Trans cohort metabolic reprogramming towards glutaminolysis in long-term successfully treated HIV-Infection

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Running Head: Metabolic reprogramming in the long term HIV-infection

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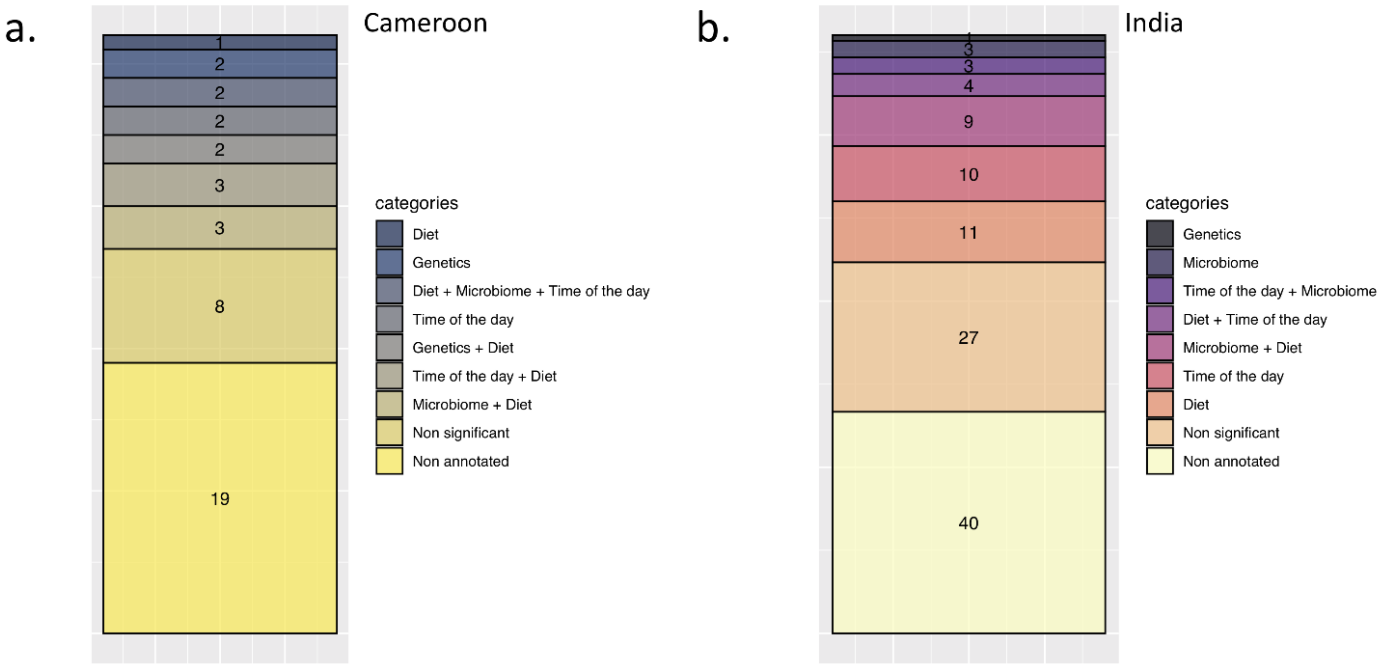
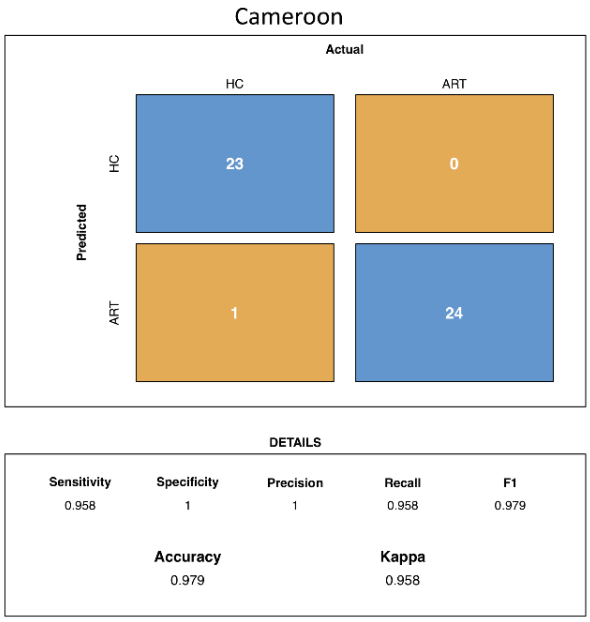
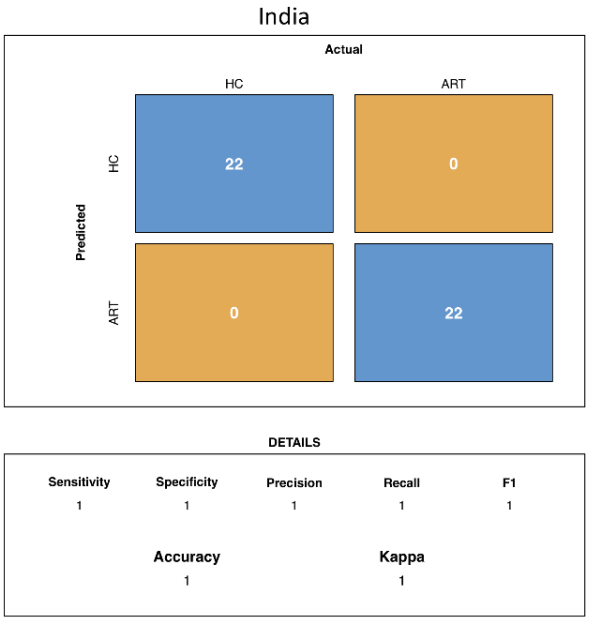


