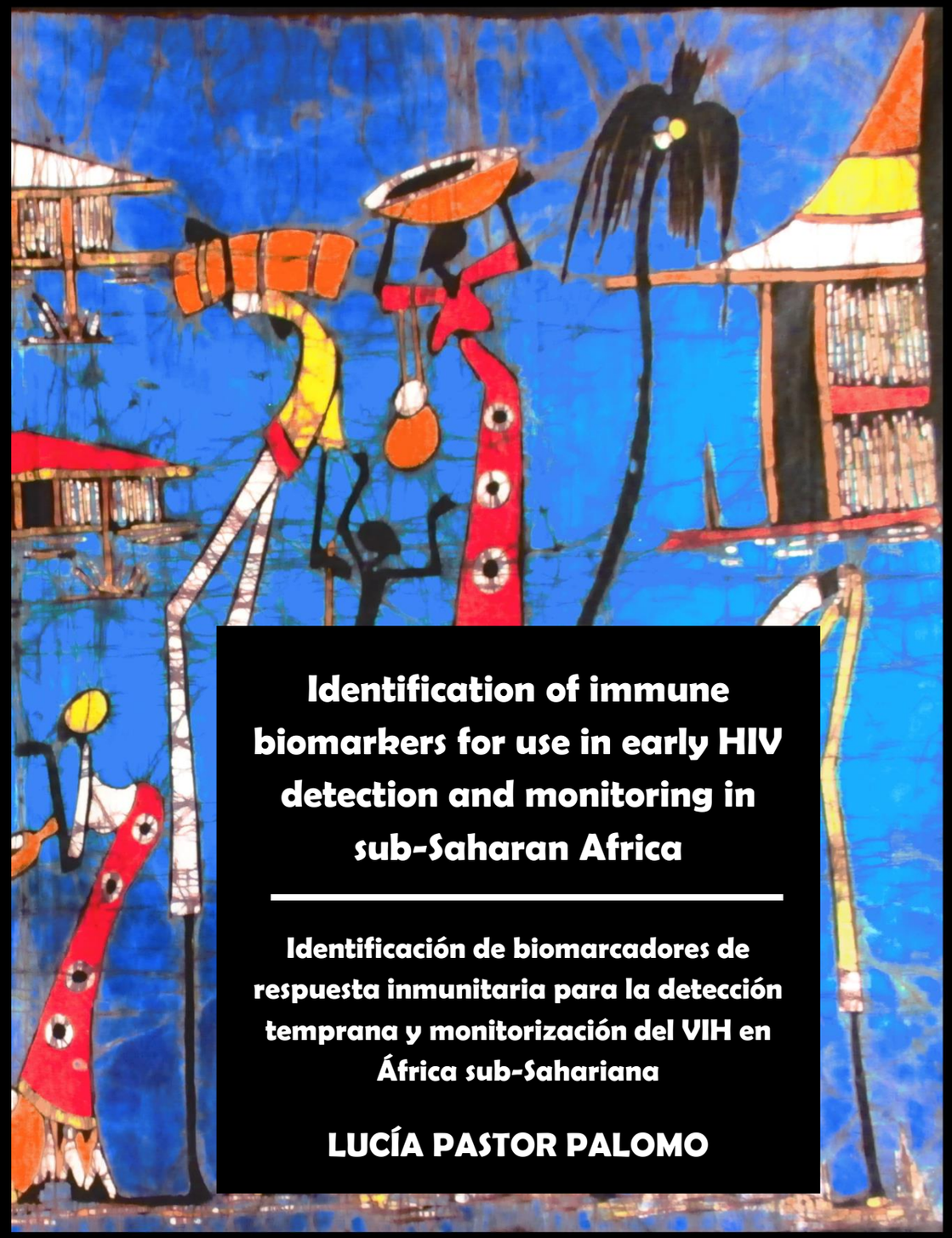
Identification of immune biomarkers for use in early HIV detection and monitoring in sub-Saharan Africa

Identificación de biomarcadores de respuesta inmunitaria para la detección temprana y monitorización del VIH en África sub-Sahariana

Lucía Pastor Palomo

Doctoral Thesis LUCÍA PASTOR PALOMO 2017



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La Dra Denise Naniche, investigadora del Instituto de Salud Global de Barcelona y del Centro de Investigação em Saúde da Manhiça , y el Dr. Julià Blanco, Investigador del Institut d'Investigació en Ciències de la Salut Germans Trias I Pujol-IGTP y del Institut de Recerca de la Sida-IrsiCaixa del Hospital Germans Trias i Pujol,

hacen constar

que el trabajo experimental y la redacción de la memoria de la Tesis Doctoral titulada 'Identification of Immune Biomarkers for Use in Early HIV Detection and Monitoring in Sub-Saharan Africa' han sido realizados bajo su dirección por

LUCÍA PASTOR PALOMO

y consideran

que la memoria resultante es apta para optar al grado de Doctor en Medicina con mención Internacional por la Universitat de Barcelona.

Y para que quede constancia, firman el presente documento

Dr Denise Naniche

Dr Julià Blanco

Barcelona, 14 de Julio del 2017

A mis padres,
por el trabajo y la dedicación de acompañarme hasta aquí.

If we knew what it was we were doing,
it would not be called research, would it?

Albert Einstein

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ABBREVIATIONS AND ACRONYMS

AHI	Acute HIV infection
AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral therapy
ARV	Antiretroviral
CRP	C-reactive protein
CCR5	Chemokine Receptor 5
CTL	Cytotoxic T-lymphocyte
CXCR4	CX-Chemokine Receptor-4
DC	Dendritic cell
DNA	Deoxyrribonucleic Acid
ELISA	Enzyme-linked Immunosorbent assay
Env	Envelope
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
Gp	Glycoprotein
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen-D related
HTC	HIV testing and counseling
IBD	Intestinal Bowel Disease
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin

IP-10	interferon gamma inducible protein 10 (CXCL10)
LMIC	low and middle-income countries
NK	Natural killer
PD-1	Programmed cell death protein-1
PHI	Primary HIV infection
POC	Point of care
PrEP	pre-exposure prophylaxis
RDT	Rapid diagnostic tests
RT-PCR	Real-time polymerase chain reaction
RNA	Ribonucleic Acid
SIV	Simian immunodeficiency virus
SSA	Sub-Saharan Africa
TNF	Tumor necrosis factor
Th	T helper
Tregs	regulatory T-cells
UNAIDS	United Nations Programme on HIV and AIDS
VF	Virological failure
VL	Viral load
WB	Western blot
WHO	World Health Organization

SUMMARY

BACKGROUND AND RATIONALE

Acute HIV infection (AHI) is the period between the acquisition of the human immunodeficiency virus (HIV) and the development of HIV-specific antibodies that define seroconversion. AHI is characterised by high HIV viral replication and, in most cases, a transient non-specific febrile illness that typically occurs around 2 weeks after the HIV-transmission event. Primary HIV infection (PHI) is generally considered the period up to 6 months after infection and is a rapidly evolving phase characterized by the stepwise gain in positivity for the detection of HIV-RNA and HIV-specific antibodies. Different HIV antigen specificities appear in sequence after HIV transmission as do immunoglobulin G (IgG) subclass responses. As such, using different diagnostic tools, PHI has been categorised into 'Fiebig stages' that are useful in approximating infection date with relative accuracy.

As a result of high viremia in bodily fluids, individuals are considered hyper-infectious during AHI. In areas of high HIV incidence, this phenomenon could contribute greatly to fuelling the worldwide HIV pandemic. Despite the importance of early diagnosis and treatment to reduce onward transmissions and prevent substantial irreversible immunological damage, AHI represents a 'window period' during which persons infected with HIV are commonly undiagnosed. Routine second generation HIV-rapid test algorithms provide negative or indeterminate results for up to 6-8 weeks after infection. During this time, HIV can only be diagnosed by detecting the presence of the virus itself. The current gold-standard test for confirming viremia is HIV-RNA viral load (VL) testing. However, technical and financial constraints make this technique very limited in low-income areas such as Sub-Saharan Africa, where the prevalence of AHI among febrile patients may reach 3%.

VL testing is also used to monitor the efficacy of anti-retroviral treatment (ART). Achieving effective ART monitoring is a key determinant to ensure viral suppression and reach the UNAIDS 90-90-90 targets. Although considerable international efforts have resulted in a dramatic increase in ART coverage in the last years, relatively little progress has been achieved in the development of simple, accurate and affordable tools that allow proper surveillance of ART efficacy. VL monitoring is important for timely diagnosis of virological failure (VF) to allow early adherence interventions, prevent further transmissions and avoid delays in regimen switches that could lead to disease progression or emergence of drug resistances. Detecting virological failure depends on VL testing; whose availability is very limited in low and middle-income countries (LMIC) due to cost and operational constraints. The alternative to VL has often been clinical and/or immunological monitoring, which frequently results in patients remaining on failing ART as well as unnecessary regimen switches. Indeed, recent cross-sectional surveys reported that around 25%-35% of individuals on ART in Mozambique had detectable HIV viremia levels.

As viremia increases during PHI, there is a striking cascade response of inflammatory cytokines. Significant efforts have been made to characterise host and viral proteins present during AHI aiming to identify biomarkers of progression or key pathological pathways that could be targeted to minimize HIV-induced immune damage over the course of infection. Subsets of T cells can be defined by their specificity, surface phenotype or degree of maturation, and any or all of these parameters can be affected by HIV infection. During PHI, many cells of the immune system show signs of extensive activation and a progressive loss of resting subsets. Generally, untreated HIV-infection is characterized by progressive CD4 T-cell depletion and CD8 T-cell expansion. The profound CD4 T-cell depletion is linked directly to the risk for opportunistic infections and mortality, while CD8 T-cell activation and exhaustion have been observed to be strong correlates of disease progression. Such alterations of CD4 and CD8 homeostatic mechanisms lead to progressive loss of the naïve and memory T-cell pool, resulting in an imbalance in T-cell phenotypes. Similarly, after HIV infection, accelerated aging of T cells or immunosenescence has been also associated with risk of adverse clinical events in HIV infected individuals.

The challenges of identifying individuals during the AHI phase have resulted in a lack of critical information that constrains the development of therapeutic interventions. In this thesis, we provide a longitudinal characterization of T-cell subsets and the expression of soluble inflammatory biomarkers over the first year after PHI in a cohort of Mozambican adults and compare these changes with Chronic HIV-infection (CHI). Additionally, we assess the predictive power of these soluble biomarkers as surrogates of viremia for detection of AHI in seronegative febrile individuals and for identification of virological failure in ART-treated subjects.

METHODS

This thesis is based on research conducted at the Barcelona Institute for Global Health (ISGlobal)/ Hospital Clinic-Universitat de Barcelona in Spain, the AIDS Research Insitute/ Germans Trias i Pujol Research Institute (IGTP) and at the Centro de Investigaçao em Saude de Manhica (CISM) in Mozambique.

To longitudinally analyze individuals during PHI, a screening based on reported-fever and pooled VL- testing was used to identify AHI in HIV-seronegative adults presenting at the Manhiça District Hospital (MDH), Mozambique. HIV-uninfected and chronically HIV infected individuals, both ART-naïve and on first-line ART, were also recruited at MDH in the context of the study. Plasma levels of inflammatory and immune biomarkers were subsequently determined by Luminex and ELISA, anti-HIV antibodies were analysed by flow-cytometry and Western Blot (WB) and T-cell phenotyping was performed through multi-panel flow-cytometry analysis.

To evaluate biomarkers in treated HIV infected individuals, samples from a resistance survey study performed in 2013 were retrospectively analysed.

The thesis is presented as a collection of four articles, of which three are accepted for publication in peer-reviewed international journals, and one is under review for publication.

KEY RESULTS

The findings from the studies that constitute this thesis provide a further characterization of the dynamics of immune response biomarkers over the different stages of HIV infection among adults in Mozambique.

Immune response biomarkers across Fiebig staging at primary HIV infection

A total of 85 AHI individuals were identified in our cohort as seronegative or indeterminate for rapid test and positive for plasma HIV-RNA among Mozambican adults seeking health care at the MDH. This represents an AHI prevalence of 3% among seronegative individuals reporting febrile illness. Soluble biomarkers, including inflammatory cytokines and general and HIV-specific antibody subtypes, were determined over different Fiebig stages at PHI, together with clinical and immunological characteristics. We compared cytokine levels between individuals at pre- (Fiebig I-IV) and post-seroconversion stages (Fiebig V-VI) at the screening visit. Thus, we identified a signature of four cytokines composed of BAFF, MCP-1, sCD163 and MIG that is highly associated with the PHI phase prior to development of the HIV-specific humoral response as determined by standard Western Blot serology.

Longitudinal analysis of soluble and cellular biomarkers along the HIV infection process

After longitudinal follow up of the PHI individuals, T-cell subsets and the expression of soluble inflammatory and immune biomarkers were characterized over the first year after of infection. Although plasma HIV viremia, CD4 and CD8 T cell counts undergo a rapid stabilization after HIV infection, several immunological parameters, including Th1Th17 CD4 T cells and activation or exhaustion of CD8 T cells continue to decrease even after 9 months post-infection. Importantly, no sign of immunosenescence was detected over the first year of HIV infection and no significant changes were observed in the Tregs population. Levels of IP-10, MCP-1, BAFF, sCD14, TNFR2 and TRAIL were significantly overexpressed at the first month of infection and underwent a prompt decrease in the following months. However, MIG and CD27 levels started to increase 1 month after infection and remained over-expressed for almost one year post-infection. Early levels of plasma TNFR2, sCD27, BAFF, IL-10 and sCD14 cytokines were significantly associated with later levels of exhausted CD4 T-cells or with CD8 T-cell activation.

Biomarkers as an accurate tool to identify acute HIV infection in febrile individuals

In order to evaluate whether levels of a single or a combination of biomarkers had predictive accuracy to identify AHI among HIV-seronegative adults presenting with reported fever at the MDH, plasma levels of 49 inflammatory biomarkers from AHI (n=61) and non-HIV infected outpatients (n=65) were compared. The cytokine IP-10 demonstrated the best predictive accuracy for AHI detection (AUC=0.88 [95%CI 0.80-0.96]). A cut-off value of IP-10≥161.6pg/mL provided a sensitivity of 95.5% (95%CI 85.5-99.5) and a specificity of 76.5% (95%CI 62.5-87.2) for AHI identification. Thus, an IP-10-based screening for subsequent AHI identification with VL could reduce the number of VL determinations necessary by 75%. After a cost-effectiveness analysis of this IP-10-based approach, we concluded that the implementation of an IP-10 screening test could avert from 21 to 84 new infections and save from US$176,609 to US$533,467 to the health system per 1,000 tested patients.

IP-10 predictive power to detect virological failure in treated patients

Due to the strong association with VL, we hypothesized that plasma IP-10 levels could be a surrogate marker of detectable viremia in ART-treated individuals. Consequently, we found that IP-10 levels were significantly higher in ART-treated subjects with detectable VL (108.2 pg/mL) as compared to those with undetectable VL (38.0 pg/mL) (U-test p<0.0001) in a cohort of 316 HIV-infected individuals on ART for more than a year. An IP-10 univariate model demonstrated high accuracy for prediction of detectable viremia (AUC=0.85 [95% Confidence Interval (CI) 0.80-0.90]). Using a cut-off value of IP-10≥44.2 pg/mL, the IP-10 model identified viremic ART-treated subjects with 91.9% sensitivity (95% CI 83.9-96.7) and 59.9% specificity (95% CI 52.0-67.4). Accordingly, we found that such IP-10 screening for potential virological failure would reduce by 43% the number of VL determinations required to monitor the same number of patients and this approach could potentially save 38% of VL-derived costs to the health system.

CONCLUSIONS AND RECOMMENDATIONS

AHI prevalence in febrile seronegative adults presenting at the MDH was found to be 3% as reported in 2008, having thus remained unchanged in this population in the last 5 years. After quantification of the soluble biomarkers across the different Fiebig stages described in PHI, we identified a signature of four cytokines composed of BAFF, MCP-1, sCD163 and MIG that was highly associated with the pre-seronversion phase as determined by WB serology. These effectors could provide clues for the development of vaccine or immunomodulatory strategies aimed at reducing the irreversible immune damage inflicted during PHI.

Throughout characterization of cellular and soluble immune biomarkers along the different phases of HIV infection, we found that activated and effector memory CD8 T-cells together with Th1Th17 cells continued to decay several months after control of viremia. These findings indicate that balance in the T-cell compartments occurs months after viremia or CD4 count stabilize, suggesting persistent immune dysfunction in many different T cell subsets and raises the potential need for early initiation of therapy that could limit immunological damage.

From the 49 soluble biomarkers that were assessed in febrile seronegative individuals, IP-10 demonstrated the best predictive power for AHI detection, providing a sensitivity of 95.5% and a specificity of 76.5%. Implementation of an IP-10-based screening for subsequent AHI diagnosis with VL is a cost effective strategy that could avert up to 84 new infections and save up to US$500,000 to the health system per 1,000 tested patients.

IP-10 is also an accurate biomarker to screen individuals on ART for virological failure (VF), identifying 91.9% of patients with detectable viremia with a specificity of 59.9%. Employing IP-10 as a screening tool to target the individuals on ART most likely to require VL testing would reduce by 43% the number of VL determinations required to control viral suppression and it could save 38% of VL-derived costs to the health system.

Thus, IP-10 quantification could be developed as a screening tool to identify both AHI in febrile seronegative individuals and VF in patients on ART with subsequent viral load testing. The implementation of these algorithms would facilitate AHI diagnosis and ART-monitoring in LMIC, as sub-Saharan Africa. Nevertheless, further research is necessary to explore the impact of other common HIV comorbidities on HIV-induced levels of IP-10, validate the IP-10 predictive power and optimize model cut-off values in other populations.

RESUMEN

ANTECEDENTES Y JUSTIFICACIÓN

La fase aguda de la infección por el virus de la inmunodeficiencia humana (VIH) es el periodo comprendido entre la adquisición del virus y el desarrollo de anticuerpos específicos frente al VIH que definen la seroconversión y se conoce como AHI por sus siglas en inglés (*Acute HIV Infection*). La AHI se caracteriza por un alto nivel de replicación viral y, en la mayoría de los casos, un cuadro transitorio de fiebre no específica que suele producirse alrededor de 2 semanas después del evento de transmisión. El término *infección primaria por el VIH* (PHI, por sus siglas en inglés) engloba generalmente el periodo hasta 6 meses después de la infección y es una fase de evolución rápida caracterizada por la aparición secuencial en plasma de proteínas y ARN virales y anticuerpos específicos frente al VIH. Diferentes antígenos del VIH aparecen secuencialmente después de la transmisión, así como las respuestas VIH-específicas de diferentes subclases de inmunoglobulina G (IgG). Así, utilizando diferentes herramientas de diagnóstico, la PHI se ha categorizado en los llamados estadios Fiebig, que proporcionan una buena aproximación del tiempo de infección.

Durante la AHI y como resultado de un alto nivel de virus en los fluidos corporales, los individuos se consideran híper-infecciosos. En las zonas de alta incidencia de VIH, este fenómeno podría contribuir en gran medida a la pandemia mundial del VIH. A pesar de la importancia del diagnóstico precoz y el inicio temprano del tratamiento para reducir las potenciales transmisiones y prevenir un sustancial daño inmunitario irreversible, el AHI representa un "periodo ventana" durante el cual las personas infectadas con el VIH no son diagnosticadas habitualmente. Los algoritmos rutinarios de prueba de VIH (*test de segunda generación*) proporcionan resultados negativos o indeterminados hasta aproximadamente 6-8 semanas después de la infección. Durante este tiempo, la infección por VIH sólo puede ser diagnosticada mediante la detección del virus en sí. La actual prueba de referencia para confirmar la detección de virus en sangre o viremia es la prueba de carga viral (CV), que consiste en detectar el ARN del virus en plasma. Sin embargo, las restricciones técnicas y financieras hacen que esta técnica sea de acceso muy limitado en las zonas de escasos recursos como el África Subsahariana, donde la prevalencia de AHI entre los pacientes con síntomas febriles puede llegar al 3%.

La prueba de CV también se usa para monitorizar la eficacia del tratamiento antirretroviral (TARV). Conseguir una monitorización eficaz del TARV es un determinante clave para asegurar la supresión viral y alcanzar los objetivos del 90-90-90 de ONUSIDA. Aunque los esfuerzos internacionales han dado lugar a un aumento dramático en la cobertura de TARV en los últimos años, se han logrado relativamente pocos avances en el desarrollo de herramientas sencillas, precisas y asequibles que permitan una adecuada vigilancia de la eficacia del TARV en países de baja renta. Además, la monitorización de la CV es importante para el diagnóstico de fallo virológico (FV) y el desarrollo de intervenciones tempranas que

fomenten la adherencia al tratamiento. Así, se posibilita la prevención de futuras transmisiones y se evita retrasos en los cambios de régimen de TARV que podrían conducir a la progresión de la enfermedad o a la aparición de resistencias a los fármacos. La detección del FV depende de las pruebas de CV, cuya disponibilidad es muy limitada en los países de renta baja debido a los elevados costes de procesamiento y las dificultades logísticas. Una alternativa común a la CV es la monitorización clínica y/o inmunológica, lo que con frecuencia tiene consecuencias negativas en los pacientes, los cuales permanecen en fallo terapéutico por más tiempo del deseado o sufren interrupciones innecesarias del régimen de TARV. De hecho, estudios transversales recientes han descrito que entre el 25% y el 35% de las personas en TARV en Mozambique tenían niveles detectables de viremia en sangre.

A medida que aumenta la viremia durante la fase de AHI, se desarrolla una fuerte respuesta en cascada de citoquinas inflamatorias. Se han realizado esfuerzos significativos para caracterizar las proteínas víricas y de respuesta inmunitaria presentes en el huésped durante la AHI con el objetivo de identificar tanto biomarcadores de progresión de la enfermedad, como procesos patológicos clave, contra los cuales se podrían dirigir terapias encaminadas a minimizar el daño inmunitario inducido por el VIH durante el curso de la infección. Además de cambios en los niveles de citoquinas, se han descrito importantes alteraciones en la función de diferentes células del sistema inmunitario, especialmente las células T CD4 (dianas del VIH) y T CD8 (responsables de la respuesta inmunitaria antiviral). Los subgrupos de células T pueden definirse por su especificidad, por los fenotipos de superficie celular o por el grado de maduración, y cualquiera de estos parámetros pueden verse afectados por la infección del VIH. Durante la PHI, muchas células del sistema inmunitario muestran signos de activación y una pérdida progresiva de subgrupos celulares en estado de reposo. De forma general, la infección por el VIH en ausencia de tratamiento se caracteriza por una depleción progresiva de células T CD4 y una expansión de células T CD8. La destrucción de las células T CD4 está directamente relacionado con el riesgo de infecciones oportunistas y mortalidad, mientras que la activación y el agotamiento de las células T CD8 han demostrado ser potentes marcadores de la progresión de la enfermedad. Tales alteraciones en los mecanismos homeostáticos de células T CD4 y CD8 conducen a una pérdida funcional progresiva, lo que resulta en un desequilibrio en los fenotipos de células T. De manera similar, después de la infección por el VIH, el envejecimiento acelerado de las células T o inmuno-senescencia también se ha asociado con el riesgo de eventos clínicos adversos en individuos infectados con el VIH.

La dificultad en identificar a los individuos durante la fase de AHI ha limitado la disponibilidad de información crítica de los procesos inmunitarios iniciales, lo cual ha restringido el desarrollo de intervenciones terapéuticas tempranas. En esta tesis, se proporciona una caracterización longitudinal de los subconjuntos de células T y la expresión de biomarcadores inflamatorios solubles durante el primer año de infección por el VIH en

una cohorte de adultos mozambiqueños y se compara estos cambios con la infección por el VIH en la fase crónica. Además, también se evalúa el poder predictivo de estos biomarcadores solubles para la detección de la AHI en individuos seronegativos con fiebre y para la identificación de FV en individuos tratados.

MÉTODOS

Esta tesis se basa en una investigación realizada en el Instituto de Salud Global de Barcelona (ISGlobal)/Hospital Clínic-Universitat de Barcelona, en el Instituto de Investigación del Sida-IrsiCaixa/Instituto de Investigación Germans Trias i Pujol (IGTP) y en el Centro de Investigación en Salud Manhica (CISM), Mozambique.

Para poder identificar los individuos en fase de AHI y realizar su posterior seguimiento longitudinal se realizó un cribado de los pacientes que se presentaban con sintomatología febril en el Hospital Distrital de Manhiça (HDM), Mozambique, mediante la prueba de CV con estrategia de "*pooling*". En el contexto del estudio también se reclutaron individuos no infectados por el VIH y crónicamente infectados, tanto sin tratamiento como en primera línea de TARV. Los niveles plasmáticos de biomarcadores inflamatorios e inmunitarios fueron determinados posteriormente por Luminex y ELISA y los anticuerpos anti-VIH fueron analizados por citometría de flujo y Western Blot. El fenotipado de células T se realizó mediante análisis multicolor por citometría de flujo.

Para evaluar los biomarcadores en los individuos infectados por el VIH en TARV, se analizaron retrospectivamente muestras del estudio PREVIR, el cual se desarrolló en 2013 con el objetivo de describir la prevalencia de resistencias al TARV.

La tesis se presenta como una colección de cuatro artículos, de los cuales tres ya han sido aceptados para publicación en revistas internacionales, y uno se encuentra en revisión para su publicación.

RESULTADOS PRINCIPALES

Los hallazgos de los estudios que constituyen esta tesis proporcionan una nueva caracterización de la dinámica de biomarcadores de respuesta inmunitaria en las diferentes etapas de la infección por el VIH entre adultos de Mozambique.

