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Co-circulation of Three HTLV-2 Subtypes and Distinct Immune signatures in Asymptomatic People Living with HTLV in the Western Brazilian Amazon

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Abstract

Background

HTLV is endemic in the Brazilian Amazon, but the diversity of circulating strains in Amazonas remains poorly characterized, and it is still unclear whether asymptomatic carriage is associated with a distinct systemic immune profile. We combined phylogenetic characterization with serum cytokine and chemokine profiling to address both questions.

Methods

Forty prospective blood donors with confirmed HTLV-1/2 infection and 30 HTLV-negative blood donors were included. The 5′ LTR region was amplified by nested PCR, and 40 amplicons underwent Sanger sequencing and maximum-likelihood phylogenetic analysis. Serum cytokines, chemokines, and growth factors were quantified by multiplex immunoassay. Immune profiles were evaluated using univariate comparisons, correlation analysis, principal component analysis, PERMANOVA, and sensitivity analyses.

Results

All HTLV-1 sequences belonged to the Cosmopolitan subtype, Transcontinental subgroup (HTLV-1aA; n = 27), whereas HTLV-2 segregated into three co-circulating subtypes: 2a (n = 1), 2b (n = 4) and 2c (n = 8). After correction for multiple testing, five mediators were higher in carriers than in controls, with moderate to large effect sizes (CCL5, IL-13, CXCL10, IL-18 and IL-4) and no growth factor differed. CCL5, CXCL10 and IL-13 were elevated in both viral types and IL-18 in HTLV-2 only, but no mediator differed between HTLV-1 and HTLV-2. Carriers also showed increased network connectivity among chemokines and type 1 and type 2 cytokines, and a significant global shift in mediator composition (PERMANOVA $R^2 = 0.047$, $p = 0.002$).

Conclusion

Our findings reveal the co-circulation of three HTLV-2 subtypes in Amazonas and a distinct circulating immune mediator profile in asymptomatic carriers, mainly driven by CCL5, CXCL10 and IL-13. Molecular surveillance should therefore extend into structured clinical follow-up, as asymptomatic infection does not imply immunological quiescence.

Keywords: HTLV-1; HTLV-2; Asymptomatic carriers; Genetic diversity; Molecular surveillance; Cytokines; Chemokines; Brazilian Amazon.

Introduction

Human T-cell lymphotropic virus types 1 and 2 (HTLV-1/2) are deltaretroviruses first identified in 1980 and 1982, respectively [1,2]. Transmission occurs through sexual contact, transfusion of infected cellular blood components, organ transplantation, and mother-to-child transmission, primarily through breastfeeding [3–6]. Although many infected individuals remain asymptomatic or present only non-specific symptoms, HTLV-1 infection can lead to severe diseases such as adult T-cell leukemia/lymphoma (ATLL) and a chronic neurological disorder known as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [[7-9].

It is estimated that 5–10 million people are infected with HTLV-1 worldwide, with Brazil accounting for the largest absolute number of infected individuals [10]. Since mandatory serological screening for HTLV-1/2 was implemented in Brazilian blood banks, substantial progress has been made in the epidemiological surveillance of these infections [11–13]. However, while screening identifies infected individuals, it does not provide information on the molecular characteristics of the viruses they carry. As a result, studies describing the molecular diversity of circulating strains remain scarce, particularly in the Western Amazon.

Molecular characterization of the long terminal repeat (LTR) region enables the assignment of isolates to subtypes and subgroups and is the standard approach in HTLV molecular epidemiology. The Cosmopolitan subtype HTLV-1a predominates in Brazil, whereas HTLV-2 is genetically more heterogeneous, comprising subtypes 2a, 2b, 2c, and 2d with distinct geographic distributions [14–16]. In the Brazilian Amazon, this diversity has been characterized mainly in Indigenous communities and in the eastern portion of the region, where HTLV-2c predominates [17,18]. Which subtypes circulate in urban populations of the Western Amazon, and whether this diversity is restricted to HTLV-2c, has not been established.

Beyond viral genotype, the host immune response plays a central role in HTLV persistence and disease progression, with cytokine and chemokine dysregulation linked to immune activation, proviral burden, and inflammatory manifestations [19–21]. However, systemic immune alterations in individuals identified through blood-donor screening remain poorly characterized, particularly among HTLV-2 carriers and Amazonian populations, as most available evidence comes from patients with established disease at referral centers. Unlike these clinical cohorts, blood donors do not routinely undergo standardized neurological or rheumatological assessments, and systematic follow-up after a positive screening result is often lacking, limiting the evaluation of their clinical status and risk of progression. Whether this apparent clinical silence also reflects immunological quiescence therefore remains unresolved.

The clinical heterogeneity of asymptomatic HTLV infection has become increasingly evident, as detailed assessments have identified neurological and systemic abnormalities in individuals previously considered free of disease [21]. Data are even more limited for HTLV-2, whose systemic immune profile has rarely been studied, and it is unclear whether different viral subtypes elicit distinct host responses. To address these gaps, we conducted molecular subtyping and comprehensive serum immune profiling in blood donation candidates with confirmed HTLV-1/2 infection in Amazonas, Brazil.

Methods

Ethics statement

This study was approved by the Research Ethics Committee of the Hematology and Hemotherapy Foundation of Amazonas (HEMOAM) (protocol no. 5,348,608/2021; CAAE no. 57153922.5.0000.0009). Written informed consent was obtained from all participants before enrollment. The study was conducted in accordance with Brazilian Resolution No. 466/2012 and applicable institutional and national requirements..

Study group

Blood donation candidates screened at HEMOAM were tested for HTLV-1/2 by chemiluminescent immunoassay (CLIA). Of 100 CLIA-reactive samples, 52 were confirmed as HTLV-1/2 positive by repeat CLIA, Western blot (WB), FC-Simplex and FC-Duplex, according to the Brazilian Ministry of Health Clinical Management Guidelines for HTLV Infection [23]. All 52 confirmed samples were submitted to nested PCR of the 5′LTR region, of which 40 (76.9%) yielded amplicons of sufficient yield and quality for Sanger sequencing. These 40 participants — 27 with HTLV-1 and 13 with HTLV-2, aged 19–78 years (25 females and 15 males) — formed the study group for both the phylogenetic and the immunological analyses.

Participants were classified as having no symptoms documented during routine predonation screening. This screening did not include standardized neurological or rheumatological examinations; therefore, the classification should not be interpreted as a comprehensive assessment for HTLV-associated manifestations. The control group consisted of 30 HTLV-1/2-seronegative blood donors (18 females and 12 males, aged 18–60 years), recruited from the same institution during the same period and negative for all other mandatory blood-donor screening markers. The

screening and analysis workflow, with the number of samples at each stage, is summarized in Supplementary Figure S1.

Sample collection and DNA amplification

Venous blood was collected into EDTA and serum separator tubes (BD Vacutainer® EDTA K2 and BD SST™ II Advance) for whole-blood and serum samples, respectively. Genomic DNA was extracted from buffy coat by the phenol-chloroform method with Brazol, as previously described [24]. DNA concentration and purity were assessed on a NanoDrop™ 1000 spectrophotometer.

The 5′LTR regions of HTLV-1 and HTLV-2 were amplified by nested PCR. HTLV-1 amplification was performed in a final volume of 25 µL with 300 ng of genomic DNA under the conditions described by Razavi Pashabayg et al. [25], whereas HTLV-2 amplification followed the protocol described by Pontes et al. [26]. Amplification was confirmed by electrophoresis on 1.5% agarose gels in 1× TAE buffer stained with SYBR™ Safe DNA Gel Stain (Invitrogen) at a final concentration of 1× and visualized on a blue-light LED transilluminator, yielding amplicons of approximately 579 bp for HTLV-1 and 788 bp for HTLV-2.

Sequencing, phylogenetic, and phylogeographic analyses

PCR products were purified using the Virus RNA-DNA Purification Kit (Cellco) and sequenced in both directions with the BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Chromatograms were inspected in BioEdit, low-quality ends were trimmed, and forward and reverse reads were assembled into consensus sequences. The amplicons of approximately 579 bp for HTLV-1 and 788 bp for HTLV-2 correspond to the amplified fragment; because read quality declined toward the primer-proximal ends, the consensus recovered at high quality in both directions was shorter, and it is this region that was analyzed.