Supplementary Fig 1: Bar plot representing the number of detected metabolites associated significantly with each environmental category in Cameroon (a) and India (b) cohorts.

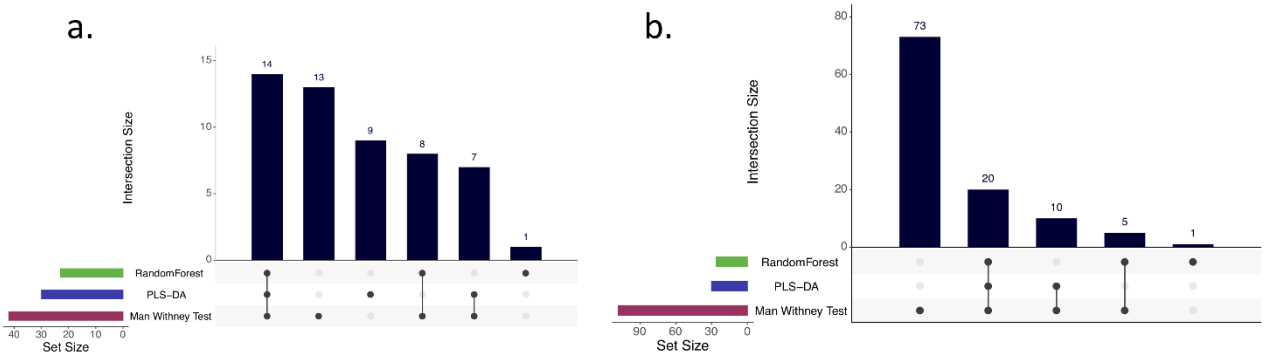
a.



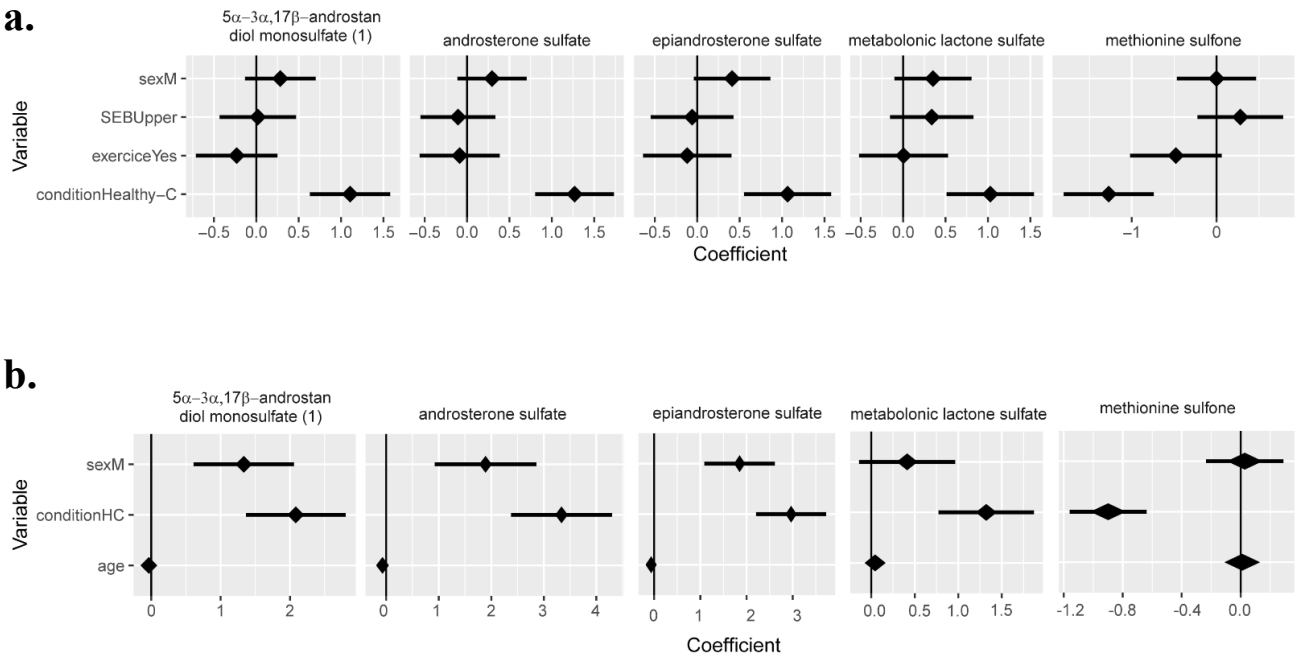
b.