Expresión de biomarcadores de respuesta inmunitaria a lo largo de los diferentes estadios Fiebig en la infección primaria por el VIH

Entre los adultos mozambiqueños que buscaban atención médica en el HDM, se identificaron un total de 85 individuos en fase de AHI. Estos individuos se diagnosticaron al presentar un resultado negativo o indeterminado para el test rápido de anticuerpos contra el VIH y positivo para la detección de ARN de VIH en plasma. Esto representa una

prevalencia de AHI del 3% entre los individuos seronegativos que reportaron sintomatología febril. Posteriormente se cuantificaron los niveles de diversos biomarcadores solubles (incluyendo citoquinas inflamatorias y subtipos de anticuerpos genéricos y específicos contra el VIH) y se describieron las características clínicas e inmunológicas de los individuos categorizados en los diferentes estadios Fiebig. Consecutivamente, se compararon los niveles de biomarcadores entre los individuos en estadios pre- (Fiebig I-IV) y pos-seroconversión (Fiebig V-VI) en la visita de cribado. Así, se consiguió identificar un patrón de citoquinas compuesto por BAFF, MCP-1, sCD163 y MIG que está muy asociado con la fase de PHI previa al desarrollo de la respuesta humoral específica contra VIH según el análisis de serología determinado por tecnología Western Blot.

Análisis longitudinal de biomarcadores solubles y celulares a lo largo del proceso de infección por el VIH

Después del seguimiento longitudinal de los individuos en PHI, se caracterizaron los subconjuntos de células T y la expresión de biomarcadores inflamatorios e inmunitarios solubles durante el primer año después de la infección. A pesar de que los niveles de viremia en plasma y los recuentos de células T CD4 y CD8 experimentaron una rápida estabilización después de la infección por el VIH, varios parámetros inmunológicos, incluyendo las células Th1Th17 CD4, la activación y el agotamiento de las células T CD8 continuaron disminuyendo incluso después de 9 meses tras la infección. Es importante destacar que no se detectó ningún incremento en los niveles de inmunosenescencia durante el primer año de infección por VIH y no se observaron cambios significativos en la población de Tregs. Los niveles de IP-10, MCP-1, BAFF, sCD14, TNFR2 y TRAIL fueron significativamente más elevados durante el primer mes de infección y sufrieron una rápida disminución en los meses siguientes. Sin embargo, los niveles de MIG y CD27 comenzaron a aumentar un mes después de la infección y permanecieron sobre-expresados hasta casi un año. Los niveles iniciales de las citoquinas plasmáticas TNFR2, sCD27, BAFF, IL-10 y sCD14 se asociaron significativamente con niveles posteriores de células T CD4 agotadas o de células T CD8 activadas.

Biomarcadores solubles como herramienta para detectar la infección aguda por el VIH en individuos con síntomas febriles

Para evaluar si los niveles de un único o una combinación de biomarcadores tenían capacidad predictiva para identificar AHI entre los adultos seronegativos presentados con síntomas febriles en el HDM, los niveles plasmáticos de 49 biomarcadores inflamatorios en individuos en fase de AHI (n=61) se compararon con los de pacientes febriles no infectados por el VIH (n=65). Así, la citoquina IP-10 demostró tener la mejor precisión para la detección de AHI (AUC=0.88 [Intervalo de Confianza (IC) del 95% 0.80-0.96]). Un valor de

corte de IP-10≥161.6pg/mL proporcionó una sensibilidad del 95.5% (IC 95% 85.5-99.5) y una especificidad del 76.5% (IC 95% 62.5-87.2) para la identificación de AHI. De este modo, el cribado de pacientes con sintomatología febril basado en los niveles plasmáticos de IP-10 y el posterior diagnóstico de AHI mediante CV podría reducir el número de determinaciones de CV necesarias en un 75%. Tras el análisis de coste-efectividad de esta estrategia combinada, concluimos que la implementación de una prueba de detección de los niveles de IP-10 podría evitar de 21 a 84 nuevas infecciones y ahorrar entre 176,609US$ y 533,467 US$ al sistema de salud por cada 1,000 pacientes analizados.

Capacidad predictiva del IP-10 para detectar fallo virológico en pacientes en TARV

Debido a la fuerte asociación con la CV, hipotetizamos que los niveles plasmáticos de IP-10 podrían ser un buen marcador de los niveles de viremia en pacientes tratados. En una cohorte de 316 individuos en TARV durante más de un año, encontramos que los niveles de IP-10 eran significativamente más elevados en los sujetos con CV detectable (108.2 pg/mL) en comparación con aquellos con CV indetectable (38.0 pg/mL; p<0.0001). El modelo univariado de IP-10 demostró una alta precisión para la predicción de viremia detectable (AUC=0.85 [IC 95% 0.80-0.90]). Utilizando un valor de corte de IP-10≥44.2 pg/mL, el modelo IP-10 identificó a los individuos virémicos en TARV con una sensibilidad del 91.9% (IC 95% 83.9-96.7) y una especificidad del 59.9% (IC 95%: 52.0-67.4). En consecuencia, se concluyó que la detección de potenciales casos de FV basada en los niveles de IP-10 podría reducir en un 43% el número de CV necesarias para controlar el mismo número de pacientes y este enfoque podría ahorrar un 38% de los costes derivados de las determinaciones por CV para el sistema de salud.

CONCLUSIONES Y RECOMENDACIONES

Un 3% de los individuos seronegativos que reportaron síntomas febriles en el HDM se encontraban en fase de AHI, porcentaje similar al que se describió en el año 2008 en esta misma población. Esto indica que la prevalencia de AHI en esta población se ha mantenido sin cambios en los últimos 5 años. Después de la cuantificación de los biomarcadores solubles a lo largo de los diferentes estadios de Fiebig, se identificó un patrón de cuatro citoquinas compuesto por BAFF, MCP-1, sCD163 y MIG que estaba altamente asociado con la fase de pre-seronversión determinada por serología de Western Blot. Estos biomarcadores podrían proporcionar pautas para el desarrollo de vacunas o estrategias inmuno-moduladoras dirigidas a reducir el daño inmunitario irreversible infligido durante la PHI.

Tras la caracterización de biomarcadores inmunitarios celulares y solubles a lo largo de las diferentes fases de la infección por el VIH, se encontró que las células T CD8 activadas y exhaustas junto con las células Th1Th17 continuaron decayendo varios meses después de la estabilización en los niveles de viremia. Estos hallazgos indican que la estabilización

inmunitaria se produce meses después de que la viremia o el recuento de células CD4 se estabilicen, lo que sugiere una disfunción inmunitaria persistente en diferentes subgrupos de células T y corrobora la necesidad de iniciar tratamiento temprano para intentar limitar el daño inmunitario.

De los 49 biomarcadores solubles que se evaluaron en individuos seronegativos con síntomas febriles, IP-10 demostró tener el mejor poder predictivo para la detección de AHI, proporcionando una sensibilidad del 95.5% y una especificidad del 76.5%. La implementación de un cribado de pacientes con sintomatología febril basado en IP-10 para el posterior diagnóstico de AHI con CV es una estrategia rentable que podría evitar hasta 84 nuevas infecciones en países de alta incidencia de VIH y ahorrar más de 500,00US$ al sistema de salud por cada 1,000 pacientes analizados.

El IP-10 es también un biomarcador preciso para detectar los casos de FV entre individuos en TARV, identificando el 91.9% de los pacientes con viremia detectable con una especificidad del 59.9%. Así, el empleo de IP-10 como herramienta de cribado para detectar los individuos en TARV con mayor probabilidad de requerir CV reduciría en un 43% el número de CV necesarias para controlar la supresión viral y podría ahorrar un 38% al sistema de salud.

Por lo tanto, la cuantificación de IP-10 podría desarrollarse como una herramienta de detección para identificar tanto AHI en individuos seronegativos febriles y FV en pacientes en TARV mediante confirmación posterior por CV. La implementación de estos algoritmos facilitaría el diagnóstico de AHI y la monitorización del tratamiento en áreas de escasos recursos, como el África sub-Sahariana. Sin embargo, se necesitan nuevas investigaciones para explorar el impacto de otras comorbilidades comunes del VIH en los niveles de IP-10, validar el poder predictivo de IP-10 y optimizar los valores de corte del modelo en otras poblaciones.

INTRODUCTION

A. INTRODUCTION

1. The HIV pandemic: a global health challenge

The human immunodeficiency virus (HIV) is one of the world's leading infectious killers, claiming more than 35 million lives since the beginning of the epidemic[1]. At the end of 2015, there were approximately 36.7 million [34.0–39.8 million] people living with HIV[1,2], up from 33.3 million in 2010, the result of continuing new infections, people living longer with HIV, and general population growth. This translates to an estimated 0.8% [0.7-0.9%] of adults aged 15–49 years worldwide living with HIV, although the burden of the epidemic continues to vary considerably between countries and regions (Figure 1)[3]. Sub-Saharan Africa (SSA) remains most severely affected, with nearly 1 in every 25 adults (4.4%) living with HIV and accounting for about 70% of the people living with HIV and almost 90% of HIV-infected children worldwide[2] (Figure 2)[4]. In the SSA countries most affected, HIV-induced acquired immune deficiency syndrome (AIDS) has raised death rates and lowered life expectancy among adults between the ages of 20 and 49 by about twenty years (Figure 3)[5].

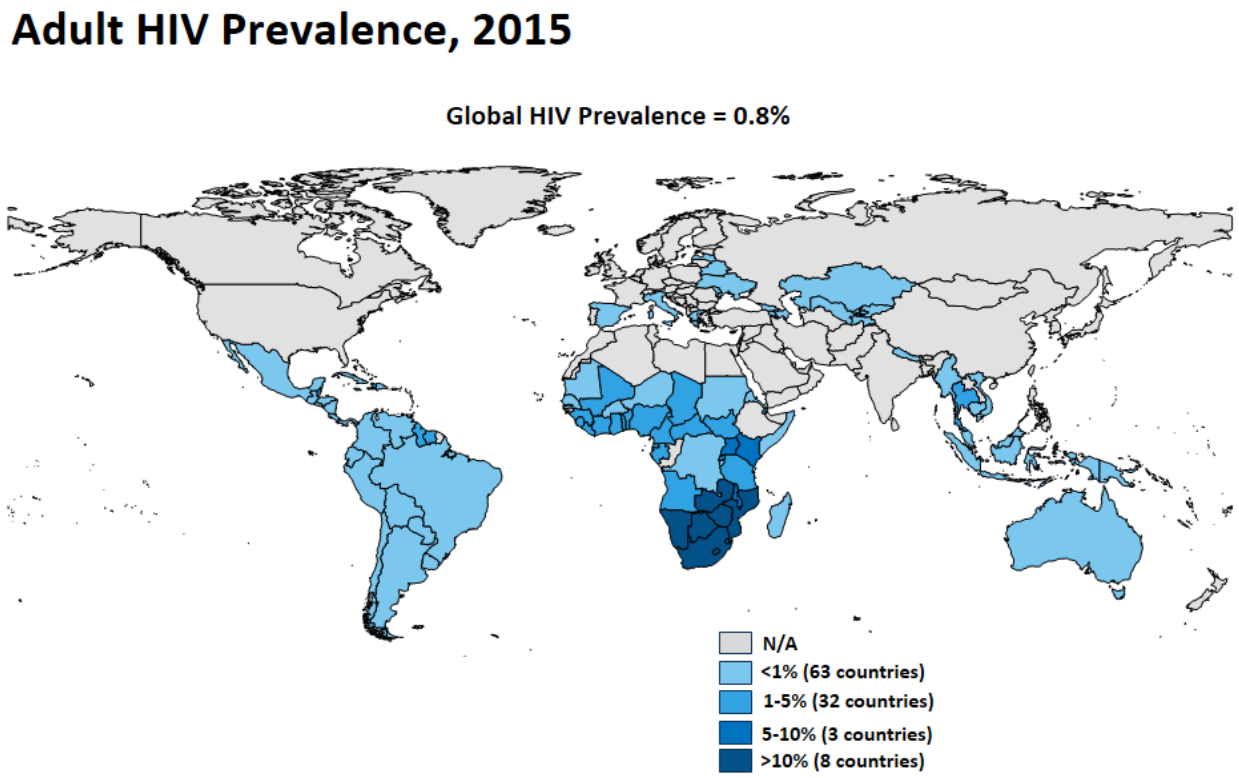
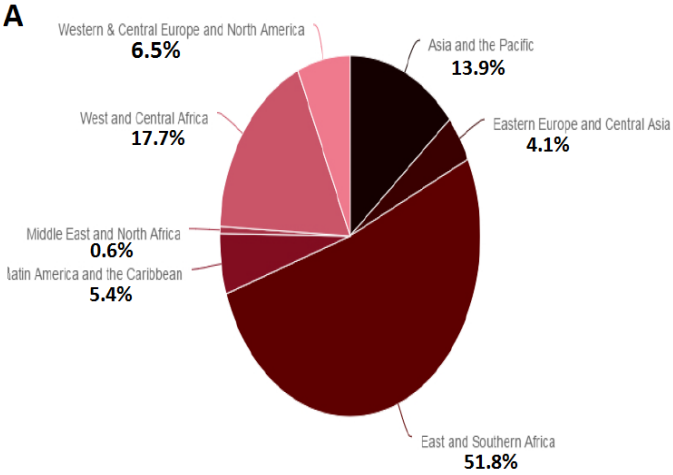


Figure 1. Estimate of adult HIV prevalence in 2015. Prevalence includes adults ages 15-49. Adapted from Kaiser Family Foundation[3], based on United Nations Programme on HIV and AIDS (UNAIDS) global reports[2]. N/A, data not available.

A



B

	Adults and children living with HIV	Adult & child deaths due to AIDS
Eastern and southern Africa	19.0 million [17.7 million – 20.5 million]	470 000 [390 000 – 560 000]
Western and central Africa	6.5 million [5.3 million – 7.8 million]	330 000 [250 00 – 430 000]
Middle East and North Africa	230 000 [160 000 – 330 000]	12 000 [8700 – 16 000]
Asia and Pacific	5.1 million [4.4 million – 5.9 million]	180 000 [150 000 – 220 000]
Latin America and the Caribbean	2.0 million [1.7 million – 2.3 million]	50 000 [41 000 – 59 000]
Eastern Europe and central Asia	1.5 million [1.4 million – 1.7 million]	47 000 [39 000 – 55 000]
Western and Central Europe and North America	2.4 million [2.2 million – 2.7 million]	22 000 [20 000 – 24 000]
TOTAL	36.7 million [34.0 million – 39.8 million]	1.1 million [940 000 – 1.3 million]

Figure 2. People living with HIV by UNAIDS regions. (A) People living with HIV (all ages) by region and (B) regional HIV and AIDS statistics in 2015. *Source UNAIDS Estimates 2016, adapted from AIDSinfo website*[4].

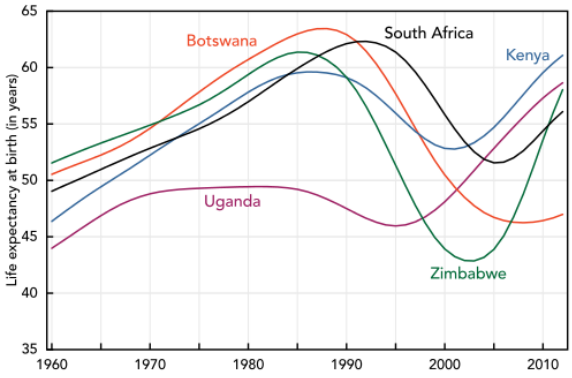


Figure 3. Graph of life expectancy at birth for some Sub-Saharan countries showing the fall in the 1990s primarily due to the AIDS pandemic. *Source The World Bank Estimates 2016, adapted from The World Bank website*[5].

Illnesses related to HIV-induced AIDS remain one of the leading causes of death globally and are projected to continue as a significant global cause of premature mortality in the coming decades. Although important progress has been achieved in preventing new HIV infections and in lowering the annual number of AIDS related deaths[6], the number of people living with HIV continues to increase, especially in some areas of the world and in vulnerable populations[7]. Worldwide, women represent half of all adults living with HIV and HIV is the leading cause of death among women of reproductive age[2]. Gender inequalities, differential access to service, and sexual violence increase women's vulnerability to HIV, and, in heterosexual relationships, women are biologically more susceptible to HIV[8,9]. Young people (ages 15-24) account for about a third of new HIV infections globally[8], significantly affecting those in their most productive years (Figure 4A). Most infections are transmitted heterosexually (Figure 4B) and AIDS is thus primarily a sexually transmitted disease[10]. However, men who have sex with men, injecting drug users, sex workers, transgender people, and prisoners are disproportionally affected by HIV in some countries[8] (Figure 4C).

Two genetically different but related types of HIV, HIV-1 and HIV-2, have been isolated from patients with AIDS[11]. Each type is believed to represent an independent transmission of simian immunodeficiency viruses into humans[10]. HIV-1 is the most common type associated with AIDS, whereas HIV-2, although accounting for less than 10 % of the global pandemic and considered less virulent and transmissible than HIV-1, causes a similar disease[10,12]. The HIV-1 group M viruses predominate and can be further subdivided into different subtypes based on genetic sequence data, taking precedence: subtype B as the dominant form in Europe, America, Japan, and Australia and subtype C as the dominant form in Southern and Eastern Africa and Central Asia[10,12]. HIV-2 principally resides in West Africa[10,12]. The ensuing discussion and thesis work relates primarily to HIV-1 subtype C but the information is generally applicable to other HIV-1 subtypes.

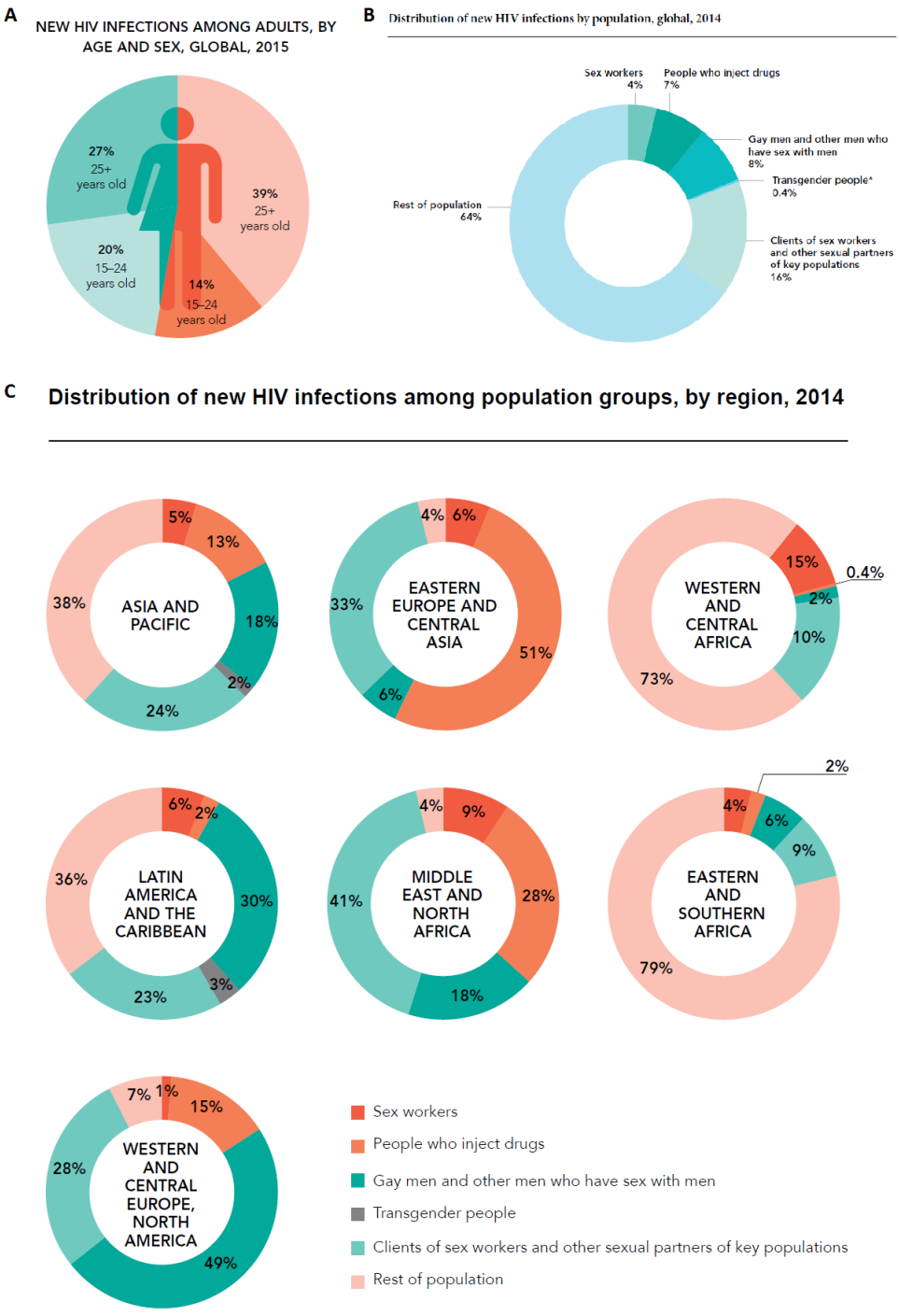


Figure 4. Distribution of new HIV infections among population groups. (A) Distribution of new HIV infections among adults by age and sex in 2015; (B) distribution of new HIV infections by global population in 2014; (C) distribution of new HIV infections among population groups by region in 2014. *Adapted from UNAIDS 2016 estimates*[2].

A cure for HIV infection has not yet been found but with effective treatment with antiretroviral (ARV) drugs, patients can control virus replication despite viral persistence. The role-out of antiretroviral therapy (ART) in the last decade has contributed greatly to the reduction of AIDS-related deaths[2] (Figure 5). However, many people living with HIV or at risk for HIV do not have access to prevention, care, and treatment[7]. Thus, the HIV/AIDS pandemic continues to be a major global health priority.

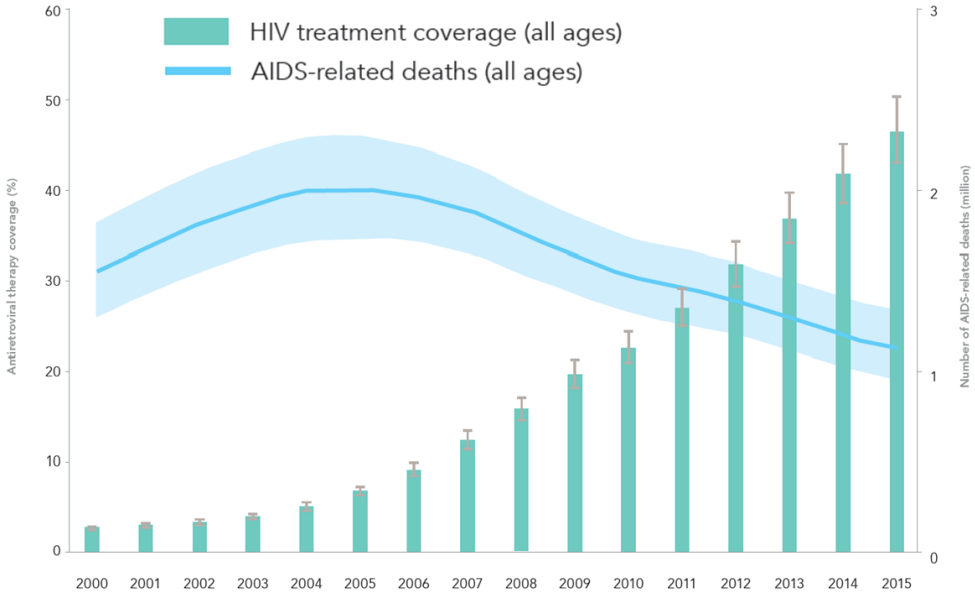


Figure 5. Global antiretroviral therapy coverage and number of AIDS-related deaths, 2000–2015. *Adapted from UNAIDS global AIDS-update 2016[2].*

2. HIV pathogenesis and treatment

2.1. Clinical course of HIV infection and progression to AIDS

The HIV belongs to the family of animal retroviruses, characterized by their enveloped structure surrounding a viral core containing two copies of single-stranded ribonucleic acid (RNA)[11,13]. The typical modes of transmission are mother to child, through blood contamination and, most commonly, through sex; HIV being able to be transmitted as both free virus particles or associated with immune cells[14]. HIV has the ability to infect cells of the immune system, destroying or impairing their function[13,15]. As the infection progresses, the immune system weakens, the person becomes more susceptible to infections and in the absence of treatment, further progression results in AIDS[16].

At the beginning of the infection, HIV crosses the mucosal barriers where the virus may be captured by dendritic cells (DCs) that will migrate to the nearest lymph nodes to present antigens to specific CD4 T-cells[17] (Figure 6). Single-genome amplification and sequencing of the first detectable virus has shown that around 80% of mucosally transmitted HIV clade B and C infections are initiated by a single virus[18,19]. After some days post-infection, viral replication can be detected in the lymph nodes and plasma viremia is increased in the infected individuals[20]. Then, both humoral and cell-mediated immune responses are mounted and the viral load (VL) is decreased to stable but still detectable levels[17]. During the early stages of HIV infection, memory CD4 T-cells in mucosal lymphoid tissues are the principal target of HIV infection and subsequent cell death[14]. Concomitantly, the HIV-reservoir is established by infection of non-activated cells that will keep the virus in a latent state[21,22]. HIV mainly infects cells from the immune system bearing CD4 molecules and co-receptors chemokine receptor-5 (CCR5) or CX-chemokine receptor-4 (CXCR4) on their surface, determining the viral tropism[11,23]. Although these cell types are mainly CD4 T-lymphocytes and macrophages, HIV can also infect monocytes, DCs, CD8 T-cells, B-cells and natural killer (NK) cells[11]. Along the infection process, there is generally a transition from virus that uses CCR5 and is predominantly macrophage tropic in early HIV-infection to virus that binds to CXCR4 and is T-cell line tropic late in the disease[24]. The CXCR4-tropic strains tend to be more virulent, presumably because the wider expression of CXCR4 in CD4 T-cells makes them infect and deplete more T-cells than CCR5 strains[25]. The importance of CCR5 in HIV infection in vivo is supported by the finding that individuals who do not express this receptor because of genetic mutations are resistant to HIV infection[26–28], as long as they are not infected by a strain with ability to use CXCR4 receprtor[29,30].

Under normal circumstances, the body may continue to regenerate the CD4 T-cells that are being destroyed. However, continuous attempts of the immune system to control HIV replication and regenerate the loss of CD4 T-cells induce a sustained condition of immune activation, that will erode immune system increasing immune exhaustion and immunosenescence. Failure to regenerate the progressive destruction of CD4 T-cells by the thymus and the continued immune exhaustion collapse the immune system. At the point when the number of CD4 T-cells necessary to assure proper immune responses is not maintained, HIV-infection leads to AIDS and the appearance of opportunistic infectious[13].

