Nanoflow-nanospray mass spectrometry metabolomics reveals disruption of the urinary metabolite profiles of HIV-positive patients on combination antiretroviral therapy

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Abstract

Background: The use of combination antiretroviral therapy (cART) has substantially improved the outlook for patients with HIV infection. However, lifelong exposure to cART is also associated with adverse metabolic changes and an enhanced risk of renal, hepatic and cardiovascular dysfunction. This study investigated disruptions of the urinary metabolome of cART-exposed patients, thereby furthering our understanding of some of the side effects of pharmaceutical intervention.

Methods: HIV-positive patients were recruited from an HIV clinic and divided into cART-naïve and cART-exposed groups. HIV-negative patients were recruited from a sexual health clinic. All 89 subjects were white males. Targeted biochemistry analyses were performed on plasma samples. Urine samples were collected following an overnight fast and analysed with a highly sensitive untargeted metabolomic method using nanoflow/nanospray liquid chromatography-time of flight mass spectrometry. Datasets were analysed using projection modelling to detect metabolite markers of cART exposure.

Results: Metabolites or parent compounds of all cART drugs were detected in urine extracts of all but one of the cART-exposed patients confirming adherence to the pharmaceutical regimen. Analysis of urine samples from patients on cART revealed significant reductions in selected bile acids, lipid, nucleoside and androgen metabolites. However, plasma concentrations of free or conjugated testosterone were unchanged indicating possible disruption of androgen transport or excretion in urine of patients on cART.

Conclusions: Discovery-based metabolomics reveals the potential to identify novel markers of cART intervention and metabolite disruption in HIV-positive patients, which may enable the efficacy, compliance and side effects of these pharmaceutical mixtures to be investigated.

Introduction

The introduction of combination antiretroviral therapy (cART) has transformed HIV infection from a life-threatening disease into a chronic condition. Multiple co-morbidities not associated with AIDS, such as cardiovascular, renal, bone and hepatic diseases, are becoming more prevalent in HIV-positive individuals compared to those without the infection.[1] Although the ageing process and the virus itself might play an important role in the development of non-AIDS comorbidities, the cumulative effects of cART have been associated with the onset and progression of non-AIDS co-morbidities in HIV infection. For instance, nucleotide reverse transcriptase inhibitors (NRTIs), such as tenofovir disoproxil fumarate (TDF), have been linked with proximal tubular renal disease and reduced bone mineral density[2,3], while abacavir has been found to increase the risk of ischaemic heart disease in cohort studies.[4] Protease inhibitors (PIs), another class of antiretrovirals frequently used in clinical practice have been widely associated with complications, such as dyslipidemia, abnormal fat distribution, and insulin resistance.[5-7]

Currently, it is not possible to predict which patients are more likely to develop a particular non-AIDS comorbidity after starting cART. Therefore, there is an urgent need for biomarkers that can predict and guide therapy in people living with HIV affected by non-AIDS comorbidities. The use of a discovery-based untargeted metabolomics approach may help to further elucidate mechanisms of action, metabolism and toxicity associated with the use of cART.[8] This analytical approach allows the profiling of a wide range of metabolites in a patient's biofluids, such as urine and blood, and therefore has great potential for the discovery of biomarkers of pharmaceutical efficacy and toxicity in HIV infection. To date, the effect of cART exposure on urinary metabolite profiles has not been studied using trace analytical methods, yet analysis of this biofluid may provide a useful non-invasive screen for pharmaceutical efficacy and toxic side effects arising from exposure to combinations of NRTIs and PIs.

This study aims to investigate the effect of two cART regimens on the urinary metabolome in comparison with cART-naïve HIV-positive and negative patients. Urine samples were extracted and analysed using, for the first time, highly sensitive nanoflow liquid chromatography-nanoelectrospray–mass spectrometry methods, which enable sensitive non-targeted analysis of urine samples to detect disruption in metabolite profiles associated with cART intervention.

Methods

Study population

Fasted urine and plasma samples were collected between 2010 and 2011 from HIV-positive patients participating in a longitudinal study investigating reduced bone mineral density in HIV-positive men from a single HIV outpatient clinic in Brighton, UK.[9] HIV-negative men were recruited from a single sexual health clinic in Brighton. All subjects were white males and exclusion criteria were men (HIV-infected and HIV-negative) <18 years old, patients unable to give written, informed consent, patients who have had imaging with contrast in the previous 48 hours, or are infected with hepatitis C virus, showing moderate to severe hepatotoxicity, renal insufficiency or reduced bone mineral density at the time of sampling. Participants were classified into 3 study groups: HIV-positive on cART; HIV-positive cART-naïve and HIV-negative. For all study groups, demographic and HIV parameters, including age, ethnicity, current and nadir CD4 cell count, current HIV viral load, time since HIV diagnosis and time since initiating cART were obtained from the main study clinic database. Ethical approval was given by the NHS Research Ethics Committee (NREC 09/H1107/101) and all patients gave written, informed consent.

cART regimens and urine sample collection

HIV-positive patients on cART were on a combination of TDF, emtricitabine and the PI booster ritonavir. Additionally, they were also on either the PI atazanavir or darunavir, which was the only way in which the cART regimens differed. The characteristics and structures of the pharmaceuticals used in cART are given in Supplementary Table S1 and Figure S1.