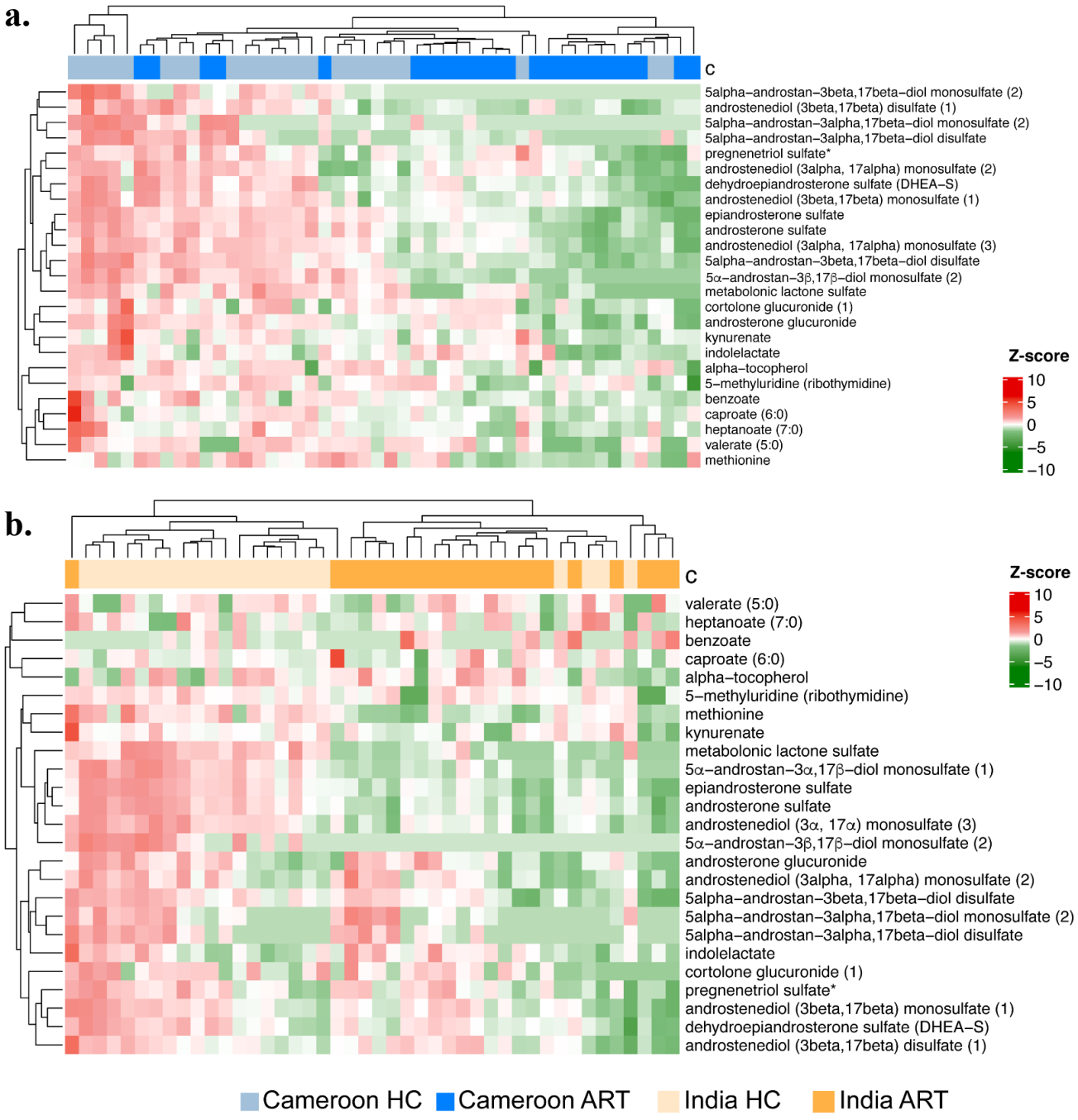
Supplementary Fig 2: Confusion matrices for Cameroon (a) and India (b) random forest models.