Thus, the clinical course of HIV infection proceeds in several phases, generally defined by adaptive immune control: (1) an acute retroviral syndrome in which the virus rapidly spreads in the infected host; (2) an asymptomatic, chronic phase, in which adaptive immune response partly control viral replication to a relatively stable level, commonly referred to as VL set point[17]; and (3) clinical AIDS characterized by immune deficiency and the appearance of opportunistic infections (Figure 7)[16]. Several studies have shown that VL set point predicts progression to AIDS and death and it is achieved within months after infection, although differ greatly among individuals[31–33].

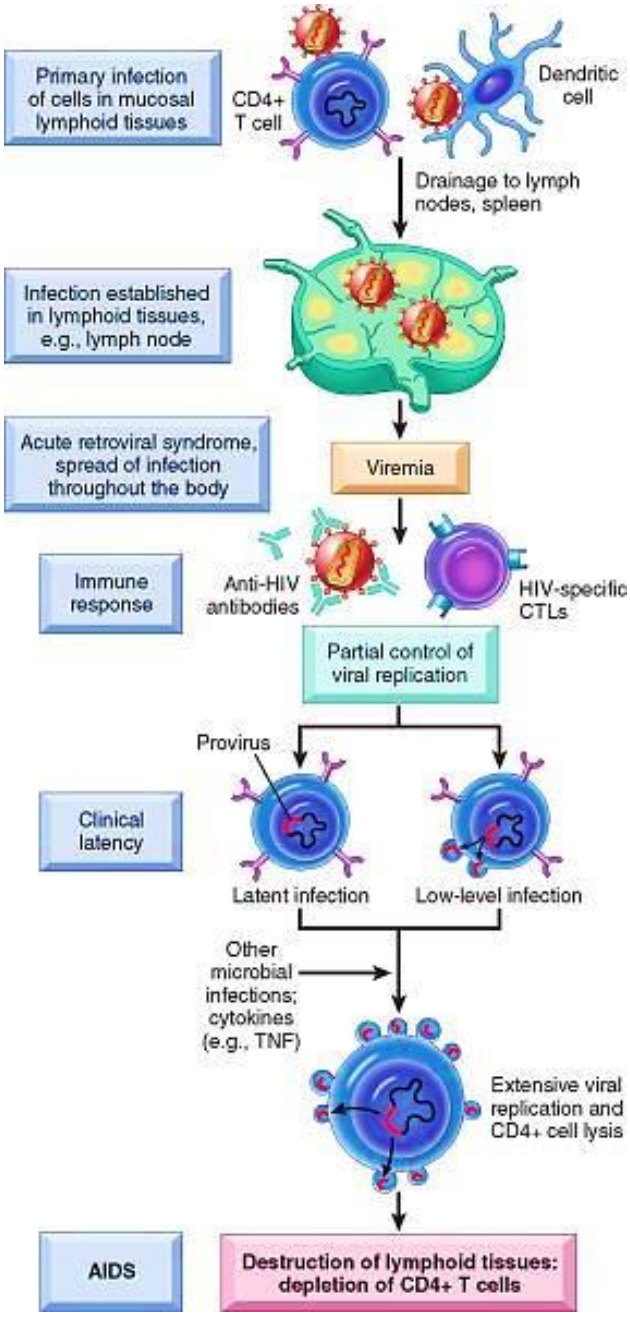


Figure 6. Pathogenesis of HIV-1 infection. The initial infection starts in mucosal tissues and spreads to lymph nodes. Viral replication leads to viremia and widespread seeding of lymphoid tissue. When CD4 T-cell numbers decline, the patient develops clinical symptoms and AIDS. CTL, cytotoxic T-lymphocyte. *Adapted from Robbins Pathologic Basis of Disease, 8th ed*[13].

HIV-infected individuals are clinically monitored through assessment of the CD4 T-cell count in peripheral blood. AIDS diagnosis is made when the HIV infected individual has a CD4 T-cell count less than 200 cells/mm^3 or develops one or more opportunistic illnesses (Figure 7)[12,15]. If not treated, the rate at which HIV-infected individuals progress to AIDS is variable and can be divided into different categories[13]. The majority of infected individuals are considered to be intermediate progressors, characterized by disease progression to AIDS approximately 9 years after primary infection, with significant variations according to age[34,35]. In the absence of therapy, death usually occurs within 1 year after the onset of clinical AIDS and the risk of death increases with decreasing CD4 T-cell counts[35]. However, an individual with HIV infection who is diagnosed and treated early has nearly the same life expectancy as that of an HIV-negative person, as long as they maintain good treatment adherence and continuous access to care[34].

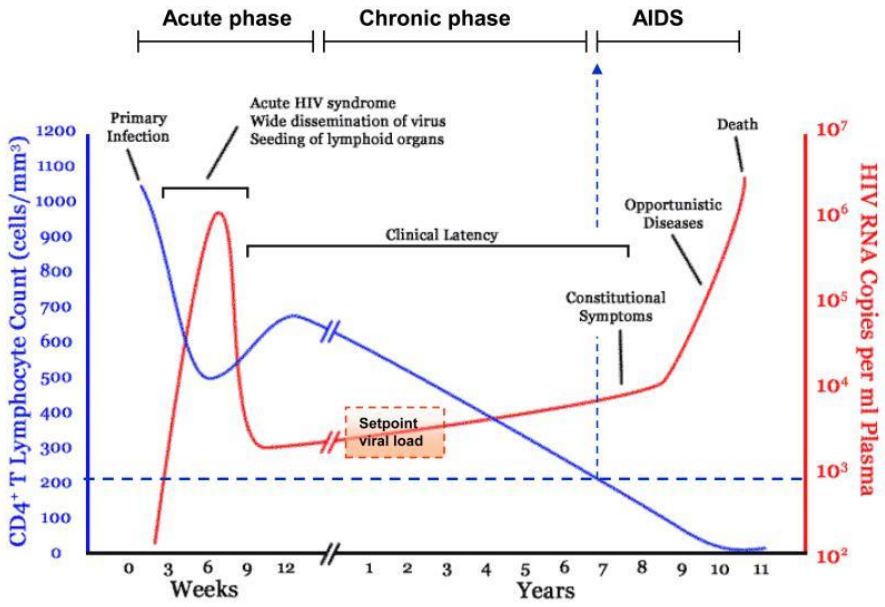


Figure 7. Clinical course of HIV infection. During the early period after primary infection there is dissemination of virus, development of an immune response to HIV, and often an acute viral syndrome. During the period of clinical latency, viral replication decays to a set point level and the CD4+ T-cell count gradually decreases, until it reaches a critical level below which there is a substantial risk of AIDS-associated diseases. *Adapted from An. Et al. Trends in Immunology 2010*[36].

2.2. HIV life cycle and antiretroviral therapy

The life cycle of HIV encompasses different steps beginning with viral entry and retrotanscription to deoxyrribonucleic acid (DNA) inside the target cells, integration of the

provirus into the host cell genome, activation of viral transcription, and production and release of new infectious viruses (Figure 8)[13]. The advent of new ARV drugs that target the main viral proteins: reverse transcriptase, protease, and integrase, has changed the clinical face of AIDS. These drugs are given in combination ART to reduce the emergence of viral mutations that confer resistance to any single drug[37].

Over 25 antiretroviral drugs from six distinct drug classes have been developed for the management of HIV infection[12]. Standard ART regimens combine two nucleoside reverse transcriptase inhibitors (emtricitabine or lamivudine together with one of abacavir, tenofovir, or zidovudine) with a non-nucleoside reverse transcriptase inhibitor, protease inhibitor, or integrase inhibitor (Figure 8). Several effective nucleoside reverse transcriptase inhibitor-sparing regimens can be used as second line treatment if intolerance or resistance to nucleoside reverse transcriptase inhibitors develops[12]. When a combination of at least three effective drugs is used in an adherent patient, HIV replication is reduced to below the detection levels usually within 3 months and stays undetectable[38]. Once the virus is suppressed, the progressive loss of CD4 T-cells is halted and after several years the peripheral CD4 T-cell count returns to a normal level, as long as ART was not initiated under conditions of severe immune-depression (CD4 T-cell count<200 cells/mm^3)[39]. Immune activation is also reduced but not completely abolished by ART. This fact has consequences in the pathogenesis of vascular disease[40] , chronic inflammation[41] and immune exhaustion[42] in treated HIV-infected individuals.

Seminal studies have shown that treatment must be continued in HIV-infected individuals in order to maintain suppressed viremia, elevated CD4 T-cells counts and reduced HIV-associated morbidity and mortality[37,43–45], as well as to reduce the risk of developing drug resistance[37,46,47]. Other studies have obtained favorable outcomes with CD4-guided or fixed-cycle treatment interruptions, suggesting ART interruption as a way to reduce drug toxicity, patient treatment exhaustion and even as a cost-saving strategy in low and middle-income countries (LMIC)[48–50]. However, these latter studies found that AIDS-related illnesses were more frequent in the treatment interruption arms. Nevertheless, international guidelines on the use of ARV drugs maintain that treatment should be uninterrupted in HIV-infected patients[51,52]. Regarding the best time for treatment initiation in HIV-infected individuals, conclusive results from recent clinical trials have demonstrated a clear benefit in patients that initiate ART as early as possible after HIV-diagnosis. Early ART initiation prevents the development of HIV-related clinical events[53] and progression to AIDS[54,55], limits the size of HIV reservoir[22,56–58], diminishes the HIV-related immune activation[59] and the early immunological damage at the intestinal level[57,59], as well as reducing further transmissions[53,60]. Based on this evidence, the World Health Organization (WHO) updated the ART guidelines in 2015 to recommend ART initiation after diagnosis regardless of T-CD4 cell counts or disease stage and to support long-term adherence to ART in all population groups[61,62].

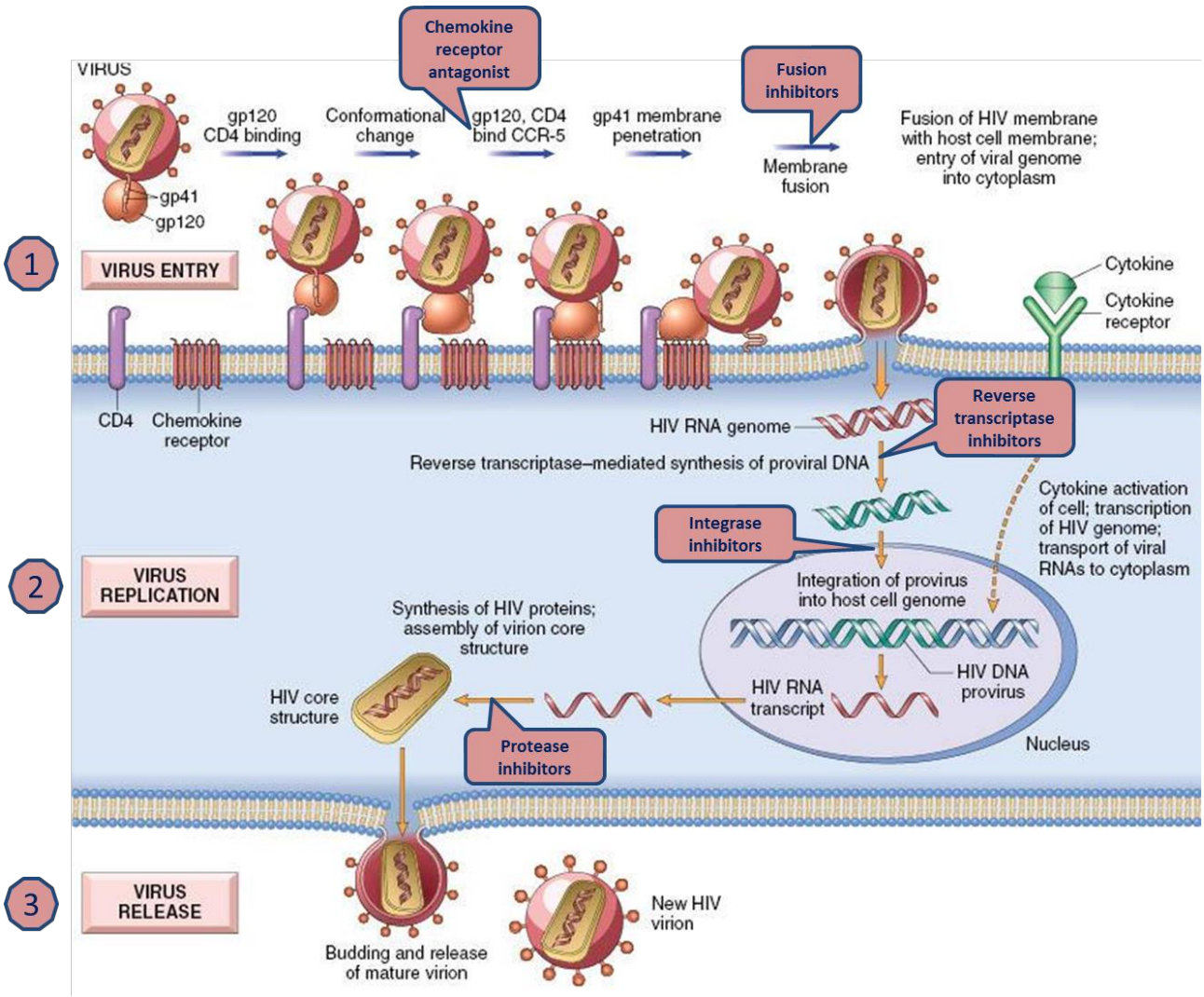


Figure 8: HIV life cycle showing the sites of action of different classes of antiretroviral drugs. The main stages of the HIV life cycle are: 1) virus entry into target cells after binding and fusion with the cell membrane; 2) virus replication, including reverse transcription to DNA, integration of provirus into host cell genome, replication, synthesis and assembly of viral proteins; and 3) budding and virus release. *Modified from Robbins basic pathology; Vinay Kumar, Abul K. Abbas, Jon C. Aster. 10th ed.*[13].

Despite considerable advances in the understanding of HIV pathogenesis and treatment[63,64], there are no effective cure strategies. This is mainly due to the virus' persistence in long-lived latently infected[65,66] resting memory T-cells. These and other cell types[67] make up anatomical reservoirs of HIV including the gastrointestinal (GI) tract[68], lymphoid tissue or tissue macrophages[69], and the central nervous system[70]. Although there have been impressive advances in the development of a protective vaccine[69], HIV's elevated mutation rate tremendously complicates the task[71]. Recent efforts have focused

on eliciting broadly neutralizing antibodies against the most conservative portion of HIV envelope (Env) glycoproteins (gp)[72–74]; however, there is still no evidence of interventions providing lasting protection against HIV infection[75]. Thus, prevention, testing and treatment remain the mainstays in the fight against the HIV pandemic.

3. Acute HIV infection

3.1. Characteristics and stages of primary HIV infection

Acute HIV infection (AHI) is the first stage of infection, often called the 'window period', defined as the time from HIV transmission until the development of HIV-specific antibodies that define seroconversion[76,77]. The term 'recent infection' is generally used to describe the phase up to 6 months after infection. Early or primary HIV infection (PHI), which includes AHI, is regarded as the interval between virus acquisition and the establishment of a VL set point[78,79]. Throughout this thesis, the term early or PHI is used to refer to recent infection, therefore including AHI.

AHI is characterized by high viral replication, reaching plasma VL levels only comparable to those encountered during the end stage of AIDS (see Figure 6)[36]. Along with high viremia, in most cases, a transient non-specific febrile illness occurs around 2-4 weeks after the transmission event[80–82], typically before or at the time of peak viremia[83]. AHI is a rapidly evolving phase characterized by intensive immune responses preceding the development of HIV-specific antibodies. Peak Plasma VL occurs around 21–28 days after infection[17,83], followed by a slower decrease as HIV-specific cellular and humoral responses start to develop (Figure 9A)[84]. Different HIV antigen-antibody specificities appear in sequence after HIV transmission as do immunoglobulin (Ig) G subclass responses. As such, based on a sequential gain in positivity for different HIV diagnostic tools, patients have been categorized into Fiebig stages I–VI[85], that are useful in approximating time since infection with relative accuracy[86] (Figure 9A). The time between infection and the first detection of viral RNA in plasma is commonly referred to as the eclipse phase and lasts approximately 10 days[36]. During the eclipse phase, only HIV-RNA is detectable in blood (Fiebig stage I); 7 days later, p24 antigen becomes detectable by enzyme-linked immunosorbent assay (ELISA) (Fiebig stage II); 5 days later, IgM HIV-specific antibodies become detectable with sensitive ELISA (Fiebig stage III); another 3 days later some HIV-specific antibodies become detectable by Western blot (WB) which will be considered indeterminate (Fiebig stage IV); in another 6 days WB converts to clearly positive (Fiebig stage V) and, approximately 40 days later, antibodies to the p31 antigen become detectable in the WB (Fiebig stage VI)[77]. Thus, at the end of Fiebig stage V, within 100 days following infection, patients leave acute infection phase to enter the early chronic stage of infection as the plasma VL begins to plateau[17,77,82].

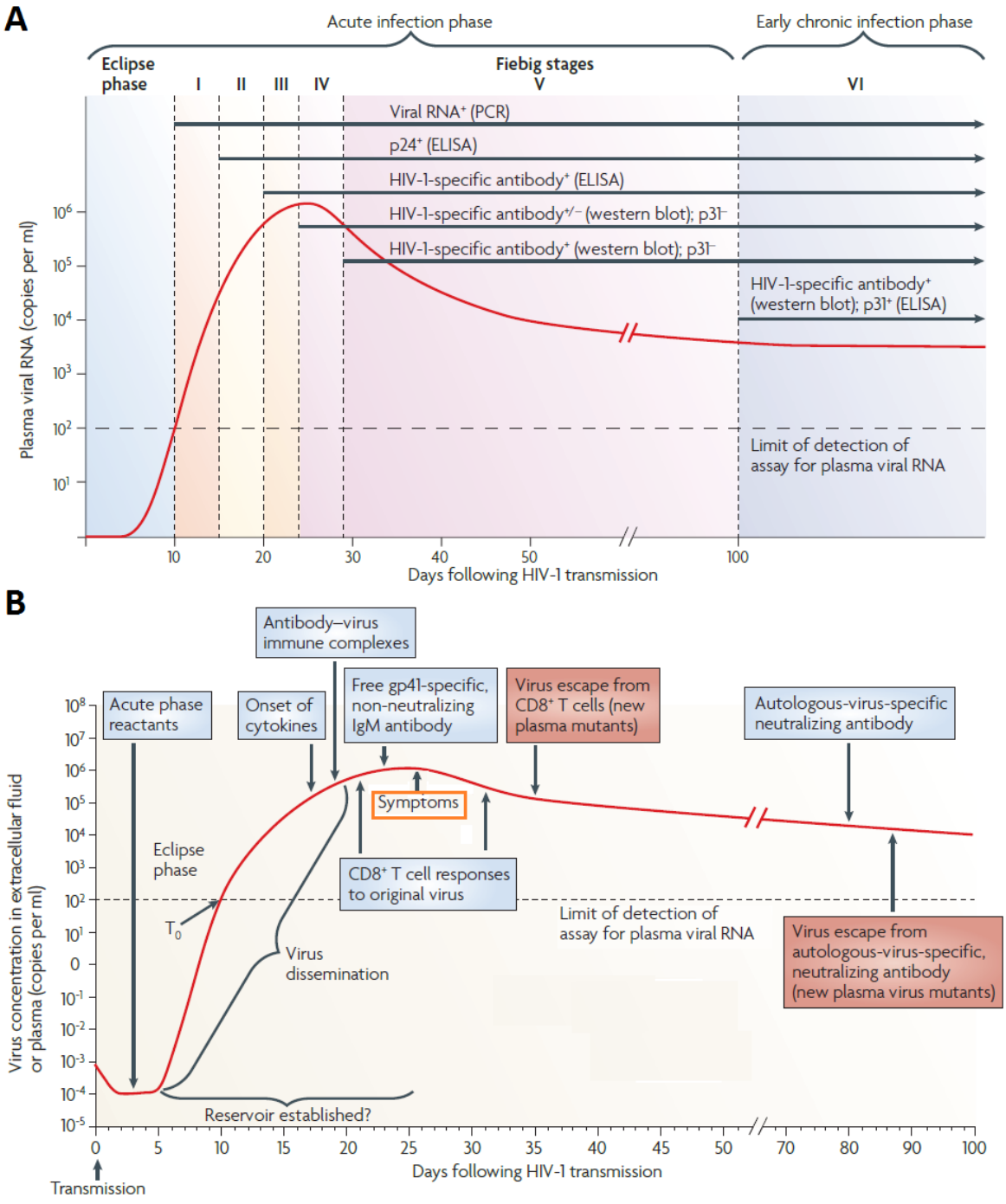


Figure 9. Phases during the acute HIV infection process. (A) Clinical stages in early HIV-infection defined by the stepwise gain in positivity for the detection of HIV-antigens and HIV-specific antibodies in diagnostic assays. (B) Primary innate and adaptive immune responses detected after HIV transmission. *Modified from McMichael et al. Nature Reviews Immunology 2010*[17].

The first detectable immune responses to HIV infection are mediated by the innate (non-antigen specific) mechanisms characterized by increases in levels of acute-phase proteins in plasma, which are observed during the eclipse phase when virus replication is still largely restricted to the mucosal tissues and draining lymph nodes (Figure 9B). Within days, as plasma viremia increases exponentially, viral reservoirs are established[21] and the first antibody–virus immune complexes are detected. Expansion of the earliest HIV-specific CD8 T-cell responses also commences prior to peak viraemia[84], followed by detection of the first non-neutralizing IgM antibodies against the HIV-Env gp41. However, as the HIV-specific immune response is mounted, HIV develops immune escape mechanisms. As a result, complete virus escape from the first CD8 T-cell responses often occurs within a few days[84]. The earliest HIV neutralizing antibodies are detected around 80 days after infection, prior to the onset of the VL set point, but virus mutants capable of escaping from neutralizing antibodies can be detected in plasma one week later[87–89].

3.2. Gastrointestinal damage and immune activation in early HIV infection

Early after infection, HIV induces a massive destruction of mucosal CD4 T-cells, particularly in the gut-associated lymphoid tissue (GALT)[90], where most of these cells reside[91,92]. Models of pathogenic simian immunodeficiency virus (SIV) infection in macaques[14,93], as well as studies in humans[94], have shown that during AHI up to 80% of CD4 memory T-cells in the GALT is depleted within the first 3 weeks of infection. This depletion is accompanied by a mucosal inflammation and a deregulation of the epithelial barrier maintenance and digestive/metabolic functions[95], resulting in an enteropathy that continues throughout the entire disease course in the absence of ART[90] (Figure 10). This GI damage is associated with microbial translocation, which is defined as the passage of microbial particles/debris from the intestinal lumen to systemic circulation[96]. These changes coincide with the up-regulation of immune activation, inflammation and apoptosis-associated genes in the GALT[95]. Hence, the imbalance between immune responses and mucosal repair and regeneration finally results in a GI epithelial barrier dysfunction[95,96] defined by an increased permeability to bacterial products. This condition represents a considerable assault to the immune system and a near-complete restoration of mucosal immune system can only be achieved by very early treatment initiation[59,97]. Similarly, in the absence of viral suppression, innate lymphoid cells are also depleted irreversibly in the blood during AHI and only early treatment initiation may preserve immune homeostasis[98].

Translocation of microbial products from intestinal lumen to peripheral blood contributes to the systemic inflammation and immune activation[99]. Paralleling the establishment of a VL set-point[96], an immune-activation set point has also been defined in chronic HIV infection, based on the level of activated CD8 T-cells[100]. The level of HIV-induced immune activation has attracted interest as it is closely associated with the exhaustion of the

immune system[90,101] and it can determine the rate at which CD4 T-cells are lost over time independent of VL[100,102].

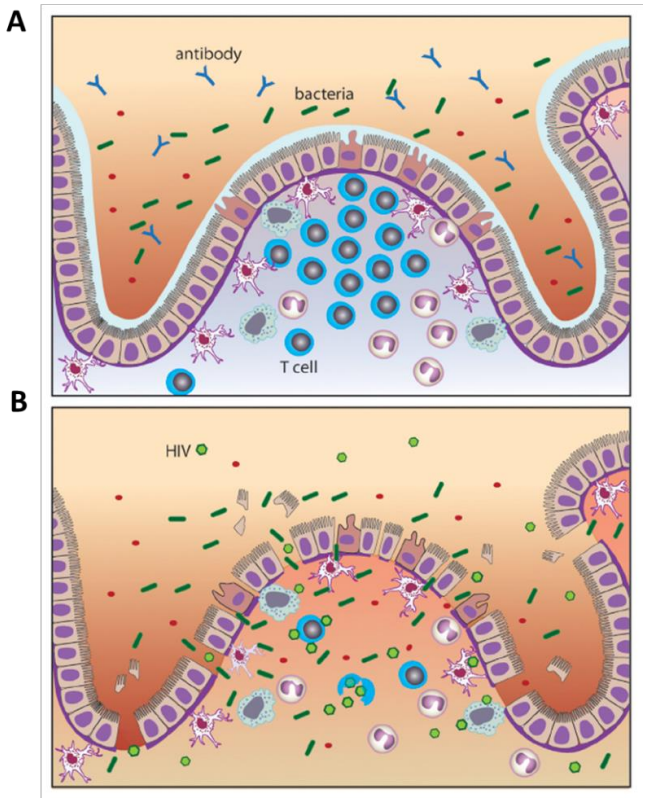


Figure 10. (A) Healthy mucosa vs. (B) HIV-infected mucosa. Gastrointestinal chronic inflammation and bacterial translocation is well established at the early stages of HIV infection. *Adapted from Deeks SG et al. Immunity 2013*[96]

.Interestingly, chronic immune activation is not observed in naturally SIV-infected Sooty mangabeys. Indeed microbial translocation does not seem to occur in these animals despite high levels of virus replication and intensive CD4 T-cell depletion in the GALT during AHI[99]. In contrast to HIV in humans, SIV in Sooty mangabeys rarely progress to AIDS[103,104] thus suggesting a role for chronic immune activation in HIV-related complications and AIDS progression[105]. Similarly, in natural non-pathogenic SIV infection of African green monkeys, after initial acute inflammation in the GALT, activation is rapidly attenuated in the chronic phase and the mucosa is preserved, even in the presence of persistently high levels of VL[104–107]. Several studies have also shown a positive correlation between markers of CD8 T-cell activation and HIV disease progression[100,108]. Interestingly, HIV-infected individuals that control viremia in the absence of ART, often termed 'elite controllers', exhibit low levels of immune activation and microbial translocation[99,109]. It is thus hypothesized that retaining

GALT integrity and maintaining low immune activation may protect the host from continued CD4 T-cell depletion and progression to AIDS[17,82,100].