Consensus sequences were aligned with GenBank reference sequences using MAFFT v7.526 with the L-INS-i strategy, separately for each target. The HTLV-1 alignment comprised 68 entries, the 27 study sequences and 41 references, and 560 positions including gaps, with 197 variable and 87 parsimony-informative sites, no undetermined bases (N) or other ambiguous codes, and 11.54% gaps. The HTLV-2 alignment comprised 62 entries, the 13 study sequences and 49 references, the latter including the STLV-2 sequence used to root the tree, and 587 positions

including gaps, with 288 variable and 82 parsimony-informative sites, seven N across three sequences, no other ambiguous codes, and 9.50% gaps.

Maximum-likelihood analyses were conducted in IQ-TREE v3.1.2, with HKY+R2 for HTLV-1 and HKY+F+R2 for HTLV-2 selected by ModelFinder under the Bayesian information criterion and branch support assessed by 1,000 SH-aLRT and 5,000 ultrafast bootstrap replicates (UFBoot2) with BNNI optimization. The HTLV-1 topology was inferred unrooted and oriented for visualization along the split separating HTLV-1a from non-HTLV-1a lineages. Repeating the HTLV-2 analysis on the region common to all study sequences, and with positions containing undetermined bases excluded, returned identical subtype assignments, including that of the single HTLV-2a sequence.

An exploratory temporal and phylogeographic analysis were subsequently performed for HTLV-2c sequences with available sampling-year and geographic information to assess temporal signal and geographic structure. Sampling years were used as tip dates, and geographic origin was coded as Amazonas or Pará. Root-to-tip genetic distance was regressed against sampling year, with significance evaluated using 10,000 unrestricted permutations and permutations restricted within geographic state to assess date–location confounding.

Bayesian analyses were performed using BEAST v2.7.7 with the BEAST_CLASSIC v1.6.4 package, an HKY+Γ4 substitution model, an uncorrelated lognormal relaxed clock, and a constant-size coalescent prior, with geography modeled as a two-state discrete trait. Three independent MCMC chains of 1×10^6 states were evaluated after discarding 10% as burn-in, using effective sample sizes (ESS) and the potential scale reduction statistic (R-hat). Sensitivity to alternative molecular-clock priors was also assessed.

Reference sequences were selected from GenBank using a purposive strategy aimed at providing broad geographic and phylogenetic context for the study sequences. Particular emphasis was placed on Brazilian and Amazonian references, complemented by sequences from other geographic regions to broaden geographic representation. Reference sequences representing different HTLV-1 subtypes and Cosmopolitan subgroups, as well as HTLV-2 subtypes, were included to contextualize subtype assignment. The reference datasets were not intended to constitute exhaustive or geographically balanced samples of global viral diversity. Consequently, geographic affinities were interpreted in the context of the available reference sequences rather than as evidence of population-level geographic distributions or migration routes.

Analysis of the immunological profile

Cytokine and chemokine concentrations were quantified using the Invitrogen™ ProcartaPlex™ Human Th1/Th2 Cytokine & Chemokine Panel 1, 20-plex (Thermo Fisher Scientific), according to the manufacturer's instructions. The panel comprised GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12p70, IL-13, IL-18, TNF-α, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (eotaxin), CXCL1 (GROα), CXCL8 (IL-8), CXCL10 (IP-10), and CXCL12 (SDF-1α). Acquisition was performed on a Luminex™ 200™ Instrument System (Invitrogen, Thermo Fisher Scientific) based on xMAP® technology, using xPONENT® software, and concentrations were interpolated from five-parameter logistic standard curves. All samples were processed in a single batch, and values below the limit of quantification were handled as described in the statistical analysis section.

Statistical analysis

Values reported as below the limit of quantification (<LOQ), including non-quantifiable readings recorded as zero, were imputed as half the analyte-specific LOQ. This single rule was applied consistently to all analyses, both univariate and multivariate. Categorical variables were summarized as counts and percentages, and continuous variables as medians and interquartile ranges. Two-group comparisons used the Mann–Whitney U test, while comparisons among Control, HTLV-1, and HTLV-2 groups used the Kruskal–Wallis test followed by Dunn's post hoc test. Associations between immune mediators were assessed using Spearman's rank correlation coefficient.

Because left-censoring may differ between groups and generate apparent distributional differences, the robustness of univariate comparisons to the censoring rule was evaluated in two ways. First, a two-part analysis compared detectability between groups (Fisher's exact test) and, separately, concentrations among quantifiable samples only (Mann–Whitney U). Second, all univariate comparisons were repeated with censored values assigned tied lowest ranks instead of half-LOQ imputation. Detectability did not differ between groups for any analyte (all $p \geq 0.42$), and the mediators significant after FDR correction were identical under both approaches. This equivalence is structural rather than coincidental: because the univariate comparisons are rank-based, they are invariant to the value substituted for censored observations, provided it lies below the limit of quantification. We confirmed that half-LOQ imputation, assignment of tied lowest ranks, retention of zeros, and per-analyte exclusion of censored observations yielded identical p- and q-values for all 20 analytes. The imputation rule therefore affects only the multivariate analyses, in which a numerical value is required for every participant.

Heatmaps were generated from mediator Z-scores, with hierarchical clustering of participants. Spearman correlation networks were constructed separately for the Control and HTLV groups and visualized using Cytoscape v3.8.2. Only associations remaining significant after Benjamini–Hochberg correction ($q < 0.05$) were included, with edge weights representing correlation magnitude; no inverse association remained significant in either group, so all retained edges were positive. Network connectivity was characterized by node degree, defined as the number of connections per mediator. To assess the robustness of network differences to unequal sample sizes, the HTLV group was randomly subsampled to the size of the Control group ($n = 30$) in 1,000 iterations; networks were reconstructed for each subsample using the same association criteria, and the resulting edge-count and node-degree distributions were compared with those of the Control network.

IL-2 was excluded from the multivariate analyses, including module analyses, because approximately half of its measurements were below the LOQ (50.0% in controls, 39.0% in carriers), limiting its reliability for quantitative comparisons, but was retained for descriptive visualization. Mediator concentrations were $\log_{10}$-transformed, mean-centered, and scaled to unit variance. Principal component analysis included all participants, as the imputation procedure left no missing values. Global differences between groups were assessed by PERMANOVA using Euclidean distances calculated from the same transformed and standardized matrix, with 9,999 permutations. Homogeneity of multivariate dispersions was assessed by PERMDISP, computed as distances to the group spatial median with the $\mathrm{sqrt}(n/(n-1))$ bias adjustment and tested by 9,999 permutations. Mediator contributions to PC1 and PC2 were evaluated from PCA loadings, and the six mediators with the highest combined contributions, calculated as the sum of squared loadings across both components, were highlighted in the loading plot.

To estimate the contribution of individual mediators to the global multivariate signal, complete and reduced models were compared after removing CCL5, CXCL10, and IL-13 from the global profile; CCL5 and CXCL10 from the chemokine module; and IL-13 from the type 2/regulatory module. Effect retention was calculated as the ratio of the R^2 of the reduced model to that of the complete model. Confidence intervals for R^2 were obtained by a group-stratified bootstrap, resampling participants with replacement within each group at the observed group sizes and taking the 2.5th and 97.5th percentiles across 2,000 replicates. Statistical analyses were performed in R version 4.3, using the stats package for rank-based comparisons, Spearman correlations, PCA, hierarchical clustering, and multiple-testing adjustment, and the vegan package for PERMANOVA and PERMDISP. Network visualization was performed using Cytoscape

v3.8.2. P-values were adjusted using the Benjamini–Hochberg procedure, with a false discovery rate (FDR) < 0.05 considered statistically significant.

Results

Molecular and phylogenetic characterization of HTLV-1 and HTLV-2.

Bidirectional sequences were obtained for all 40 participants. After trimming of low-quality ends, individual sequence lengths ranged from 456 to 516 nucleotides for the 27 HTLV-1 sequences and from 382 to 562 nucleotides for the 13 HTLV-2 sequences, and the sequences of each target overlapped a common alignment region of 508 and 392 positions, respectively (Table S4). Phylogenetic analysis of the 27 HTLV-1 sequences showed that all isolates clustered within the Cosmopolitan subtype, Transcontinental subgroup (HTLV-1aA), together with the corresponding GenBank reference sequences (Fig. 1). The sequences were deposited in GenBank under accession numbers PZ665096–PZ665122.