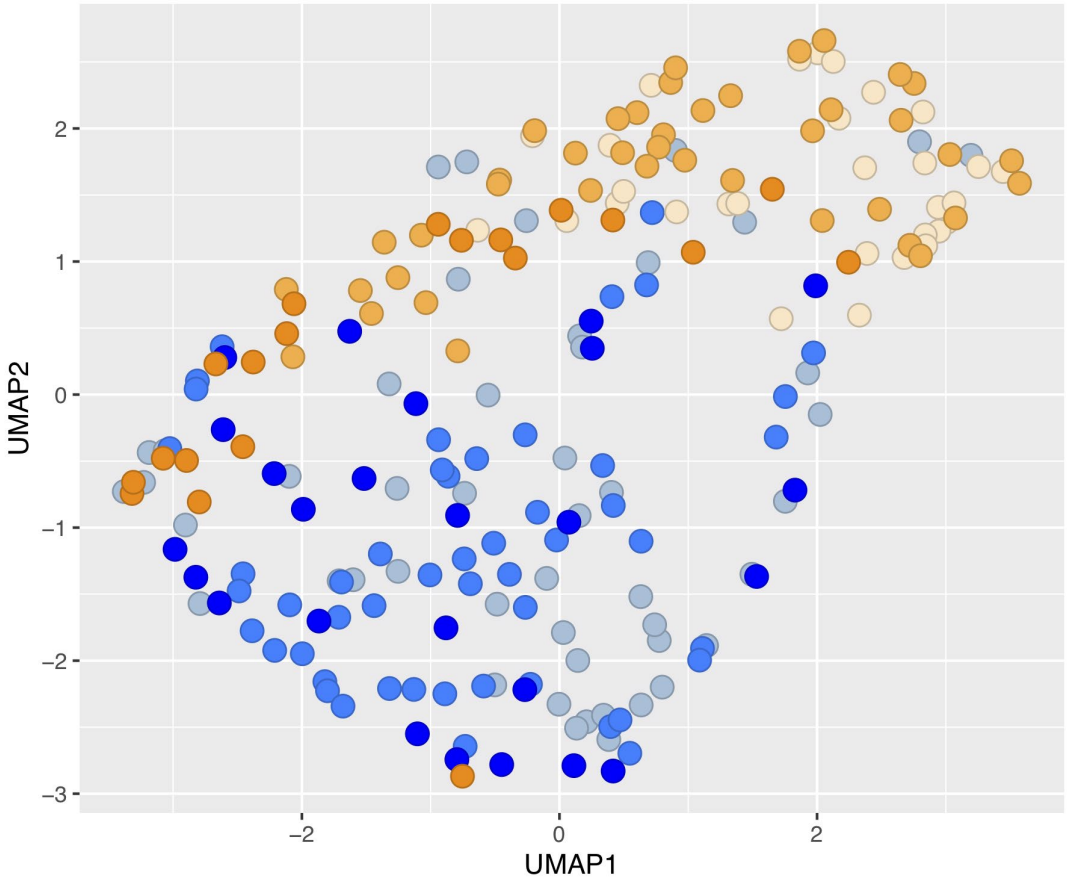
Supplementary Fig 3: Upset plot of metabolites with differential abundance between HC and ART patients identified by 3 different methods in Cameroon (a) and Indian (b) cohorts. Horizontal bars show the number of metabolites found with each method. Vertical bars display intersects between methods as indicated in the matrix below the graph.