3.3. AHI diagnosis and early ART initiation

The AHI period represents a 'window of opportunity' for prevention strategies which can bring tremendous benefit both at the individual and public health levels. As previously described, early HIV-diagnosis and treatment may prevent substantial irreversible immunological damage[96,98], preserve mucosal function[57,59], diminish the viral reservoir[22,56,57,110], attenuate the immunologic activation[41,59,96] and stop progression to AIDS[53–55]. But also, AHI represents a critical narrow stage for preventing further HIV transmissions. As a result of high viremia in bodily fluids and high levels of genital shedding of the virus, individuals are considered hyper-infectious during the acute phase[111,112]. Although the precise contribution of early infection to population HIV incidence varies across settings and local transmission dynamics, numerous modelling[79] and phylogenetic studies[113,114] indicate that this early period plays a disproportionate role in the spread of HIV. Previous studies in Malawi[115], Uganda[111] and Quebec[116] have suggested that 38.4%, 43.8% and 49% of onward transmissions, respectively, occur in the first 6 months after infection. However, there was some concern as to the risk of overestimating transmission because of unmodeled heterogeneity in transmission rates between couples and by inconsistent censoring [117]. More recent studies from Swiss[60] and US[113] cohorts have both concluded that approximately 44% of the new transmissions occurred during the first year of infection. Thus, the AHI stage may be crucial in fuelling the worldwide HIV pandemic.

Despite the importance of early HIV-infection, there are immense difficulties in identifying AHI patients for treatment initiation[118]. Patients are often asymptomatic, or experience a non-specific febrile illness; a diagnostic dilemma compounded in the Africansetting by a significant symptom overlap with malaria in high HIV endemic areas[77,119,120]. To diagnose AHI, the presence of the virus itself must be detected by nucleic acids or proteins in the absence of HIV-specific antibodies. Time from HIV infection to seroconversion highly depends on the sensitivity of the serology test employed and slightly varies according to different studies[77]. Routine second-generation HIV rapid diagnostic test (RDTs) algorithms provide negative or indeterminate results for up to 6-8 weeks after infection[121]. Newly available fourth-generation assays for the detection of HIV-1 p24 antigen and HIV-1/2 antibodies have the potential to identify infected individuals in the acute phase. However, recent data show that the HIV-1 antigen detection component of most fourth generation assays may be lacking in sensitivity[122] and, in any case, remains less sensitive than nucleic acid amplification technology[77]. Thus, current gold-standard test for confirming viremia is real-time polymerase chain reaction (RT-PCR) for plasma HIV-RNA[121].

4. Immune response biomarkers during HIV infection

4.1. Expression of inflammatory and immune activation cytokines during early and chronic HIV infection

At the beginning of the HIV-infection, as viremia increases, there is a striking cascade response of acute phase reactants and inflammatory cytokines that has been referred to as the 'cytokine storm' (Figure 11)[17,123]. Studies in this area have demonstrated inconsistencies in type and timing of cytokine observations, including reports of significant up-regulation of cytokines which are not observed or contested by others[118,123–128]. The discrepancies may be due in part to differences in the cytokine measurement assays, but likely also reflect the highly dynamic nature of AHI in a short time of frame, thus leading to high variability between cross-sectional samplings[129,130]. The very first measurable innate immune response, detectable just prior to appearance of plasma HIV-RNA, is acute phase protein serum amyloid A[128]. Thereafter, generally, levels of interleukin (IL)-15, type I interferon (IFN)-α and interferon gamma inducible protein 10 (CXCL10/IP-10) are increased rapidly after infection, followed by an increase in IL-10, IL-18, Tumor necrosis factor (TNF) and IFNγ[17,123–127]. The cellular sources of this acute-phase cytokines have not been definitively identified, but most likely include infected CD4 T-cells, DCs[131,132], monocytes and macrophages[99], NK cells and NK-T cells[132,133].

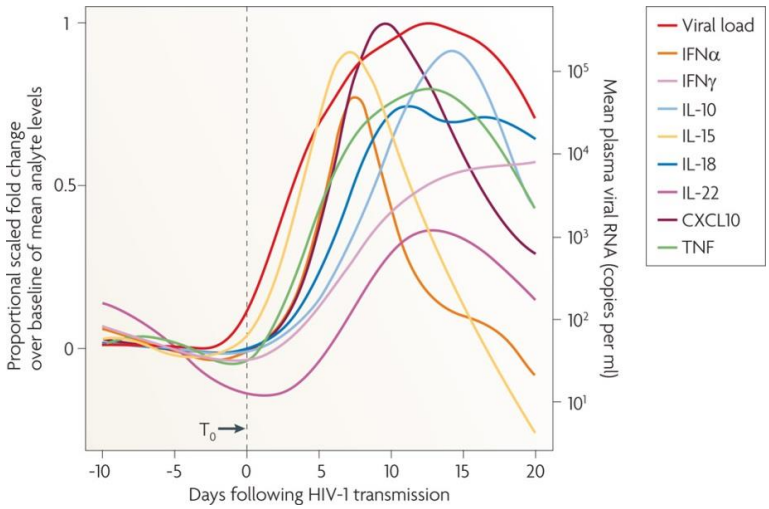


Figure 11. The cytokine storm in acute HIV-1 infection. The relative kinetics of elevation of acute-phase proteins, cytokines and chemokines in the plasma during acute HIV-1 infection. *Adapted from McMichael et al. Nature Reviews Immunology 2010*[17,123].

Some of these cytokines have antiviral activity; like IFNα[134], which probably derives from plasmacytoid DCs[135], supporting the idea that this cell subset undergoes activation as plasma viremia increases in AHI. Plasmacytoid DCs-derived IFNα together with IL-15 and IL-18 enhance innate and adaptive immune responses after HIV-infection. However, the activation induced by innate immune cells and the resulting production of pro-inflammatory cytokines can stimulate HIV replication and mediate immunopathology[131]. IFNα and TNFα also have pro-apoptotic effects and can thereby contribute to a loss of activated DCs and the bystander destruction of CD4 T and B-cells[130].

Significant efforts have been made to characterize early cytokines response aiming to identify biomarkers of progression or key pathological pathways that could be targeted to minimize HIV-induced immune damage over the course of infection[125,128–130]. Some cytokine storm components, including IL-12, IFNγ, IP-10, IL-7 and IL-15, can predict VL set point[126,130], T-cell activation and CD4 T-cell count[126,127,136] or the subsequent disease progression[126,127,136,137]. It has also been shown that immune activation, as measured by levels of cytokine markers, particularly elevated levels of IL-10 and IP-10, are associated with increased susceptibility to HIV-infection and HIV infectiousness[138]. Furthermore, a recent study suggested that rapid disease progressors have earlier and more robust cytokine response than slow disease progressors, suggesting that a faster and stronger cytokine storm during AHI could promote disease progression[139]. Additionally, during chronic infection, release of several inflammatory biomarkers including soluble (s)CD14, sCD163, C-reactive protein (CRP) and IL-6 are associated with disease progression and mortality, in patients both on and off ART[129,140–143].

As a whole, soluble immune response markers in plasma and other body fluids reflect the individual's immunological status and may therefore be helpful in assessing risk of HIV transmission and acquisition[138], as well as disease progression[126,129,130]. Besides, cytokines could potentially be also used as adjuvants in the development of HIV-vaccines or as therapy either to suppress viral replication or to prevent deleterious immune effects of infection[144].

4.2. Development of HIV-specific antibodies

The first detectable humoral response after HIV infection is in the form of immune complexes 8 days after plasma virus detection (Figure 9B); whereas the first free plasma anti-HIV antibodies appear 13 days after detectable plasma viremia. These antibodies are from the IgM subtype and are directed against Env subunit gp41 which will be followed by IgG and IgA isotypes[145]. Env gp120-specific antibodies delayed an additional 14 days[146]. IgG antibodies against inner viral proteins appear at a median time of 18 days (p24, p55) and 33 days (p17) following detectable plasma HIV-RNA[145],while antibodies against HIV-integrase (p31) are the last elicited appearing at a median time of 53 days after detectable

viremia[145]. The kinetics of antibody generation and Ig class switching during HIV infection may vary according to individual factors, subtype and dose of infecting virus[17]. Previous studies have shown that the most abundant anti-Env HIV plasma antibodies present during both early and chronic HIV infection are of the IgG1 subclass. During AHI, the second most abundant subclass of anti-Env is IgG3 [147]. This initial antibody response to the viral antigens is non-neutralizing and has little effect on the control of initial viral replication[82,146]. Antibodies that neutralize the transmitted founder virus and select for viral escape are not detected until at least 3 months after infection and are mainly directed to Env variable regions[87,148], accounting for the extensive variation in the env gene that is observed in early HIV infection[87,149]. However, by the time this potentially effective antibody response has developed, it is much too late to influence the course of the infection[87]. Thus, the human immune system is capable of generating multiple neutralizing antibodies in response to a constantly evolving viral population that exposes new targets as a consequence of a continuous escape[150]. However, antibodies that show some degree of neutralization of heterologous virus eventually arise in only 10-25% of patients years after infection[151,152], indicating that both genetic factors and maturation of the antibody response are necessary for the generation of certain broad neutralizing activity. However, highly potent broadly neutralizing antibodies against a diversity of viral isolates are only identified in and estimated 1% of HIV infected individuals, the so-called 'elite neutralizers'[73,153].

4.3. T-cell populations during HIV infection

Subsets of T-cells can be defined by their specificity, surface phenotype, degree of maturation, location, or functions they exert upon stimulation, and any or all of these parameters can be affected by HIV infection[154]. During early HIV infection, many cells of the immune system show signs of extensive activation and a progressive loss of resting subsets[90]. Apart from the above described increased plasma levels of pro-inflammatory cytokines, other features characterizing immune activation include cell-surface expression of CD38 and the human leukocyte antigen-D related (HLA-DR) markers in CD4 and CD8 T cells and high level of activation-induced apoptosis of uninfected cells [101]. The apoptotic condition is reflected by the expression of exhaustion cell markers like programmed cell death protein-1 (PD-1), Tim-3 and Lag-3 proteins, which were shown to be predictive of disease progression[155,156].

Generally, untreated HIV-infection is characterized by progressive CD4 T-cell depletion and CD8 T-cell expansion. HIV can inhibit proliferation of immature thymocytes and/or directly infect leading to impaired production of CD4 T-cells[157]. This hampered supply of naive T-cells together with the profound CD4 T-cell depletion as a consequence of HIV active proliferation, is linked directly to the risk for opportunistic infections and mortality[158]. Likewise, CD8 T-cell activation was seen to be the strongest correlate of disease progression[100,102,108,159], where CD8 expression of HLA-DR and CD38 are significantly higher

in HIV-infected patients compared with HIV-negative older and younger controls[158]. The subsequent alterations in immune homeostatic mechanisms may lead to progressive loss of the naïve and memory T-cell pool, resulting in an imbalance in T-cell phenotypes[160]. Indeed, naïve CD4 and CD8-T cells (CD45RA+ CCR7+) have been found to be significantly reduced in HIV-infected patients together with central memory T-cells (CD45RA- CCR7+)[158], while terminally differentiated effector T-cells (CD45RA+/-CCR7-) have been observed in elevated proportions over the different stages of HIV infection[161]. The phenotypic and functional alterations in T-cells observed during human aging are characterized by the increased expression of CD57 cell marker[162] and the loss of CD28[163]. Similarly, after HIV infection, accelerated aging of T-cells or immunosenescence may occur due to the continuous highly productive viral replication and cell stimulation[160] (Figure 12) and it has been associated with poor CD4 T-cell restoration after ART initiation[164,165] and faster disease progression[166]. Besides, different studies showed that ART reversed, at least to some extent, many (but not all) of the changes described in chronic HIV infection[167,168].

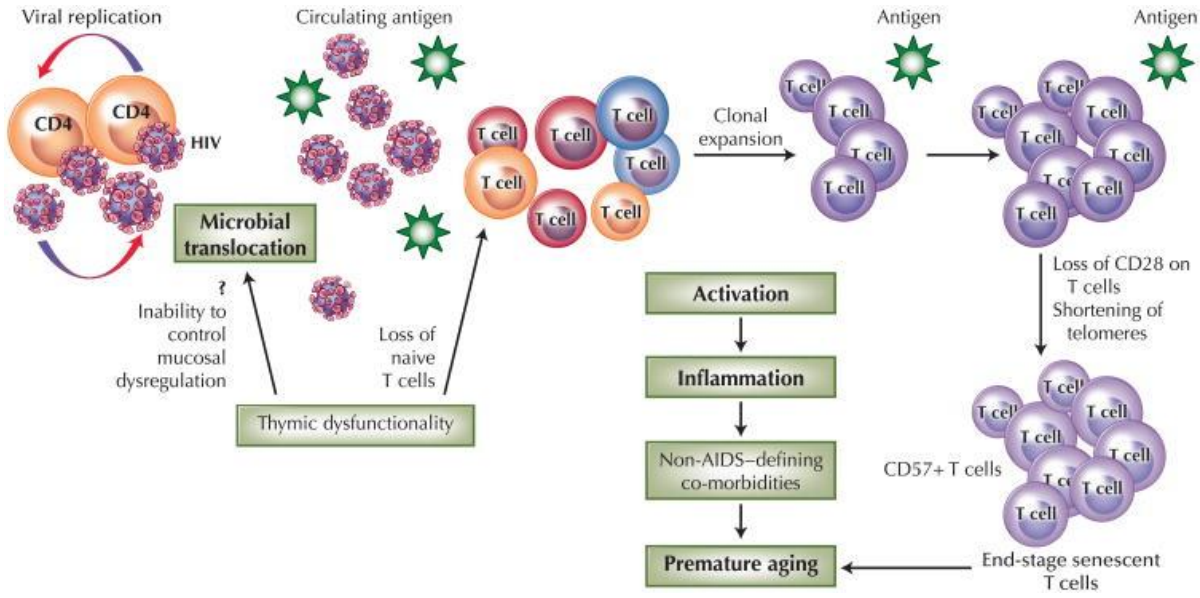


Figure 12. Accelerated aging model in HIV infection. Activated cells undergo clonal expansion in response to the persistent antigen, resulting in differentiation and accumulation of nonfunctional end stage senescent cells. *Adapted from Desay et al. Current HIV/AIDS reports 2010*[158].

Differentiation of CD4 T-cells into phenotypes with diverse effector roles is dependent on the expression of specific transcription factors in response to the predominant cytokine

environment. Initially, two subsets were defined: Th1 cells, which produce IFNγ and mediate protection against intracellular pathogens; and Th2 cells, which secrete different types of ILs against extracellular pathogens[169]. More recently, two further subsets have been identified: regulatory T-cells (Tregs), which induce tolerance against self-antigens and prevent autoimmunity[170–172]; and Th17 cells, which produce IL-17 and IL-22 and are involved in epithelial barrier integrity and protection against extracellular bacteria, fungi and mycobacteria[173]. Since mucosal Th17 cells have been observed to be irreversibly depleted during the first stages of AHI and only preserved by prompt ART initiation[59], in untreated patients, the loss of Th17 cells in the GALT may contribute to microbial translocation and sustained inflammation during HIV progression[174].

Tregs express low levels of IL-7 receptor CD127, high levels of IL-2 receptor CD25 and the nuclear transcription factor FoxP3[175], whereas Th17 cells express IL-23R, CCR6 and the transcription factor ROR-γt [176,177]. As pro-inflammatory Th17 cells and immune regulatory Tregs have antagonistic functions, several studies have defined a link between Th17 cells and Tregs. In SIV infection of nonhuman primates, the loss of Th17 cells, the increase in the frequency of Tregs[178,179] and the imbalance between these Th17/Treg cells was critical in determining systemic immune activation and the outcome of the infection process[179]. Similarly, an unbalance between these two subsets has been associated with disease progression during the chronic HIV-infection[180,181]. Some studies have reported an increased Tregs frequency in lymphoid tissues during progressive HIV disease[182,183] as shown for SIV infection while others have reported a gradual decline in Treg frequency in peripheral blood during HIV-infection associated with increased immune activation[181,184–186]. Additionally, during early HIV-infection, the Th17/Treg ratio in untreated patients negatively correlates with CD8 T-cell activation set point in the absence of systemic microbial translocation[187] and it has been recently suggested a potential link between this ratio and key HIV-specific CD8 T-cell responses against the infection[188]. Thus, early changes in Treg and Th17 cells seem to have a relevant role in predicting disease progression.

5. HIV monitoring and current international guidelines

5.1. WHO recommendations for HIV diagnosis and clinical management

5.1.1. WHO guidelines for HIV diagnosis

According to the latest UNAIDS 'Consolidated guidelines on HIV prevention, diagnosis, treatment and care for key populations', updated in 2016[189]; HIV testing and counseling (HTC) is the essential first step in enabling people with HIV to know their status and obtain HIV prevention, treatment and care services. Even for those who test negative, HTC is an important opportunity to put those at risk for HIV in contact with primary prevention programs and to encourage retesting and adherence along the HIV care cascade (Figure 13)[190]. To improve the quality of service delivery and the acceptability and uptake of HTC, for many settings WHO recommends the use of RDTs rather than conventional laboratory based diagnostics such as enzyme immunoassay. RDTs allow provision of same-day results and appropriate referral and follow-up. Most RDTs do not require venipuncture specimen collection, but instead can be performed with simple finger-prick collection procedures. Thus, HTC can be offered in a variety of settings, including primary care clinics and by a variety of providers, including outreach workers. WHO recommends standardized testing strategies to maximize the accuracy of test results while minimizing cost: specimens are first tested with a highly sensitive assay, and specimens that are non-reactive are considered HIV-negative and those reactive should be tested again using a different highly specific assay to confirm the HIV-positive results[191]. For reactive specimen on the first but non-reactive on the second, individuals should be asked to attend for re-testing after 14 days. Rapid point of care (POC) third-generation HIV-antibody tests that use whole blood are available and more sensitive than second-generation tests. In light of the opportunities and challenges in detecting AHI, WHO has not yet established a clear strategy or any specific recommendation to identify or treat people during this phase of infection[51].

5.1.2. WHO guidelines for ART initiation

In 2013, WHO published consolidated guidelines on the use of ARV drugs for HIV treatment and prevention across all age groups and populations recommending treatment initiation in individuals with CD4 T-cell counts of 500cells/mm^3 or less[192]. Based on new scientific evidence and lessons from implementation, a comprehensive revision of these guidelines was released in 2015[61,62] and updated in 2016[51]. These last updated WHO guidelines recommend ART initiation in everyone living with HIV regardless of WHO clinical stage or CD4 T-cell count and also incorporate the use of daily oral pre-exposure prophylaxis (PrEP) as a prevention choice for people at

substantial risk of HIV. The latest recommendation for 'test and treat' is based on evidence from clinical trials and observational studies released since 2013 showing that earlier use of ART results in better clinical outcomes for people living with HIV compared with delayed treatment[54,55,110].

New WHO guidance on preferred ART regimens for adults allows for a reduced dosage of efavirenz to improve tolerability and reduce costs and for the option of using the integrase inhibitor class of drugs in first-line ART.

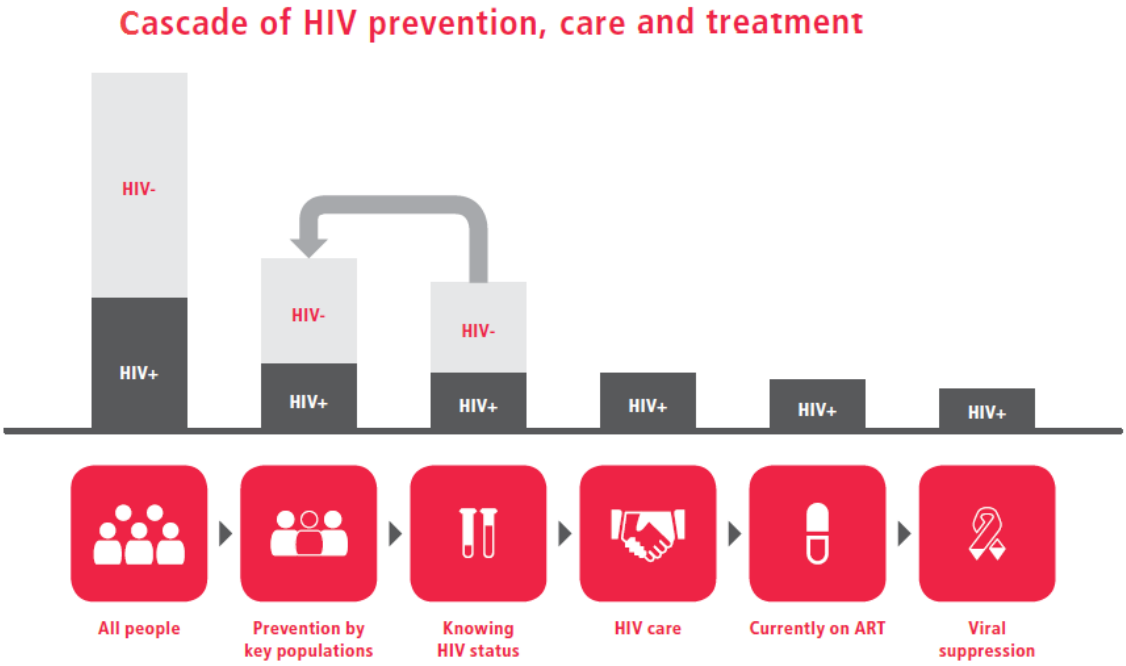


Figure 13. The HIV care cascade. The term 'cascade' emphasizes that a sequence of services is needed to achieve the desired impacts on HIV-prevention and treatment and highlights the gradual attrition of coverage of the eligible population over the steps of the sequence. Monitoring the cascade of services requires a consolidated set of indicators covering the entire sequence. *Adapted from WHO Consolidated strategic information guidelines for HIV in the health sector 2015*[190].

5.1.3. WHO guidelines for ART monitoring

Monitoring the response to ART is important for timely detection of treatment failure and to allow early adherence interventions, prevent further transmissions and avoid delays in regimen switches that could lead to disease progression or accumulation of HIV drug resistance mutations[193]. Continuation on a failing regimen can be associated with more complex mutation patterns, development of cross-resistance to reverse-

transcriptase inhibitors backbone that constitute the base of first and second-line treatments and thus, promote onward transmission of drug-resistant HIV to ART-naive patients. Many countries are moving to implement VL testing as the preferred means of monitoring people on ART and new POC-VL testing technologies offer further potential to expand this approach[51]. Additionally, in settings where routine VL monitoring is available, CD4 T-cell count monitoring can be stopped in individuals who are stable on ART and virologically suppressed[62]. Since 2013, WHO recommends routine HIV-RNA VL testing at 6 months after ART initiation and every 12 months in stable patients as the preferred monitoring approach to supervise treatment adherence and assure viral supression[192]. According to the latest WHO guidelines, virological failure (VF) is defined by a persistently detectable VL exceeding 1000 copies/mL (two consecutive VL measurements within a 3-month interval with adherence support between measurements) after at least 6 months of starting a new ART regimen[62]. After diagnosis of VF patients should be switched to a second-line ARV regimen consistent with ART optimizing principles, availability of fixed-dose combinations, tolerability and the risk of resistance mutation[62].

5.2. UNAIDS 90-90-90 targets and disparities in Sub-Saharan Africa

In 2014 UNAIDS worked with partners to obtain a global consensus on the creation of a new target for HIV treatment scale-up beyond 2015. These targets include that, by 2020, 90% of people (children, adolescents and adults) living with HIV will know their status, 90% of all people with diagnosed HIV infection will receive sustained ART and 90% of people on treatment will have suppressed VL. However, by the end of 2016 still almost half of the people living with HIV do not know their HIV status and often test late starting treatment when they already are significantly immunocompromised[7].

With the increasing availability of more accurate diagnostic testing, more effective drug regimens and better knowledge of the dynamics of HIV transmission and viral reservoirs, early diagnosis during recent HIV infection has been viewed as an opportunity for treatment and prevention interventions, with a potentially important public health impact[53,194]. Use of fourth-generation assays with pooled VL substantially improves AHI case finding, and although these laboratory-based diagnostic tools have been incorporated into screening algorithms in the USA and Europe[195,196], the technologies are rarely available in settings with scarce resources. Even though the prevalence of AHI among febrile patients in some low-income areas as SSA may reach 3%[119,120], logistics difficulties and financial constraints make VL testing very limited[53,77,81]. Despite the necessity of further development of affordable POC-VL devices and international AHI diagnostic strategies, prominent international guidelines, such as those from the WHO, have not yet made any specific recommendation regarding AHI detection for early ART initiation in LMIC[62,189,197].

Despite the latest WHO recommendations on universal eligibility for ART, national guidelines still vary across the different countries (Figure 14). Implementation of 'test and treat' strategy will mean that more people will start ART earlier. In June 2016, UNAIDS sources for Global AIDS response programme estimated that 18.2 million [16.1 million–19.0 million] people were accessing ART[7]. If these recommendations reach global implementation according to UNAIDS targets, this number will increase to 30 million by 2020. As this expansion takes place, programmes must maintain the capacity to respond to the needs of all patients[62]. In many settings long waiting times, ARV stock outs and overworked staff in central hospitals are significant barriers to access to services and retention in care, particularly in rural areas in SSA. As a result, initiation of ART at a late stage of disease is common in these countries, where high rates of attrition due to delays or failure to complete ART eligibility assessments have been described[198–200]. Thus, WHO recommends decentralization of ART services in order to deliver all HIV services closer to the individual[51].

