Variation in antiviral immunity and inflammation pathways precedes HIV-1 infection in a high-risk African cohort

Mwikali Kioko[1]*, Shaban Mwangi[1], Lynn Fwambah[1], Amin S. Hassan[1,2], Jason T. Blackard[3], Philip Bejon[1,4], Eduard Sanders[5,6], Thumbi Ndung'u[7,8,9,10], Eunice W. Nduati[1,4,11†], Abdirahman I. Abdi[1,4,11†]

[1]Bioscience Department, Kenya Medical Research Institute-Wellcome Trust Research Programme, Kilifi, Kenya

[2]Institute for Human Development, Aga Khan University, Nairobi, Kenya

[3]Division of Gastroenterology & Hepatology, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA

[4]Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK

[5]Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

[6]The Aurum Institute, Johannesburg, South Africa

[7]Africa Health Research Institute, Durban, South Africa

[8]HIV Pathogenesis Programme, The Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban, South Africa

[9]Ragon Institute of Mass General Brigham, Massachusetts Institute of Technology and Harvard University, Cambridge, MA, USA

[10]Division of Infection and Immunity, University College London, London, UK

[11]Pwani University Biosciences Research Centre, Pwani University, Kilifi, Kenya

*To whom correspondence should be addressed: Mwikali Kioko: P.O Box 230-80108, Kilifi, Kenya, +254711776932, kmwikali@kemri-wellcome.org

†These authors share senior authorship.

Conflict-of-interest

The authors have declared that no conflict of interest exists.

ABSTRACT

Background

Susceptibility to human immunodeficiency virus type 1 (HIV-1) infection varies between individuals, but the biological determinants of acquisition risk remain poorly defined.

Methods

We conducted a case-control study nested within a high-risk cohort in Kenya. We compared the plasma extracellular RNA collected before HIV-1 acquisition with matched uninfected controls to identify immunological processes linked to infection risk.

Results

Individuals who later acquired HIV-1 exhibited upregulation of immune processes that facilitate viral infection, including T cell suppression, type II interferon and Th2 immune responses. In contrast, processes associated with antiviral defence and tissue repair, such as neutrophil and natural killer cell responses, type I interferon responses, wound healing, and angiogenesis, were downregulated.

Conclusion

These findings highlight dampened antiviral immunity prior to exposure as a correlate of increased risk for subsequent HIV-1 acquisition.

Trial number

Not applicable

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INTRODUCTION

Susceptibility to human immunodeficiency virus 1 (HIV-1) infection varies significantly across populations and individuals (1-3). For example, analysis from multiple studies showed that sub-Saharan Africa has a higher risk of HIV-1 transmission per sexual contact compared to higher-income regions (1). Although these differences may reflect low access to antiretroviral drugs in sub-Saharan Africa at the time, intra-population differences in susceptibility have been documented in a longitudinal study of high-risk Kenyan adults, in which only 7% were infected during follow-up despite likely widespread exposure (4). This variability stems from a diverse range of factors, including behavioural differences, viral load, characteristics of circulating viruses (including HIV-1 subtype), and host-related factors such as genetic diversity and environmental exposures such as sexually transmitted infections (STIs) that can modulate basal immune status (2, 5, 6). However, the specific host biological factors and pre-existing pathogens associated with HIV-1 acquisition are not fully known.

Identifying biological determinants of HIV-1 susceptibility is crucial for developing diagnostic biomarkers and interventions (7, 8). High-throughput omic techniques, including proteomics and transcriptomics,

are increasingly employed to understand host mechanisms predisposing to HIV-1 infections (4, 9). Transcriptomics offers a sensitive method to detect subtle differences in gene expression, providing insights into the host's immune response and immunodulatory pathogens (10).

All cells secrete a diverse population of RNA collectively called extracellular RNAs (exRNAs) into biofluids such as plasma, saliva, and urine (11, 12). The majority of these exRNAs are secreted within membrane-bound vesicles called extracellular vesicles (EVs), which protect them in the harsh extracellular space (11-18). Additionally, the profiles of circulating exRNAs largely reflect the biological state of the secreting cells, which provides a more holistic view of systemic biological processes (19-21) and pathogen signals (22-26) relative to the cellular RNA obtained from peripheral immune cells. Therefore, analyzing plasma-derived exRNA from pre-infection samples may provide valuable immune correlates of HIV-1 acquisition.

Here, we highlight transcriptional immune correlates of HIV-1 susceptibility by retrospectively analysing plasma-derived exRNA collected before HIV-1 infection in a case-control study nested within a longitudinal cohort of HIV-negative high-risk individuals in coastal Kenya (27).

RESULTS

Plasma exRNA highlights immunological pathways associated with HIV-1 acquisition risk

The primary objective of this study was to identify pre-infection transcriptional correlates of HIV-1 acquisition in high-risk adults. To achieve this, we took advantage of a long-term longitudinal cohort of high-risk individuals on the Kenyan coast, for whom the dates of HIV infection have previously been estimated (4, 27-29) as summarised in Figure 1 and described in detail in the Methods. We compared plasma-derived exRNA from individuals who later acquired HIV-1 (cases; n=32), collected approximately 3±2 months prior to the estimated date of infection, to that from matched negative controls (n=64) (Figure 1). This analysis identified 767 genes with differentially increased abundance and 774 genes with significantly decreased abundance in HIV-1 cases at a false discovery rate (FDR) of less than 5% (Figure 2A). Next, we performed principal component analysis (PCA) and supervised heatmap clustering on the differentially enriched genes and found that the transcriptional profiles of EVs distinguished controls from the HIV-1 cases (Figure 2B, C). The differentially increased genes included the endothelial nitric oxide synthase (*NOS3*), angiotensin-converting

enzyme 2 (*ACE2*), interleukin 17 and 21 receptors (*IL17RA*, *IL17RD*, *IL21R*), the viral-sensing Toll-like receptor 7 (*TLR7*), and the inhibitor of *IRF3*- and NF-kB-dependent antiviral response gene (*ILRUN*) (30) (Figure 2C). In contrast, the differentially decreased genes featured the pro-angiogenic factor *VEGFA*, the interferon regulatory factors (*IRF1*, *IRF3*, *IRF4*, and *IRF5*), and the p53 negative regulator *MDM2* (Figure 2C).