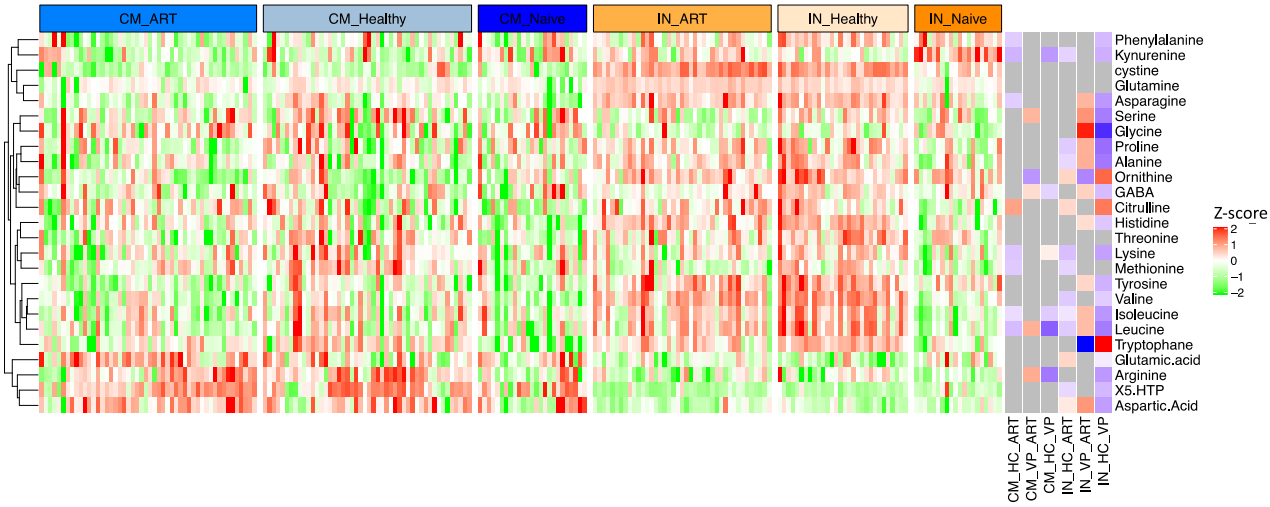
Supplementary Fig 4: Linear regression coefficients in Cameroon (a) and Indian (b) cohorts.



Supplementary Fig 5: Heatmaps of potential biomarkers and first neighbors in HC and ART in Cameroon (a) and Indian (b) cohorts. Data were log-transformed and z-score transformed.

