fold change) > 1 was finally selected as significantly changed metabolites. The above statistical criteria have been designed to select mainly biologically relevant metabolites and avoid nonspecific noisy signals in the study.

Result visualizations were provided for the performed statistical analyses, including volcano plot in differential metabolite test, scatter plot with confidence ellipse in PCA, scatter plot with confidence ellipse and variable importance dot plot in PLS-DA/OPLS-DA, and variable mean decrease accuracy dot plot in RF.

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The differentially expressed metabolites between groups were mapped into biochemical pathways, and the major disturbed metabolic pathways were compared between groups using Student’s t-test. Pathways with p < 0.1 were considered significantly modified and further investigated. Heat maps and correlation matrix were generated for visualization. Pearson correlations were conducted to study the associations among deferentially expressed metabolites, clinical data, and cytokine data.

**Results**

1. **Demographic characteristics**

The metabolomic features were studied in three types of participants, including group 1 of HIV-infected INRs, group 2 of HIV-infected IRs, and group C of healthy controls (HC). In HIV-infected groups 1 and 2,
sequential samples were evaluated including a pre-ART baseline point (A, groups A1 and A2) and a post-ART point (B, groups B1 and B2). In addition, fasting blood from HIV seronegative subjects (n = 25) was obtained in a single visit (Flow chart in Fig. 1). All participants were balanced among age, gender, as well as the baseline viral load and CD4 + T cell counts (Table 1). The ART regimens were also similar during follow-up (Table 1). Overall, the average time of treatment and follow-up in all HIV-1-infected patients was 6.81 ± 3.10 years. However, verification of clinical information revealed that three subjects developed lymphoma or used other adjuvant agents to improve CD4 count in follow-up. Therefore, their associated samples and metabolite information was removed after follow-up analysis. Clinical and demographic characteristics were also balanced among groups (see in Table S1).

2. Altered plasma metabolite summary

2.1 PCA and PLS-DA analysis

From the original list of detected metabolites in the metabolomics analysis, 770 out of 938 metabolites were selected, which are endogenous and with confirmed identity. The 770 metabolites could be divided into six categories: lipids, amino acid, carbohydrate, nucleotide, cofactors and vitamins, peptide. PCA and PLS-DA models were performed to determine variation between groups. In PCA model, a clear mode of separation between PLWH and HC was found, more concentrated in HC and more scattered in HIV patients (Fig. 2a-2c, A-C, A-B, B-C). However, the differences between INRs and IRs before and after ART in PCA analyses were less obvious (Fig. 2d-2e). A similar separation between the groups was also observed in the PLS-DA analyses (Figure S1, Supplementary Materials).

2.2 Significant metabolites summary

The Welch's t-test and PLS-DA method identified significantly different metabolite levels between groups. When comparing untreated HIV patients with the healthy controls, 55 metabolites were up-regulated and 30 were down-regulated. In the 55 up-regulated metabolites, 60% were lipid metabolites and 16% were amino acid metabolites. In the 30 down-regulated metabolites, 68% were lipid metabolites and 18% were amino acid metabolites (Figure S2a). After long-term ART, HIV patients had 50 significantly higher and 28 lower levels of metabolites compared with the HC. In the comparisons between pre-ART and after-ART HIV patients (A vs. B), after-ART HIV patients had seven down-regulated metabolites and 31 up-regulated metabolites. Two of the seven down-regulated metabolites were 2'-deoxyuridine and N-carbamoylaspartate, and the rest were lipids (Figure S2b). Of the significantly upregulated metabolites in the HIV-infected patients compared with healthy controls (B vs. C), 60% were lipid, 16% were amino acids and 10% were nucleotide (Figure S2c).

To find metabolites with different levels between IRs and INRs, significant metabolites were compared at baseline and post-ART (pre-treatment: 1A-2A, after-treatment: 1B-2B, Figure S2d and Figure S2e). Three significant metabolites were prominent in the comparison before treatment and three in the comparison after treatment. Maltotetraose was down-graduated while dihydroorotate and beta-cryptoxanthin were up-graduated in 1A compared with 2A. Maltotetraose and N, N-dimethyl-5-aminovalerate were lower while
decadienedioic acid (C10:2-DC) was higher in 1B compared with 2B. The upset diagrams summarizing metabolites comparisons were shown in Fig. 3a and 3b. Maltotetraose, which was a glucose residue at the reducing end, was the only metabolite regulated in over all groups at the same time. It remained increased in all HIV-infected patients compared with HC, particularly prominent before ART initiation. In addition, INRs were associated with lower levels of maltotetraose than IRs no matter they received ART or not. Meanwhile, there are other two significant metabolic bio-chemicals including higher N-stearoylsphinganine (d18:0/18:0), higher 1-stearoyl-2-oleoyl-GPS (18:0/18:1) and commonly regulated in HIV-infected patients compared with HC. They were also higher in PLWH before ART compared with those after ART. Whereas the 5-hydroxyhexanoate showed up-regulation in HIV-infected patients compared with HC but was down-regulated after ART initiation (differential metabolites for each comparison in Table S2).