Urine osmolality was measured using a Model 3320 osmometer (Advanced Instruments Inc., Massachusetts, U.S.A) and an aliquot of the remaining sample was frozen at -80 °C in 10% methanol prior to sample extraction for metabolomics analyses.

Targeted plasma and urine biochemistry

All blood plasma and urine chemistries were performed on a Cobas Integra 800 analyser (Roche Diagnostics, UK). Urine total protein concentration was measured by turbidimetry after alkaline denaturation and precipitation with benzethonium chloride, with a measuring range of 40–2000 mg/L. Imprecision (analytical error) was 3.9% at 55 mg/L and 2.5% at 260 mg/L. Urine albumin was measured by immunoturbimetric assay using polyclonal sheep anti-human albumin antisera with a working analytical range of 12–200 mg/L. Imprecision was 2.0% at 20 mg/L and 2.3% at 120 mg/L. Samples with a concentration above these upper limits were automatically re-run at dilution. Urine creatinine concentration was determined by enzymatic (creatininase) colorimetry with a measuring range of 0.1–40.0 mmol/L and imprecision was 1.8% at 5.8 mmol/L and 3.6% at 25.6 mmol/L.

Metabolomics analyses of urine samples

Full details of urine extraction methods and their analyses are given in the supplementary information. Briefly, urine was extracted as previously described by our laboratory [10] and extracts were reconstituted to give a final osmolality of 1360 mOsm prior to MS analysis.[11] Quality control (QC samples) and sample extracts were analysed by nanoflow ultrahigh performance liquid chromatography-nano electrospray ionisation time of flight mass spectrometer (nUHPLC-nESI-TOFMS) as described in recent studies.[12] This analytical methodology allows the profiling of a wide range of small molecule metabolites present in urine samples, including steroids, lipids, neurotransmitters, peptides and nucleotides.[10] MS datasets were processed and analysed by multivariate modelling, including principal components analyses (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), to identify metabolite differences between treatment groups. Discriminating metabolites of cART intervention were detected using S-plots from OPLS-DA models which were constructed for atazanavir versus cART-naïve patients and for darunavir versus cART-naïve patients. Following the exclusion of ions associated with the parent cART drugs and their metabolites, further OPLS-DA models and S-plots were constructed between the cART-naïve and cART groups to identify endogenously derived metabolites that were markers of cART treatment. Metabolite identities were determined from their accurate mass, isotopic fit and comparison of fragmentation data with authentic standards or metabolite databases.

Statistical analyses

Univariate statistical analysis of urinary marker metabolites (and targeted blood biochemistry) was performed using SPSS version 22 (IBM, New York, USA). Metabolites were tested for normality and for significance at $p<0.05$ using a one-way ANOVA with a Games-Howell test (because of unequal variances) to determine p-values. Non-normally distributed data were $\log_{10}$ transformed prior to ANOVA. A false discovery rate of 5% was used to check for the occurrence of type 1 errors arising from multiple testing. [13] Linear regression modelling was used to adjust for the confounding effects of age and urine protein/creatinine ratio (uPCR) (see results in Table 1) on the association of metabolite disruptions in the different patient groups .

Results

Patient data and targeted blood biochemistry

In total, samples from 89 patients were analysed, comprising 13 cART-naïve HIV-negative patients, 26 cART-naïve HIV-positive patients and 50 HIV-positive patients on cART. Of these 50 patients, 20 were on atazanavir and 30 were on darunavir. All patients, except one, were on the specified cART regimen for at least a month prior to sampling: those on atazanavir for a (mean (SD)) period of 16.8 (11.0) months and darunavir for 12.9 (7.0) months.

Clinical data for patient groups are summarised in Table 1. All subjects were white males between the ages of 20-71. All HIV-positive patients on cART had a plasma HIV viral load <40 RNA copies/ml in contrast to the high and variable viral load in the cART-naïve HIV-positive group (Table 1). Nadir CD4 counts were lower in those on cART, but at the time of urine sampling there was no difference in CD4 cell counts measured between those on cART and those that were cART-naïve. There were also no clear indications of major kidney or liver toxicity for those on atazanavir or darunavir. However, the uPCR, a marker of kidney injury, was significantly increased in those on cART compared to the HIV-negative patients, but values in all study groups were within the reference range. The urine albumin creatinine ratio (uACR), another marker of kidney disease, was unchanged in those on cART. Bilirubin and alkaline phosphatase (ALP) levels were, as expected,

increased in the atazanavir group compared with the cART-naïve and darunavir groups; this is a common side effect of atazanavir medication due to disrupted biliary excretion.[14]

Plasma triglyceride and cholesterol levels were significantly increased in the darunavir group compared to the cART-naïve and HIV-negative groups, respectively. There was no significant variation in free or total testosterone levels in plasma, however, steroid hormone binding globulin (SHBG) levels were significantly higher in patients on cART compared to the HIV-negative group.