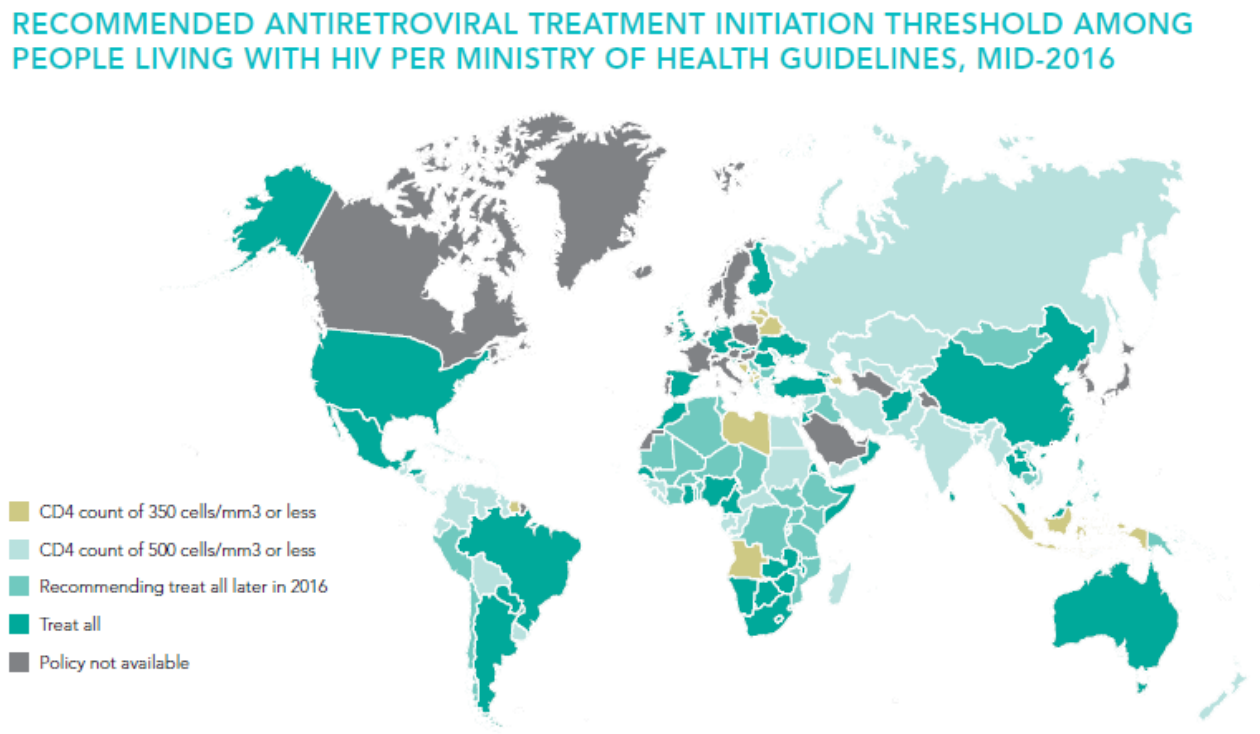


Figure 14. Global map according to the recommended criteria for ART initiation in 2016. *Adapted from UNAIDS Prevention Gap report*[7]

ART monitoring through VL is important for timely diagnosis of VF to allow early adherence interventions and prevent further transmissions[193]. Not only pooling-plasma VL[201–203], but also combination of finger-prick dried blood spots and a pooling strategy were shown to be a feasible and efficient option to reduce costs associated to VL monitoring while maintaining accuracy[204]. However, despite the international recommendations and demand for this assay, VL testing is still not widely available in many LMIC due to high cost and implementation constraints [205–207]. The alternative to VL has often been clinical and/or immunological monitoring, which frequently results in patients remaining on failing ART as well as unnecessary regimen switches [208]. As seen in several studies in SSA, almost 80% of subjects with detectable viremia have lost most first-line drug options available due to drug resistance[209–212]. New drugs with complementary resistance profiles and formulations with enhanced pharmacokinetic properties are urgently needed to construct effective second-line ART in LMIC.

As a consequence of the these limitations, by the end of 2015, of the estimated 36.7 million people living with HIV globally, only about 60% [56–65%] knew their HIV status, 46% [43–50%] were on ART, and 38% [35–41%] had achieved viral suppression, according to data reported to UNAIDS by 86 countries and considering that only 22% of people on ART worldwide reported to ever been tested for VL[7] (Figure 15A).Thus, the global gap to achieving the 90–90–90 target in 2015 was around 10.9 million people living with HIV who did not know their status, 12.7 million people in need of ART, and 13.0 million people living with HIV who were not virally suppressed (Figure 15B).

5.3. Characteristics of the HIV epidemic in Mozambique

The HIV national prevalence in Mozambique among adults between 15-49 years in 2015 was still extremely high, estimated to be 10.5% [8.3-13.3%][213]; however, the number of new HIV infections in the country has reduced by almost 40% in the last decade (Figure 16A)[4,213]. The recent roll-out of ARVs, the changes in the HIV national guidelines and the community testing campaigns have resulted in a dramatic increase of HIV-infected people receiving treatment in the last 5 years. The estimated ART coverage among pregnant women for prevention of mother to child transmission went from 21% in 2010 to 95% in 2015, while the general ART coverage increased from 16% to 53%, resepectively[4,213] (Figure 16B). However, recent country reports on HIV prevention strategies shown that among young people aged 15–24, 22.8% had a sexual intercourse before the age of 15 and only 34.9% both rejected major misconceptions about HIV transmission and correctly identify ways of preventing sexual transmission[4,213]. Additionally, data from national demographic and health surveys indicates that among people aged 15-49, 28% reported discriminatory attitudes towards people living with HIV, 26.9% of those who had more than one partner in the past year used a condom during their last sexual intercourse and 27.7% of ever-married

or partnered women experienced physical or sexual violence from a male intimate partner in the past year[4,213].

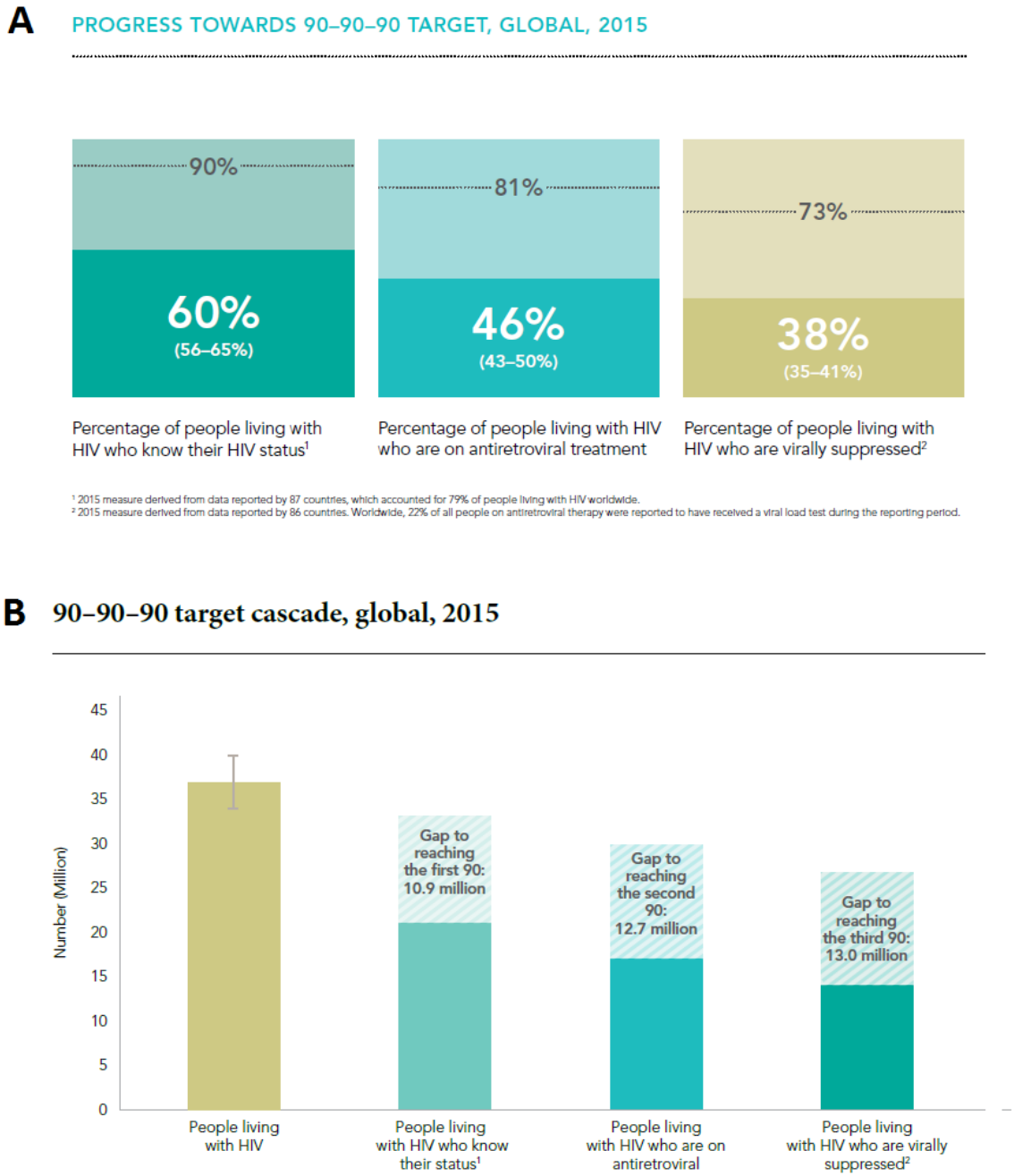


Figure 15. Global Progress Towards 90-90-90 targets by 2015. *Adapted from UNAIDS Prevention Gap Report 2016*[7].

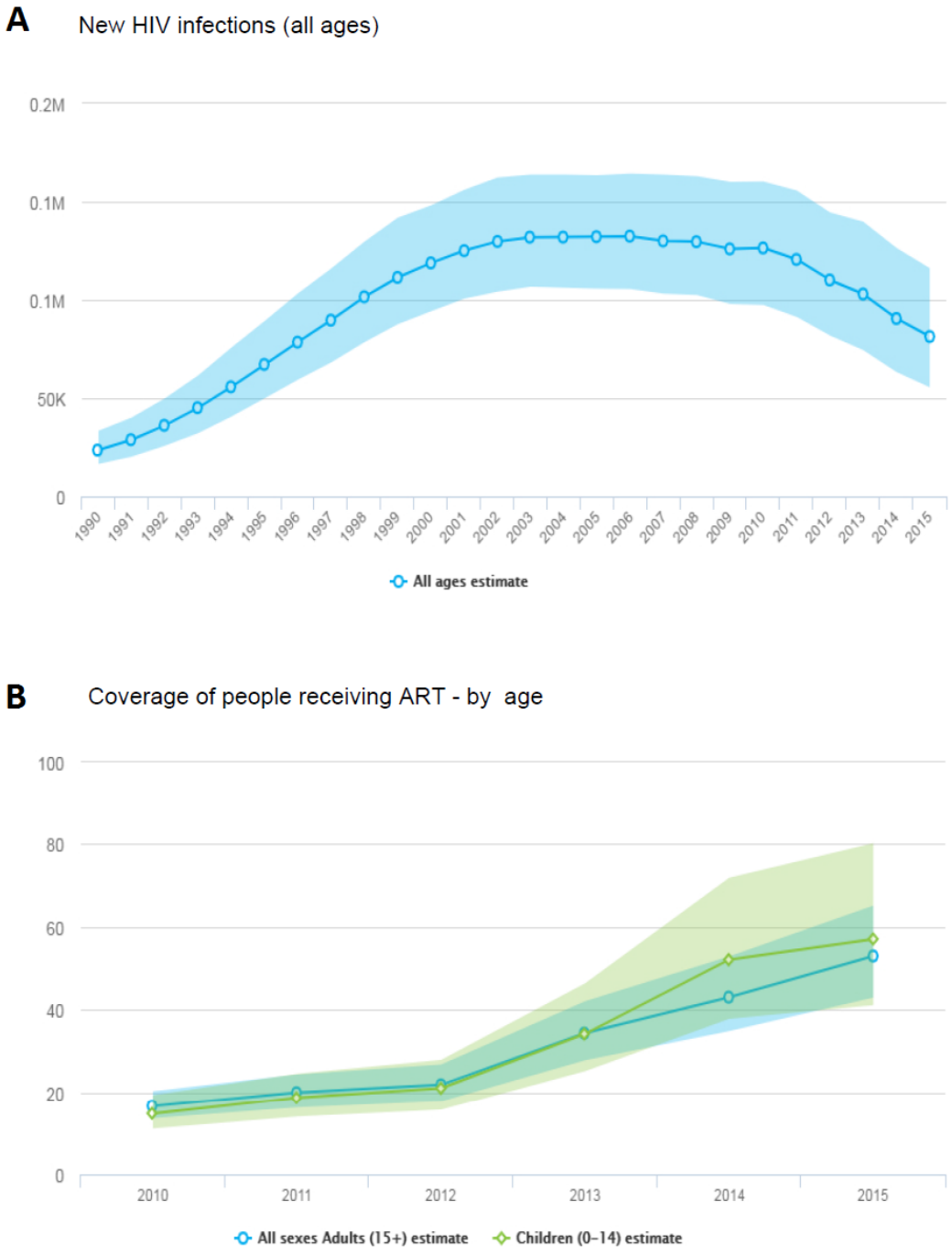


Figure 16. UNAIDS 2015 HIV estimates in Mozambique. (A) People living with HIV over the last 25 years; (B) new HIV infections in Mozambique over the last 25 years; (C) ART coverage of pregnant women over the last 5 years and (D) ART coverage by age over the last 5 years. Source UNAIDS Estimates 2015[213], adapted from *AIDSinfo website*[4].

HIV prevalence varies greatly between country regions in Mozambique (Figure 17)[214]. Manhiça is a rural area in the Maputo province (Southern Mozambique) where the studies presented in this thesis were developed. Community based studies in adults in Manhiça, performed in 2010[215] and 2012[216], reported nearly 40% of HIV prevalence and 30% among pregnant women attending the antenatal clinics at the Manhiça District Hospital (MDH).

Studies in this area have also estimated a prevalence of 9% of mother to child transmission at first month of age and of 27% in the first year[217]. Moreover, recent studies conducted in the Manhiça community have shown that around 3% of the HIV-seronegative outpatients presented with reported fever at MDH were AHI-individuals[120,218]. ARVs are provided free of charge and the national coverage in adults in need of therapy was approximately of 53% in 2015[7219]. At the time when the studies were performed, HIV national guidelines recommended ART initiation in patients with a CD4 T-cell count ≤350 cells/mm^3 or presenting any AIDS-associated syndrome and in pregnant women. The most prevalent ART regimen was zidovudine/lamivudine/nevirapine[220] and no routine VL monitoring was provided after ART initiation, thus switches to second-line ART were based on clinical and immunological criteria.

The demographic structure of the Manhiça population has a predominance of young people, and close to half the population under the age of 15 years. As a consequence of labor migration to Maputo city and the neighboring South Africa, this population presents a reduced proportion of adult males from age 20 onwards. The full geographic and socio-demographic characteristics of the study community has been previously described[221].

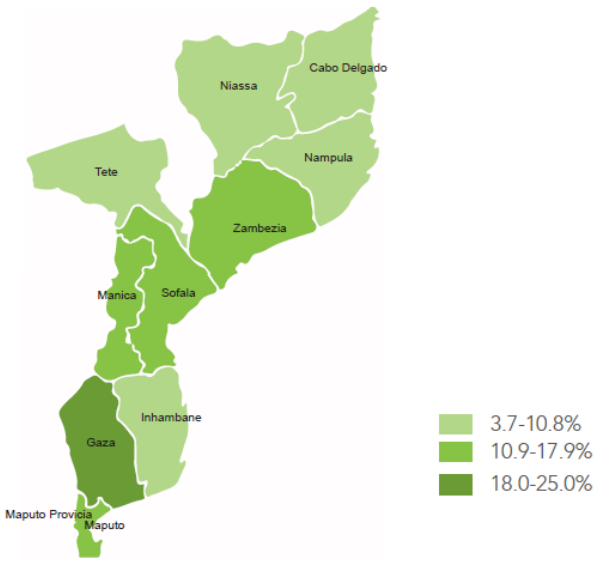


Figure 17. HIV prevalence by region in Mozambique in 2009. Data source INSIDA 2009 report, figure adapted from UNAIDS 2014 Mozambique report[214].

HYPOTHESIS AND OBJECTIVES

B. JUSTIFICATION, HYPOTHESIS AND OBJECTIVES

JUSTIFICATION

AHI is the initial period after HIV acquisition characterized by high viremia, high transmission potential and irreversible immune damage. Despite the importance of early HIV diagnosis and treatment, there are still immense difficulties identifying patients at the earliest stages of infection, particularly in the SSA setting Thus, studies seeking further characterization of immune and clinical responses during PHI are required in the SSA context in order to improve the knowledge of the early pathogenesis events and design early intervention strategies in these specific settings.

Although considerable international efforts have resulted in a dramatic increase in ART coverage in the last years, relatively little progress has been achieved in the development of simple, accurate and affordable tools that allow early HIV diagnosis and ART monitoring in LMIC. Current gold standard test for confirming viremia and diagnose either AHI or VF is HIV-RNA VL, however its availability is very limited in LMIC due to cost and implementation constraints.

HYPOTHESIS

The hypothesis is that the expression pattern of immune biomarkers may be used as a surrogate of viremic and clinical responses to HIV. Characterization of these biomarkers during early HIV infection can provide the basis to develop accessible tools to detect AHI or predict immune responses in HIV-infected patients living in LMIC. Moreover, the levels of immune biomarkers maybe employed to monitor response to ART and identify those with VF.

OBJECTIVES

I. General objective

The overarching goal of this research project is to describe the dynamics of soluble and cellular immune biomarkers over different stages of HIV infection in order to develop new tools for HIV diagnosis and monitoring in resource-limited countries and shed light in the early pathogenic events following HIV-acquisition.

II. Specific objectives

1. To describe the prevalence of AHI in HIV-seronegative outpatients presented with reported fever at the MDH throughout a VL-pooling-based strategy (Article 1).
2. To determine the expression pattern of innate and humoral biomarkers over the different stages of PHI and define a signature specific for pre- and post- seroconversion phases (Article 1).
3. To describe the kinetics of activated, exhausted and senescent T-cell lymphocytes, as well as specific T-cell lineages during early and chronic HIV infection and compare them with the clinical response (Article 2).
4. To analyze the association between soluble innate biomarkers and T-cell phenotypes during early HIV infection (Article 2).
5. To identify a soluble immune biomarker with predictive power to detect AHI among HIV-seronegative outpatients with undifferentiated fever (Article 3).
6. To evaluate the cost-effectiveness of detecting AHI with a biomarker-based algorithm in low-income settings (Article 3).
7. To identify a VL-surrogate biomarker that can be employed to accurately screen patients on ART for VF (Article 4).
8. To assess the potential benefit of implementing a biomarker as a screening tool to target the individuals on ART most likely to require VL testing (Article 4).

MATERIALS AND METHODS

C. MATERIALS AND METHODS

1. Thesis research context

This thesis is based on the research work undertaken at the Barcelona Institute for Global Health (ISGlobal)/ Hospital Clinic-Universitat de Barcelona in Spain, the AIDS Research Insitute/ Germans Trias i Pujol Research Institute (IGTP) and at the Centro de Investigacao em Saude de Manhica (CISM) in Mozambique, with whom ISGlobal has a longstanding partnership. The CISM is associated with the Mozambican Ministry of Health, the national malaria and HIV control programmes, the district and local health service authorities and the director and personnel from the Manhica District Hospital (MDH).

The thesis work is being directed by the group of 'HIV/Tuberculosis' from ISGlobal/Hospital Clínic (Dra. Denise Naniche) with proved experience in epidemiology and cohort studies in Mozambique together with the group of 'Cell Virology and Immunology' from IrsiCaixa/IGTP (Dr Julià Blanco) which presents a strong component in basic research of the immunological and cellular response to the HIV infection.

The presented work is based on patient cohorts from 2 major studies:

I. The Gastro-intestinal biomarkers in AHI Mozambican Adults study (GAMA): a prospective cohort of primary HIV-infected adults enrolled and followed up for 12 months between March 2013 and November 2014 at the MDH, Maputo, Southern Mozambique. This study was developed with two major objectives: 1) to assess the correlation of gastro-intestinal and immune response biomarkers in early HIV infection with the progression of the disease and 2) to identify novel biomarkers for use in determining HIV incidence by comparing its expression levels between recent and long standing HIV infected people. The screening, recruitment and follow up visits of the study participants were coordinated at the field by the author of this thesis, together with the supervision of data collection and sample processing at the laboratory facilities.
II. Determinants of the effectiveness of first-line ART in HIV-1-infected adults in Manhiça, Mozambique (PREVIR): a cross-sectional cohort for detecting drug resistances in ART-treated adults enrolled between February and March 2013 at the MDH. The recruitment of the study participants, data collection and sample processing was supervised at the field by María Rúperez, co-author of the Article 3 included in this thesis.

GAMA and PREVIR studies were approved by local institutional review boards at Barcelona Clinic Hospital (GAMA 2011/6264; PREVIR 2012_7955) and by the Ministry of Health of Mozambique (GAMA 461/CNBS/12; PREVIR 427/CNBS/12). Written informed consent was obtained from patients prior to participation.

The GAMA study received support from the Bill and Melinda Gates Foundation [OPP1068252] and the Spanish Ministry of Science (Mineco) [SAF-2011-27901]. The PREVIR study was supported through an investigator-initiated grant from Gilead Sciences. The development of automated analysis of flow cytometry data was funded by the Institute of Health Carlos III (ISCIII), (Grant DTS15/00185) .The author of this thesis was supported by the Spanish Ministry of Health through a grant from the ISCIII, [FI12/00096]. The printing of this thesis was supported by the PhD student program at ISGlobal. The CISM receives core funding from the Spanish Agency for International Cooperation and Development (AECID), and the HIV/AIDS day hospital (at the MDH) from the Agencia Catalana de Cooperacio al Desenvolupament (ACCD).

2. Study area and research facilities

The District of Manhiça is located within the Maputo province, at the very southern part of Mozambique (Figure 18). It is estimated that at the time of the studies there were around 170,000 inhabitants in the district. Manhiça, capital of the District, is a semi-rural town set on a plateau that borders the flood plains of the Incomati River.

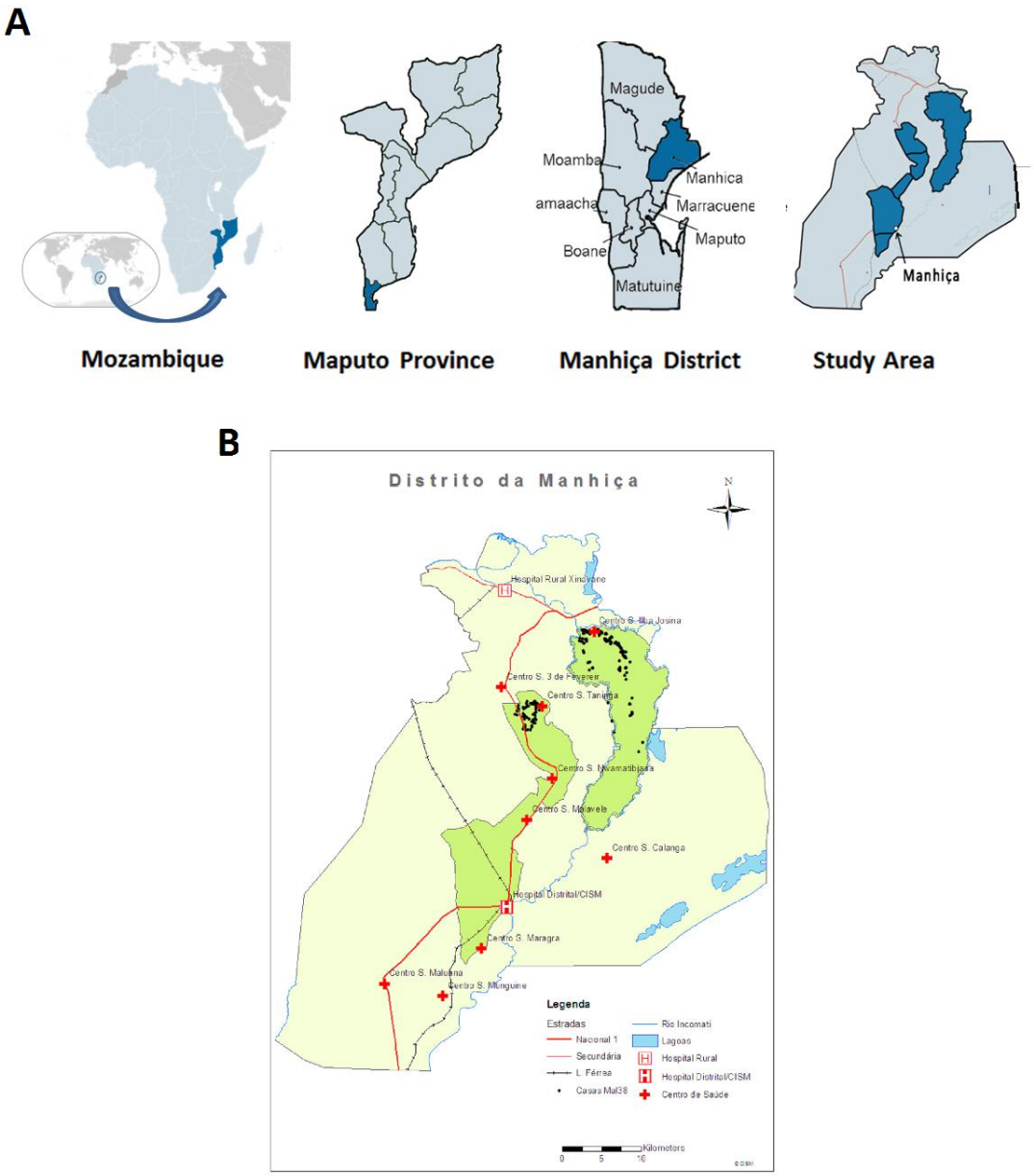


Figure 18. Manhiça study area. A, Mozambique, Maputo province, Manhiça district and CISM study area; B, detail of the Manhiça district.