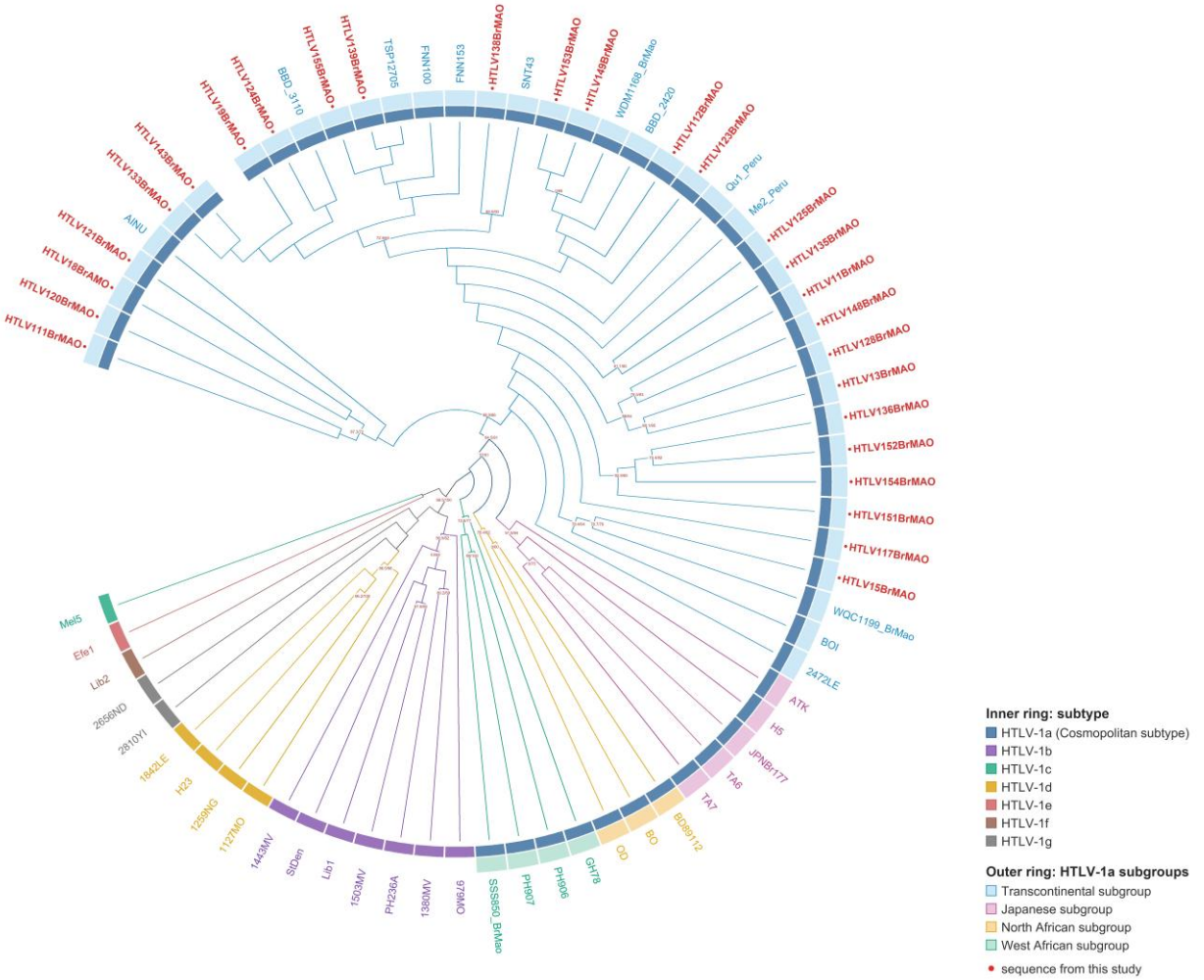


Figure 1. Maximum-likelihood phylogenetic tree based on the LTR region of HTLV-1. The tree was inferred using IQ-TREE v3.1.2 under the HKY+R2 model. Node labels indicate SH-aLRT/UFBoot support values, shown for UFBoot ≥60%. Branches and terminal labels are colored according to HTLV-1 subtype: HTLV-1aA, HTLV-1aB, HTLV-1aC, HTLV-1aD, HTLV-1b, HTLV-1c, HTLV-1d, HTLV-1e, HTLV-1f, and HTLV-1g. Sequences generated in this study are highlighted in red.

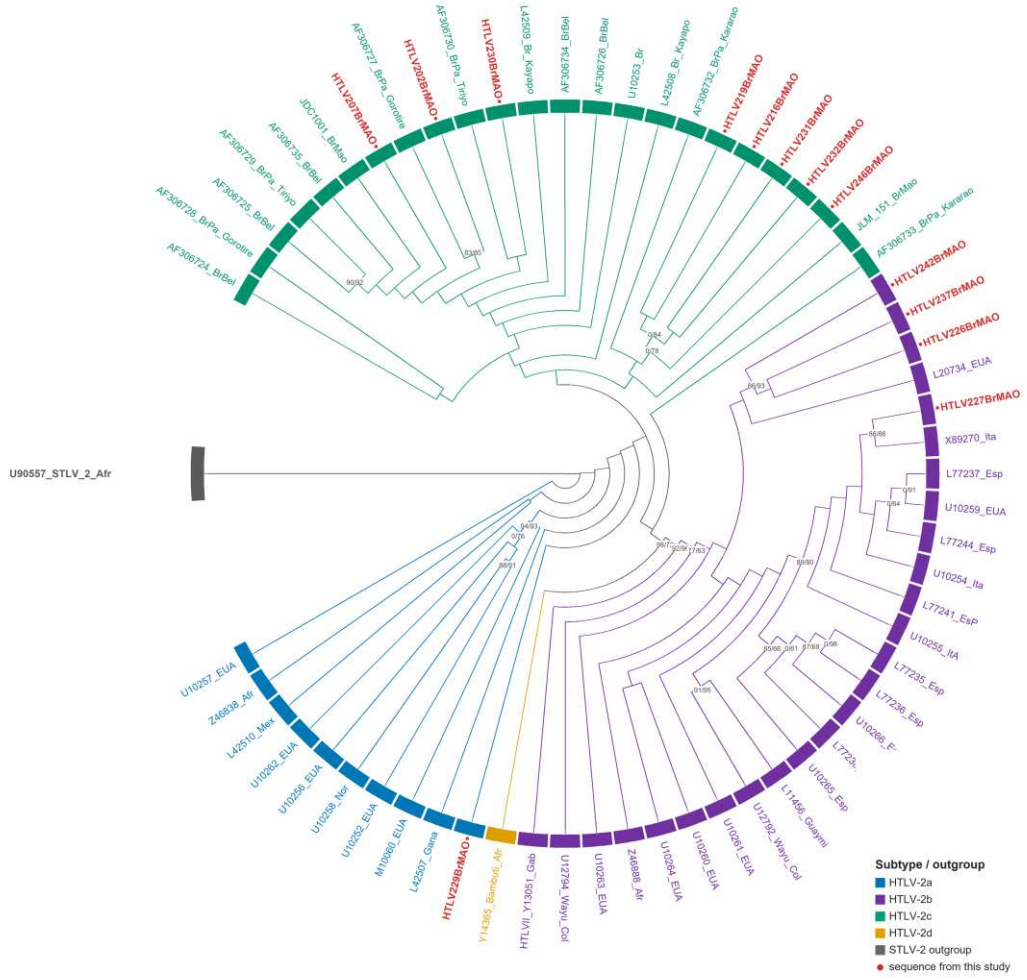
Among the 13 HTLV-2 sequences, one was classified as HTLV-2a, four as HTLV-2b, and eight as HTLV-2c, demonstrating the co-circulation of three HTLV-2 lineages in Manaus (Figure 2A). The sequences were deposited in GenBank under accession numbers PZ665123-PZ665135. HTLV-2c sequences clustered with reference sequences from Pará. Within the reference set analyzed, HTLV-2b study sequences showed affinities with Italian and US references, whereas the single HTLV-2a sequence was nearest to the Ghanaian reference L42507.

Relationships between the study sequences and geographically annotated reference sequences in the maximum-likelihood tree are summarized in Figure 2B. These relationships describe the selected reference set and do not establish geographic origin, migration routes, or timing.In a separate exploratory temporal analysis of HTLV-2c sequences, sampling year was

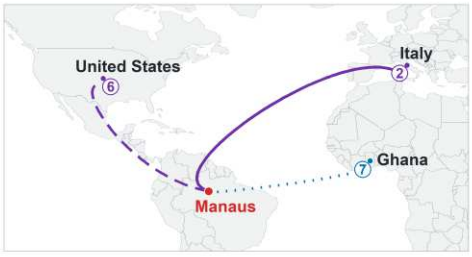
strongly associated with sampling location. Root-to-tip divergence was associated with sampling year in the unadjusted analysis ($R^2 = 0.132$; $p = 0.012$), but this association was not retained when evaluated using permutations restricted within geographic state ($p = 0.321$). Sampling year and location were strongly confounded ($r = 0.986$), limiting the interpretation of the apparent temporal signal.