Metabolomic analysis of the effect of cART treatment

Analyses of the QC samples revealed highly repeatable data indicating acceptable extraction and MS instrumental performance (see Supplementary Results). The urinary metabolomic profiles of individuals taking the two cART regimens were compared to cART-naïve HIV-positive and HIV-negative groups using PCA modelling. The data sets comprised 6705 and 9043 MS features in positive and negative mode respectively. The grouping of patient samples on plots of both scores of data obtained in + and – nESI modes were similar and revealed no discrimination between the metabolomic profiles of urine from cART-naïve and HIV-negative patients (Figure 1 and Supplementary Figure S2). Patients on cART regimens containing either atazanavir or darunavir clustered separately and away from the cART-naïve group. In +nESI, the two cART treatment groups were separated from the cART-naïve group on the first component and from each other on the second component, whereas in -nESI discrimination was only observed on the first component.

Identification of cART drugs and metabolites

Discriminating metabolites of cART intervention were detected using S-plots from OPLS-DA models (see Figure 2 and Supplementary Figure S3 for examples). The majority of the discrimination between the cART-naïve and exposed groups was driven by ions associated with the cART drugs and their metabolites. A total of 16 structures were identified, four of which were the parent drug compounds, and the remaining were metabolites of three PIs (Tables 2 and Supplementary Table S2). Identification of the metabolites was supported by fragmentation patterns which were compared to those of metabolite structures that had already been detected in rodent urine, human blood and hepatocyte studies. [15-18] Analysis of the OPLS-DA

loadings plot for each cART parent compound confirmed that all but one urine sample contained cART drugs at the time of sample donation. As none of the parent drugs nor any of their metabolites were detected in urine extracts from this patient in the darunavir group, this sample was excluded from further statistical analyses.

Effect of cART intervention on the endogenously derived metabolome

Following the exclusion of ions associated with the parent cART drugs and their metabolites, further OPLS-DA models were constructed to identify endogenously derived metabolites that were associated with cART treatment. Interrogation of the S-plots uncovered 13 endogenous metabolites that discriminated between the sample groups after accounting for a false discovery rate of 5%. These included 5 bile acids/alcohols, 4 conjugated androgens, and a nucleoside, lipid, peptide and unidentified metabolite. Details of the determination of metabolite identity following fragmentation of the molecular ion are given in Supplementary Table S3. The effect of age and uPCR as potential confounding variables influencing the association of the urinary metabolites with cART status was analysed using linear regression modelling. After adjusting for confounders, the analysis revealed that 12 metabolites were significantly decreased in urine of cART exposed groups patients compared to either the cART-naïve and/or HIV-negative groups. One metabolite, a tetra-peptide, was significantly increased in urine from in the atazanavir cART group compared with the cART-naïve group (Table 3).

Discussion

Metabolism of cART drugs

An advantage of using a global metabolomics analysis of urine samples is that both exogenously-derived compounds, such as administered drugs and other xenobiotics, are detected as well as changes in endogenously-derived biochemical metabolites. In this study, the parent compounds of all three PIs and two NRTI drugs were detected in urine samples from cART-exposed groups. The PIs were also highly metabolised by each patient, whereas only the parent NRTIs, tenofovir or emtricitabine, were detected in urine extracts

in keeping with previous studies indicating that these compounds are poorly metabolised in the humans. [19,20] In agreement with previous *in vitro* studies two metabolites of ritonavir were detected in urine extracts (the oxidised and deacylated products), however the glutathione conjugate was not detected and this metabolite may have preferentially been excreted by the biliary route.[15] The detection of a number of metabolites of atazanavir and darunavir in this study agreed with previous work showing that these pharmaceuticals are extensively metabolised in humans via oxidation, carbamate hydrolysis, deacylation (atazanavir) and glucuronidation (darunavir).[16,17]

The ability to detect the parent cART drugs and a wide range of their transformation products in urine extracts suggest that in future studies, links between pharmacogenetics, metabolism and drug efficacy could be investigated, which may lead to a more personalised approach to cART intervention. In addition, it is also possible to identify individuals who have not taken the medication, which was evident for one patient in our study from the darunavir group where neither darunavir nor any of its metabolites or any other cART drugs were detected in their urine sample.