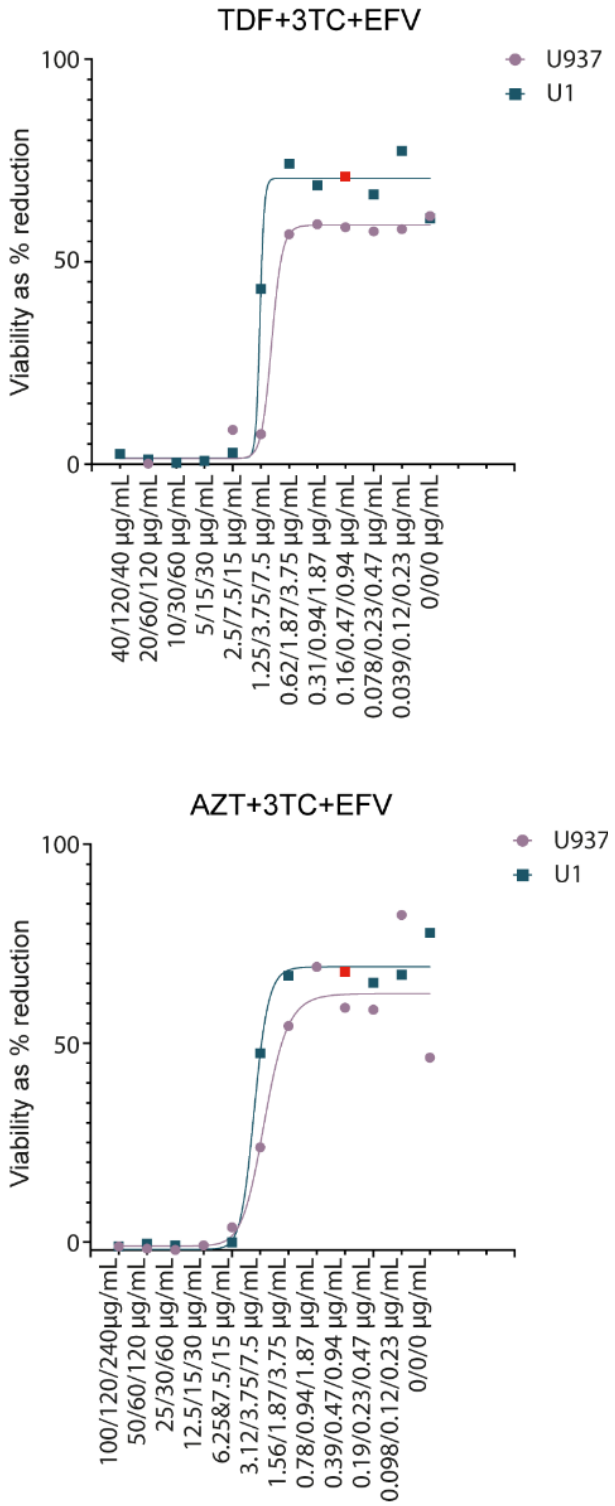


Supplementary Fig 6: UMAP visualization of AA (targeted metabolomics) for samples from Cameroon and Indian cohorts together. Cameroon HC = light blue; Camerron cART = steel blue; Cameroon naïve = blue, India HC = light orange; India cART = dark yellow, Indian naïve = Orange

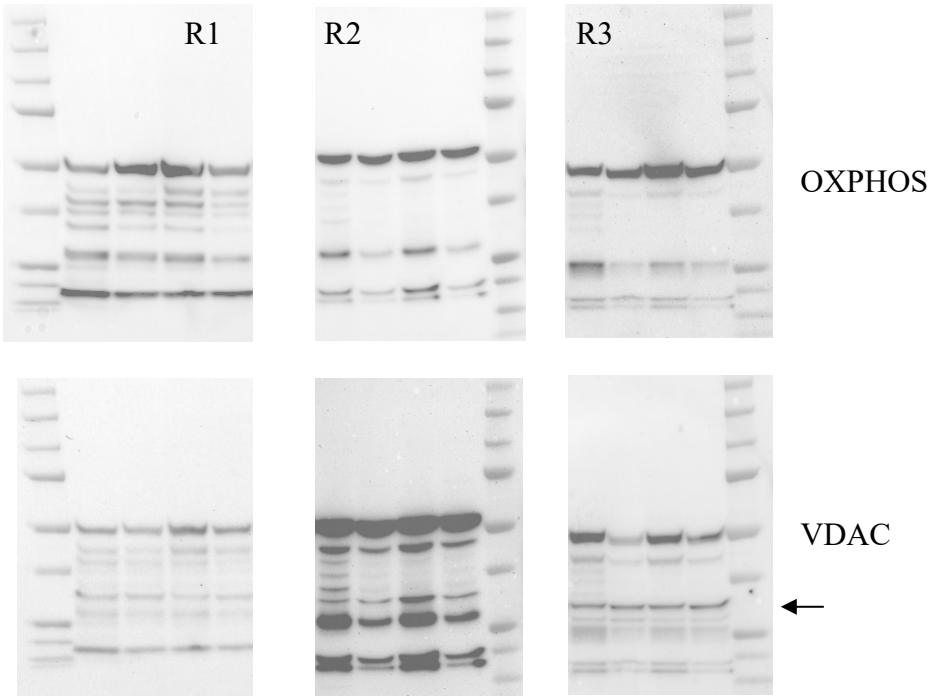


Supplementary Fig 7: Heatmap targeted metabolomics (amino acids) for Cameroon and India

Supplementary Fig 8: Dot plot representing pathway analysis for all comparisons in U937 and U1 corrected for U937 cells. For each cell line, pathways for comparisons Control vs Prostatin (C-P), Control vs DON (CON-C), Prostratin vs Prostratin+DON (PDON-P), DON vs Prostratin+DON (PDON-CDON. Size of the bubble represents the number of proteins and the number, the rank of the pathway based on FDR.



Supplementary Fig 9: Cytotoxicity of antiretroviral regimens TDF+3TC+EFV and AZT+3TC+EFV in monocytic cell lines, U937 (purple) and U1 (blue). The concentration of regimen used in Fig 5 is shown in red.



Supplementary Fig 10. Uncropped and unedited blot images

Supplementary Table 1. Demographic and clinical data for cohorts that were used for the targeted analysis.