Since its foundation in 1996, the CISM research agenda is focused on the most pressing public health problems in the country such as malaria, HIV/AIDS, tuberculosis, diarrheal diseases, pneumonias and maternal and reproductive health, among others. Since then, the CISM is running a continuous health demographic surveillance system (HDSS) for vital events and migrations. The Manhica study area at the time of this thesis covered 500 km2

(one fifth of the whole Manhica district, see Figure 17). At the time of the studies the HDSS covered over 94,000 inhabitants and around 20,000 households. All households in this area are geo-positioned using global positioning system (GPS) and all individuals in the HDSS receive a Permanent Identification number (Perm-ID) allowing monitoring of longitudinal demographic information, which is collected electronically through three basic procedures: (i) annual household visits (ii) maternity and morgue visits to record births and deaths, and (iii) contact with key community informants. By linking the information obtained through its morbidity surveillance system to the demographic data, CISM has published in recent years detailed descriptions of the health status of the community[221].

The CISM's laboratory (Figure 19A) provides infrastructure and diagnostic support to the center's research projects and healthcare activities performed at the community or at the MDH. The geographical location of the CISM near South Africa allows for regular shipping and maintenance of equipment, so that molecular and immunological experiments can be done onsite. The CISM has performed HIV-PCR for diagnosis of HIV-exposed children in the District and currently plays a pivotal role as the country prepares to scale-up VL monitoring, since it is one of the few laboratories in the country with capacity to perform VL testing. The laboratory works in accordance with Good Clinical Laboratory Practices (GCLP) and standardized operating procedures (SOP). Study samples are labeled and archived using barcodes and managed by a laboratory information system based on Servolab® Software version 4. The laboratory has extensive sample storage capacity including temperature-monitored -80°C freezers and liquid nitrogen tanks, being the whole center secured throughout backup power generators. The CISM's laboratory activities are distributed into the following areas:

1. Clinical analyses: hematology/biochemistry.

2. Microbiology: general bacteriology and parasitology (Figure19F).

3. Immunology: this unit performs blood processing as well as a wide range of immunological assays and flow-cytometry analysis (Figure19E).

4. Molecular biology: this unit has the necessary equipment to extract and amplify genetic material (DNA and RNA), allowing performance of both standard and qPCR techniques (including HIV-RNA VL detection using the Abbott m2000 system). It also diagnoses microorganisms by ELISA and GeneXpert technology and study molecular markers of resistance using Pulsed Field in Gel Electrophoresis (PFGE).

5. Tuberculosis: fully equipped biosafety level 3 TB laboratory.

6. Quality assurance & biosafety: This unit ensures that appropriate quality regulations are properly followed.

The MDH is adjacent to CISM and is among the main referral health centers in the area. It has an outpatient clinic, a mother and child health clinic, an emergency room and a ward with 153 beds (Figure 19B and C). There are 6 peripheral health posts in the area, used only for primary health care. Both the hospital and the health posts are easily accessible and all pediatric outpatient consultations are free, except for a standard subsidized fee for outpatient medication that is taken home.

Figure 19. (A) CISM research center; (B) Manhiça District Hospital (MDH); (C) antenatal care and outpatient wards in MDH; (D) study consulting room; (E) laboratory of immunology in CISM and (F) laboratory of bacteriology in CISM.

3. Methodology of the thesis

The thesis is presented as a collection of four articles, three of them accepted for publication in peer-reviewed international journals, and one manuscript under review for publication.

GAMA screening cohort and recruitment procedures performed at MDH and CISM facilities are detailed in Article 1 together with subsequent biomarker quantification developed in ISGlobal/Irsicaixa; while GAMA complete follow-up cohort, microbiological characterization and PBMCs phenotyping characteristics are presented in Article 4. Briefly, HIV-seronegative individuals presenting to the outpatient clinic of MDH with reported fever, flu-like symptoms or malaria suspicion were tested for pooled-VL in the context of the GAMA cohort. AHI was defined as HIV seronegative by rapid test and VL positive by HIV-RNA RT-PCR testing. Plasma, PBMCs and stool samples were collected at enrolment and visits 2, 3, 4, 6, 9 and 12, together with clinical and immunological data and microbiological evaluation. The levels of immune response biomarkers were subsequently quantified at ISGlobal/Irsicaixa laboratories in Barcelona by Luminex or ELISA and PBMCs phenotyping was performed through flow-cytometry. Measurements were compared between AHI-infected individuals, HIV-negative adults and long-standing HIV-infected individuals (Figure 20).

Characteristics of PREVIR cohort were previously described[220]. HIV-1-infected adults on first-line ART attending routine visits at the MDH, were evaluated for VF in a cross-sectional study. Subsequently, plasma levels of IP-10 were quantified by ELISA at Irsicaixa and IP-10 was assessed for its predictive power to identify patients with detectable viremia.

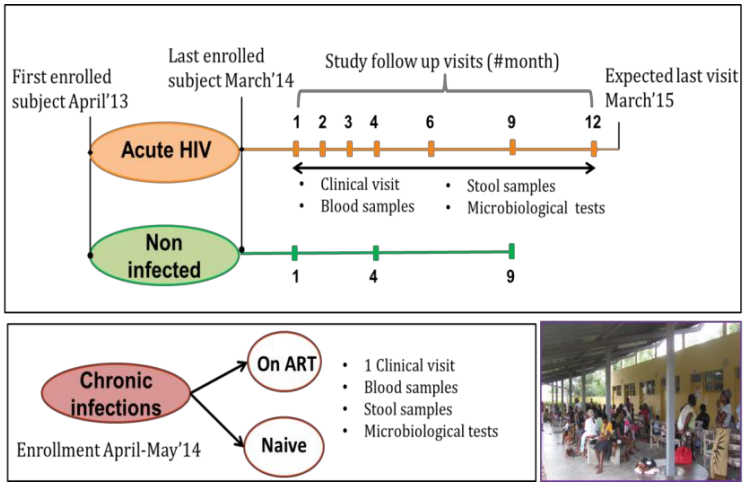


Figure 20. **GAMA study profile**. The study included samples from a longitudinal cohort of early HIV infected individuals. Enrollment was initiated in April 2013 and finalized in March 2014. Follow up continued until March 2015. Early HIV infection included samples from screening and visits at 1, 2, 3, 4, 6, 9, and 12 months after HIV detection.

RESULTS

ARTICLE 1

A Cytokine Pattern That Differentiates Preseroconversion From Postseroconversion Phases of Primary HIV Infection

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A cytokine pattern that differentiates pre- from post- seroconversion phases of primary HIV infection

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Running tittle: Cytokine patterns in primary HIV infection
Keywords: Acute HIV Infection; Sub-Saharan Africa; Cytokines; HIV pathogenesis; Fiebig; antibodies

ABSTRACT

Background: During acute HIV infection (AHI) HIV actively replicates but seroconversion has not yet occurred. Primary HIV infection (PHI) is characterized by a transient non-specific febrile illness, a massive inflammatory response and the progressive appearance of anti-HIV specific antibodies. In this study we have identified patterns of inflammatory biomarkers associated with the innate immunological reaction prior to completion of a full humoral response.
Methods: A symptom-based screening was used to identify AHI in the Manhiça District Hospital in Mozambique. Plasma levels of biomarkers were determined by luminex and ELISA. Anti-HIV antibodies were analysed by flow-cytometry and Western blot. Statistical analyses used Random Forest and logistic regression models.
Results: Of 3116 rapid test seronegative or indeterminate individuals, 85 (2.7%) had positive plasma HIV viral load and were enrolled as PHI, of which n=45 (52.9%), n=8 (9.4%), n=12 (14.1%) and n=20 (23.5%) were classified as Fiebig I-III, IV, V and VI stages, respectively by Western-blot. Comparison of individuals at early (Fiebig I-IV) and late (Fiebig V-VI) immune stages identified significant differences in the expression level of plasma BAFF, MCP-1, sCD163 and MIG. This cytokine signature classified patients in the pre-seroconversion phase with a sensitivity of 92.5% and a specificity of 81.2%
Conclusions: Identification of a cytokine signature specific for the pre-seroconversion stage of PHI may help to understand the earliest HIV pathogenic events and identify new potential targets for immunotherapy aimed at modulating the cytokine response to HIV infection.

INTRODUCTION

Acute HIV infection (AHI) is commonly defined as the period between the HIV transmission event and the development of an antibody response to HIV antigens defined as seroconversion[1]. Recent infection generally is considered the phase up to 6 months after infection during which anti-HIV-1 antibodies are detectable. Throughout this article, the term primary HIV infection (PHI) is used to refer to both acute and recent HIV-1 infection[2].

AHI is characterised by high HIV viral replication, massive gut-associated lymphoid tissue (GALT) CD4+ T cell destruction, immune activation and the establishment of viral reservoirs[3]. As a result of high viraemia in bodily fluids and high levels of genital viral shedding, individuals are considered hyper-infectious at this stage[4,5]. Current HIV antibody tests typically become positive within 3–6 weeks of infection and 1–3 weeks after the onset of acute HIV symptoms[6]. This seronegative window is thus of crucial importance for early treatment outcome, vaccine development and public health[7,8].

Despite the importance of AHI, there are immense difficulties identifying AHI patients for treatment initiation. Patients are often asymptomatic, or experience a non-specific febrile illness; a diagnostic dilemma compounded in the African setting by a significant symptom overlap with malaria[9,10]. To diagnose AHI, the presence of the virus itself must be detected by nucleic acids or proteins in the absence of HIV-specific antibodies. Fourth-generation HIV assays have demonstrated good sensitivity and specificity for detecting HIV infection and AHI but remain less sensitive than nucleic acid amplification technology[11]. Thus, current gold-standard test for confirming viraemia is RT-PCR for plasma HIV RNA[12], while the most widely accepted assay for confirmation of HIV antibody testing is Western Blot (WB) or indirect immunofluorescence assay (IFA)[13].

PHI is a rapidly evolving phase characterized by the stepwise gain in positivity for the detection of HIV RNA and HIV-specific antibodies. Different HIV-1 antigen specificities appear in sequence after HIV-1 transmission as do immunoglobulin G (IgG) subclass responses[3]. As such, using different diagnostic tools, PHI has been categorised into 'Fiebig stages'[14] that are useful in approximating infection date with relative accuracy[15].

As HIV viraemia increases, there is a striking cascade response of acute phase reactants and inflammatory cytokines which has been referred to as the 'cytokine storm'[3]. Although many of the cytokines present are common inflammatory pathway effectors, their study can shed light on key pathogenic pathways occurring prior to the generation of the humoral response[16–20]. We hypothesize that the expression patterns of these early response cytokines may be different between pre and post-seroconversion stages of the PHI phase. These effectors could provide clues for vaccine or immunomodulator development aimed at reducing the irreversible immune damage inflicted during AHI.

METHODS

Study population

The study population was enrolled between 2013 and 2014 at the Manhiça District Hospital (MDH) in the district of Manhiça, Southern Mozambique. The present analysis is a sub-study of a prospective cohort of primary HIV infected adults enrolled and followed up for 12 months in the study of gastrointestinal inflammation (GAMA cohort). This study was approved by local institutional review boards at Barcelona Clinic Hospital (2011/6264) and by the Ministry of Health of Mozambique (461/CNBS/12). Written informed consent was obtained from patients prior to participation.

Adults over 18 years of age, who were residents of the established District Surveillance System (DSS) study area, and who presented to the outpatient clinic of the MDH for non-specific febrile symptoms or voluntary HIV counselling and testing (VCT) were invited to participate in the study.

HIV diagnosis

Blood was collected via finger-prick for HIV rapid antibody testing with Determine HIV 1/2 (Abbott Laboratories, Chicago, IL). Positive results were then confirmed with a more specific Unigold rapid test (Trinity Biotech Co., Wicklow, Ireland) following the national guidelines. Individuals with a positive HIV serology in both rapid tests were referred for clinical management and were not eligible for enrolment into the study. Primary HIV infection was diagnosed in patients with a negative or indeterminate rapid test serology (first test negative or first test positive and second test negative) and positive HIV viremia by RT-PCR testing on frozen plasma using Abbott Real Time HIV-1 assay (Illinois, U.S.) according to manufacturer's instructions, with a sensitivity of detection of 150 copies/ml. . HIV-RNA was quantified by applying a multi–level pooling scheme of 10 samples/pool as described and validated in previous work [21]. A subgroup of HIV-negative time-matched controls was selected via computer randomisation.

Clinical follow-up

Individuals identified with PHI and the control subgroup of HIV-negative individuals were invited to attend a study visit 1 month after the screening date to start the follow up in the context of the GAMA cohort. In this paper, the 1 month visit sample was only employed to establish the reference level for the biomarkers in a non-febrile non-HIV infected group and the coinfections and CD4 and CD8 T-cell counts in both study groups. Demographic and clinical data was collected in a specific questionnaire. Medical consultation and HIV counselling was provided. CD4 and CD8 T cell counts were determined using CD3, CD8, CD4 and CD45 fluorochrome-labelled antibodies on fresh whole blood in a single platform system using *Trucount tubes* (BD Biosciences, New Jersey, U.S.). Clinical and microbiological evaluation was performed, testing for the most prevalent infections in the area.

Quantification of immunoglobulins and definition of primary HIV infection phases (see *Detailed Methodology* section in the *Supplementary data*).

HIV specific antibodies present in plasma screening samples were assessed by Western Blot assay (WB) for sg-p120, gp41, p31, p24, p17 antigens and sgp105 and gp36 antigens for HIV type 2 infection using INNO-LIA™ HIV I/II Score (Innogenetics N.V. Gent, Belgium). Patients with a negative or indeterminate WB result were considered to be in the pre-serconversion acute stage and patients with a positive WB result were considered to be in the post-seroconversion recent stage of HIV infection. Thus, in our study cohort seropositive status was defined as positive WB result at screening. Viral Load (VL) and WB results at screening visit were employed to categorize individuals into Fiebig stages I-III (VL positive, WB negative), IV (VL positive, WB indeterminate), V (VL positive, WB positive with p31 band negative), VI (VL positive, WB positive with p31 band positive) according to previous work[14].

Seven plasma antibody isotypes (IgA, IgM, IgG1, IgG2, IgG3, IgG4 and total IgG) were quantified in the screening samples by an in house ELISA. HIV specific antibodies of different subtypes (IgG1, IgG2, IgG3, IgG4) for HIV antigens (gp41-Env, p24-Gag and p31-integrase) were performed by multi-parametric flow cytometry.

Quantification of plasma cytokines (see *Detailed Methodology* section in the *Supplementary data*).

Multiplex cytokine profiling was performed for a total of 54 cytokines in screening plasma samples with particular interest in biomarkers of intestinal damage (10 biomarkers), T, B and monocyte cells function (23 biomarkers) and general biomarkers of inflammation and cell death (21 biomarkers) *(Supplementary table 1).* Determinations were performed by ELISA assay or Luminex multi-analyte profiling technology.

Statistical analysis

Group comparisons by Fiebig stage were performed using the non-parametric Kruskal-Wallis test and Chi-squared test for categorical variables. Multiple tests were adjusted using the Bonferroni correction. Post hoc pairwise comparisons between Fiebig stages were performed by Dunn's test with Bonferroni adjustment. Comparisons between PHI and non-HIV infected controls were based on the non-parametric Mann-Whitney U-test. Identification of cytokines with the best performance in distinguishing between pre- and post-seroconversion stages was performed using Random Forest analysis. Random forest is a supervised learning method based on the ensemble of multiple classification trees [22]. Relation between early and late Fiebig stages with respect the selected cytokines were explored by Principal Component Analysis (PCA). Patterns of the selected cytokines by PHI phases was assessed by multivariate logistic regression. Outcome was a binary variable where 1 represents pre-seroconversion AHI group (Fiebig I-IV) and 0 represents recent HIV infected group (Fiebig V-VI). In both PCA and regression model, MIG, MCP-1 and BAFF

values were log transformed for a better adjustment of the data. Clinical variables (age, gender, VL and other co-infection) were tested for inclusion in the model by backward step-wise elimination approach (inclusion criteria was p-value<0.05 in multivariate analysis). Statistical analyses were performed using R-3.2.2 and Stata14 software.

RESULTS

Identification of primary HIV infected individuals

Among the 4011 outpatients screened at the MDH, 3000 (74.8%) presented with fever-like symptoms and 1011 (25.2%) for voluntary HIV testing. Seven hundred and ninety-nine (20%) were seropositive on rapid testing, and a further 92 (2.3%) were excluded prior to serological testing, mainly due to residence outside of the study area (*Figure 1)*. A majority (64.4%) of screened patients were under the age of 30, and most (62.8%) were female. These age and gender tendencies were similar between seropositive patients and those subjects who met criteria for inclusion in the study *(data not shown).*

Of the 3116 rapid test seronegative or indeterminate patients who entered pyramid pooling for HIV RNA testing, 87.1% were individuals presented for non-specific febrile symptoms. Ninety-three had a detectable level of HIV-RNA and 4 patients were excluded for presenting an inconclusive HIV-RNA result. Eight of these rapid test-seronegative HIV-RNA-positive cases were found to be positive for antibodies against all the HIV antigens by subsequent Western Blot testing and excluded *(Figure 1).* Eighty-five individuals thus fulfilled criteria for PHI; a prevalence of 2.73% (95% confidence interval 2.18-3.36) among all enrolled patients, and 3.02% (95% confidence interval 2.41-3.74) among those who were symptomatic at presentation.

Characteristics of the primary HIV infected population

At the screening visit, the median age of PHI cases was 24 years (IQR 20-30), 58% were female and most (96%) presented with a non-specific febrile syndrome to the outpatient ward including reported fever, flu or headache (*Table 1*). There was no significant difference in age, gender balance or malaria co-infection between the 85 PHI cases and the 3019 HIV-negative subjects. PHI cases reported a significantly greater total number of symptoms than did HIV-negative patients, and were significantly more likely to report fever in the last 24 hours (p-value=0.024), and intestinal complaint (diarrhoea or abdominal pain) in the last week (p-value<0.001). Fifty-six PHI cases (70%) returned to commence follow-up and n=58 HIV-negative participants were randomly selected to attend the 1 month follow-up visit as a reference group. There was no significant difference in age, gender or HIV-RNA between PHI patients who returned and those who were lost to follow-up *(Supplementary table 2).* After one month of follow-up, median CD4 T-cell count in PHI-individuals was significantly lower than in the non-HIV infected group, 585 (IQR 460-682) vs. 955 (IQR 773-1149) cells/uL respectively (p-value<0.0001); while median CD8 T-cell count was

significantly higher, 1119 (IQR 741-1650) vs. 591 cells/uL (IQR 417-746) respectively (p-value<0.0001).

Fiebig staging of PHI patients

The majority of PHI cases (74.1%) was seronegative at presentation *(Table 1),* with the remainder indeterminate on rapid tests. Patient HIV-RNA levels at presentation were high, with a median of Log_{10} 6.3copies/mL (IQR 5.2-7.3) (*Table 1*). Classification according to Fiebig[14] revealed that the majority of the 85 PHI cases were in Fiebig I-III, 45 (52.9%) whereas 8 (9.4%), 12 (14.1%) and 20 (23.5%) were in Fiebig IV, V, and VI respectively. There was no significant difference in age, gender balance or initial complaint between Fiebig groups *(Table 1).* The later Fiebig stages did, however, have a higher prevalence of malaria infection (p-value=0.049), and higher rate of intestinal complaints in the previous week (p-value=0.010), when compared with the earlier stages. As expected, the proportion of PHI cases with an indeterminate result for HIV rapid test was significantly higher in Fiebig VI (85%, p-value <0.001) and the median HIV-RNA level lower (p-value=0.0001) compared with the earlier Fiebig stages.

Table 1. Clinic and demographic characteristics of study population according to HIV-status and Fiebig stage. Comparisons for proportions were performed by chi2 test^{x} and continuous variables by Mann and Whitney U-test* for the two group comparison and global comparison by Kruskal Wallis test**. p-value obtained from (1) comparison by study group or (2) comparison by Fiebig group.

	HIV-neg at Screening (n=3019)	PHI at Screening (n=85)	p-value [1]	Fiebig I-III (n=45)	Fiebig IV (n=8)	Fiebig V (n=12)	Fiebig VI (n=20)	p-value [2]
Age (years) [Median (IQR)]	26 (20-35)	24 (20-30)	0.083*	23 (19-32)	25 (20-35)	23 (19-31)	26 (20-28)	0.8994**
Gender [F (%)]	1900 (63)	50 (58)	0.441^{x}	28 (62)	3 (37)	8 (67)	11 (55)	0.543^{x}
Initial complaint [n (%)]								
Headache	80 (3)	1 (1)	0.401^{x}	1 (2)	0 (0)	0 (0)	0 (0)	
Febrile syndrome	2385 (79)	75 (88)	0.039^{x}	41 (91)	8 (100)	11 (92)	15 (75)	
Flu symptoms	153 (5)	6 (7)	0.412^{x}	2 (4)	0 (0)	0 (0)	4 (20)	0.395^{x}
Voluntary testing	395 (13)	3 (4)	0.010^{x}	1 (2)	0 (0)	1 (8)	1 (5)	
Others	6 (0)	0 (0)	1^{x}	0 (0)	0 (0)	0 (0)	0 (0)	
Fever last 24h [n (%)]	2503 (83)	80 (94)	0.024^{x}	43 (96)	8 (100)	11 (91)	18 (90)	0.703^{x}
Malaria [n (%)]	337 (17)	7 (11)	0.234^{x}	1 (3)	0 (0)	3 (33)	3 (17)	0.049^{x}
Intestinal complaint last week [n (%)]	84 (3)	9 (11)	< 0.001^{x}	1 (2)	1 (12)	1 (8)	6 (30)	0.010^{x}
RT Serostatus [n (%)]								
Negative	-	63 (74)	-	44 (98)	8 (100)	8 (67)	3 (15)	< 0.001^{x}
Indeterminate		22 (26)		1 (2)	0 (0)	4 (33)	17 (85)	
Viral Load (RNA Log 10 copies/mL) [Median (IQR)]	-	6.3 (5.2-7.3)	-	7.1 (6.2-7.5)	7.0 (6.3-7.4)	5.6 (4.9-6.3)	4.8 (4.3-5.5)	0.0001**

Kinetics of subclass HIV-specific humoral responses during PHI

In order to quantify the nascent HIV-specific IgG response by Fiebig stage in our cohort, we assessed levels of IgG1, IgG2, IgG3, and IgG4 specific for p24, gp41 and p31 antigens. The levels of all antibodies against gp41 and p24 detected for subjects in Fiebig I-III were similar to those in Fiebig IV. HIV-specific antibody levels significantly increased in Fiebig V and Fiebig VI as compared to Fiebig I-III and IV *(Figure 2).* As expected from WB results, in the case of antibodies against p31 antigen, only the Fiebig VI group demonstrated significantly higher median level of IgG1 compared with the rest of the Fiebig groups (p-value <0.05).

Kinetics of biomarker expression patterns during PHI

From total of 61 biomarkers assessed, 49 were quantifiable in more than 75% of the samples and were included for analysis *(Supplementary table 1).* Besides VL, nine biomarkers were differentially expressed across Fiebig groups including total plasma IgG, IgG1, sCD163, MCP-1, sCD23, BAFF, MIG, TRAIL and TNFα (p-value<0.1, *Figure 3*). Total plasma IgG and IgG1 were highly correlated (Spearman rho=0.78, p-value<0.0001); thus, only total IgG1 was maintained in subsequent analyses and 8 biomarkers were then considered differentially expressed. Patients in Fiebig I-III showed a significantly different biomarker profile compared with those in Fiebig VI (p-value<0.01). Levels of total IgG, IgG1, sCD163, sCD23 and MIG significantly increased with Fiebig stages, and showed a weak negative correlation with viral load (rho<-0.4). In contrast, MCP-1, BAFF, TRAIL and INFα biomarkers were expressed at high levels in Fiebig I-III and IV stages and decreased in Fiebig V and VI stages, significantly correlating with VL levels (p<0.0001, MCP-1 Spearman rho=0.60, BAFF rho=0.60, TRAIL rho=0.48 and INFα rho=0.47). There was no significant difference in the plasma level of these biomarkers between Fiebig I-III and Fiebig IV or between Fiebig V and Fiebig VI (p-value>0.05).

Cytokine expression profile can classify primary HIV infection into phases

In order to determine whether the subgroup of eight biomarkers was associated with the acute pre-seroconversion stage of the PHI, cases were grouped into two categories: 1) the pre-seroconversion acute group roughly covering the 1st month of PHI (Fiebig I-IV) and 2) the post-seroconversion group referring to recent PHI (Fiebig V-VI) when HIV-specific antibodies are detectable by Western Blot.

Random Forest analysis models consistently selected BAFF, MCP-1, sCD163 and MIG as having the best power to discriminate between the pre and post-seroconversion phases. Additionally, a biplot representation generated from principal component analysis (PCA) showed differential expression patterns for the selected biomarkers according to the grouping (*Figure 4A*). Multivariate regression was performed in order to assess the power of the four cytokines to distinguish between the two categories (*Figure 4B*). Based on regression diagnostics measures, five outlier observations were excluded from model fitting. Clinical variables (age, gender, VL or other co-infection) showed no significant

association or interaction with the selected biomarkers (data not shown) and were thus eliminated from the model.

BAFF and MCP-1 were positively associated with the pre-seroconversion group while sCD163 and MIG were negatively associated *(Fig. 4B).* The multivariate model was capable of correctly identifying 49 out of the 53 individuals in the pre-seroconversion phase, and 26 out of the 32 in the post-seroconversion phase. This goodness of fit allowed the model to identify patients in the pre-seroconversion phase with a sensitivity of 92.5% and a specificity of 81.2%.

DISCUSSION

This study has identified a signature of four cytokines composed of BAFF, MCP-1, sCD163 and MIG that is highly associated with PHI prior to development of the HIV-specific humoral response as determined by WB [14,15,23] . BAFF and MCP-1 are highly expressed in the first month of PHI whereas sCD163 and MIG rise several weeks later.