Variation in the endogenous metabolome as a result of cART

The PCA scores plot (Figure 1 and Supplementary Figure S2), as well as further modelling studies using OPLS-DA, did not reveal any discrimination between the cART-naïve and HIV-negative groups despite their different HIV statuses. Furthermore, stratification of the cART-naïve group by nadir or current CD4 count into two groups (≤250 and ≥250 cells/μL) did not result in discrimination between these sample groups. A possible explanation is that metabolomic changes associated with immunosuppression may not be detected in urine samples from HIV-positive patients. Instead, analyses of blood samples may elucidate early stage metabolomic consequences of HIV infection. Other studies analysing blood samples from cART-naïve HIV patients report disrupted lipid and carbohydrate metabolism and changes in organic acids associated with oxidative stress and disturbed mitochondrial metabolism. [8,21]

In the current study, 12 metabolites were detected which decreased in the urine of HIV-positive patients on cART. Of these, four were bile acids which, depending on the metabolite and after adjusting for

confounding variables, were detected at between 2 and 80 times lower levels in the urine from both cART groups (Table 3). In contrast, previous studies have detected increased levels of plasma bile acids in patients receiving cART or PIs.[22,23] Plasma concentrations of bile acids were not measured in our study but blood ALT levels did not suggest significant liver damage. However, plasma/hepatocyte concentrations of bile acids are also influenced by cell membrane transporters such as organic anion transporting polypeptides (OATPs) and two subtypes, OATP2B1 and OATP1B1, are inhibited by PIs including atazanavir, darunavir and ritonavir.[24-26] In addition, apical membrane transporters such as ATP-binding cassette transporter B11 (ABCB11) which export bile acids from the hepatocytes into the bile are also inhibited by PIs.[27-29] These transporters are also present in the apical membrane of proximal tubules of the kidney.[30,31] Thus, they may be involved in the excretion of bile acids into the urine, and if inhibited by PIs, this may result in decreased levels of urinary bile acids. However, in the current study only a subset of the total bile acids that were present in the urine extracts were disrupted (Supplementary Table S4), implying that these transporters may have different affinities for the various bile acids.

The results show lower concentrations of a bile alcohol, cholestanehexol glucuronide, in the urine of patients on cART. Bile alcohols are end products of cholesterol metabolism and are minor components in the bile and urine of healthy subjects. However, increased concentrations of conjugated bile alcohols have been detected in patients with primary biliary cirrhosis, an autoimmune disease.32 Similar to bile acids, they may be markers of liver function or indicate disruption of bile metabolism and transport as a result of cART intervention.

Concentrations of a number of conjugated androgens, major urinary metabolites of testosterone, were reduced in the cART- exposed groups. Reduced plasma concentrations of pregnenolone, androsterone and dehydroepiandrosterone sulphates have been reported in patients on cART.[22] It has been suggested that type 1 interferons, which are markers of innate immune activation, may downregulate sterol metabolism during anti-viral responses.[33] However, it was observed that plasma levels of free and total testosterone were not significantly different between sample groups (Table 1), which suggests that other mechanisms, such as inhibition of androgen transport in the kidney epithelium, may be responsible for decreased levels of

androgens in the urine of cART-exposed groups. In addition, levels of SHBG, an important carrier protein for sex steroids, were elevated in both groups on cART, which may have resulted in increased binding of free androgens in plasma and less availability for metabolism and conjugation.

Another important finding in our study was the identification of the nucleoside 5-deoxy-5(methylthio)adenosine which was decreased in the urine of cART-treated HIV-positive patients. This metabolite has previously been detected at elevated levels in the urine of immunocompromised children.[34,35] It is thought to regulate cell apoptosis through the inhibition of protein carboxymethyltransferase which can modulate cell signalling and protein expression via protein methylation. [36,37] This potential role in apoptosis may be relevant in HIV disease which is a major cause of CD4 cell loss. [38,39] Furthermore, 5-deoxy-5(methylthio)adenosine is involved in the activation of lymphocytes and thus may also be elevated as part of the immune response to HIV infection. [36] The possible role of 5-deoxy-5(methylthio)adenosine in HIV disease requires further investigation.

Concentrations of a C17 hydroxysphinganine type of lipid were reduced in both HIV-positive groups on cART . To our knowledge, this is the first time this metabolite structure has been detected in human biofluids. However, a C17 sphinganine has been detected in human plasma as a biomarker of kidney cancer. [40] The role of sphingolipid bases in HIV infection is currently unknown, but sphingolipids and their metabolites can initiate cellular apoptosis[41], regulate HIV viral entry into cells[42,43] and are associated with age-related changes in cholesterol/low density lipoprotein levels.[44] Concentrations of a tetrapeptide increased in patients on atazanavir, possibly as a result of TDF and reduced proximal tubular function and reduced re-uptake of low molecular weight proteins.[45,46] The effects of PI intervention on urinary protein and peptide profiles require further investigation.

To conclude, this first study using trace metabolomic analyses of urine from an HIV cohort has revealed a number of metabolite disruptions in the urine of cART-exposed patients which need to be further investigated to determine their association with the side-effects of pharmaceutical intervention. However, this study has some limitations. As all the patients were male and white, these results cannot be extrapolated to other groups of HIV-positive patients, including HIV-positive women and those of non-white ethnicity.

Additionally, due to the cross-sectional nature of the study, direct causality with pharmaceutical intervention cannot be inferred. Future studies using larger sample sizes and comparing blood and plasma metabolomic profiles in longitudinal cohorts prior to, and during, pharmaceutical intervention will aid understanding of cART-related toxicity in patients.