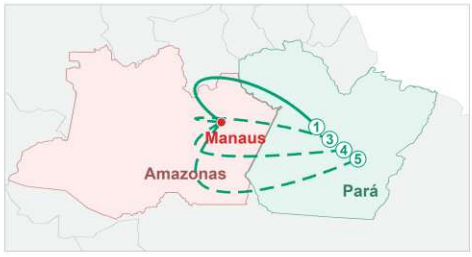
Pará sequences were collected between 1996 and 2000 and Amazonas sequences between 2015 and 2023, with no temporal overlap. Bayesian analyses showed approximately equal posterior support for Amazonas and Pará as the root location, substantial variation in root-age estimates across molecular-clock priors, and low effective sample sizes. Because sampling year and geographic origin are almost perfectly confounded ($r = 0.986$, with no temporal overlap between the two sets), the temporal and phylogeographic analyses cannot separate time from place, and the root-location and root-age estimates were unstable. The clustering of the Manaus HTLV-2c sequences with Pará references is therefore reported as a topological affinity and should be regarded as hypothesis-generating.

A



B





Relationships within the selected reference set

① **HTLV202**
Pará · Gorotire · 83/85

② **HTLV227**
Italy · X89270 · 86/86

③ **HTLV230**
Pará · Tiriyó · 74/48

④ **HTLV207**
Pará-associated clade · 0/37

⑤ **HTLV216, 219, 231, 232, 246**
Pará · Kararaô · 0/44

⑥ **HTLV226, 237, 242**
United States · L20734 · 0/24

⑦ **HTLV229**
Ghana · L42507 · distance only

Node support: SH-aLRT/UFBoot (%)

Circled numbers 1–7 link maps to the sequence list.

● Manaus study samples

—— Sister relationship (support shown)

– – Weakly supported placement

····· Nearest reference (patristic distance)

Figure 2. Phylogenetic relationships and geographic affinities of HTLV-2 study sequences. (A) Maximum-likelihood tree based on the HTLV-2 LTR region. Branches and terminal labels are colored by subtype, and study sequences are highlighted in red. The tree was rooted using the STLV-2 reference U90557_STLV_2_Afr (gray). Node labels show SH-aLRT/UFBoot support values (%), displayed when UFBoot ≥60%. Branch lengths are not drawn to scale.(B) Geographically annotated phylogenetic relationships between sequences from Manaus and selected reference sequences. Circled numbers 1–7 link map connections to the corresponding study sequences in the accompanying list. Solid lines represent sister relationships with the indicated node support, whereas dashed lines represent weakly supported placements; corresponding SH-aLRT/UFBoot values (%) are reported in the list. Dotted lines indicate nearest-reference relationships based solely on patristic distance, calculated as the sum of branch lengths connecting two tips, without implying a supported sister relationship. Line colors indicate HTLV-2 subtype. Geographic positions are schematic. Connections are non-directional and do not establish geographic origin, ancestral location, migration routes, transmission events, or timing. All relationships are conditional on the analyzed reference set.

Immunological profile of HTLV infection

Five mediators were significantly higher in the combined HTLV group than in controls after correction for multiple comparisons across the 20 analytes: CCL5 ($q < 0.001$), IL-13 ($q < 0.001$), CXCL10 ($q = 0.001$), IL-18 ($q = 0.037$) and IL-4 ($q = 0.041$). Effect sizes were moderate to large, from Cliff's $\delta = 0.35$ for IL-4 to $\delta = 0.63$ for CCL5. Three further analytes were higher in carriers before correction but did not survive it: IL-6 ($p = 0.018$; $q = 0.059$), CXCL1 ($p = 0.028$; $q = 0.079$) and IL-12(p70) ($p = 0.044$; $q = 0.109$). The remaining twelve analytes did not differ between groups: CXCL8, CCL11, CCL3, CCL4, CCL2, CXCL12/SDF-1α, IL-1β, TNF-α, IFN-γ, IL-5, GM-CSF and IL-2 (Figure 3; Table S1).

In analyses stratified by viral type, four mediators differed across the three groups after correction (Kruskal–Wallis $q < 0.05$). CCL5, CXCL10 and IL-13 were higher in both the HTLV-1 and the HTLV-2 group than in controls (all Dunn $q \leq 0.023$), whereas IL-18 was higher in HTLV-2 only ($q = 0.005$; HTLV-1 versus controls, $q = 0.083$). No mediator differed between HTLV-1 and HTLV-2 (all $q \geq 0.083$), and no other analyte differed across the three groups after correction; IL-4 reached significance before correction only (Kruskal–Wallis $p = 0.024$; $q = 0.094$; Supplementary Figure S2). Table S2 gives the full post hoc comparisons.The corresponding individual profiles are shown in Supplementary Figure S3, and Table S3 gives the full post hoc comparisons.

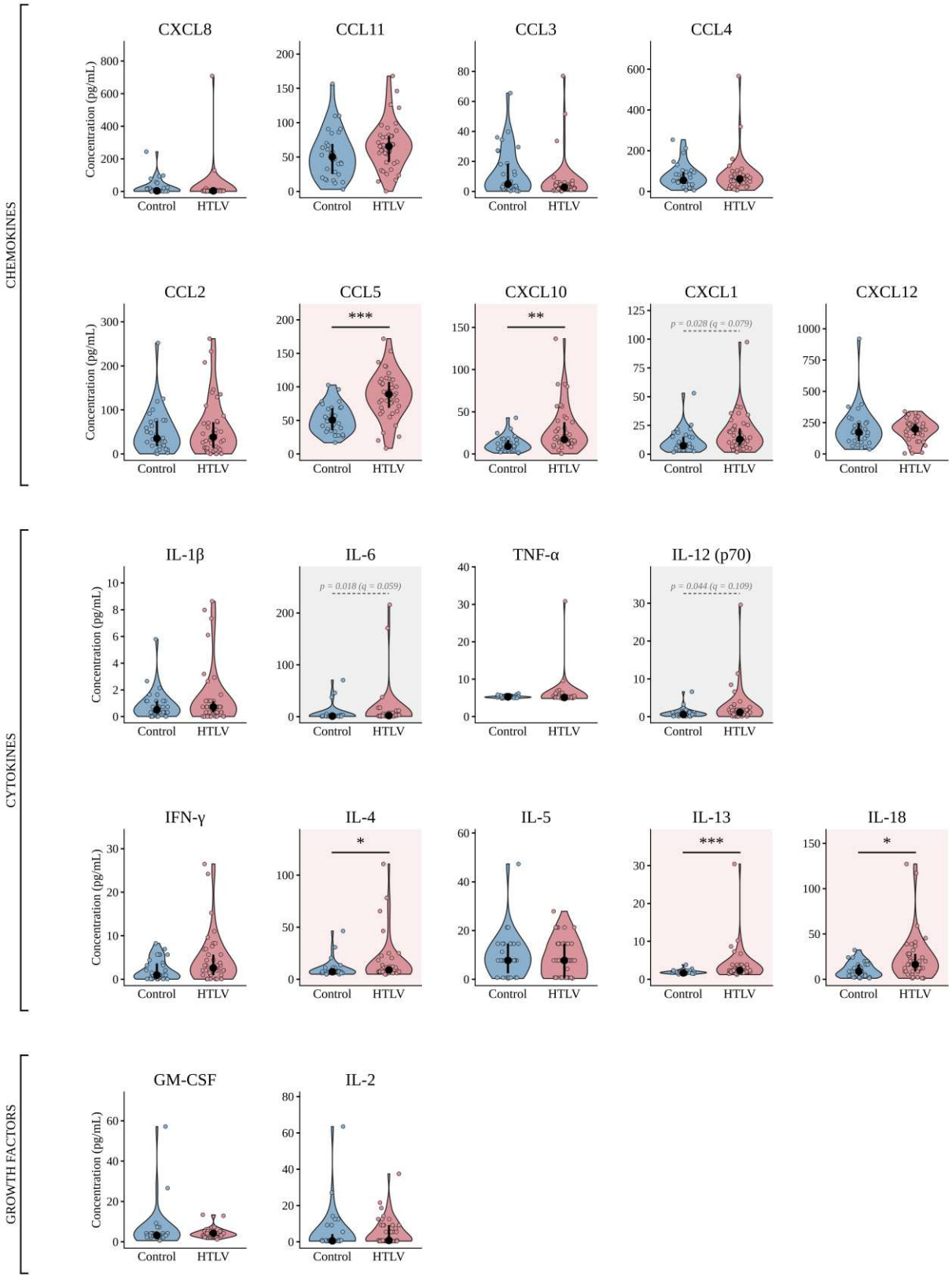

CHEMOKINES
CXCL8
CCL11
CCL3
CCL4
CCL2
CCL5
CXCL10
CXCL1
CXCL12
CYTOKINES
IL-1β
IL-6
TNF-α
IL-12 (p70)
IFN-γ
IL-4
IL-5
IL-13
IL-18
GROWTH FACTORS
GM-CSF
IL-2
Concentration (pg/mL)
Control
HTLV
***
**
*
p = 0.028 (q = 0.079)
p = 0.018 (q = 0.059)
p = 0.044 (q = 0.109)