Cell enrichment analysis demonstrated that the genes upregulated in HIV-1 cases 3±2 months prior to infection belonged to cells such as eosinophils, plasma B cells, central memory CD8-T cells, plamacytoid dendritic cells (pDCs), and Th2 cells (Figure 2D). In contrast, the downregulated genes were enriched for signatures associated with several cell types, including natural killer (NK) cells, B-memory cells, and neutrophils (Figure 2D**)**. Next, we performed pathway enrichment analysis of the 767 genes increased in HIV-1 cases, revealing an overrepresentation of genes linked to endothelial nitric oxide synthase (eNOS), IL-17 and IL-10 signalling, suppressive T-cell response, and apoptosis (Figure 2E). Conversely, the 774 genes decreased in HIV-1 cases belonged to a wide range of biological pathways, including reparative processes (wound healing and p53-signalling pathway) and

pathways related to type-I interferon (IFN), including NFKB activation by protein kinase R (PKR) and IFN-beta signalling (Figure 2E). These findings suggest that reduced type I interferon and pro-reparative immune responses, alongside elevated eNOS, suppressive T cell response, IL17 and IL10 signalling, are strongly linked to HIV-1 acquisition in high-risk adults.

Plasma exRNA clustering uncovers distinct immunological endotypes in HIV-1 cases and controls.

There could be heterogeneity in the biological mechanisms that underlie protection or susceptibility to HIV-1 infection, which is obscured when comparing the average biological signals between cases and controls. To reveal intragroup heterogeneity and biological signal, we constructed a participant similarity network (PSN) using the exRNA dataset generated from the samples collected 3±2 months prior to HIV-1 infection. Spectral clustering of the similarity network identified five endotypes of study participants - named A, B, C, D, and E - of which endotypes A, B, and C were enriched for controls, while D and E were enriched for HIV-1 cases (Figures 3A-C). We subsequently performed differential feature analysis and identified over 4000 genes whose exRNA profiles differed significantly between the endotypes, surpassing

the differential signal observed in the case-control analysis (Figure 3D, Supplemental Table 1). Pathway enrichment analysis revealed that the control endotypes were enriched for features associated with pro-reparative processes (wound healing, TGF-beta/SMAD signalling, VEGF overexpression and histamine metabolism), T cell function (T cell CD3, T cytotoxic cell surface, co-stimulatory T cell activation, granzyme-B pathway and CTLA4 signalling), mitochondrial function (protection against ROS, Keap1-Nrf2, respiratory electron transport, citric acid cycle), and type I IFN signalling (IFN Beta signalling pathway, cGAS-STING-TBK1 pathway, TLR-TRIF pathway, NFKB activation by PKR) (Figure 3E).

The two endotypes composed mainly of HIV-1 cases (Figure 3A-C) were also enriched for distinct pathways, with genes augmented in endotype D featuring those linked to eNOS signalling, regulatory T cells, CXCR4 signalling, and FAS-mediated apoptosis (Figure 3E). Finally, endotype E showed evidence of increased apoptosis, including HIV-1 mediated T cell apoptosis, TRAIL and DR3 death receptor signalling. Signatures of B-cell differentiation, IL-7 signalling, and suppressor of cytokine signalling (SOCS) were also enriched in endotype E (Figure 3E). Our endotyping analysis revealed more differentially expressed genes, and

enrichment analysis compared to case-control analysis, which suggests that different biological mechanisms could promote or impede HIV-1 infection.

The immunological processes observed at 3±2 months were conserved at 6±2 months prior to HIV-1 infection.

To investigate whether the immune profile observed 1 to 5 months prior to HIV-1 infection was also evident at earlier time points, we analysed the transcriptional profiles from 9 individuals who later acquired HIV-1 and 29 matched controls who remained uninfected, using samples collected 4 to 8 months before the cases became HIV-1 positive. We found that 2688 genes were significantly increased in HIV-1 cases, while 4521 genes were significantly decreased (Figure 4A, Supplemental Table 2). Cellular enrichment analysis of the altered genes showed significant downregulation of genes belonging to natural killer cells (e.g. *NCAM1*, *FCGR3A*), plasma B-cells (e.g. *CD38*), and pDCs (Figure 4A-B). When we performed pathway over-representation analysis, we observed that genes upregulated 6±2 months prior to HIV-1 infection featured those belonging to type II interferon signalling (e.g. *CXCR3*, *IFNG*, *IL19*, and *CXCL9*) (Figure 4C). On the other hand, genes downregulated 6±2 months prior to HIV-1 infection were enriched for

type I interferon signalling (e.g. *IRF3, IRF9, JAK1, STAT2, STAT5A, IL1B, TLR2, TLR4*), and VEGFA-VEGFR2 signalling, consistent with the 3±2 months prior to infection timepoint (Figure 4C). These observations confirm that reduced type 1 interferon-driven innate immunity, together with an elevated type II interferon state, precedes HIV-1 infection.

The presence of human pegivirus type-1 (HPgV-1) is associated with HIV-1 acquisition.