2.3 Significant pathway summary

The significantly changed metabolites between different groups were applied to pathway enrichment analysis using MetaboAnalyst software in the Human Metabolome Database. Since there were only five significantly changed metabolites between INRs and IRs, and the five metabolites belong to five different metabolic pathways, pathway enrichment was not applied to the comparison between INRs and IRs. Further biochemical analysis was mainly targeted on changed between HIV-infected patients and HCs, as well as between before and after ART HIV patients.

The significantly changed metabolites between different groups were primarily nucleotides, amino acids, and energy metabolism metabolites (Table S3). These metabolites between pre-ART HIV patients and HC belong to 21 different metabolic pathways, and five of these 21 pathways had impact > 0.1 and FDR < 0.05 and were further investigated. The most distinct pathway was glycerophospholipid metabolism with highest impact value of 0.256, followed by the pyrimidine metabolism, sphingolipid metabolism, purine metabolism and starch and sucrose metabolism pathway (Fig. 3c and table S2). Between HIV-infected patients before and after ART, the only enriched was the pathway of synthesis and degradation of ketone bodies with an impact score of 0.6. (Fig. 3d, Table S2). Purine metabolism, sphingolipid metabolism, glycerophospholipid metabolism and pyrimidine metabolism were from the top down in importance between HIV-infected patients and HCs, with an impact score of 0.21 (Fig. 3e).

3.Identification of potential biomarkers

Random forest analyses were conducted to determine potential biomarkers that can differentiate different sample groups. The RF analysis resulted in a predictive accuracy of 98.61% between pre-ART HIV patients and HCs, and a predictive accuracy of 100% between post-ART HIV patients and HCs. For patients infected with HIV before and after ART, the RF analysis had a predictive accuracy of 87.23%. The biochemical importance plots revealed the top 20 metabolites that contributed most to the distinction between baseline PLWH and HC (Fig. 4a), between baseline PLWH and post-ART patients (Fig. 4b), and between post-ART PLWH and HC (Fig. 4c). 1-stearoyl-GPS (18:0) was the top metabolite that could be used to distinguish = HIV patients from HCs (Fig. 4a). Quinolinate was the top metabolite to differentiate
HIV-patients before and after ART (Fig. 4b), As shown in Fig. 4c, stearoylcholine contributed most to the separation of post-ART patients and HCs (Fig. 4c). The top 20 metabolites differentiating pre-ART and HC groups involved nucleotide metabolism (3/20), amino-acid metabolism (3/20) and lipid metabolism (11/20). Metabolites involved in amino-acid metabolism (4/20) and lipid metabolism (11/20) were among the top 20 metabolites to separate post-ART patients and HCs. Most of the top 20 metabolites in the RF analysis to differentiate pre-ART and post-ART patients were mainly involved in lipid metabolism (12/20). The pathways revealed by the RF analysis were consistent with the pathways identified by the pathway enrichment analysis, pointing to potential biomarkers in lipid, nucleotide, and amino acid metabolisms.

4. Potential biomarkers correlated with inflammatory factors

The metabolites identified from RF and pathway enrichment analysis as potential biomarkers are further investigated for their correlations with inflammation and cardiovascular disease biomarkers. The levels of different cytokines in plasma are routinely tested to reflect systemic immune activity and disease progression. Thus, the plasma samples tested in the metabolomics analysis were also analyzed for inflammatory factors and biomarkers for cardiovascular risk. Most of these tested values had significant changes in HIV-infected patients both before and after ART. MCP-1 and CD40L were markedly different between INRs and IRs (Figure S3).

We then analyzed the correlation between the plasma inflammatory factors and changed metabolites between different groups (Fig. 5). Correlations with a p-value < 0.01 were deemed significant. Comparing the correlation heatmap Fig. 5a(A-C) with 5b(B-C), IL-10, D-dimmer, VCAM-1, TNF-RII and ICAM were positively correlated with most significantly changed metabolites between pre-ART patients and HCs (Fig. 5a), as well as between post-ART patients and HCs, but were negatively correlated with changed nucleotide metabolism metabolites. IL-8 and IL-7 were positively correlated with most of the changed lipids after ART, but negatively connected with most of the changed amino acids. There was no obvious between the plasma cytokines and significantly changed metabolites between pre-ART and post-ART samples (Fig. 5c). Two of the top candidates in the RF analysis, N-carbamoylaspartate and 2-deoxyuridine, showed negative correlation with IL-10, IL-8, D-dimer and VCAM-1, but were positively correlated with CD4 + T cells.