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Table 1: Blood and urine biochemistry of samples of study groups.

	HIV-negative (n=13)	HIV-positive, cART-naïve (n=26)	cART including atazanavir (n=20)	cART including darunavir (n=30)
Age	34±8	37±10	**46±6.7**$^{(0.001,0.006)}$	**48±7.5**$^{(0.0002,0.001)}$
CD4 count at sampling(cells /µL)	na	463(216-886)	593(114-1087)	540(105-1010)
Nadir CD4 count (cells/µL)	na	328(73-639)	**245(4-680)**$^{na,0.01}$	**242(30-385)**$^{na,0.001}$
cART status	na	naïve	On cART	On cART
Viral load (RNA copies/mL plasma)	na	**5942(40-212917)**$^{(0.0001)}$	<40	<40
Urine osmolality (mOsm)	735±241	644±281	700±178	577±259
Urine creatinine (µmol/L)	14.4±8.9	13±7.7	13±4.7	12±8
uPCR (mg/mMol)	6.7±1.2	10±4.6	**14.5±8.3**$^{0.002/0.1}$	**14±7.0**$^{0.0004/0.04}$
uACR (mg/mMol)	nd	0.9±0.7	1.0±0.9	1.5±1.9
uAPR (ratio)	nd	0.90±0.06	0.83±0.08	0.94±0.08
ALP (IU/L)	59.4±17.1	68.7±19.6	**95.5±25.2**$^{0.0003/0.002}$	73.5±18.9
ALT (IU/L)	26.2±18.2	24.5±12.2	32.4±21.7	33.21±73
Plasma bilirubin (µmol/L)	12.4±3.1	7.9±2.7	**32.5±15.4**$^{0.0001/0.0001}$	7.4±2.9
Cholesterol (mMol/L)	4.4±0.7	4.3±1.2	5.1±1.1	**5.1±0.8**$^{0.055/0.05}$
HDL (mMol/L)	1.4±3	1.2±0.3	1.1±0.3	1.1±0.3
Triglycerides (mMol/L)	0.96±0.7	1.38±0.6	1.65±0.6	**1.91±0.87**$^{0.009/0.02}$
Free testosterone (pMol/L)	462±125	442±140	401±133	369±117
Total testosterone (nMol/L)	21.7±8.3	22.3±9.6	24.1±9.6	23.2±9.5
SHBG (nMol/L)	29.5±13.4	36.8±16.0	**49.0±17.3**$^{0.006/0.08}$	**52.2±23.5**$^{0.005/0.07}$

Study groups were 100% white males. uPCR= urinary protein creatinine ratio, uACR= urinary albumin creatinine ratio, uAPR= urinary albumin protein ratio, ALP= alkaline phosphatase, ALT= alanine transaminase. Values are mean (SD) with the exception of CD4 count and viral load which are reported as the median with the range in parenthesis. Values in bold are significantly increased compared to HIV negative or the cART naïve group. P-values for comparison with HIV negative/cART naïve groups are given in superscript. na= not applicable. nd=not determined. Limit of detection of viral load was 40 RNA copies/mL plasma.

Table 2: The identity of cART pharmaceutical metabolites identified in urine extracts

Metabolite	Metabolite identity/transformation	Retention time	Experimental mass	Theoretical mass	Formula
Ritonavir	Parent compound	24.09	721.3186	721.3191	$C_{37}H_{48}N_6O_5S_2$
Ritonavir M1	Hydroxylation	17.96	737.3163	737.3155	$C_{37}H_{48}N_6O_6S_2$
Ritonavir M2	Deacylation	17.28	580.3333	580.3321	$C_{32}H_{45}N_5O_3S$
Darunavir	Parent compound	18.41	548.2439	548.2430	$C_{27}H_{37}N_3O_7S$
Darunavir M1	Carbamate hydrolysis	12.00	392.2014	392.2008	$C_{20}H_{29}N_3O_3S$
Darunavir M2	Carbamate hydrolysis and hydroxylation	10.09	408.1971	408.1957	$C_{20}H_{29}N_3O_4S$
Darunavir M3	Glucuronide of M2	9.67	584.2279	584.2278	$C_{26}H_{37}N_3O_{10}S$
Darunavir M4	Glucuronide of parent	14.11	722.2585*	722.2595*	$C_{33}H_{45}N_3O_{13}S$
Atazanavir	Parent compound	19.43	705.3964	705.3976	$C_{38}H_{52}N_6O_7$
Atazanavir M1	Deacylation	14.49	538.3255	538.3241	$C_{26}H_{43}N_5O_7$
Atazanavir M2	Carbamate hydrolysis	12.59	647.3929	647.3921	$C_{36}H_{50}N_6O_5$
Atazanavir M3	Carbamate hydrolysis	13.06	647.3912	647.3921	$C_{36}H_{50}N_6O_5$
Atazanavir M4	Hydroxylation	15.41	721.3910	721.3925	$C_{38}H_{52}N_6O_8$
Atazanavir M5	Keto metabolite	21.18	719.3770	719.3768	$C_{38}H_{50}N_6O_8$
Tenofovir	Parent compound	5.27	288.0884	288.0862	$C_9H_{14}N_5O_4P$
Emtricitabine	Parent compound	5.16	248.0522	248.0505	$C_8H_{10}FN_3O_3S$

*Refers to m/z of [M-H]- ion, all other ions reported are [M+H]+.