Figure 3. Serum concentrations of chemokines, cytokines, and growth factors in the HTLV and control groups. Data are presented as violin plots, with individual participants plotted as points; black circles and bars indicate the median and interquartile range. Panels shaded in pink identify the five analytes that differed between groups after correction for multiple comparisons across the 20 analytes by the Benjamini–Hochberg procedure (CCL5, CXCL10, IL-4, IL-13 and IL-18) with asterisks indicating the adjusted p-values (*$q \leq 0.05$; **$q \leq 0.01$; ***$q \leq 0.001$; ****$q \leq 0.0001$). Panels shaded in grey identify three further analytes that were significant before correction but did not survive it (CXCL1, IL-6 and IL-12(p70)for which the dashed bracket gives the unadjusted p and the adjusted q. Control, n = 30; HTLV, n = 40.

Reorganization of correlation patterns among immunological mediators

Following the identification of differences in serum concentrations of immunological mediators, correlation patterns among analytes were evaluated in the control and HTLV groups to investigate potential differences in the coordination of the immune response associated with infection.

The control network contained 22 significant correlations compared with 46 in the HTLV network ($q < 0.05$; Figure 4A). All retained associations were positive, and the additional connections in carriers were distributed across chemokines, pro-inflammatory / type 1 cytokines and type 2 / regulatory mediators (Figure 4B). TNF-α (3 to 10), IL-13 (1 to 7), IFN-γ (2 to 7), CXCL1 (0 to 4) and IL-6 (3 to 6) showed the largest increases in node degree in the HTLV network relative to the control network (Figure 4C). Repeated subsampling of the HTLV group to the size of the control group (n = 30; 1,000 resamplings) yielded a median of 36 significant correlations, exceeding the control network in every resampling (empirical p = 0.02), indicating that the greater connectivity observed in carriers was not attributable to the larger sample size.

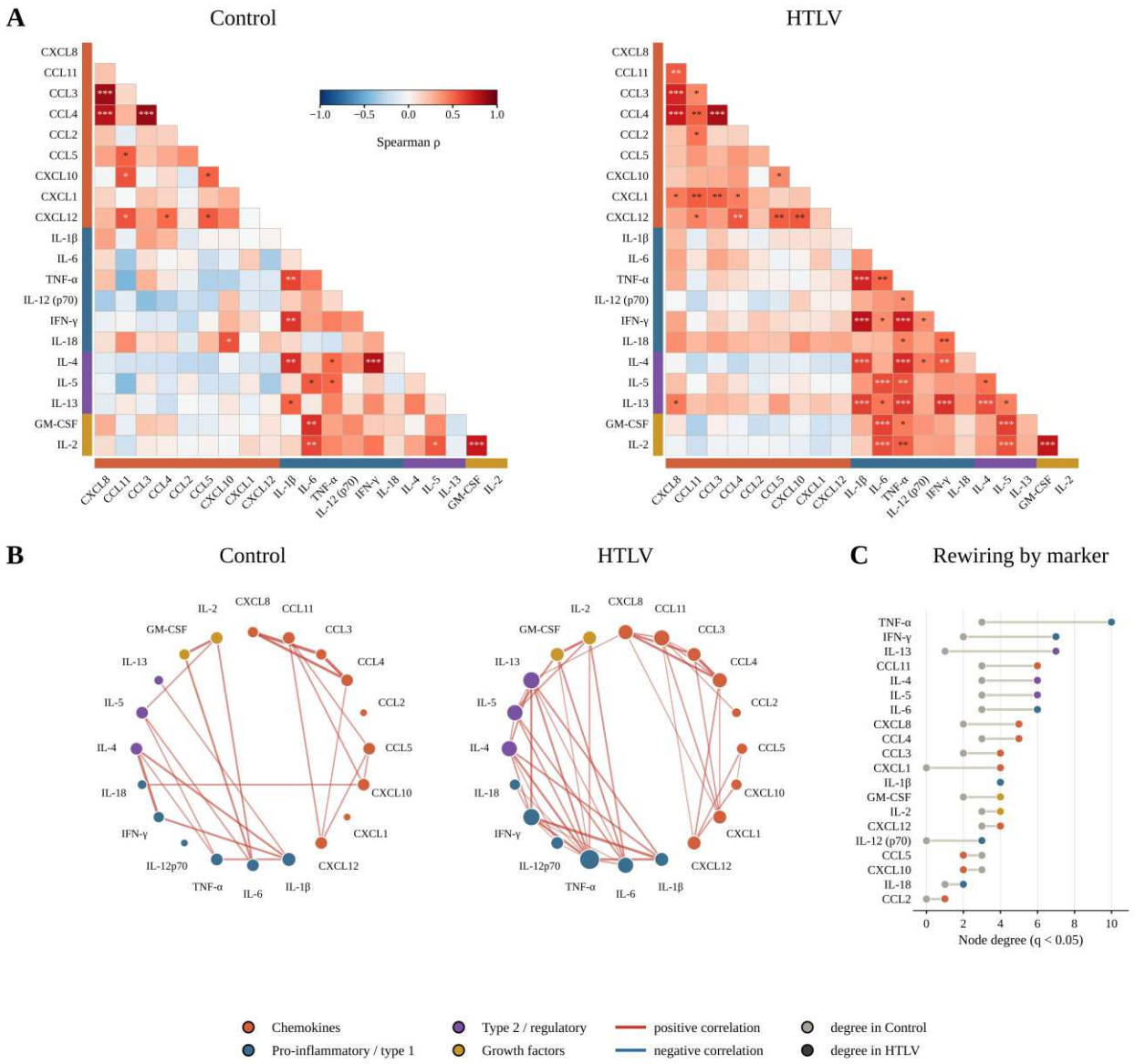


Figure 4. Correlation patterns and connectivity among immunological mediators in the Control and HTLV groups. (A) Spearman correlation matrices for the Control and HTLV groups, shown as lower triangles with mediators ordered by functional class. Colors indicate correlation direction and magnitude, from negative (blue) to positive (red)**.** Asterisks indicate statistical significance (*q < 0.05; **q < 0.01; ***q < 0.001). (B) Correlation networks showing the associations that remained significant after Benjamini–Hochberg correction, with node size proportional to degree and mediators grouped by functional class. (C) Node degree per mediator in each group; the Control network contained 22 significant correlations and the HTLV network 46.

Global remodeling of the immune profile associated with HTLV infection

Hierarchical clustering of the individual profiles is shown in Figure 5A. The mediators grouped by functional class, with chemokines, type 1 cytokines and type 2 cytokines forming separate blocks, whereas the samples did not separate into clusters corresponding to infection status. Within this structure, carriers showed higher relative intensity across the chemokine and type 2 cytokine blocks. Five mediators differed significantly between carriers and controls after FDR correction, with effect sizes ranging from moderate to large: CCL5/RANTES (Cliff's δ =

0.63), IL-13 (δ = 0.57), CXCL10/IP-10 (δ = 0.53), IL-18 (δ = 0.37) and IL-4 (δ = 0.35). The remaining mediators showed small effect sizes and did not differ between groups.