5. Summary of changed metabolites HIV-infected patients before and after ART.

Through pathway enrichment analysis, we found that the levels of many amino acids, lipids, and nucleotides were greatly changed in the PLWH compared with the HC. The metabolic pathways disturbed by HIV infection and ART were shown in Fig. 6. Maltotetraose and maltooligosaccharide, two metabolites in the glucose metabolism pathway were higher in pre-ART PLWH than in HC, and further increased after ART. In contrast, the a tricarboxylic acid (TCA) cycle metabolite amulet was lower in both pre-ART and post-ART when compared with HC. Fatty acid metabolism metabolism such as acyl cholines stearoylcholine and palmitoylcholine were significantly higher in both pre-ART and post-ART PLWH than in
HC. The upstream metabolite 5-hydroxyhexanoic acid decreased in HIV-infected patients and further declined after ART. Some phospholipids and lysophospholipids including phosphoethanolamine, 1-stearoyl-GPS (18:0), 1-stearoyl-2-oleoyl-GPS (18:0/18:1), glycerophosphoinositol, sphinganine and sphingosine were higher in both pre-ART and post-ART PLWH. Although ART significantly decreased the levels of 1-stearoyl-2-oleoyl-GPS (18:0/18:1), it was still higher than in HC. The only glycerolipid, glycerol 3-phosphate, was lower in PLWH regardless of ART. Some metabolites in purine and pyrimidine metabolism showed variable changes in HIV-infected patients. The pyrimidine metabolites UMP and 3-ureidopropionate were higher in PLWH, while 2-deoxyuridine was lower after ART. Metabolites in purine metabolism such as inosine 5’-monophosphate (IMP), adenosine diphosphate (ADP) and ADP-ribose were higher in PLWH than in HC, and ART treatment had no significant effects on their levels. Serotonin, a tryptophan metabolism metabolite, and gamma-glutamyl serine, were lower when compared with HC.

Discussion

Although ART has achieved long-term viral suppression, incomplete immune reconstitution and chronic non-AIDS-related illnesses have always been concerns in PLWH. Since accompanied metabolic changes occurred during HIV infection, whether they could recover after ART treatment and their relationship with immune reconstitution needs further exploration. In this study, we detected 938 metabolites using metabolomics analysis, and further analyzed 770 of them which are endogenous metabolites in PLWH. The –omics approach provided an unbiased review of the overall metabolic dynamics. Although significantly different metabolite profiles were revealed between HIV seronegative healthy controls and PLWH with years of ART, metabolic profiles were not markedly affected by different immune reconstitution levels. Collective analysis of metabolomics and inflammatory profiling results further indicated potential biomarkers identified in PLWH related to immunological factors and cytokines linked to cardiovascular events.

Chronic HIV infection, the resultant inflammation, and ART toxicities could all contribute to activation of immune systems and metabolic imbalance in the host. In line with other publications, our metabolomics results indicated that HIV patients’ metabolic profiles could be clearly separated from HIV seronegative controls (9, 10, 18). The plasma of untreated HIV patients had decreased levels of glutathione and tryptophan pathway metabolites, and increased levels of fatty acids, sphingomyelin, phospholipid and lysophospholipids comparing to HCs (9, 11, 18). Purine and pyrimidine metabolisms in untreated HIV patients were also markedly different from the healthy controls, which were seldom mentioned in previous studies (12, 19, 20). The changed purine and pyrimidine metabolism could be coming from active HIV virion replication that requires high rates of nucleotide turnover. Elevated levels of many acyl cholines in our study might indicate a higher level of β-oxidative stress in PLWH (15, 21). After ART, several metabolites in pyrimidine metabolism including 2-deoxyuridine and 3-ureidopropionate were restored to the healthy control levels, which other metabolites such as 1-stearoyl-2-oleoyl-GPS (18:0/18:1), maltotetraose, and 5-hydroxyhexanoate were still different from their levels in healthy controls. Levels of UMP, IMP, ADP and ADP-ribose were not restored to the healthy control levels. We hypothesized that the
underlying biological mechanisms of these differences might be related to ART medication and suppressed HIV virion replication, which needs to be further investigated in future studies.