	Cameroon				India				
	control	**PLWH on cART**	**Naive**	**p-value**	**control**	**PLWH on cART**	**Naive**	**p-value**	**Test**
number	50	50	25		30	41	20		
Age in years, median (IQR)	46.50 (40.00-53.00)	49.50 (40.50-56.75)	49.0 (40.0-56.0)	0.5828	46.00 (40.00-53.75)	45.00 (42.00-49.00)	43.00 (40.75-45.25)	0.242	Kruskal-Walis
Gender, male, n (%)	23 (46)	21 (42)	12 (48)	0.8645	13 (43.33)	25 (60.98)	9 (45)	0.2708	Chi square
CD4 current, median (IQR)	-	563.0 (464.2-793.5)	495.0 (382.0-558.0)	0.0031*	-	669.0 (465.0-779.0)	300.5 (213.2-527.0)	0.0004**	Mann-Whitney
CD8, median (IQR)	-	635.0 (386.0-798.8)	669 (385-900)	0.7065	-	796.5 (628.2-1,119.0)	1,278 (872-1,585)	0.0050*	t-test
Viral load in copies/mL, median IQR)	-	40.0 (40.0-40.0)	44,153 (21,412-215,343)	<0.001	-	149 (149-149)	55,700 (13,450-149,500)	<0.001	Mann-Whitney

Supplementary Method:

Targeted metabolomics of amino acids:

Standards and Calibration Curve

Amino acid standards (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine, glutamine, asparagine, GABA, citrulline, ornithine, taurine, tryptophan, 5-HTP, kynurenine and norvaline) were purchased from Sigma (St. Louis, MO, USA). Isotopically labeled amino acid standards (alanine ($^{13}C_3$, ^{15}N), arginine ($^{13}C_6$, $^{15}N_4$), aspartic acid ($^{13}C_4$, ^{15}N), cystine ($^{13}C_6$, $^{15}N_2$), glutamic acid ($^{13}C_5$, ^{15}N), glycine ($^{13}C_2$, ^{15}N), histidine ($^{13}C_6$, $^{15}N_3$), isoleucine ($^{13}C_6$, ^{15}N), leucine ($^{13}C_6$, ^{15}N), lysine ($^{13}C_6$, $^{15}N_2$), methionine ($^{13}C_5$, ^{15}N), phenylalanine ($^{13}C_9$, ^{15}N), proline ($^{13}C_5$, ^{15}N), serine ($^{13}C_3$, ^{15}N), threonine ($^{13}C_4$, ^{15}N), tyrosine ($^{13}C_9$, ^{15}N), valine ($^{13}C_5$, ^{15}N), Citrulline (d4), GABA ($^{13}C_4$), glutamine ($^{13}C_5$), asparagine($^{13}C_4$), ornithine (d6), tryptophan (d8), kynurenine (d4)) were obtained from Cambridge Isotope Laboratories (Andover, MA). Stock solutions of each compound were prepared at a concentration of 500 ng/µL and stored at -80ºC. A 10-point calibration curve (0.01-100 pmol/µL) was prepared by serial dilutions. 10µL of each calibration point was evaporated to dryness.

Mass spectrometry grade formic acid was purchased from Sigma-Aldrich (St Louis, MO, USA) and HPLC grade acetonitrile from Fisher Scientific (Fair Lawn, NJ, USA).

Extraction of amino acids in plasma

Amino acids were extracted by mixing 50 µL plasma with 450 µL 90:10 (v/v) Methanol: water solution containing norvaline at 2.2 pmol/µL as an internal standard. Each sample was extracted for 2 minutes using a mixer mill, incubated in the freezer for 2 hours and centrifuged at 4ºC, 14000 RPM, for 10 min. 25µL of the supernatant was transferred to micro vials and evaporated to dryness in a speed-vac concentrator. The samples were stored at -80 °C until analysis.

Amino acid derivatization with AccQ-Tag

Extracted samples were derivatized by AccQ-Tag™ (Waters, Milford, MA, USA) according to the manufacturers' instructions. Briefly, the dried extracts were resuspended in 20 µL of 20 mM HCl, and 60 µL of AccQ•Tag Ultra borate buffer spiked with all isotopically labelled internal standards at a final concentration of 0.833 pmol/µL was added to each sample. Finally 20 µL of freshly prepared AccQ•Tag derivatization solution was added and the samples were immediately vortexed for 10 seconds.

The dried calibration curves were prepared in a similar way using the same spiked Ultra borate buffer.

Samples were kept at room temperature for 30 minutes followed by 10 minutes at 55ºC. For each batch quality control samples and procedure, blanks were included.

Amino acids Quantification by LC-ESI-MSMS

Derivatized samples were analyzed using a 1290 Infinitely system from Agilent Technologies (Waldbronn, Germany), consisting G4220A binary pump, G1316C thermostated column compartment and G4226A autosampler with G1330B autosampler thermostat coupled to an Agilent 6460 triple quadrupole mass spectrometer equipped with a jet stream electrospray source operating in positive ion mode.