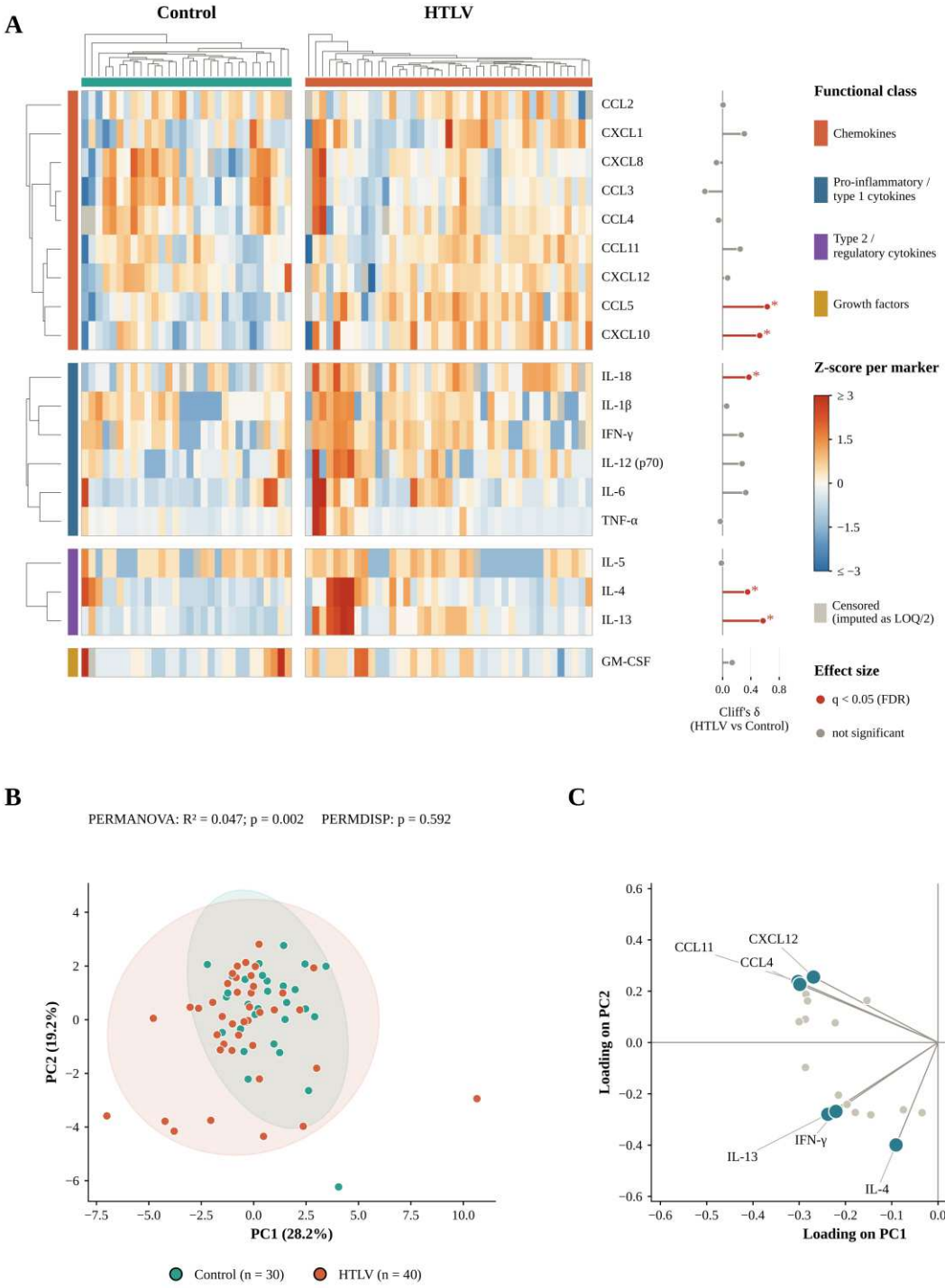


Figure 5. Global structure of the serum immune profile in the Control and HTLV groups. (A) Marker × sample heatmap of standardized mediator values (Z-scores), with mediators organized by functional class and participants

ordered by hierarchical clustering within each group (average linkage; Euclidean distance between participants, correlation distance between mediators). Red and blue indicate values above and below the marker mean, respectively, and gray cells represent non-quantifiable values imputed as LOQ/2. The adjacent plot shows Cliff's δ for each mediator (HTLV versus Control), aligned row by row with the heatmap; red points and asterisks mark the five mediators that remained significant after Benjamini–Hochberg correction. (B) Principal component analysis of the same matrix, with 95% confidence ellipses; the PERMANOVA and PERMDISP results for this comparison are shown above the panel. (C) Mediator loadings on PC1 and PC2, with the six largest combined contributors highlighted and labelled. IL-2 was excluded from the multivariate panels because 31 of 70 measurements were non-quantifiable.

Clustering participants without reference to group membership gave the same picture: no cluster corresponded to infection status, and the two- and three-cluster solutions matched group membership no better than chance (adjusted Rand index ≈ 0; Supplementary Figure S3). Local structure was nevertheless detectable, with 64.8% of participants having their nearest neighbour in the same infection status compared with 50.5% expected by chance (permutation $p = 0.021$; 47.9% versus 35.8% across the three groups, $p = 0.043$).

Principal component analysis was then used to assess the profile as a whole. The first two components explained 47.4% of the total variance (PC1, 28.2%; PC2, 19.2%), and the scores of the two groups overlapped substantially, with a partial displacement of carriers relative to controls (Figure 5B). PERMANOVA showed a significant association between infection status and overall mediator composition: infection explained 4.7% of the multivariate variation in the Control versus HTLV comparison ($R^2 = 0.047$; $p = 0.002$) and 5.6% when Control, HTLV-1 and HTLV-2 were analyzed separately ($R^2 = 0.056$; $p = 0.015$). Multivariate dispersion did not differ between groups in either comparison (PERMDISP, $p = 0.592$ and $p = 0.748$), indicating that the association reflected a displacement of group centroids rather than unequal within-group variability. The small proportion of variance explained is consistent with the overlap seen in both the ordination and the heatmap.IL-4, CCL11, CCL4, CXCL12, IL-13 and IFN-γ made the greatest combined contribution to PC1 and PC2 (Figure 5C), distributed across both axes of variation.

Contribution of functional modules and influence of key mediators

Considering that CCL5/RANTES, CXCL10/IP-10, and IL-13 showed the most consistent differences in the univariate analyses, a sensitivity analysis was performed to assess whether these mediators accounted for the observed multivariate effects. Complete models were compared with reduced models after removing CCL5, CXCL10, and IL-13 from the overall profile, CCL5 and CXCL10 from the chemokine module, and IL-13 from the type 2/regulatory module (Figure 6).

Removal of CCL5, CXCL10, and IL-13 approximately halved the multivariate effect in the overall profile, and the association no longer reached significance after FDR correction in any comparison. R^2 decreased from 0.047 to 0.024 for the combined HTLV analysis and from 0.045 to

0.021 for the HTLV-1 comparison, corresponding to 52% and 48% of the initial effect being retained (q = 0.35 and q = 0.47, respectively; Table S3). The residual signal was therefore consistent in direction but not distinguishable from noise at the present sample size.

The functional modules showed greater dependence on the leading mediators. In the chemokine module, removal of CCL5 and CXCL10 retained 33% of the initial effect in the Control versus HTLV comparison and 34% in the Control versus HTLV-1 comparison. In both analyses, statistical significance was lost, indicating that these two mediators accounted for a substantial proportion of the multivariate signal in this module. Similarly, removal of IL-13 from the type 2/regulatory module retained 40% and 31% of the initial effect in the Control versus HTLV and Control versus HTLV-1 comparisons, respectively, with loss of statistical significance.

These findings suggest a central contribution of IL-13 to the multivariate structure of this module. Multivariate dispersion also differed between groups in this module (PERMDISP, p = 0.015 for Control versus HTLV and p = 0.021 for Control versus HTLV-1), in contrast to the homogeneous dispersion observed for the global profile and for the chemokine module. The PERMANOVA results for the type 2/regulatory module therefore cannot be attributed to a difference in group centroids alone.

In the Control versus HTLV-2 comparison, the type 2/regulatory module showed the largest initial effect (R^2 = 0.128). After removal of IL-13, 58% of the effect was retained (R^2 = 0.074), although the association was no longer significant after adjustment for multiple comparisons (q = 0.35; nominal p = 0.040). This result suggests that other mediators may also contribute to the observed effect, although it should be interpreted cautiously given the smaller HTLV-2 sample size. Direct comparisons between HTLV-1 and HTLV-2 showed no significant multivariate differences.