Details of fragmentation data to confirm structural identity are given in Supplementary Table S2

Table 3: Identification of metabolite markers of cART exposure.

Measured mass m/z	Ion [M+H]+ or [M-H]-	Formula	Identity	Confounder	Effect of confounder [a]R, P-value	Effect of cART treatment [a]R, P-value	Effect of cART regimen P-value after Bonferroni correction [b](fold change from estimated marginal means)			
Metabolites decreased in urine of cART-exposed groups							**Atazanavir vs. HIV-negative**	**Atazanavir vs. cART-naïve**	**Darunavir vs. HIV-negative**	**Darunavir vs. cART-naïve**
247.1662	M+H	$C_{11}H_{22}N_2O_4$	unknown	Age uPCR	0.08 0.4 0.04 0.6	0.53 $4x10^{-6}$ 0.52 $8x10^{-6}$	0.1 0.4	0.006 (-1.2) 0.01 (-1.2)	0.001 (-1.3) 0.005 (-1.3)	$2x10^{-6}$ (-1.3) $3x10^{-6}$ (-1.3)
298.0978	M+H	$C_{11}H_{15}N_5O_3S$	5-deoxy-5(methylthio) adenosine[c]	Age uPCR	0.06 0.5 0.23 0.02	0.46 $1x10^{-4}$ 0.41 0.001	0.1 1.0	$2x10^{-4}$ (-2.7) 0.002 (-2.1)	0.5 1.0	0.001 (-2.1) 0.007 (-1.8)
304.2853	M+H	$C_{17}H_{37}NO_3$	C17-hydroxy sphinganine like	Age uPCR	0.03 0.1 0.15 0.8	0.61 $1x10^{-8}$ 0.62 $1x10^{-8}$	1.0 1.0	$3x10^{-6}$ (-2.8) $1x10^{-5}$ (-2.5)	1.0 1.0	$4x10^{-8}$ (-3.5) $5x10^{-8}$ (-3.1)
407.2800	M-H	$C_{24}H_{40}O_5$	Cholic acid[c]	Age uPCR	0.17 0.1 0.01 0.8	0.41 0.001 0.36 0.007	1.0 1.0	0.006 (-2.3) 0.03 (-2.3)	1.0 0.8	0.002 (-2.4) 0.009 (-2.4)
583.3118	M-H	$C_{30}H_{48}O_{11}$	Cholic acid glucuronide	Age uPCR	0.08 0.4 0.04 0.6	0.66 $2x10^{-8}$ 0.69 $7x10^{-12}$	0.02 (-3.4) 0.01 (-3.6)	$3x10^{-9}$ (-5.5) $7x10^{-10}$ (-5.6)	0.02 (-3.0) 0.01 (-5.6)	$1x10^{-9}$ (-4.9) $5x10^{-11}$ (-5.3)
464.3011	M-H	$C_{26}H_{43}NO_6$	Glycocholic acid[c]	Age uPCR	0.09 0.3 0.14 0.1	0.67 $5x10^{-11}$ 0.70 $2x10^{-12}$	0.0002 (-30) $8x10^{-4}$ (-59)	$4x10^{-10}$ (-41) $1x10^{-10}$ (-79)	0.001 (-6.6) 0.0003 (-9.7)	$1x10^{-9}$ (-9.2) $1x10^{-10}$ (-9.2)
464.3013	M-H	$C_{26}H_{43}NO_6$	Glycocholic acid like	Age uPCR	0.01 0.8 0.14 0.1	0.58 $2x10^{-7}$ 0.63 $2x10^{-9}$	0.001 (-2.0) 0.001 (-2.2)	$5x10^{-5}$ (-2.0) $4x10^{-6}$ (-2.1)	$9x10^{-5}$ (-2.2) $6x10^{-6}$ (-2.5)	$9x10^{-7}$ (-2.2) $3x10^{-8}$ (-2.4)
629.3537	M-H	$C_{32}H_{54}O_{12}$	Norcholestanehexol glucuronide	Age uPCR	0.01 0.09 0.20 0.06	0.80 $3x10^{-18}$ 0.82 $1x10^{-19}$	$3x10^{-9}$ (-1.7) $2x10^{-9}$ (-1.7)	$6x10^{-15}$ (-1.8) $2x10^{-15}$ (-1.8)	$3x10^{-10}$ (-1.7) $1x10^{-10}$ (-1.7)	$1x10^{-16}$ (-1.8) $1x10^{-17}$ (-1.8)
367.1581	M-H	$C_{19}H_{28}O_5S$	Testosterone sulfate[c]	Age uPCR	0.08 0.4 0.03 0.8	0.32 0.03 0.36 0.008	0.03 (-1.2) 0.01 (-1.3)	0.1 0.08	0.09 0.03 (-1.3)	0.4 0.1
369.1735	M-H	$C_{19}H_{30}O_5S$	Dihydrotestosterone sulfate[c]	Age uPCR	0.01 0.9 0.23 0.03	0.45 0.0002 0.55 $2x10^{-6}$	0.03 (-1.3) 0.001 (-1.4)	0.0002 (-1.3) $6x10^{-6}$ (-1.4)	0.2 0.009 (-1.3)	0.005 (-1.3) 0.0001 (-1.3)
369.1735	M-H	$C_{19}H_{30}O_5S$	Unidentified androgen sulfate	Age uPCR	0.02 0.8 0.01 0.9	0.35 0.01 0.35 0.01	0.03 (-1.7) 0.03 (-1.6)	0.02 (-1.6) 0.02 (-1.5)	0.2 0.2	0.3 0.2
479.2283	M-H	$C_{25}H_{36}O_9$	Oxo-androsterone glucuronide	Age uPCR	0.06 0.5 0.12 0.5	0.44 0.0003 0.47 0.0001	0.02 (-1.6) 0.01 (-1.7)	0.0001 (-1.8) $7x10^{-4}$ (-1.8)	0.5 0.3	0.01 (-1.4) 0.009 (-1.4)
Metabolites increased in urine of cART-exposed groups										
539.2694	M-H	$C_{21}H_{34}N_{10}O_7$	Tetrapeptide (Gln/Arg/His/Thr)	Age uPCR	0.10 0.3 0.03 0.7	0.30 0.05 0.34 0.01	0.1 0.09	0.05 (2.2) 0.02 (2.2)	0.9 0.4	0.4 0.1