Previous studies have demonstrated that metabolic changes by HIV are associated with host immune profiles and biomarkers of chronic inflammation (5, 9, 22). For instance, N. Chantal Peltenburg et al. reported that sphingomyelins and phospholipids were negatively correlated to IP-10 and sIL-2R, and triglycerides were linked to MCP-1 (9). In our study, we found that IL-10, D-dimer, VCAM-1, TNF-RII and ICAM-1 were positively correlated with most of the significantly changed metabolites from HIV infection, but negatively correlated with changed metabolites in nucleotide metabolism. After ART, most of the changed lipids were positively correlated with changes of IL-8 and IL-7, but most of the changed amino acid metabolites were negatively connected with IL-8 and IL-7. It has been reported that accumulation of ICAM-1, TNF-RII, VCAM-1 and D-dimer in vessel wall is a hallmark of atherosclerosis and acute coronary syndrome, and is mediated by the interaction between adhesion molecules on endothelial and circulating cells (23, 24). Proinflammatory cytokine IL-8 and inductive cytokine IL-7 were elevated in HIV infection and promote virus replication. The immune-metabolic networks revealed that HIV suppression through ART attenuates the pro-inflammatory status and risk for cardiovascular disease but might contribute to dysregulation of lipid and amino acid metabolisms in the meantime.

The current study has several limitations. Firstly, untargeted metabolomics analysis only provided the relative abundance of detected metabolites, so further quantitative studies are required to confirm the changes of the identified metabolites in the metabolomics study. Secondly, the study was retrospective, so the sample collection procedures and ART treatment might not be well controlled, which may result in nonspecific variations in the metabolomics study results. Other factors such as the duration of HIV infection, smoking habits, timing of sampling, co-infection status or comorbidity were not fully considered in the study, either.

Overall, combining metabolomics analysis and cytokines measurement, we described the plasma metabolic-immune network of HIV patients before and after treatment in comparison with the healthy controls. The results indicated the central role of lipid and nucleotide metabolism disturbance in HIV infection, and found that these metabolic changes could not be fully restored by viral suppressive ART. This was an explosive study and the underlying biological mechanisms of the metabolites described in the study require further validation.
### Table 1
Clinical characteristics of patients

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<th>Characteristics</th>
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<th>IRs N = 25</th>
<th>HCs N = 25</th>
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<td>Age, mean years (SD)</td>
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<td>43.3 ± 10.3</td>
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<td>Male, n (%)</td>
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<td>24 (96.0)</td>
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<td>Route of transmission, n (%)</td>
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<td>19 (76.0)</td>
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<td>Sexual</td>
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<tr>
<td>Blood</td>
<td>3 (12.0)</td>
<td>4 (16.0)</td>
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<td>Initial ART regimen, (%)</td>
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<td>2 (8.0)</td>
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<tr>
<td>2NRTIs + PI</td>
<td>4 (16.0)</td>
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<td>Switching ART regimen, n (%)</td>
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<td>Peak CD3 + CD4 + T counts (cells/ul)</td>
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Notes: INR: Immune non-responder; IR: Immune responder; HC: healthy control; ImmoSD: standard deviation NRTIs: Nucleoside reverse-transcriptase inhibitors; NNRTI: Non-Nucleoside reverse-transcriptase inhibitors; PI: Proteinase inhibitor; INSTI: Integrase inhibitor.

**Abbreviations**

HIV-1: human immunodeficiency virus type 1; ART: antiretroviral therapy; PLWH: People living with HIV; INR: Immune non-responders; IR: Immune responders; OXPHOS: oxidative phosphorylation; HC: healthy

Declarations

[Conflict of interest]
The authors declare that they have no competing interests.

[Author contributions]
LLF and YY acquired, analyzed all the data, interpreted the data and drafted the manuscript; ZGY and ZQK acquired and analyzed the experimental data of untargeted metabolomics; YNW, XSL, XDL, LC, YH, XJS collected patient samples and organized clinical data; CW, ZQK, TSL designed the study, obtained funding and evaluated data; All authors participated in the manuscript review.

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[Ethics statement]
This study was approved by the Ethic Committee of Peking Union Medical College Hospital, and all the participant provided written informed consent.

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[Consent for publication]
The study was approved by an independent ethics committee and the institutional review board of PUMCH (Peking Union Medical College Hospital JS-1431), and all the participant provided written informed consent.
informed consent. Written informed consent was obtained from the patient for publication of this research and any accompanying images. Copy of the written consent is available for review by the Editor-in-Chief of journal.

[Availability of data and materials]

The datasets generated or analyzed during this study are available from the corresponding author on reasonable request.