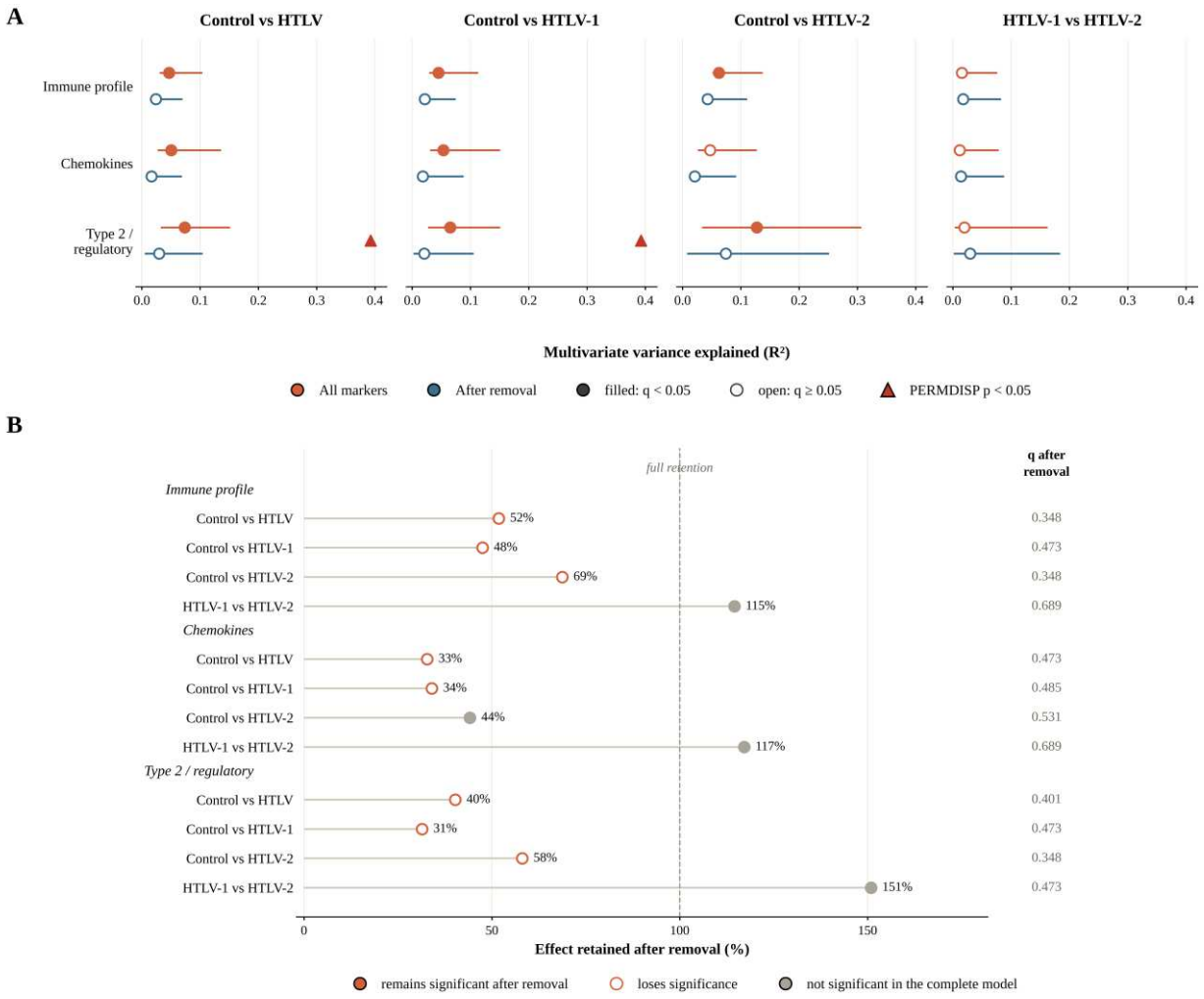


Figure 6. Sensitivity of the multivariate effect to the removal of the leading immunological mediators. (A) PERMANOVA R^2 for complete and reduced models of the overall profile, chemokine module, and type 2/regulatory module. The overall profile comprised the 19 mediators of the panel other than IL-2; the chemokine module comprised CXCL8, CCL11, CCL3, CCL4, CCL2, CCL5, CXCL10, CXCL1 and CXCL12, and the type 2/regulatory module IL-4, IL-5 and IL-13. CCL5, CXCL10, and IL-13 were removed from the complete profile; CCL5 and CXCL10 from the chemokine module; and IL-13 from the type 2/regulatory module. Red and blue dots indicate complete and reduced models, respectively. Filled and open circles indicate FDR < 0.05 and FDR ≥ 0.05, respectively. Bars represent 95% confidence intervals obtained by a group-stratified bootstrap with 2,000 replicates; triangles indicate PERMDISP $p < 0.05$. (B) Proportion of the initial multivariate effect retained after mediator removal. Filled points indicate significant associations, open points indicate loss of significance, and gray points indicate non-significant comparisons in the complete model. The dashed line indicates 100% preservation of the initial effect.

Discussion

This study jointly examined viral genetic diversity and systemic immune responses in HTLV-infected blood donation candidates, an approach rarely applied to carriers identified through blood bank screening. Two main findings emerged. First, three HTLV-2 subtypes co-circulated in an urban Amazonian setting where only HTLV-2c had previously been documented. Second, clinically asymptomatic carriers showed concurrent elevations in pro-inflammatory chemokines and type 2 cytokines, accompanied by greater connectivity among immune mediators. Phylogenetic analysis placed all HTLV-1 sequences within the Cosmopolitan subtype, Transcontinental subgroup (HTLV-1aA), the most widely distributed HTLV-1 subgroup worldwide and previously documented in northern Brazil, particularly in Pará [17,27,28,29]. The exclusive detection of HTLV-1aA in this cohort is consistent with its predominance throughout Brazil.

In contrast, HTLV-2 showed greater subtype diversity, with HTLV-2a and HTLV-2b detected alongside the predominant HTLV-2c. The latter has previously been identified in Manaus and is associated with circulation in Amazonian Indigenous populations [18,28]. Its presence in this urban cohort may reflect historical migration, population admixture, and sustained transmission within the region [28,30]. Although HTLV-2a and HTLV-2b had not previously been documented in this setting, both have been reported elsewhere in the Brazilian Amazon: HTLV-2b in a single blood donor from Belém, Pará [31], and HTLV-2a among blood donation candidates in the same state [29].

To our knowledge, this is the first description of HTLV-2a and 2b in Amazonas and the first demonstration of the simultaneous circulation of all three subtypes in a single urban population of the Brazilian Western Amazon. The nearest-reference relationships observed of the HTLV-2b sequences with reference strains from Italy and the United States, and of the single HTLV-2a sequence with a strain from Ghana, is consistent with introductions distinct from the HTLV-2c lineage historically associated with Amazonian Indigenous peoples, and with the role of Manaus as a destination for internal and international migration in the region [26]. Given the small number of HTLV-2a and HTLV-2b sequences and the short genomic region analyzed, these geographic affinities should be regarded as hypothesis-generating.

The immunological profile revealed significantly higher serum concentrations of CCL5, IL-13, CXCL10, IL-18 and IL-4 in HTLV-infected individuals compared with controls after correction for multiple comparisons; CXCL1, IL-6 and IL-12(p70) were also higher before correction but did not survive it. Stratifying by viral type did not change this picture: against

controls, CCL5, CXCL10 and IL-13 were elevated in both HTLV-1 and HTLV-2 carriers, and IL-18 reached significance in HTLV-2 but not in HTLV-1. When the two infected groups were compared directly with each other, no mediator differed significantly. Thus, the findings do not establish a viral type-specific immune profile but reveal alterations spanning inflammatory chemokines and cytokines involved in type 1 and type 2 responses. These alterations were detectable in clinically asymptomatic carriers, indicating that the absence of overt clinical.

CCL5 and CXCL10 emerged as central features of the immune profile, showing the largest effect sizes and, together with IL-13, accounting for much of the multivariate signal in sensitivity analyses. Their induction downstream of NF-κB, a pathway activated by Tax-1 and Tax-2, offers a plausible mechanistic link between HTLV infection and the observed chemokine elevations [32]. Both recruit immune cells to tissues and sustain inflammatory responses [33,34]. Previous studies have associated serum CCL5 and CXCL10 with dermatological manifestations and proviral load in HTLV-1 infection [35], while evidence supporting CXCL10 as a prognostic and surrogate marker of HAM/TSP derives primarily from cerebrospinal fluid [20,36]. Our findings extend these observations to clinically asymptomatic carriers identified through blood bank screening, showing that these chemokine alterations are detectable even in the absence of overt clinical manifestations. Their prominence across analyses supports longitudinal evaluation as peripheral blood markers of HTLV-associated immune alterations.