We next analyzed the exRNAseq data using a metatranscriptomic approach to nominate potential pathogens associated with HIV-1 susceptibility. HPgV-1 RNA abundance was significantly higher in HIV-1 cases than controls three months before HIV infection (log_2fold-change>4, FDR<0.05) but not at six months (Figure 5A). Applying more stringent criteria (>5 reads) to define HPgV1 positivity, rather than considering any detectable HPgV1 RNA level as positive, we identified 20 HPgV1 positives. HPgV-1 positivity was non-significantly higher among HIV-1 cases than controls at both three months (28% in cases versus 17% in controls; OR = 1.89, 95% CI 0.69-5.16, p = 0.29) and at six months (22% in cases versus 14% in controls; OR = 1.79, 95% CI 0.27-11.86, p = 0.61, indicating a modest enrichment of HPgV1 among individuals who later acquired HIV-1 (Figure 5B). 14 participants were

identified as HPgV-1 positive by conventional PCR, of which only 3 of them were not detected using NGS. (Figure 5C). Poisson regression analyses showed that HPgV-1 infection detected by next-generation sequencing (NGS) and PCR at 3 ± 2 months prior to HIV-1 infection was significantly associated with HIV-1 acquisition (NGS: RR = 1.99, 95% CI 1.11-3.55; PCR: RR = 2.32, 95% CI 1.32-4.08) (Figure 5D). However, after adjustment for other sexually transmitted infections, the association was reduced (NGS: RR = 1.51, 95% CI 0.88-2.61; PCR: RR = 1.66, 95% CI 0.96-2.87), indicating that HPgV-1 was not an independent predictor of HIV-1 acquisition. We next compared the endotypes by HPgV1 status, revealing that individuals clustered in endotype D were more likely to be HPgV1 positive compared to endotypes A or B (Figure 5E). To assess the impact of HPgV-1 on transcriptional alterations between HIV-1 cases and controls, we compared the transcriptional difference between HIV-1 cases and controls, before and after adjusting for HPgV-1 status. We found a high correlation ($R=0.97$, $P<0.0001$) of the $\log_2$fold changes before and after adjusting for HPgV1 (Figure S1, Supplementary Table 3**)**. Further, 120 and 201 of the upregulated and downregulated genes between HIV-1 cases and controls, respectively, showed significant differential abundance between HPgV-1 positive and negative individuals (Figure S2, Supplementary Table 4). We also compared

transcriptional changes between HPgV1-positive and -negative individuals within both the HIV cases and the control groups and found overlaps of 37 (3.7%) and 38 (4.8%) upregulated and downregulated genes, respectively (Figure S3; Supplementary Table 5). Additionally, we reanalysed previously published transcriptional data from PBMCs that were either exposed or unexposed to HPgV1 *in vitro*. The reanalysis revealed only 12 genes (6 upregulated and 6 downregulated) with concordant expression between exRNA and PBMCs (Figure S4 and Supplementary Table 6).

Finally, we assessed the genetic relatedness of the HPgV-1 genome sequences from the 3±2 months prior to infection samples relative to those from other parts of the world. We generated 11 partial HPgV-1 genomes, of which 4 were from the controls and 7 were from the HIV-1 cases. We next performed phylogenetic analysis and found that the HPgV-1 genomes clustered by geographic origin, with our partial genomes co-clustering with those from other African countries, consistent with previous studies(31) (Figure 5F).

DISCUSSION

In this study, we leveraged plasma-derived exRNA to determine pre-infection immune correlates of HIV-1 acquisition among high-risk adults in a longitudinal cohort study (27, 29). We highlight key findings, explore their biological relevance to HIV-1 susceptibility, and offer potential avenues for future research and intervention. Given that the profiles of circulating exRNA often mirror molecular activities in the tissues most affected by a specific condition (32), in this case, the mucosal sites that serve as primary portals of HIV-1 entry, we also discuss our observations within the context of mucosal immune regulation.

Our differential feature analysis showed that, three months prior to HIV-1 infection, individuals who later got infected exhibited significant alterations in exRNA profiles compared to the controls. Notably, transcripts associated with IL-17 receptor signalling, apoptosis, regulatory T-cells, and eNOS signalling were upregulated in HIV-1 cases. Higher sexual activity, particularly receptive anal intercourse (33-35), together with STIs, may promote mucosal damage, immune activation and apoptosis, events that compromise barrier integrity and facilitate viral entry. The elevated IL-17 receptor and eNOS signalling, along with Treg responses, may represent compensatory mechanisms

that restore mucosal homeostasis (36-43) but could also be induced by STIs and anal intercourse (33, 44). However, chronic activation of these pathways could sustain inflammation and tissue damage. Moreover, enhanced IL-17 receptor signalling may also drive chemokines that enhance Th17 cell recruitment at mucosal sites (45), key HIV-1 target cells (46-48). While Tregs help reduce immune activation, they are also susceptible to HIV-1 infection (49, 50) and can weaken antiviral response, collectively enhancing susceptibility to HIV-1 acquisition.

A key observation from our study was the downregulation of genes linked to type I interferon response, accompanied by an upregulation of type II interferon-associated transcripts in individuals who later acquired HIV-1. This pattern suggests a reprogramming of the immune landscape toward a less antiviral (30, 51-57) and more inflammatory state, which may increase the expression of key HIV-1 entry receptors such as CCR5 (58-60), thereby increasing susceptibility to HIV-1 infection. The suppression of type I interferon response may be driven by elevated IL-17 signalling, given that type I interferon and Th17 responses are known to act antagonistically (61). Indeed, individuals with a gain-of-function mutation in type 1 interferon signalling are predisposed to fungal infection due to impaired Th17 responses (62, 63), while chronic

hyperactivation of Th17 responses has been associated with increased susceptibility to viral infections (45, 64, 65).