Linear regression modelling was used to adjust for the confounding effects of age and urine protein/creatinine ratio on metabolite concentration. [a]R=partial regression coefficient. [b]Values in parenthesis are the fold change in metabolite intensity from estimated marginal means adjusted for the covariate. [c]identity of metabolite confirmed with an authentic standard and other compounds identified from exact mass and fragmentation data. A negative fold change is where the metabolite intensity is reduced in cART recipients relative to cART naive patients. The mean (SD) of metabolite intensities and their mass spectra data are given in Supplementary Table S3.

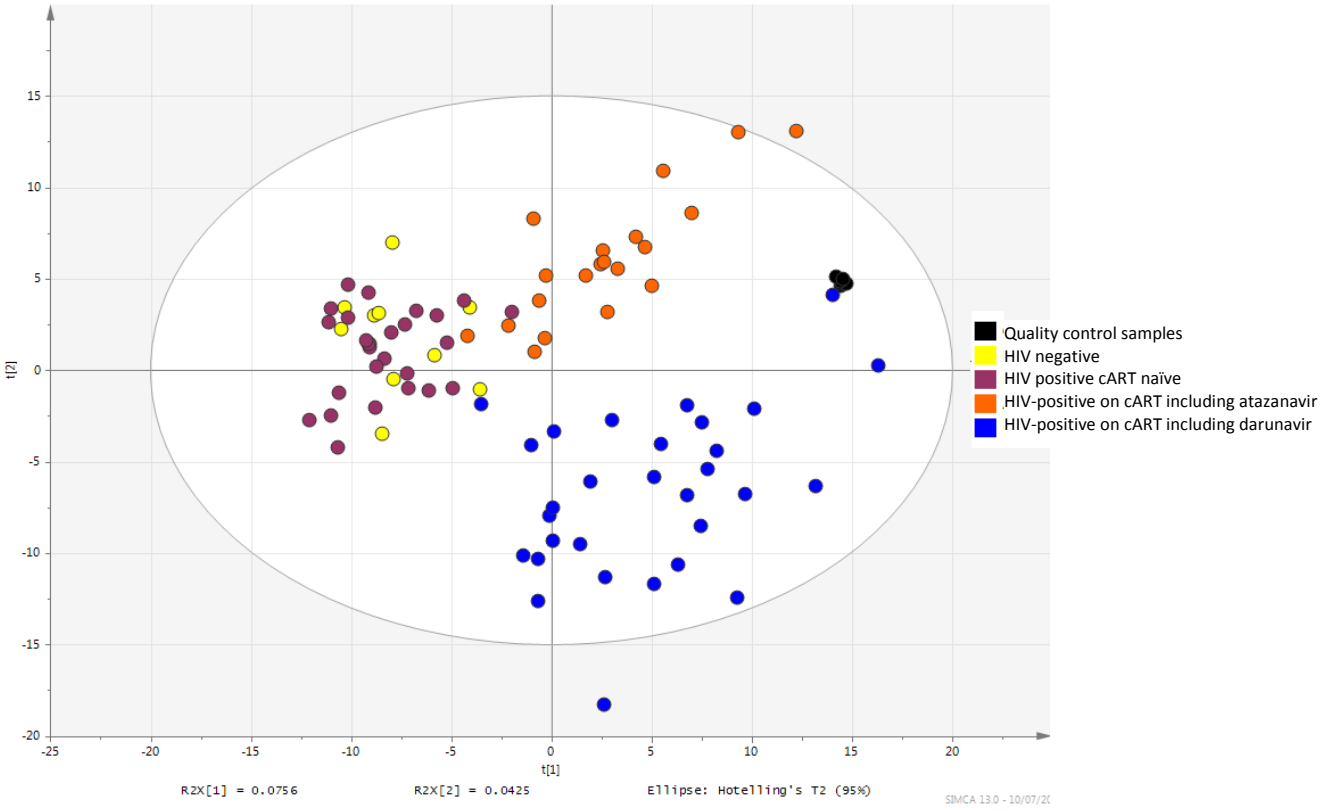

Figure 1: PCA scores plot analysis of the effect on the urinary metabolome of HIV and cART status (+nESI mode).

cART-exposed groups received the NRTIs tenofovir disoproxil fumerate and emtricitabine, and the ritonavir protease inhibitor as well as either atazanavir or darunavir (labelled