This inflammatory profile also included a type 2 component, with higher IL-13 levels in both HTLV-1 and HTLV-2 carriers. CXCL1, which promotes neutrophil recruitment [35], showed a parallel increase that did not persist after correction for multiple comparisons. In HTLV-1-infected lymphocytes, Tax transactivates IL-13 expression, and IL-13 has been proposed to act as an autocrine growth stimulus [37]. Its effects on antiviral responses, including IFN-γ production and cell survival, further suggest potential roles in the host–virus interaction [37,38]. Although these mechanisms were not examined here, the shared increase in IL-13 is consistent with an immunomodulatory component accompanying the inflammatory response, extending the observed signature beyond pro-inflammatory mediators alone.

IL-12(p70), a cytokine that promotes type 1 responses, was also higher in carriers, although this difference did not persist after correction for multiple comparisons. The direction of the finding contrasts with reports of reduced IL-12 expression in HTLV-1 infection [39] but is consistent with the observations by Furuya (1999) [40]. Differences in population characteristics, clinical status, sample type, and analytical methods may contribute to this discrepancy. In the present cohort, the concurrent elevation of IL-13 and IL-4 without any corresponding reduction in type 1 mediators argues against interpreting the profile as a simple shift toward Th2 polarization. Together, these

findings suggest a mixed systemic immune signature in clinically asymptomatic infection, revealing immune alterations that may not be apparent from clinical status alone.

IL-18 showed the opposite pattern to that previously described. Reduced IL-18 has been reported in HTLV-1 carriers [39], whereas concentrations were elevated in the present cohort. Because IL-18 promotes T and NK cell activation and amplifies inflammatory responses [41], its elevation is consistent with persistent immune stimulation; the discrepancy with earlier work may reflect differences in clinical status, sample matrix and analytical platform, and highlights the heterogeneity of the immune response to HTLV across populations.

Beyond these individual mediators, the multivariate analyses provided a complementary and arguably more informative description of the host response. The correlation network analysis revealed that HTLV infection was associated not only with altered mediator concentrations but also with increased connectivity among immune mediators. Specifically, IFN-γ, IL-13, TNF-α, IL-6, and CXCL1 exhibited more connections in the HTLV group, while the overall network structure seen in controls was largely maintained. Importantly, IFN-γ and TNF-α showed increased connectivity despite no significant differences in their concentrations between groups, indicating that the coordination and integration of the immune response may be altered by infection—a feature not captured by univariate analyses. Repeated subsampling to match the control group size confirmed that the observed increase in connectivity was not due to differences in statistical power.

These changes formed a detectable, though modest, population-level signature. PERMANOVA identified a significant difference in overall mediator composition between carriers and controls, with no evidence of unequal dispersion by PERMDISP. The type 2/regulatory module was an exception, suggesting that variability among carriers may also contribute to the separation in this compartment. Across sensitivity analyses, infection status accounted for 4.7–5.6% of the total variance. Removing CCL5, CXCL10, and IL-13 abolished statistical significance across all marker sets, although the global profile retained 52% of its original effect size. This pattern highlights the contribution of these three mediators while suggesting that additional alterations contribute to the broader signature. Whether this group-level association can discriminate infection status in individuals requires predictive modeling with internal validation and evaluation in independent cohorts.

These findings challenge the assumption that asymptomatic HTLV infection is biologically quiescent. Although most HTLV-1 carriers remain free of clinically overt disease throughout life [10], emerging evidence suggests that the absence of symptoms does not necessarily imply immunological inactivity. In a long-standing Brazilian cohort, systematic re-evaluation revealed that approximately one quarter of individuals previously classified as

asymptomatic met the criteria for HTLV-1-associated multiple inflammatory disorder, an intermediate condition characterized by higher proviral loads, chronic immune activation, subtle spinal cord abnormalities, and cognitive impairment [42]. Moreover, a proportion of individuals with this intermediate syndrome subsequently progressed to overt myelopathy [22]. While these observations emerged from longitudinal clinical and neurological assessments at a referral center, our findings derive from serum mediator profiling of blood donation candidates in an endemic region. Despite differences in study populations and methodological approaches, both lines of evidence converge on a central conclusion: The absence of overt clinical manifestations does not preclude a distinct systemic immune signature in HTLV infection.

In the present study, participants were classified as asymptomatic on the basis of blood donation screening, which does not include standardized neurological or rheumatological evaluation. The systemic inflammatory profile described here is therefore compatible with two non-exclusive interpretations: a generalized immune activation inherent to chronic HTLV infection, or the inclusion of individuals already in an early inflammatory stage that donation screening cannot detect. Distinguishing between these possibilities requires standardized clinical and neurological assessment combined with proviral load measurement, neither of which was available here.

This study has limitations that should be considered when interpreting its findings. First, the cross-sectional design precludes inference about progression, and the relationship between the mediators identified here and the risk of HTLV-associated disease remains hypothetical. Second, proviral load was not measured, preventing assessment of the association between viral burden and immune activation, which is among the best-established determinants of inflammatory status in HTLV-1 infection; Third, clinical characterization was limited to donation screening, as discussed above. Fourth, the number of HTLV-2 sequences, and particularly of HTLV-2a and HTLV-2b, was small, so subtype-stratified immunological comparisons should be regarded as exploratory and were not powered to detect modest differences. These limitations restrict inferences about disease progression, but the standardized cross-sectional analyses provide robust evidence of systemic immune alterations in asymptomatic HTLV carriers and a foundation for future longitudinal studies of their clinical significance.

Conclusion

This study characterizes the genetic diversity of HTLV in the Brazilian Western Amazon, identifying HTLV-1aA and the co-circulation of the HTLV-2a, HTLV-2b, and HTLV-2c subtypes among blood donation candidates, showing that HTLV-2 diversity in the region is not restricted to the 2c subtype historically associated with Amazonian Indigenous populations. Although family contacts were not investigated, the detection of diverse HTLV lineages among asymptomatic carriers underscores the importance of sustained molecular surveillance and highlights the potential value of extending screening to sexual partners and potentially exposed family members in endemic areas.

Asymptomatic HTLV-1/2 infection was associated with a distinct systemic immune profile, with five mediators elevated in carriers after correction for multiple comparisons. Multivariate analysis revealed a significant shift in overall mediator composition, with prominent involvement of CCL5, CXCL10, and IL-13. These group-level differences persisted despite substantial interindividual heterogeneity and spanned chemokines and both type 1 and type 2 cytokines, indicating that HTLV infection is reflected in a coordinated mediator profile rather than in any single analyte.

These findings highlight the value of linking HTLV detection through blood bank screening to structured counseling, family-based screening, and longitudinal care. The immune differences observed in asymptomatic carriers provide a basis for further investigation, but their clinical significance remains uncertain. Longitudinal studies combining immune profiling, proviral load measurements, and clinical assessment are needed to determine whether these alterations persist over time and are associated with subsequent disease development.

Declarations

Ethics approval and consent to participate

The study was approved by the Research Ethics Committee of HEMOAM (protocol no. 5,348,608/2021; CAAE no. 57153922.5.0000.0009). Written informed consent was obtained from all participants before enrollment.

Consent for publication

Not applicable.

Availability of data and materials

The nucleotide sequences generated in this study were deposited in GenBank under accession numbers PZ665096–PZ665122 (HTLV-1) and PZ665123–PZ665135 (HTLV-2). The immunological datasets analyzed during this study are available from the corresponding author on reasonable request, subject to applicable ethical and privacy restrictions.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions (CRediT):

K.C.S.: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. V.K.S.A.: Methodology, Writing – original draft. F.A.S.: Methodology. U.H.S.P.: Methodology, Investigation. J.M.S.: Formal analysis, Methodology, Resources, Writing – original draft, Writing – review & editing. A.G.C.: Funding acquisition, Investigation, Methodology, Resources, Writing – review & editing. G.M.G.M.: Methodology, Writing – original draft, Writing – review & editing. J.S.B.: Methodology, Writing – review & editing. D.A.O.: Methodology, Writing – review & editing. A.T.C.: Data curation, Investigation, Methodology, Writing – review & editing. F.K.B.: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. N.R.G.: Data curation, Investigation, Methodology, Writing – review & editing. L.C.J.A.: Data curation, Investigation, Methodology, Writing – review & editing. G.S.P.: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. All authors read and approved the final version of this manuscript.

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