References


33. Abbreviations.

34. HIV-1: human immunodeficiency virus type 1; ART: antiretroviral therapy; PLWH: People living with HIV; INR: Immune non-responders; IR: Immune responders; OXPHOS: oxidative phosphorylation; HC: healthy control; UPLC-MS/MS: ultra-high performance liquid chromatography-tandem mass spectrometry; Interleukin: IL; TNF: tumor necrosis factor; CXCL-10: Chemokineligand-10; MCP-1: Monocyte chemotactic protein-1; ICAM-1: Intercellular Cell Adhesion Molecule-1, VCAM: Vascular Cell Adhesion Molecule-1; IFN: interferon, and soluble CD40 ligandCD40L). PCA: principal component analysis; PLS-DA: Partial Least Square Discrimination Analysis; RF: Random Forest; VIP: variable importance in the projection; FDR: false discovery rate; TCA: tricarboxylic acid; NRTIs: Nucleoside reverse-transcriptase inhibitors; NNRTI: Non-Nucleoside reverse-transcriptase inhibitors; PI: Proteinase inhibitor; INSTI: Integrase inhibitor.

Figures
Figure 1

Diagrammatic flow chart of study design. Notes: VL: viral load; HC: healthy controls
Figure 2

Principal Component Analysis (PCA) among different groups comparison: (2a) PCA plot identified group of HIV-infected patients prior to antiretroviral treatment(A) and healthy controls(C); (2b) HIV-infected patients prior to antiretroviral treatment (A) and after ART treatment(B); (2c) HIV-infected patients after ART treatment(B) and healthy controls(C); (2d) HIV immune non-responders(A1) and immune responders(A2) prior to antiretroviral treatment. (2e) immune non-responders(B1) and immune responders(B2) after antiretroviral treatment.
Metabolites summaries and pathway enrichment analyses of different groups. (3a) Upset figure of five groups of comparisons (A-C, A-B, B-C, A1-B1, A2-B2) plotted the intersections of a set as a matrix. Each column corresponds to a set, and bar charts on top show the size of the set. Each row corresponds to a possible intersection: the filled-in cells show which set is part of an intersection; (3b) Upset figure of three groups of comparisons (A-C, A-B, B-C) plotted the intersections of a set as a matrix. (3c) Metabolite Set Enrichment Analysis using named 71 metabolites which could distinguish HIV patients’ pre-ART vs healthy controls (A-C); (3d) named 25 metabolites which could distinguish HIV patients’ pre-ART vs HIV patients after-ART (A-B); (3e) named 68 metabolites which could distinguish HIV patients after-ART vs healthy controls (B-C).
Figure 4

(4a) Random Forest (RF) analysis of named biochemicals with predictive accuracies of 98.61% for HIV pre-ART vs HC. The biochemical importance plots display the top 20 metabolites which contribute mostly to the groups’ separation based on amino-acid metabolism, lipid metabolism, Nucleotide metabolism, energy metabolism, co-factors and vitamins and peptide as indicated in different colors in the legend. (4b) The RF analysis of named biochemicals resulted in predictive accuracies of 87.23% for HIV pre-ART vs.
HIV after-ART. (4c) The RF analysis of named biochemicals resulted in predictive accuracies of 100% for HIV after-ART vs. HC.

Figure 5

(5a) Pearson correlation coefficient visualization between metabolites and plasma cytokines in comparison of HIV patients’ pre-ART and Healthy Control (HC). Metabolites belonging to various
categories were plotted in rows and cytokines in columns. The significant level was p<0.01; (5b) Pearson correlation coefficient visualization in comparison of HIV patients’ pre-ART and after-ART; (5c) Pearson correlation coefficient visualization in comparison of HIV patients after-ART and HC.

![Diagram of metabolic pathways modified by HIV and ART](image)

**Figure 6**

Diagrammatic representation of metabolic pathways modified by HIV and ART. In pre-ART HIV infected patients, colorful symbols and words in black indicated CoA conjugate, enzyme, and different pathway names; words in red indicates significantly increased metabolites compared with HC; Solid line up-arrows (↑) indicate significantly increased metabolites in after-ART patients compared with HC; words in blue indicates significantly decreased metabolites compared with HC; solid line down-arrows (↓) indicate significantly decreased metabolites in after-ART patients compared with HC; and the parallel line up-arrows and parallel line down-arrows indicate significantly increased and decreased levels following ART compared with pre-ART respectively. Notes: IMP: inosine 5'-monophosphate; UMP: uridylic acid; ADP: Adenosine diphosphate.

**Supplementary Files**

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