Separation was achieved injecting 1 µL of each sample onto a BEH C_{18} 2.1x100 mm, 1.7 µm column (Waters, Milford, MA, USA) held at 50ºC in a column oven. The gradient eluents used were H_2O 0.1% formic acid (A) and acetonitrile 0.1% formic acid (B) with a flow rate of 500 µL/min. The initial conditions consisted of 0% B, and the following gradient was used with linear increments: 0.54-3.50 minutes (0.1-9.1% B), 3.50-7.0 (9.1-17.0% B), 7.0-8.0 (17.0-19.70% B), 8.0-8.5 (19.7% B), 8.5-9.0 (19.7-21.2% B), 9.0-10.0 (21.2-59.6% B), 10.0-11.0 (59.6-95.0% B), 11.0-11.5 (95.0% B), 11.5-15.0 (0% B). From 13.0 minutes to 14.8 minutes the flow rate was set at 800 µL/min for a faster equilibration of the column.

The MS parameters were optimized for each compound as described in Supporting Information. MRM transitions for the derivatized amino acids were optimized using MassHunter MS Optimizer software (Agilent Technologies Inc., Santa Clara, CA, USA). The fragmentor voltage was set at 380 V, the cell accelerator voltage at 7 V and the collision energies from 14-45V, nitrogen was used as collision gas. The data was quantified using MassHunter™ Quantitation software B08.00 (Agilent Technologies Inc., Santa Clara, CA, USA) and the amount of each amino acid was calculated based on the calibration curves.

Mass Spectrometry parameters

Jet-stream gas temperature was 290°C with a gas flow of 11 L/min, sheath gas temperature 325°C, sheath gas flow of 12 L/min. The nebulizer pressure was set to 20 psi and the capillary voltage was set at 4 kV. The QqQ was run in Dynamic MRM Mode with 2 min retention time windows and 500 msec cycle scans.

Table 1. Retention times (rt), MRM-transition stages monitored (precursor ion and product ions) and collision energies of analyzed compounds.

Compounds	MRM transition		rt (min)	Collision Energy (V)
	Precursor Ion	Product Ion		
alanine	260.1	171	4.1	14
arginine	345.1	171	2.8	35
aspartic acid	304.1	171	3.4	18
cystine	581	171	5.2	25
glutamic acid	318.1	171	3.6	22
glycine	246.1	171	3.1	18
histidine	326.1	171	2.6	26
isoleucine	302.1	171	7.8	18
leucine	302.1	171	7.6	18

lysine	487.2	171	5.1	26
methionine	320.1	171	6.0	22
phenylalanine	336.1	171	8.1	18
proline	286.1	171	4.4	14
serine	276.1	171	3.1	14
threonine	290.1	171	3.7	18
tyrosine	352.1	171	5.7	18
valine	288.1	171	6.1	18
citrulline	346.2	171	3.4	30
GABA	274.1	171	4.1	18
glutamine	317.1	171	3.0	22
asparagine	303.1	171	2.8	18
ornithine	473.2	171	4.7	34
tryptophan	375.2	171	8.4	26
kynurenin	379.2	171	7.6	37
Internal standards				
Norvaline	288.1	171	6.4	18
alanine (13C3, 15N)	264.07	171	4.1	14
arginine (13C6, 15N4)	355.1	171	2.8	35
aspartic acid (13C4, 15N)	309.07	171	3.3	18
cystine (13C6, 15N2)	589	171	5.2	25
glutamic acid (13C5, 15N)	324.09	171	3.5	22
glycine (13C2, 15N)	249.05	171	3.1	18
histidine (13C6, 15N3)	335	171	2.6	26
isoleucine (13C6, 15N)	309.12	171	7.9	18
leucine (13C6, 15N)	309.12	171	7.6	18
lysine (13C6, 15N2)	495.1	171	5.2	26
methionine (13C5, 15N)	326.17	171	6.0	22
phenylalanine (13C9, 15N)	346	171	8.2	18
proline (13C5, 15N)	292.09	171	4.4	14
serine (13C3, 15N)	280.6	171	3.0	14
threonine (13C4, 15N)	295.08	171	3.7	18
tyrosine (13C9, 15N)	362.12	171	5.7	18
valine (13C5, 15N)	294.1	171	6.1	18
Citrulline (d4)	350.1	171	3.4	30
GABA (13C4)	278.1	171	4.0	18
glutamine (13C5)	322.1	171	3.1	22
asparagine(13C4)	307.1	171	2.8	18
ornithine (d6)	479	171	4.6	34
tryptophan (d8)	383.2	171	8.3	26
kynurenine (d4)	383	171	7.7	37