Despite roll out of ART and HIV prevention activities, the 3.0% (95% CI 2.4-3.7) prevalence of PHI among people presenting with fever-like symptoms at the outpatient ward of this rural area of Mozambique has remained unchanged as compared to a study performed 5 years earlier which showed an PHI prevalence of 3.3% (95% CI 1.3–6.7)[21]. Given the high risk of transmission during this phase, these data represent an important threat to public health. Periodic cross-sectional PHI screening of people presenting with fever like symptoms in malaria-endemic high HIV incidence settings could act as an indirect surrogate to monitor evolution of HIV epidemic and compare tendencies between different communities or assessing the impact of prevention campaigns.

In our cohort of primary HIV infected individuals, the nascent HIV-specific antibody response showed a distinct pattern of expression by Fiebig stages.. The kinetics of antibody generation during PHI may vary according to individual factors, subtype and dose of infecting virus[3]. Previous studies have shown that anti-Env HIV-1 plasma antibodies are predominantly IgG1 subclass, followed by anti-Env IgG3 subclass during PHI[24]. Results from our cohort are largely similar. However, very few individuals in the earliest Fiebig stages (I-IV) showed positive IgG3 responses to gp41. These results slightly differ from *Yates et al* [24,25] where they found anti-gp41 IgG3 to be positive for all PHI individuals. The timing of detection of antibodies could be influenced by the extent of immune complex formation preceding detection of free antibody. This could differ by viral load, geographic location or HIV clade. In contrast to our Mozambican clade C cohort, their PHI cohort included 37% African individuals infected with HIV clade C, which could explain discrepancies.

In parallel to the stepwise HIV-specific antibody generation, AHI is a period when many other immune processes are occurring, specifically the innate immune response. A general cytokine storm occurring early after HIV infection has described peak levels of many

inflammatory cytokines prior to seroconversion [18–20,26,27]. In order to fine-tune the immune response pattern, we have assessed those previously described cytokines as well as other relevant innate immunity effector molecules. From a total of 49 analytes that were quantifiable in this cohort, 9 biomarkers demonstrated significant difference in the expression level across Fiebig groupings. Furthermore, four of these biomarkers, BAFF, MCP-1, sCD163 and MIG, exhibited the best predictive power to distinguish between individuals in pre and post-seroconversion stages of PHI. Modelling the expression profile of these four cytokines identified with high sensitivity and specificity those individuals in which the antibody generation was still not developed.

Most of the participants (87.1%) were screened for PHI through sampling of individuals presenting with febrile syndrome. While this is the most practical way of finding PHI, the cytokine and antibody expression profile in symptomatic individuals may not be generalizable to asymptomatic individuals. Although Hepatitis B, syphilis and intestinal pathogen co-infections were not found to be associated with stage of HIV infection or levels of cytokines, we cannot exclude that other infections commonly present in HIV-infected individual, could affect plasma levels of these biomarkers. Another limitation is the lack of resolution of early Fiebig stages due to sample volume restrictions. We cannot exclude that cytokine expression may differ between Fiebig I-III. A detectable gradient of cytokine expression could become visible particularly in the transition through Fiebig III, similar to Fiebig IV. However this would likely maintain or even strengthen the signature association.

To our knowledge, no cytokine signature has been previously described to be associated with the pre-seroconversion phase of PHI. In our cohort, increased pre- seroconversion expression of MCP-1 was accompanied by elevated BAFF. In contrast, sCD163 and MIG showed a delayed increase during the early infection process which paralleled the decrease in BAFF and MCP-1. Monocyte chemotactic protein (MCP-1) and B-cell activating factor (BAFF) are both produced by monocytes, macrophages and dendritic cells after infection or tissue inflammation in order to assure a proper immune response and maintenance of normal immunity[28–30]. In contrast, macrophage low-grade inflammation marker (sCD163) and monokine induced by IFN-γ (MIG) are cytokines involved in T-cell trafficking to inflammatory sites and control of T-cell proliferation[31–33]. Since BAFF and MCP-1 expression is associated with early innate responses, they may be the very first effectors in the HIV immune cascade followed closely by sCD163 and MIG, associated with the initiation of adaptive immune responses. Further studies including gut biopsies in humans or non-human primates may show whether BAFF and MCP-1 expression are associated with viral shedding or early HIV-induced GALT destruction.

There is an interest in approaching AHI as a phase of infection in which early ART coupled with adjunct targeted immune therapy could limit the destruction of the mucosal lymphoid tissue and seeding of the HIV reservoir. Approaches include boosting HIV-specific

immunity, reducing inflammation, activating latency and targeting HIV apoptosis regulatory pathways[7,34–36]. Recent studies also point out that cART initiated during early AHI either prevents loss of (at Fiebig stage I/II) or restores (at Fiebig stage III) mucosal Th17 cells[37]. Consequently, very early ART is associated with normalization of local and systemic immune activation, reversing a hallmark of HIV pathogenesis[37]. Although further analysis will be required to define whether there is a causal relationship between BAFF and MCP-1 expression and antibody generation, these cytokines might be potential targets to boost the humoral response in vaccination strategies or to accelerate the HIV-specific antibody response during PHI. Modulation of these cytokines might promote an earlier development of autologous neutralizing antibodies that may contribute to faster and long lasting control of HIV[38,39].

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Author's contributions

DN and JB study design. LP, EP, LF and DR recruited subjects and collected clinical data. LP, EP LF, LL and CJ performed laboratory analysis at the field. LP and JC performed biomarker quantification at the laboratory and validation of the data. LP, EP and VU performed statistical analyses. LP, EP, JC, JB and DN interpreted the data. LP and EP drafted the paper. DN, JB and IM coordinated the work and supervise manuscript writing. All authors read and approved the final version of the manuscript.

Competing Financial Interests

All authors do not have any commercial or other associations that might pose a conflict of interest with the data presented in this paper.

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FIGURES

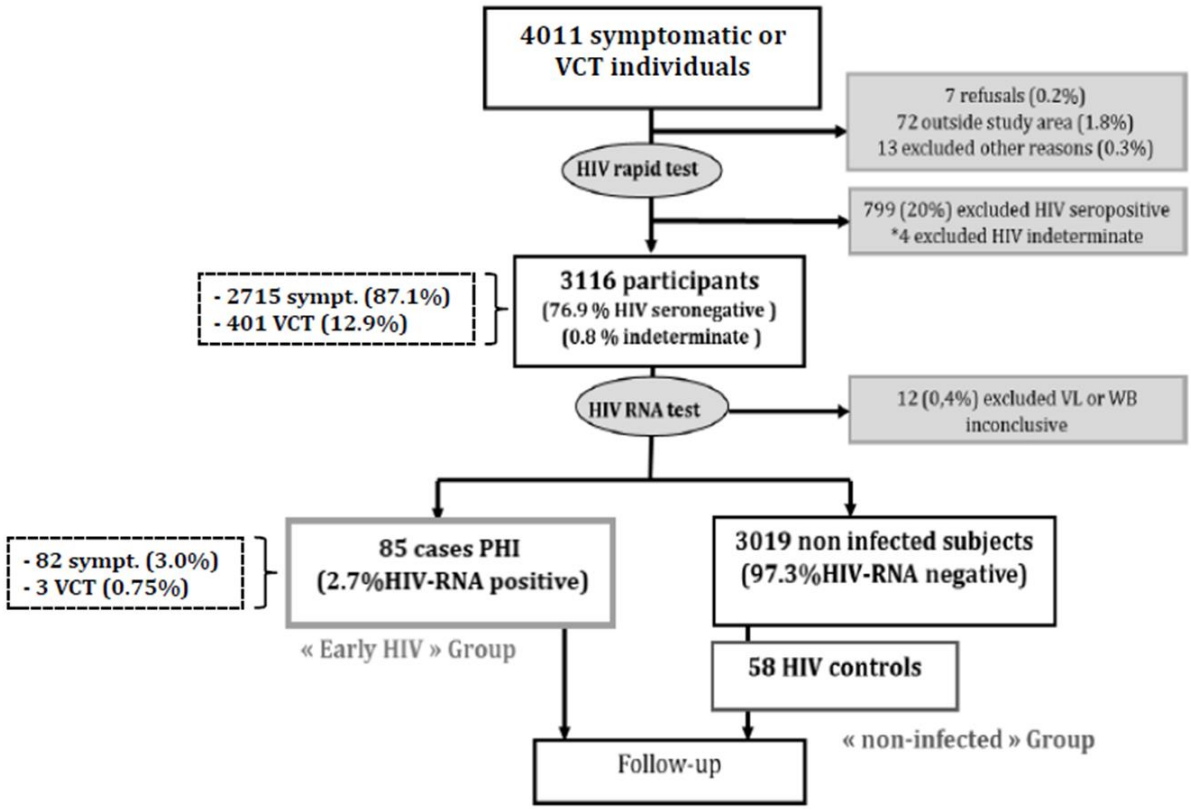


Figure 1: Screening profile: VL=viral load, WB=Western Blot test, Sympt.=symptomatic patients, VCT=patients presented for voluntary counselling and testing. *Study protocol was changed to include rapid test indeterminate patients, however four individuals were excluded prior to this change.

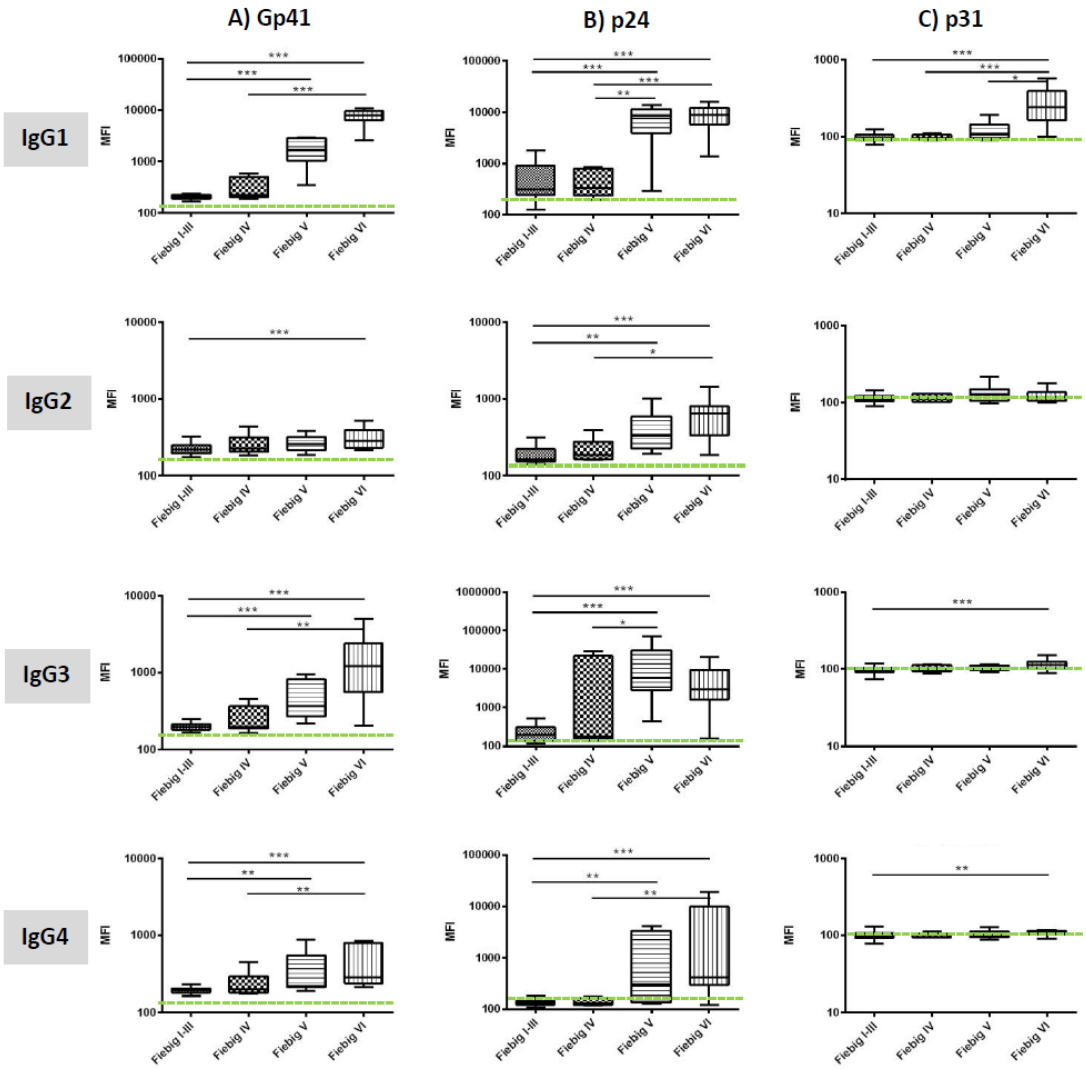


Figure 2. HIV-specific antibody expression according to Fiebig stage. The median MFI (mean fluoresence intensity) is shown for antibodies specific for A) gp41, B) p24 and C) p31. Box as IQR, middle line as median, whiskers as Tukey values (1.5 IQR). Values are Log_{10} scale for a better adjustment of the data. Nonparametric pairwise multiple comparisons were performed by using Dunn's test. Bonferroni-adjusted significance is indicated as *** if p<0.001, ** if p<0.01 and * if p<0.05. Median values for the non-febrile non-HIV infected reference group are indicated as dotted lines.

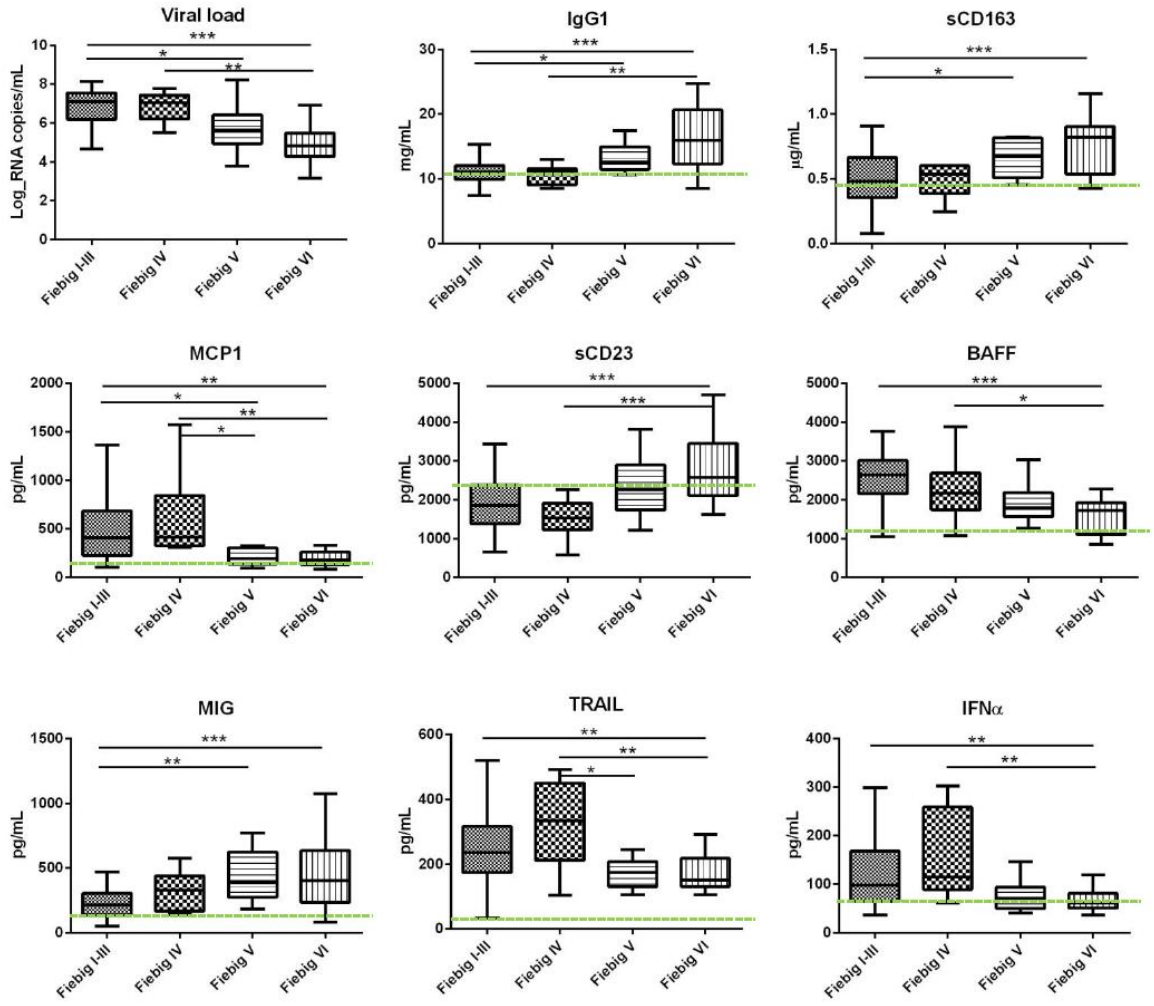
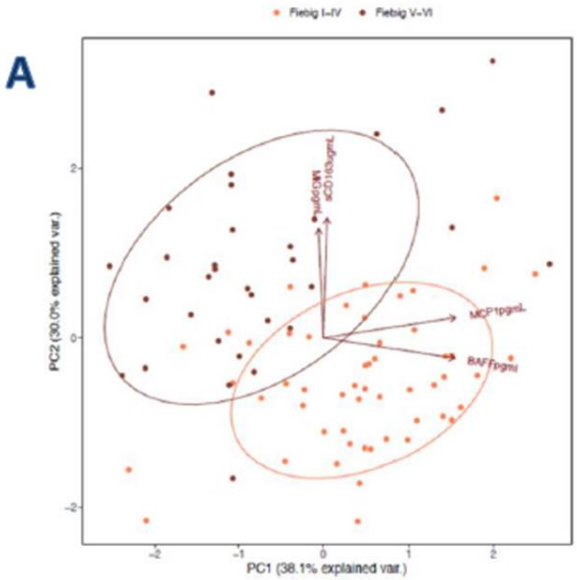


Figure 3. Expression of selected parameters varies by Fiebig stage. Nine biomarkers were differentially expressed across Fiebig groups including total plasma IgG, IgG1, sCD163, MCP1, sCD23, BAFF, MIG, TRAIL and TNFα (p-value<0.1), as was VL (p-value<0.001) by Kruskall-Wallis range comparison (Bonferroni adjusted p-value <0.01). Median levels of selected parameters are represented as middle line, box as IQR and whiskers as Tukey values (1.5 IQR). Comparison of individual difference in expression level by Fiebig group was performed by Dunn's comparison test adjusted by Bonferroni for multiple comparisons as shown above. Significance is indicated as *** if p<0.001, ** if p<0.01 and * if p<0.05. Median values for the non-febrile non-HIV infected reference group are indicated as dotted lines.



B

	Coefficient	Std. Error	p-value
(Intercept)	-23.9	13.6	0.0801
sCD163	-10.2	4.1	0.0135*
log10_MIG	-8.7	3.1	0.0050**
log10_MCP1	9.7	3.9	0.0123*
log10_BAFF	8.9	4.5	0.0510

Figure 4. Cytokines with best discriminatory power to differentiate between early and late Fiebig stages. A. Component analysis of cytokine levels. Principal component analysis of BAFF, MCP-1, MIG and sCD163 levels in patients in pre- or post- seroconversion stages (Fiebig I-IV, Fiebig V-VI respectively). B. Multivariate logistic-regression to identify cytokines associated with pre-seroconversion stage of HIV infection (Fiebig I-IV). Significance of the variable in the model is indicated as ** if $p<0.01$ and * if $p<0.05$. In both PCA and regression model, MIG, MCP1 and BAFF values were log transformed for a better adjustment of the data.

SUPPLEMENTARY DATA

Detailed Methodology

General antibody-subtypes quantification

96 well MicroWell™ MaxiSorp™ flat bottom plates (Nunc) were coated with polyclonal affinipure goat anti-human IgA antibody α-Chain Specific (Jackson Immunoresearch), mouse anti-human IgM antibody (clone G20-127) (BD Pharmingen™), mouse anti-human IgG1 antibody (clone HP6069) (Life technology,) mouse anti-human IgG2 antibody (clone G18-21) (BD Pharmingen™), mouse anti-human IgG3 Hinge (clone HP6050) (SouthernBiotech), mouse anti-human IgG4 antibody (clone G17-4) (BD Pharmingen™) or polyclonal affinipure $F(ab')_2$ fragment goat anti-human IgG, Fcγ Fragment Specific. 0.05% Tween®20 (Sigma Aldrich) 1% BSA (Sigma Aldrich) in PBS was used as blocking solution. Standard was prepared from N-Prot Standard Serum (Siemens, Healthcare Diagnostics). HRP-Goat anti human Ig Fc-specific (Jackson-Immunoresearch) was used as detection antibody, citrate diluted-OPD (Sigma Aldrich) as substrate and 4N H_2SO_4 as stop solution. Optimal density was measured at 492 and 620nm.

Specific HIV antibody-subtypes quantification

p31 was amplified from RNA isolated from a plasma sample of a HIV-1 subtype C infected patient using the following primers: NcoI integC sense: 5´ TATATCCATGGCTTTTTTAGATGGGATAGAT-3´ and XhoI Integrase as: 5´ TTTACTCGAGATCCTCATCCTGTCTACCTG-3´. A Gp41 subtype C fragment containing the HR1, HR2 and MPER domains was amplified from the plasmid p97ZA012.1 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) using the following primers: gp41-s 5´-AAAAACCATGGCAGCACTAGGAGCTTTGTTC-3´ and gp41-as 5´-TTTATCTCGAGTATTTTTATATACCACAGCC-3´ Both p31 and gp41 amplimers were cloned into the pET21 d(+) vector (Novagen). Recombinant proteins was produced in BL21 DE3 cells (Invitrogen) and purified by immobilized metal ion affinity chromatography by using Ni Sepharose 6 Fast flow (GE Healthcare). p24 (isolate 92BR025) was purchased from Sino Biological Inc. Proteins were coupled to MagPlex Microspheres (Luminex) following the manufacturer instructions. BSA and a F(ab)2 Goat anti-human IgG Fc specific (Jackson-Immunoresearch) were included as negative and positive controls, respectively. Bound antibodies was detected using the following biotin conjugated monoclonal antibodies and Streptavidin-PE (Jackson Immunoresearch): anti-huIgG1 (clone HP6069, Thermo Scientific), anti-huIgG2 (clone G18-21, BD Biosciences), anti-humanIgG3 (clone HP6050, SouthernBiotech), anti-human IgG4 (clone JDC-14, BD Biosciences) and Goat anti-human IgA (Jackson Immunoresearch).

Cytokines quantification

Detection of IL-1RA, FGF-Basic, MCP-1, G-CSF, IFN-γ, IL-12, IL-13, IL-7, VEGF, MIG, RANTES, Eotaxin, MIP-1β, IP-10, IL-2R, IFN-α, IL-15, GM-CSF, TNF-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10,

MIP-1α, IL-17, IL-8, EGF and HGF were determined by using *Human Cytokine Magnetic 30-Plex panel* (Invitrogen) according to manufacturer's instructions. Detection of CD40L, IL-21, IL-22 and IL-23 was performed by using the Bio-Plex Pro Human Th17 cytokine assay (*Biorad)* according to manufacturer's instructions. Detection of BAFF, CD27 and TNFR2 was performed by using a *Human Magnetic Luminex Screening Assay* (*R&D)* according to manufacturer's instructions. Detection of sCD14, LBP, FABP2, CRP, CD163, CXCL16, CD23, B7H1, PD-L2, TRAIL, FasL, TGF-b1 (R&D); ASCA IgG and ASCA IgA (Orgentec); EndoCab IgG and EndoCab IgA(Hycult biotech) and Zonulin (Immundiagnostik) was assessed by ELISA according to manufacturer's instructions. Overflow and under limit of detection values were validated for every analyte as the double and the half of the detection limit respectively.

Supplementary table 1. Biomarker assessed grouped by main pathway involved and technique employed. Note that those biomarkers in italics were not quantifiable in more than 75% of the samples and were therefore excluded from the analysis

Pathway	Specific biomarkers	Assay
Plasma IBD biomarkers	**N=10**	
Enterocyte damage	Intestinal fatty acid binding protein (FABP2)	ELISA
Intestinal permeability	Zonulin, IgA_ASCA, IgG_ASCA	ELISA
Translocation	EndoCab_IgG, EndoCab_IgM, sCD14, LBP	ELISA
Angiogenesis	*VEGF-A, IL-17*	Luminex
Plasma serological markers	**N=22**	
Total Ig	IgG1, IgG2, IgG3, IgG4, IgA, Ig M, total_IgG	ELISA
HIV-specific Serology	IgG1, IgG2, IgG3, IgG4, IgA specific for HIV antigens: gp41-Env, p31-integrase, p24-Gag.	FACS, beads
Plasma general markers	**N=44**	
Monocyte Function/Mobility	MCP1,sCD163, CXCL10 (IP-10), Eotaxin, MIG, *RANTES*, MIP-1b, MIP1a, *IL-8, IL-15*	Luminex, ELISA
T cell function	IFNγ, IL-2R, *IL-2,* IL-4, IL-5, *IL-7,* IL-10, IL-13, *IL1B*	Luminex, ELISA
B cell function	sCD40L, *IL-21,* BAFF, sCD23	Luminex, ELISA
Inflammation	CRP, CXCL16, G-CSF, GM-CSF, IL6, *IL-22, IL-23*, IL-12, IL1RA, *FGFbasic*	Luminex, ELISA
Cell death, Grown Factor, Pleiotropic	B7H1, FAS Ligand, PD-2 Ligand, TRAIL, EGF, HGF, CD27, TGFβ1, TNFα, IFNα	ELISA

Supplementary table 2. Age, gender and viral load comparison between PHI cases continuing follow-up and those lost to follow-up. There was no significant difference in age, gender balance or viral load between acute HIV patients who returned for enrolment and those who were lost to follow-up. *(1)* Mann-Whitney U Test, *(2)* Chi-Square Test and *(3)* Independent Samples T Test.