Our endotyping analysis identified five distinct endotypes, reflecting significant heterogeneity in the biological mechanisms at play. Three endotypes - A, B, and C -predominantly comprising controls, displayed immune profiles consistent with effective antiviral immunity (66, 67) and restrained immune activation, characterised by enhanced type I interferon response, T-cell function, TGF-beta/SMAD signalling, and oxidative phosphorylation. These features likely contribute to efficient antiviral defence (57) and maintenance of mucosal health. For example, increased TGF-beta could confer protection against HIV-1 infection through maintaining an effective mucosal immune system impervious to viral entry (68, 69) or inhibiting the pro-HIV type II IFN immune response (70). In contrast, the two susceptibility endotypes, D and E, were enriched for regulatory T-cells and FAS-mediated signalling, TRAIL, and SOC3 pathways, signatures that suppress antiviral immunity and enhance mucosal disruption (71-73). Together, these findings suggest that pre-infection immune heterogeneity, particularly involving interferon balance and T-cell function interplay, critically shapes HIV-1 acquisition

risk and may inform precision prevention strategies. However, a larger study is necessary to identify the true heterogeneity of HIV-1 risk.

Our metatranscriptomic analysis identified HPgV-1 (also called human pegivirus C type 1 or GB virus C [GBV-C] or *Pegivirus hominis*) as significantly associated with HIV-1 acquisition, albeit less pronounced when adjusting for other STIs. HPgV-1 is a flavivirus that infects lymphocytes and NK cells and is transmitted by blood transfusion, sexual exposure, and mother-to-fetal transmission (74). While our data suggests that HPgV-1 is a correlate of HIV-1 acquisition, its predictive value is influenced by the presence of other STIs. This suggests that HPgV-1 may not directly drive susceptibility but instead reflects a permissive host immune environment conducive to sexually transmitted viral infection, thus representing a biomarker for HIV-1 risk.

Interestingly, during established HIV-1 infection, HPgV-1 has been linked to slower progression to acute immunodeficiency syndrome (AIDS) (75-79). A plausible explanation, which is consistent with our data, is that HPgV-1 exploits an immune milieu characterised by reduced type I and elevated type II interferon response (80), an immune balance that favours viral acquisition but limits immunopathology (81-84). However,

a direct role for HPgV-1 in modulating host immunity cannot be ruled out, as suggested by other studies (26, 85-87).

The retrospective design of our study represents a key limitation. Concurrent collection of mucosal samples alongside blood would have allowed direct validation of the immunological signatures inferred from exRNA analyses against local mucosal responses. Consequently, some of our interpretations, although supported by existing literature, remain speculative and require confirmation through prospective studies.

In summary, we highlight the strength of plasma exRNAseq in uncovering pre-infection biological correlates of HIV-1 acquisition. Future research should focus on validating the predictive value of HPgV-1 in larger cohorts and exploring its utility in predictive models and targeted interventions. In conclusion, understanding the biological drivers of HIV-1 susceptibility among high-risk populations could enhance the development of prevention and treatment strategies.

METHODS

Sex as a biological variable

Samples from cases and controls were obtained from both men and women. In our study, sex was not considered a biological variable of interest.

Study design and population

3±2 months prior to HIV-infection samples: A case-control study nested in a historic HIV-1 high-risk cohort from Coastal Kenya was conducted. HIV-1 negative high-risk volunteers, including men-who-have-sex-with-men (MSM) and female sex workers (FSW) aged ≥18 years, were recruited and followed from 2006 to 2011 for HIV-1 vaccine preparedness studies. Volunteers were screened for incident HIV-1 infection during follow-up using RT-PCR, p24 antigen, and HIV-1-specific antibody assays as previously described (4, 28). For any volunteer testing HIV-1 positive, an estimated date of infection (EDI) was calculated either to be: 10 days before a positive HIV-1 RNA test (if antibody negative), 14 days before a p24 antigen positive test (if RNA test was missing), or midway between the last negative and first positive HIV-1 specific antibody test (if both RNA and p24 tests were missing). Cases were defined as volunteers who tested HIV-1 positive, while

controls were those who remained negative at the end of a similar follow-up period (4). Plasma samples from cases were collected 3±2 months prior to the EDI, with controls matched 2:1 to cases based on sex, age, risk group, follow-up duration, and plasma sample availability.

6±2 months prior to HIV infection samples: Plasma samples collected 6±2 months before the EDI were retrieved. Controls were matched 2:1 to HIV cases based on age, sex, risk group, follow-up duration in the study and the availability of plasma samples collected at around the same calendar date as that of the index case ±2 months.

Isolation of extracellular RNA

Nanofiltration and ultracentrifugation were used to isolate exRNA, aiming to primarily enrich for those encapsulated in small EVs, as described previously (19) In brief, 13.5 ml of prefiltered PBS was combined with 300 µL of plasma in a 15 ml Falcon tube. The diluted plasma was filtered through a 0.22 µm (Millipore) filter to exclude cell debris and centrifuged at 150,000 x g for 2 hours at 4 °C without breaks. The pellets were treated with RNAse A for 15 min and washed at 150,000 x g for two hours at 4°C. The impact of the RNase treatment was evaluated by comparing the exRNA profile before and after treatment using bioanalyzer/Agilent TapeStation (Figure S5). The

supernatant was discarded while the pellets were digested using 250 µl of RNA lysis solution (Bioline) and stored at -80°C until needed. ExRNA was extracted from the lysed pellets using the Isolate II RNA Mini Kit (Bioline) as directed by the manufacturer.