respectively). QC represent quality control samples (n=8). No discrimination was detected between the HIV-negative and HIV-positive cART naïve patients. However, both groups on cART discriminated from the cARTnaïve and HIV negative patients on the first component and cART groups clustered separately from each other on the second component.

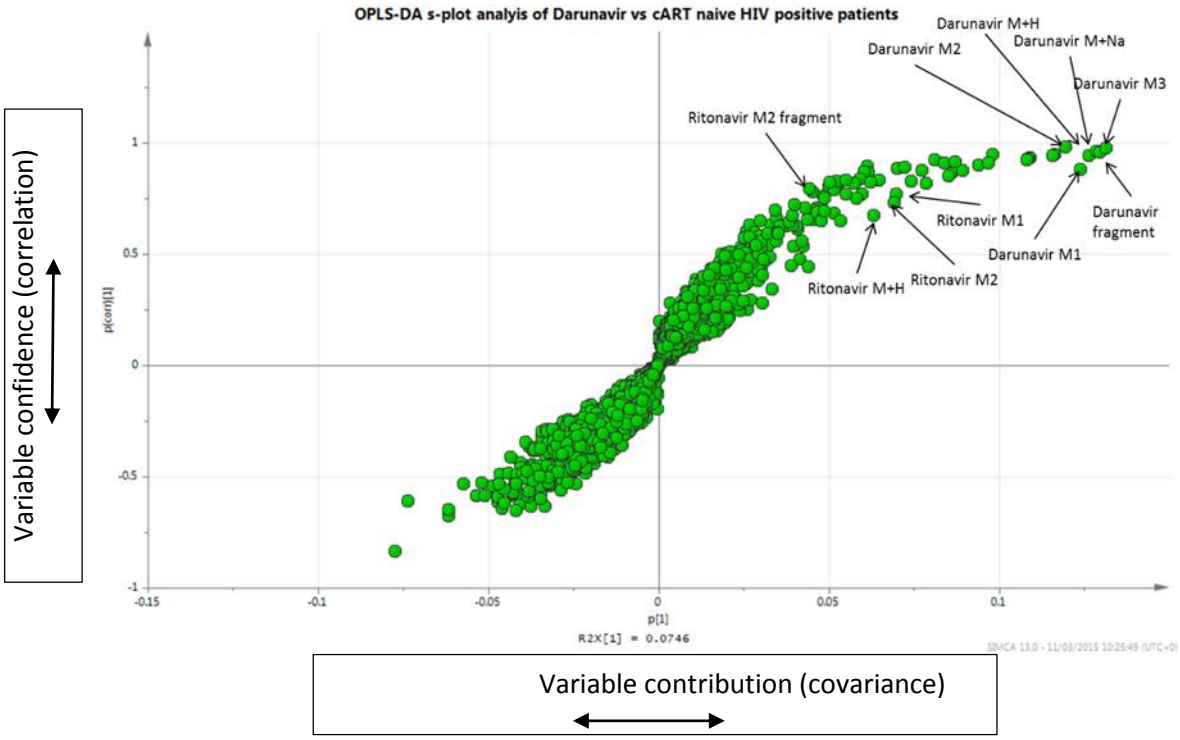

Figure 2: OPLS-DA S-plot analysis of metabolite profiles in urine samples of patients on cART containing darunavir compared to cART-naive patients.

Samples were analysed in +nESI mode. Each dot represents a spectral signal corresponding to retention time/m/z ion of a metabolite. Signals in the bottom left corner of the plot decreased in the cART-exposed when compared to the cART-naïve group. Signals in the top right corner increased in the cART group and were associated with ions corresponding to cART pharmaceuticals and their metabolites (see Table 2 and Supplementary Table S2 for cART metabolite identities).