Bead-assisted flow cytometry

Evaluation of small EV markers in our pellets was performed using bead-assisted flow cytometry (Figure S5), as we previously described (88). Briefly, 50 µL of EVs in PBS were incubated with 1 µL of aldehyde/sulfate latex beads (Invitrogen) in a total volume of 1 mL PBS for 12 hours at room temperature on a rotary mixer. Following incubation, 110 µL of 1 M glycine was added to block unreacted sites, and the mixture was incubated for an additional 30 minutes at room temperature. Beads were pelleted by centrifugation at 2000 × *g* for 5 minutes and washed once with 1 mL PBS. The pellet was resuspended in PBS supplemented with 0.5% fetal bovine serum (PBS + 0.5% FBS) and stained with 1× anti-CD9-APC (Cat. No. 341648, BD Biosciences) and 1× anti-CD63-PE (Cat. No. 55705, BD Biosciences). Negative controls included beads incubated with (i) antibody cocktail without EVs, and (ii) isotype control antibodies: PE mouse IgG1 (Cat. No. 556650, BD Biosciences) and APC mouse IgG1 (Cat. No. 550854, BD Biosciences). Stained beads were

washed twice with 500 µL PBS + 0.5% FBS and pelleted by centrifugation at 2000 × *g* for 10 minutes. Data acquisition was performed using a BD Fortessa flow cytometer.

cDNA library preparation

We used our previous protocol(19, 88) to prepare the cDNA libraries for sequencing. Briefly, Superscript III (Invitrogen) was used to produce the first strand from the total exRNA. Before synthesizing the second strand, the first strand reaction was cleaned using RNAcleanXP beads. dTTP was replaced with dUTP while synthesizing the second strand to generate double-stranded cDNA. The cDNA was fragmented, end-repaired and ligated to adapters. The cDNA was treated with USER followed by 19 cycles of PCR amplification to add Illumina primers and increase yield. Sequencing was performed using the NextSeq 550 genome analyzer.

Quantification of HPgV-1 using PCR

HPgV-1 RNA was converted to cDNA using Superscript III reverse transcriptase (NEB). HPgV-1 positive samples were detected by amplicon-targeted PCR amplification of the 5' untranslated region (UTR) with the antisense primer 5' - ATG CCA CCC GCC CTC ACC CGA A - 3' (nucleotides [nt] 494-473 according to GenBank accession number

AY196904) and the sense primer 5’ - AAA GGT GGT GGA TGG GTG ATG - 3' (nt 67-87) using Q5® High-Fidelity DNA Polymerase (New England Biolabs). Amplification conditions were 50°C for 59 minutes, 10 minutes at 94°C, then 35 cycles of 30 seconds at 94°C, 1 minute at 55°C, and 1 minute at 72°C, followed by 20 minutes at 72°C. First-round polymerase chain reaction (PCR) products were used in nested PCR with the antisense primer 5’ – CCC CAC TGG TCY TTG YCA ACT C - 3' (nt 362-341) and sense primer 5' – AAT CCC GGT CAY AYT GGT AGC CAC T - 3' (nt 107-131). After 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C, PCR products were visualized by agarose gel electrophoresis for the presence of a 256 nt band.

Statistics

Gene body read coverage depicted in Figure S7 was calculated using the *RSEQC* tool. Transcript quantities in the units of raw read counts and transcripts per million (TPM) were estimated by aligning the data to the human transcriptomes using *salmon* and *tximport*. Comparison between cases and controls was performed using *edgeR*. Raw read counts were normalized using the relative log expression (RLE) method, and the likelihood ratio test was chosen. *P*-values were adjusted for multiple testing using the Benjamini-Hochberg statistical procedure, and an FDR

threshold of less than 5% was set as the cut-off for significance. Endotyping was conducted using spectral clustering, while differences in gene expression between the endotypes were determined in *edgeR* as described above. Cellular overrepresentation was performed using protein signatures derived from a previously published study(89) while pathway genesets were obtained from *Literature Lab*(90) and *Wikipathway*(91). Pathogen classification was performed using Kraken2, while a comparison of pathogen abundance between cases and controls was performed using *edgeR*. In parallel, the predictive value of HPgV-1, as measured by both sequencing and PCR, was also assessed by calculating risk ratios, with or without adjustment of other STIs. HPgV-1 phylogenetic tree was generated by first performing multiple sequence alignment using *nextalign* followed by tree reconstruction using *iqtree*. Unless stated otherwise, all visualizations were carried out using *ggplot2* and *ComplexHeatmap* R packages.

STUDY APPROVAL

The samples used in this study were collected using the IAVI protocol B, reviewed by the Kenya Medical Research Institute Ethical Review Committee. Participants provided their written informed consent.

DATA AVAILABILITY

This study did not produce unique reagents or materials. RNAseq data have been deposited at GEO under the accession number GSE287060 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE287060). Datapoint values for all graphs are available in the Supporting Data Values file.

AUTHOR CONTRIBUTIONS

A.I.A. E.W.N. and T.N. jointly conceived the project and secured funding. P.B. guided data analysis and review of the manuscript. E.S. designed and ran the cohort and contributed to the study design and review of the manuscript. L.F. and A.S.H. participated in the study design and sample selection. M.K. and S.M. performed the laboratory experiments. M.K. performed data analysis and wrote the initial draft. J.B. designed HPgV-1 primers and assisted in project conception. A.I.A. and E.W.N. jointly supervised the project. All authors read and reviewed the final draft.

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SUPPLEMENTAL TABLES

Supplemental Table 1: excel file containing additional data related to Figure 3.

Supplemental Table 2: excel file containing additional data related to Figure 4.

Supplemental Table 3: excel file containing additional data related to Figures S1

Supplemental Table 4: excel file containing additional data related to Figures S2

Supplemental Table 5: excel file containing additional data related to Figures S3

Supplemental Table 6: excel file containing additional data related to Figures S4

Figure and legends

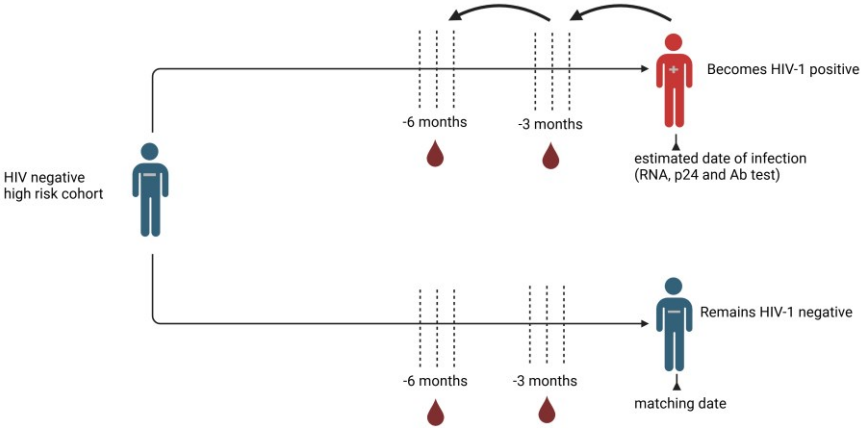

Figure 1: Schematic representation of our study design. Three and six-month prior to HIV infection, samples were selected from a historic high-risk cohort study conducted on the Kenyan coast between 2006 and 2011. Cases were defined as those who tested HIV positive during follow-up using RT-PCR, p24 antigen, and HIV-1-specific antibody assays. Controls were those who remained HIV-negative during follow-up and were matched to the cases based on sex, age, risk group, follow-up duration, and availability of samples.

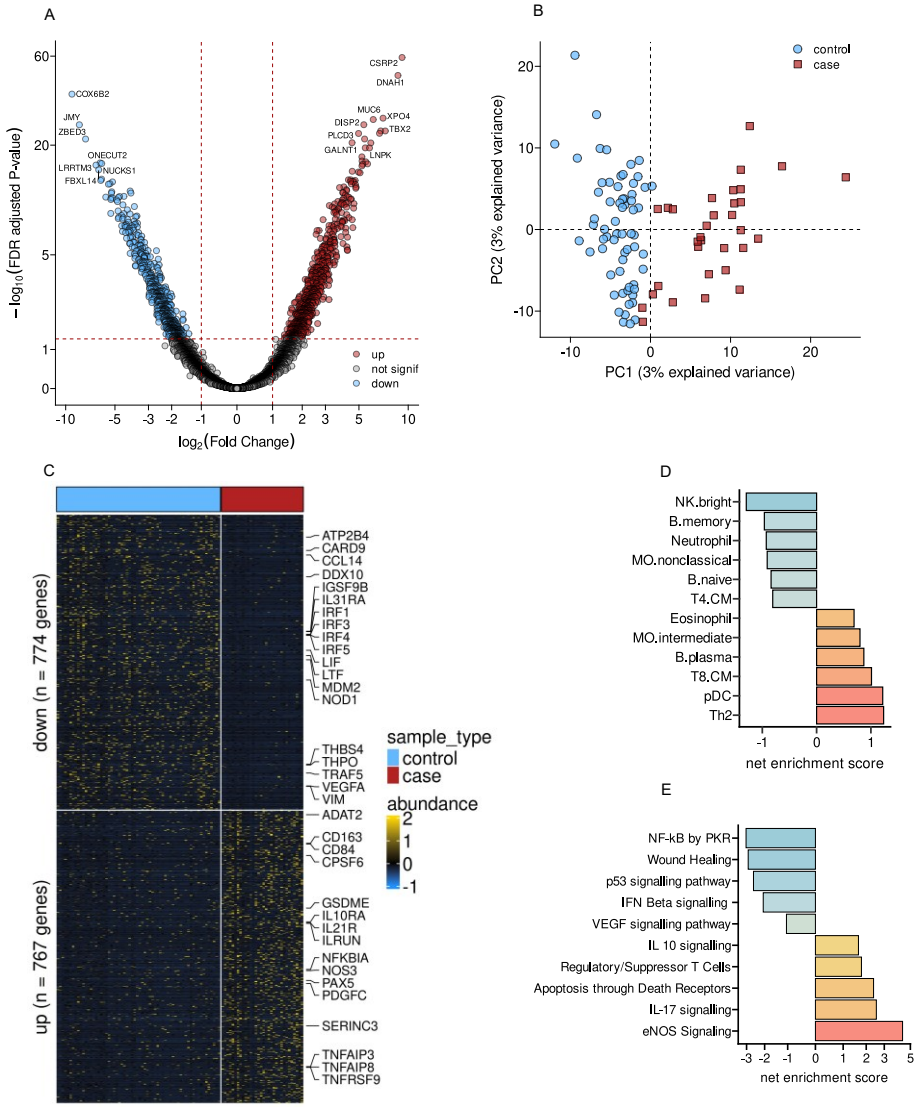

Figure 2: Cases exhibited a deregulated immunological profile three months prior to HIV-1 infection

(**A**) Volcano plot showing differentially altered genes between 32 cases and 64 controls, three months prior to cases being HIV positive. Red dots represent genes upregulated in cases, blue represents downregulated genes, and grey represents unaltered genes. (**B**) The differentially altered genes can distinguish HIV-1 cases from those who remained negative. (**C**) Supervised heatmap clustering showing differences in gene expression between cases and controls. (**D**) Gene enrichment analysis showing transcriptional alteration at the cellular level. Genes belonging to neutrophils were downregulated, while those belonging to eosinophils and memory Tregs (mTregs) were upregulated. (**E**) Pathway gene enrichment analysis shows that immunosuppressive biological processes, such as IL10 signalling and regulatory T cells, were upregulated in the cases, while inflammatory and reparative processes were downregulated.

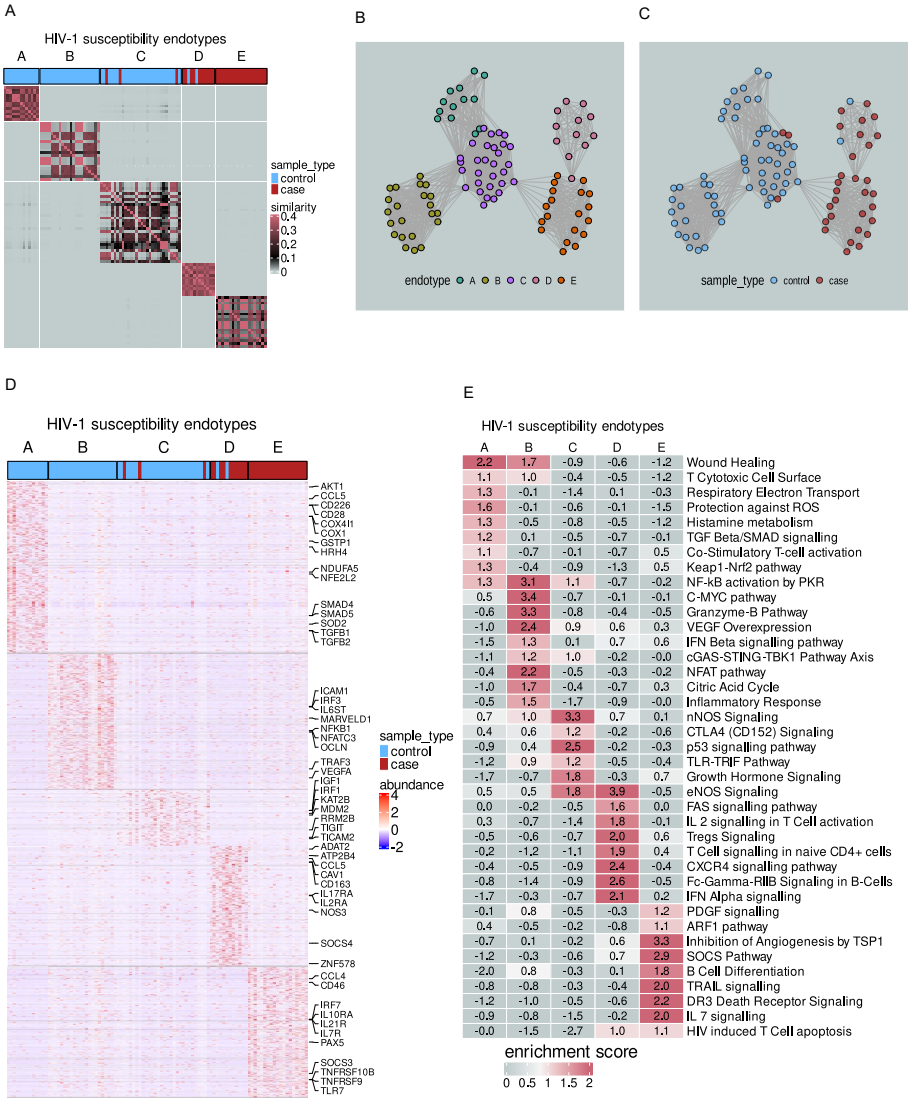

Figure 3: Cases and controls cluster into distinct immunological endotypes three months prior to HIV-1 infection

(**A**) Patient similarity matrix showing that EVs-RNAseq data, three months prior to HIV-1 infection, splits controls and cases into three and two endotypes, respectively. (**B** and **C**) Patient similarity network colored by (**B**) endotype and (**C**) sample type. Each node represents a study participant, and each edge links two similar samples. (**D**) Heatmap clustering shows that the identified endotypes have distinct transcriptional profiles. (**E**) Heatmap showing the top pathways enriched in each endotype.

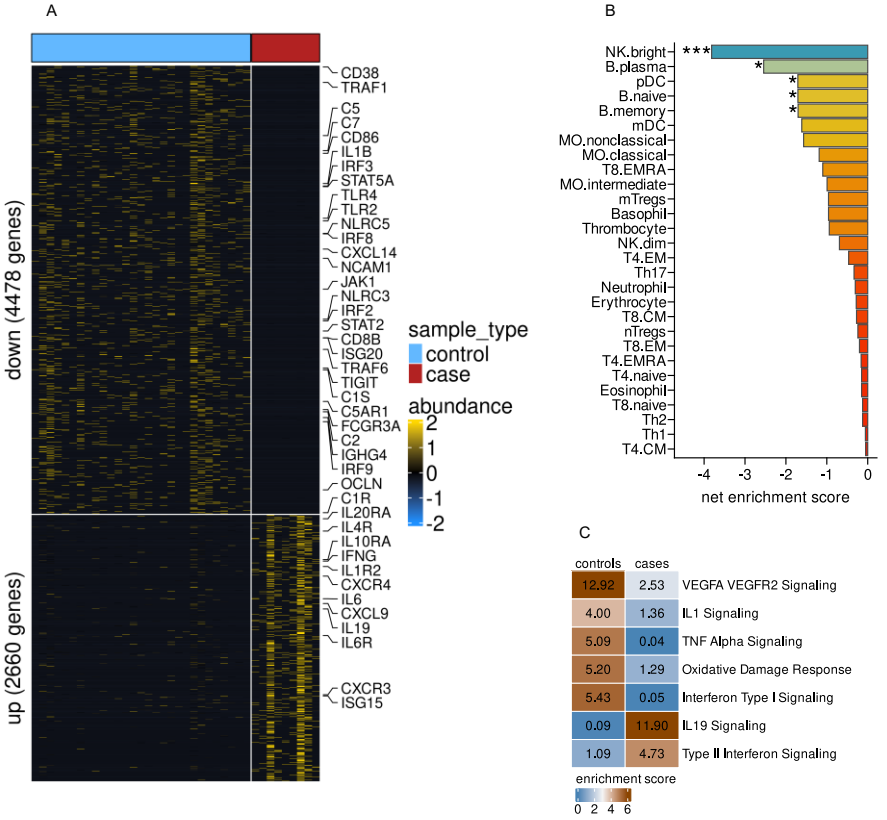

Figure 4: The immunosuppressive transcriptional profile is also evident six months prior to HIV-1 infection.

(**A**) Heatmap showing differential gene expression between 9 cases and 29 controls six months prior to HIV-1 infection. (**B**) Genes belonging to NK cells and plasma B-cell subsets are severely downregulated in HIV-1 cases relative to controls six months prior to infection. (**C**) Type II interferon response is upregulated in HIV-1 cases six months prior to infection, while type I interferon response pathways are upregulated.

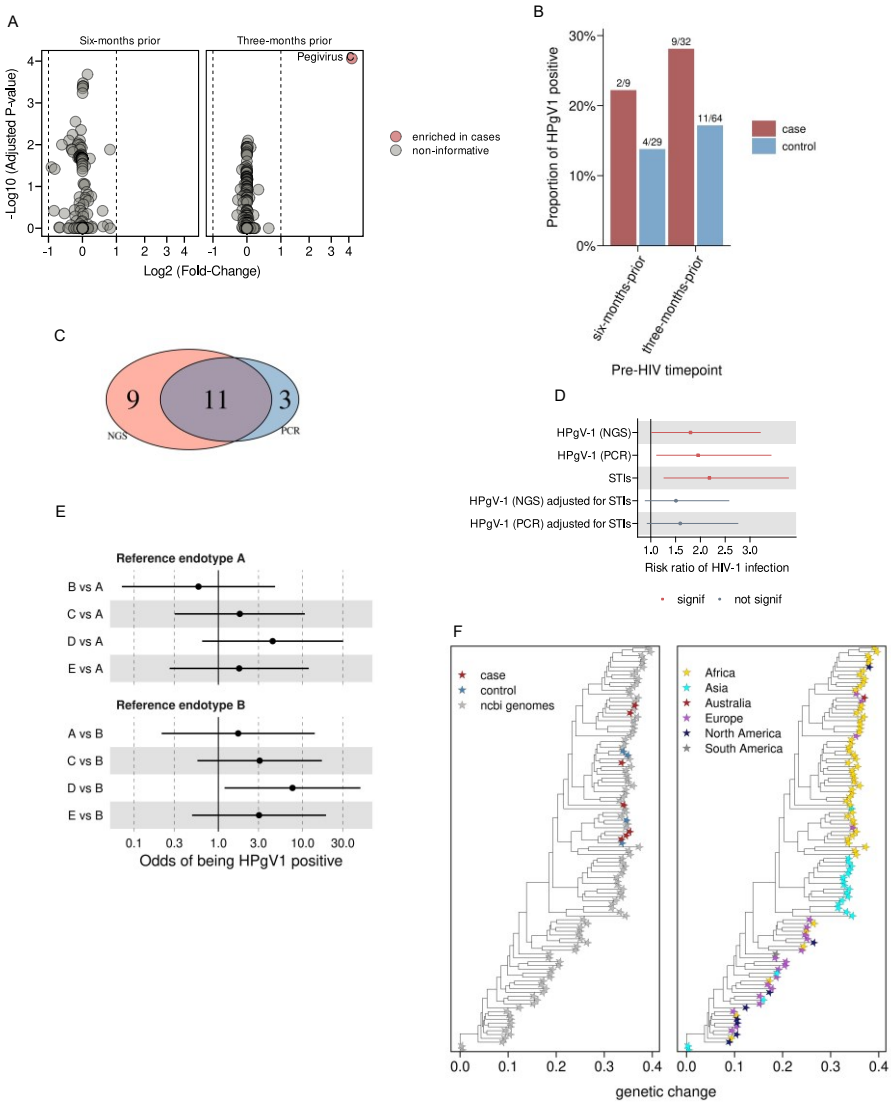

Figure 5: HPgV-1 infection predicts HIV-1 acquisition.

(**A**) HPgV-1 RNA is more abundant in cases compared to controls at six months prior to HIV-1 infection, but not at six months. (**B**) Barplots

showing the proportion of HPgV1 positive in HIV1 cases and controls. (**C**) Venndiagram showing overlap of HPgV-1 detection using next-generation sequencing (NGS) and conventional PCR. (**D**) The presence of HPgV-1 three months prior to infection is a non-independent predictor of HIV-1 infection. (**E**) Forest plots comparing HPgV1 status between the endotypes described in Figure 2. (**F**) HPgV-1 genomes exhibit regional clustering.

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