	PHI enrolled n (%) or median (IQR)	**PHI LTFU** n(%) or median (IQR)	p-value
Total (n)	44	13	
Age (years)			
Median (IQR))	26 (11.0)	25 (10.0)	
17-30	33 (75.0)	10 (80.0)	0.939*(1)*
31-40	7 (16.1)	2 (10.0)	
41-50	3 (7.1)	1 (6.7)	
50+	1 (1.8)	0 (3.3)	
Gender			
Female	27 (61.4)	5 (38.5)	0.144*(2)*
Male	17 (38.6)	8 (61.5)	
Viral load			
Median (IQR)	6.19 (2.15)	6.80 (2.80)	0.332*(3)*

ARTICLE 2

Dynamics of CD4 and CD8 T-cell Subsets and Inflammatory Biomarkers during Early and Chronic HIV infection in Mozambican Adults.

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Under revision

Dynamics of CD4 and CD8 T-cell Subsets and Inflammatory Biomarkers during Early and Chronic HIV infection in Mozambican Adults

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Running tittle: soluble and cell biomarkers during HIV infection

Keywords: AIDS; HIV pathogenesis, T-cell exhaustion; T-cell activation; senescence; cytokines; acute-HIV-infection; sub-Saharan Africa.

ABSTRACT

During primary HIV infection (PHI), there is a striking cascade response of inflammatory cytokines and many cells of the immune system show altered frequencies and signs of extensive activation. These changes have been shown to have a relevant role in predicting disease progression; however, the challenges of identifying PHI have resulted in a lack of critical information about the dynamics of early pathogenic events.

We studied soluble inflammatory biomarkers and changes in T-cell subsets in individuals at PHI (n=40), Chronic HIV infection (CHI, n=56) and HIV-uninfected (n=58) recruited at the Manhiça District Hospital in Mozambique. Plasma levels of 49 biomarkers were determined by Luminex and ELISA. T-cell immunophenotyping was performed by multicolour flow-cytometry.

Although plasma HIV viremia, CD4 and CD8 T cell counts underwent rapid stabilization after PHI, several immunological parameters, including Th1-Th17 CD4 T cells and activation or exhaustion of CD8 T cells continued decreasing until more than 9 months post-infection. Importantly, no sign of immunosenescence was observed over the first year of HIV infection. Levels of IP-10, MCP-1, BAFF, sCD14, TNFR2 and TRAIL were significantly overexpressed at the first month of infection and underwent a prompt decrease in the

subsequent months while, MIG and CD27 levels began to increase 1 month after infection and remained over-expressed for almost one year post-infection. Early levels of cytokines were significantly associated with subsequently exhausted CD4 T-cells or with CD8 T-cell activation.

Despite rapid immune control of virus replication, the stabilization of the T-cell subsets occurs months after viremia and CD4 count plateau, suggesting persistent immune dysfunction and highlighting the potential benefit of early treatment initiation that could limit immunological damage.

Author summary

After HIV infection, there is large uncertainty as to when the initial burst of immunologic activity stabilizes, particularly in a sub-Saharan African setting. Detailed information regarding the dynamics of innate response biomarkers and functionally distinct T-cell subsets during early HIV infection can shed light on important pathogenic events. Herewith, we describe the kinetics of the immunologic responses in a longitudinal cohort of Mozambican adults identified during acute HIV infection and compare them with those of chronically infected individuals. We found that levels of most inflammatory soluble biomarkers, CD4 counts and VL stabilized between 3 and 5 months after HIV infection. However, the equilibrium in certain T-cell subsets took longer to be reached. We observed that both activated and effector memory CD8 T-cells reached stable levels several months after control of viremia. Moreover, the frequency of systemic Th1Th17 cells within the T cell population continued decreasing until at least 7 months after infection. These results point to a prolonged imbalance in various T-cell compartments which may translate into specific immunologic alterations. Exploration of T-cell specific dysfunctions may pave the way for the development of adjunct therapies to enhance immune recovery and diminish immune activation during early HIV infection.

INTRODUCTION

During primary HIV infection (PHI), many cells of the immune system show signs of extensive activation and a progressive loss of resting subsets [1]. Several T-cell subsets can be defined by their specificity, surface phenotype or degree of maturation, and any or all of these parameters can be affected by HIV infection [2]. Prior to changes in T-cell subsets, as HIV viremia increases during PHI, there is a striking cascade response of pro-inflammatory cytokines, which has been referred to as the 'cytokine storm' [3]. Although many of the cytokines present are common inflammatory effectors, their study can shed light on key pathogenic pathways associated with disease progression [4–9].

Generally, untreated HIV-infection is characterized by progressive CD4 T-cell depletion and CD8 T-cell expansion. The profound CD4 T-cell depletion is linked directly to the risk for opportunistic infections and mortality [10]. Likewise, CD8 T-cell activation [11–14] and exhaustion [15,16] have been observed to be strong correlates of disease progression. The subsequent alterations in immune homeostatic mechanisms may lead to a progressive loss of the naïve and memory T-cell pool, resulting in an imbalance in T-cell phenotypes [10,17]. Similarly, after HIV infection, accelerated aging of T cells or immunosenescence may occur due to the continuous highly productive viral replication and cell stimulation [17,18]. Importantly, immunosenescence has also been associated with risk of adverse clinical events in HIV infected individuals [19].

Besides interfering in T-cell maturation, HIV infection also affects T-cell functional diversity. A switch from Th1 to Th2 has been widely described [20], as well as changes in Th17 and Treg cells. Th17 cells are CD4-T cells involved in epithelial barrier integrity and protection against extracellular pathogens [21]. Th17 cells have been seen to be irreversibly depleted in the gut-associated lymphoid tissue (GALT) during the first stages of PHI and have only been preserved by prompt ART initiation [22]. Regulatory CD4 T cells (Tregs) induce tolerance against self-antigens and prevent autoimmunity [23–25]. Interestingly, some studies have reported an increased Treg frequency in lymphoid tissues during progressive HIV disease [26,27] as shown for SIV infection [28,29], while others have shown a gradual decline in Tregs in peripheral blood associated with increased immune activation [30–33].

The challenges of identifying PHI have resulted in a lack of critical information that constrains the development of therapeutic interventions [34]. In this study, we provide a longitudinal characterization of different T-cell subsets and the expression of soluble inflammatory cytokines over the first year after PHI in a cohort of Mozambican adults and compared these changes with chronically HIV-infected (CHI) and HIV-uninfected subjects. Additionally, we explore the association between the various T-cell phenotypes and the plasma biomarker levels at the different stages of infection.

RESULTS

Characteristics of the study population

In the context of this study, we recruited 57 PHI patients identified during the screening process, as described in methods. From these, 40 individuals attended a follow-up visit 1 month later and 26, 21, 14, 14, 13 and 11 of these patients continued visits at 2, 3, 4, 6, 9 and 12 months after screening, respectively. There was no significant difference in age, gender balance or HIV-RNA viral load (VL) between PHI patients who returned for enrolment and those who were lost to follow-up [35]. HIV-uninfected individuals were randomly-selected from screened individuals and 58 subjects representing the control population attended a visit one month later. During the cross-sectional recruitment of CHI individuals, 26 patients were included in the ART-naïve group and 30 patients in the ART group. The demographic and clinical characteristics of the 40 PHI individuals who started follow-up, the HIV-uninfected and the CHI groups, are summarized in Table 1. Significant differences were found for age and body mass index (BMI, P<0.0001) but no differences were found for the clinical variables between the study groups (P>0.05).

Table 1. Clinical and demographic characteristics of study population according to HIV-status. Comparisons for proportions were performed by Fisher exact test$^{\chi}$ and continuous variables by Kruskal Wallis test**.

	1st follow-up visit PHI (n=40)	HIV-uninfected (n=58)	CHI-naive (n=26)	CHI-ART (n=30)	P-value
Age (years) [Mean (SD)]	**27.2 (9.2)**	**27.9 (9.5)**	**38.2 (13.4)**	**42.9 (8.8)**	**0.0001****
Gender [F (%)]	**24 (60.0%)**	**46 (79.3%)**	**19 (73.1%)**	**19 (63.3%)**	**0.162$^{\chi}$**
BMI (kg/m2) [Mean (SD)]	**20.3 (3.1)**	**21.5 (4.1)**	**24.5 (4.6)**	**24.1 (3.2)**	**0.0001****
time on ART (years) [Median (IQR)]	-	-	-	**2.6 (0.9-4.5)**	-
Pregnant [n (% F)]	**3 (12.5%)**	**7 (15.2%)**	**0 (0%)**	**3 (15.8%)**	**0.348$^{\chi}$**
Fever last 24h [n (%)]	**5 (12.5%)**	**3 (5.3%)**	**4 (15.4%)**	**1 (3.3%)**	**0.246$^{\chi}$**
Intestinal complaint last week [n (%)]	**12 (30%)**	**15 (25.9%)**	**4 (15.4%)**	**2 (6.7%)**	**0.067$^{\chi}$**
Co-infections *					
Hepatitis B [n (%)]	**5 (12.5%)**	**2 (3.5%)**	**2 (7.7%)**	**3 (10.0%)**	**0.400$^{\chi}$**
Syphilis [n (%)]	**3 (7.5%)**	**4 (6.9%)**	**0 (0%)**	**1 (3.3%)**	**0.481$^{\chi}$**
Malaria [n (%)]	**2 (5%)**	**0 (0%)**	**1 (3.9%)**	**0 (0%)**	**0.242**
Intestinal infection [n (%)]	**6 (15%)**	**11 (19%)**	**2 (7.7%)**	**3 (10.0%)**	**0.552$^{\chi}$**

* Co-infections were assessed as described in S1 File.

Among the 57 PHI identified, 28, 5, 7 and 17 were categorized into Fiebig I-III, Fiebig IV, Fiebig V and Fiebig VI stages, respectively, and were adjusted for time since infection as described in methods (S1 Fig). After categorizing by Fiebig stage, and adjusting for time since infection, median VL in the PHI group was 6.9 RNA Log10 copies/mL (IQR 6.2–7.5) at month one (M1) and significantly decreased to 5.1 RNA Log10 copies/mL (IQR 4.7–5.6) at month 2 post-infection (M2), (P=0.0001, Fig 1A). In the CHI-naïve patients, median VL was 4.5 RNA Log10 copies/mL (IQR 3.9-4.9).

In order to evaluate the dynamics of the different parameters along the first year of HIV-infection, two approaches based on non-parametric modeling and linear regression modeling were performed as described in methods. Longitudinal analysis confirmed the rapid decrease in VL with either a non-parametric models or a nadir VL-set point. A biphasic exponential decay model revealed a second phase of VL decay with a lower but significant slope until 6 months after infection (M6) (Fig 1B).

Regarding the dynamics of CD4 and CD8 T cells from M2, we observed that at M2 median CD4 T-cell count in PHI-individuals was significantly lower than in the HIV-uninfected group, 565 (IQR 387-675) vs. 955 (IQR 773-1149) cells/mm^3, respectively (P=0.0001, Fig 1C). CD4 T cells also showed an initial decrease that stabilized at months 5-6 post-infection (M5-6) in non-parametric longitudinal analysis, while significant linear decay was observed overtime in the regression model (p=0.033 for the slope, Fig 1D). Median CD8 T-cell count was significantly higher in PHI at M2 than in HIV-uninfected controls, 1175 (IQR 771-1683) vs. 591 cells/mm^3 (IQR 417-746) respectively (P=0.0001, Fig 1E). Both longitudinal models show that the initial increase in CD8 T cells is followed by a significant decay that also stabilized at M5-6, remaining stable and high until CHI (P=0.002, Fig 1F). In the CHI-naïve group, median CD4 T-cell and CD8 T-cell count were 595 (IQR 466-729) and 1029 (IQR 685-1562) respectively; while in the CHI-ART group median CD4 T-cell and CD8 T-cell count were 474 (IQR 377-590) and 830 (IQR 617-1061), respectively.

CD4 Th1Th17 and Treg changes during the different stages of HIV-infection

The frequency of functionally distinct CD4 T cells was analyzed by the cell surface expression of CD127 and CD25 (for Treg) and CD183 (CXCR3) and CD196 (CCR6) as described in methods. This latter combination identifies Th1Th17 cells as CD183+CD196+, while CD183+CD196- cells are mostly Th1 and CD183-CD196+ cells contain the Th17 population (S2 Fig) [20]. No major changes were observed in CD4 CD183+CD196- or CD183-CD196+ cells during PHI (data not shown). However, the frequency of CD183+CD196+ (Th1Th17 cells) cells in peripheral blood mononuclear cells (PBMCs) significantly decayed overtime until 7-8 months post-infection (M7-M8, P=0.0127, Fig 2A and B). The percentage of activation in Th1Th17 cells at M2 (as measure by CD38 and HLA-DR co-expression) was significantly increased compared to HIV-uninfected (P<0.0001) and continued to increase

along the first year post-infection (data not shown). Looking at the maturation stage of the Th1Th17 cells, a significant increase in the naive pools (TN, P=0.0002) and a significant decrease in the effector memory (TEM, P=0.0007) was observed at M2 compared to HIV-uninfected. Since the definition of Th1Th17 cells involves cell surface expression of CXCR3, the receptor for IP-10, we assessed the relationship between CXCR3 expression and IP-10 levels. Although a significant negative correlation was observed between IP-10 plasma levels and circulating CD4 T cells (rho=-0.49, P=0.0282); such association was positive and borderline significant between plasma IP-10 levels and CXCR3+ CD4 T cell frequencies (rho=0.43, P=0.0574), and no association was observed between plasma IP-10 and the frequency of Th1Th17 CD4 T cells.

No significant oscillations were observed during PHI for the CD4 Tregs (Fig 2C), but comparing to non-HIV infected individuals, CD4 Treg frequency was significantly higher in the CHI-ART group (P=0.0267). Consistently, regression models showed no significant difference of Treg levels during the first year post-infection (Fig 2D).

Intensive loss of resting CD8 subsets early after HIV infection

Dynamics of the CD4 and CD8 T-cell maturation subsets showed a different profile. Although differences were not significant, the CD4 T-cell compartment showed a prompt increase in the resting phenotypes (TN and central memory T cells [TCM]) and a decrease in the effector phenotypes (TEM and effector RA+ T cells [TEMRA]) at M2 (Fig 3A) compared to HIV-uninfected, that normalized several months after infection. These longitudinal changes observed in the CD4 T-cell compartment over the first year of infection, were not significant for any subset. When comparing CHI-naïve and CHI-ART groups, CHI-ART group showed a non-significant tendency towards higher levels of TEM and lower significant levels of TN (P=0.0413).

Conversely, we observed a marked loss of the TN and TCM CD8 T-cell pool after HIV infection concomitant to an increase in the frequencies of the TEM subset (Fig 3B). Comparing to HIV-uninfected individuals, CD8 TN significantly decreased in PHI at M2 and in CHI-naïve groups (P<0.0001) and CD8 TEM significantly increased (P<0.0001, P=0.0002, respectively). No significant changes were observed in TCM; however, TEMRA was significantly increased in CHI-ART compared to PHI at M2 (P=0.0027). Longitudinal analysis confirmed these changes (data not shown).

T-cell activation phenotypes along HIV infection

The dynamics of activation (CD38+HLA-DR+ cells) exhaustion (CD279+ cells) and immunosenescence (CD57+ cells) were also analyzed in CD4 and CD8 T cells. As for maturation markers, most relevant changes were noticed in CD8 T cells. Although both activated and exhausted CD4 T cells were significantly increased at M2 when compared to

HIV-uninfected individuals (P<0.0001, P=0.0056, respectively), and slowly but significantly decayed over the first year of infection (S3A and B Fig). Senescent CD4 T cells (CD57+) showed no significant changes in the PHI group as compared to HIV-uninfected individuals but they were significantly increased in the CHI-ART group (P=0.000, S3C Fig).

The analysis of activation in CD8-T cells showed a significant increase at M2 as compared to HIV-uninfected that remained in CHI-naïve subjects (P<0.0001) and normalized in CHI-ART (Fig 4A). The CD8 T-cell activation observed in the first months of infection was significantly reduced over time with a slow but significant decrease until months 9-11 (M9-11) (P<0.0001, Fig 4B). Exhausted CD8 T cells also showed a transient but less marked significant increase as compared to HIV-uninfected individuals (P<0.0001, Fig 4C), with a significant and slow decrease over time (P=0.0056, Fig 4D). Along the course of HIV infection, the frequency of senescent CD8 T-cells was significantly higher in the CHI-ART group as compared to the HIV-uninfected group (P<0.0001, Fig 4E). However, no significant changes over time were observed in the frequency of senescent CD8 T-cells by linear regression models (Fig 4F). There were no significant differences in percentages of activated, exhausted or senescent CD8 T-cells associated with the presence of co-infection in any of the study groups.

Dynamics of soluble inflammatory cytokines and association with T- cell phenotypes in PHI

As described in methods, the kinetics of 13 cytokines with expression levels most significantly different between febrile PHI and HIV-uninfected individuals [36] were characterized in detail during the first year after infection (Fig 5A and 5B and S4 Fig). Levels of IP-10, MCP-1, BAFF, sCD14, TNFR2 and TRAIL were highly overexpressed at the first month of infection (M1) and underwent a prompt decrease in the subsequent months. This decrease was more gradual in the case of IP-10, which remained overexpressed even 5 months post-infection. On the contrary, MIG, sCD27 and sCD23 levels started to increase 1 month after infection and remained over-expressed for almost one year post-infection, while GSCF had a later up-regulation at 7 months of infection.

Correlations between the plasma cytokine levels at M1 and activated or exhausted CD4 and CD8 T-cell phenotypes at 2 months post-infection were assessed as described in methods (Fig 5C). We observed a significant positive correlation between M1 TNFR2 and sCD27 levels and the frequency of exhausted CD4 T cells and CD8 T cells at M2 (rho=0.77, P<0.0001; rho=0.54, P= 0.0157 respectively). Similarly, we saw a significant association between M1 levels of BAFF (rho=0.50, P=0.0241), IL10 (rho=0.44, P=0.05) and sCD14 (rho=0.44, P=0.05) and the frequency of activated CD8 T-cells at M2. However, after adjustment by multiple-testing, only the significance of TNFR2 with exhausted CD4 T-cells levels was maintained (P=0.0135). We did not observe significant differences in the

expression level of these 13 selected cytokines by the presence of any co-infection in the PHI or the CHI groups; however, BAFF, MCP-1, MIG and TRAIL expression levels were significantly lower among the individuals included in the HIV-uninfected control group with a co-infection detected (P=0.0254, P=0.0418, P=0.0001, P=0.0032, respectively).

DISCUSSION

We conducted a systematic analysis of the clinical, virologic, and immunologic characteristics of the different stages of HIV infection in HIV-infected Mozambican adults. Soluble biomarker quantification and T-cell immunophenotyping revealed that while most inflammatory biomarkers, CD4 counts and VL stabilized early after HIV infection, certain T-cell subsets took longer to reach a stable level.

Several studies have shown that during AHI up to 80% of CD4 memory T-cells in GALT is destroyed within the first 3 weeks of infection [37–39]. Particularly, depletion of memory CD4 Th17-cells in GALT occurs at the first stages of acute HIV infection [22]. However, data from our PHI cohort show that these changes are not evident in circulating cells. For the specific case of systemic Th1Th17 cells, their frequency in PBMCs is similar to uninfected individuals at 2 months after infection but decreases steadily until 9-11 months after infection displaying and maintaining an activated phenotype early after infection. Since the definition of Th1Th17 cells involves cell surface expression of CXCR3, the receptor for IP-10, and this cytokine has been associated with the recruitment of CXCR3+ CD4 T cells to HIV replication sites [40], we assessed the relationship between CXCR3 expression and IP-10 levels. Although a significant negative correlation was observed between IP-10 plasma levels and circulating CD4 T cells, such association was not observed between plasma IP-10 and the frequency of CXCR3+ or Th1Th17 (CD183+CD196+) CD4 T cells. In contrast to Th17 cells, studies have reported both an increased [26,27] and a gradual decline in Treg frequency in peripheral blood during progressive HIV-infection [30–33]. Although we did not observe significant changes in the Treg compartment during PHI, we detected a trend towards an increase after HIV-infection.

Surprisingly, ART seemed to lack beneficial effects in restoring the CD4 T-cell maturation profile as the CHI-ART group showed higher levels of TEM and lower levels of TN than did CHI-naive. This is probably due to older age and poor immunological recovery that associate with a skewed CD4 T cell maturation [41,42].

CD8 T-cell activation has been described to be the strongest correlate of disease progression [11–14]. Recently, the magnitude and kinetics of CD8 T-cell activation during early acute HIV infection has been observed to impact VL set point [43]. Still, we observed in our study that both activated and effector memory CD8 T-cells peaked at month 2 after

infection and reached stable levels only at 9-11 months post-infection, several months after viremia stabilization. These results indicate that, despite the rapid immune control over virus replication, homeostasis in the CD8 T-cell compartment requires longer to be achieved. Thus, most alterations observed in the CD8 T-cell compartment during HIV-infection are not exclusively viremia driven. Our data also show an early increase of the exhaustion marker PD-1 (CD279) that slowly decays paralleling activation in CD8 T cells. Importantly, despite these profound alterations, no relevant increases of the expression of CD57 were observed in CD8 T cells during PHI, suggesting that this marker of replicative immunosenescence could be associated with longstanding HIV infection, as confirmed by its highest expression in CHI individuals.

Significant efforts have been made to characterize early cytokine responses with the aim of identifying biomarkers of progression or key pathological pathways that could be targeted to minimize HIV-induced immune damage [5,7,8,44]. In this study we provide additional data showing associations between early cytokine levels and specific T-cell phenotypes 2 months after infection. TNFR2 and sCD27 levels were associated with exhausted CD4 T-cells and CD8 T-cells, respectively, while BAFF, IL-10 and sCD14 were associated with CD8 T-cell activation. Tumor necrosis factor receptor-2 (TNFR2) is involved in cell survival that can result in cell proliferation, while CD27 participates in generation and long-term maintenance of T-cell immunity. Thus, by function, it is not surprising that these two cytokines are associated with exhausted T-cell subtype levels. B-cell activating factor (BAFF), interleukin-10 (IL-10) and sCD14 are produced by monocytes and macrophages after infection or tissue inflammation in order to assure a proper immune response [45,46], so their association with the subsequent CD8 T-cell activation could indicate a way of controlling the cellular response to HIV-infection.

Adjustment by Fiebig stage at the screening allowed us to approximate time since infection according to previous categorization [49–51]. However, this approximation may add potential uncertainty to the biomarker levels detected during the first 3 months after infection when the very intense immune responses are occurring [49–51]. Additionally, age and BMI were significantly higher in the CHI groups, comparing to the PHI and control HIV-uninfected population. This is explained by the high HIV-incidence rate in young population in the Sub-Saharan setting and the national ART recommendations in place at the moment of the study. According to the HIV guidelines in Mozambique in 2013-2014, HIV-infected patients initiated ART if CD4 T-cell counts were ≤350 cells/mm^3 or presenting an AIDS-associated disease, features more common at late stages of HIV-infection. This fact might impact immune recovery [52] and along with age and BMI differences could have affected the biomarker comparison with CHI-groups, especially at the analysis of T-cell maturation stages [53], senescence [17] and cytokine expression [54] whose levels have been described to be increased by age.

Due to the study design, T-cell phenotyping data was not available for the first month of infection. This would have allowed a further characterization of the first responses in the T-cell compartment and provided additional data in the T-cell specific phenotypes. Similarly, the loss to follow-up along the longitudinal visits may have resulted in insufficient power to detect additional significant differences. The high loss to follow up also hampered the possibility to study the associations between soluble and cellular markers with clinical disease progression in our cohort. Such a high attrition rate is common in these scarce-resource rural settings [47]. Attendance of scheduled visits is complicated by high rates of migration, long distances to health centers and difficulties missing work which threat continuity of care. Moreover, PHI individuals are usually asymptomatic after peak viremia [48], so patients do not feel the need to return to the hospital until they have further progressed to AIDS.

The high burden of infectious diseases prevalent in the study area could have impacted the T-cell phenotypic characteristics and cytokine dynamics and expression levels. However, we did not observe any significant difference according to the co-infection status in the stage of CD8 activation for any of the study groups. We did find that cytokine expression levels in those individuals that were positive for intestinal, malaria, hepatitis B or syphilis infection were significantly higher for BAFF, MCP-1, MIG and TRAIL as compared to those negative for all the tested infections, but only in the HIV-uninfected group. Thus, further studies could evaluate the specific effect that additional co-infections could have in the dynamics of these cellular and plasma biomarkers in the HIV-infected individuals. The cytokine levels for PHI patients prior to onset of symptoms were not available. Our results thus describe the cytokine levels after the start of the 'cytokine storm' from at approximately 10 (95%CI 7-21) days post-infection [49–51], when VL and cytokine levels are already close or pass to their peak.

This characterization of biomarker expression in plasma and T-cells during the different stages of HIV infection provides an in depth description of the immune responses following HIV acquisition in a population of Mozambican adults. Several studies have provided description of the cytokine [3–9] or T-cell phenotypes [34,48,55] during acute and PHI. However, our longitudinal study offers new insight into potential associations between innate and cellular responses. Early ART stops progression to AIDS [56,57], diminishes the size of viral reservoir [58], prevents intestinal damage [22] and reduces further transmissions [59]. In our study, we also show that stabilization of specific T-cell phenotypes occurs months after viremia or CD4 count stabilize in the course of infection, adding more evidence to the arguments for treatment initiation regardless of CD4 counts or viremia levels. Previous studies have seen that ART initiation at the earliest stages of acute HIV infection does not normalize the CD4/CD8 ratio even after 2 years of treatment [34], suggesting some degree of persistent immunological dysfunction. Our data shows that homeostasis in the CD8 T-cell compartment and initiation of Th1Th17 decay in PBMCs

occurs months after viremia and CD4 count reach the set point level. This indicates that many HIV-related changes observed in the CD8 T-cell and CD4 T-cell compartment may not be exclusively driven by viremia levels and additional immune responses could account for these T-cell alterations. This raises the potential need for additional therapies that could enhance immune recovery and reduce immune activation.

METHODS

Study population

The study population was enrolled between 2013 and 2014 at the Manhiça District Hospital (MDH) in the district of Manhiça, Southern Mozambique. The present analysis is a sub-study of a prospective cohort of primary HIV-infected adults enrolled and followed up for 12 months in the Gastro-intestinal biomarkers in acute-HIV infected Mozambican adults study (GAMA)[35]. This study was approved by local institutional review boards at Barcelona Clinic Hospital (2011/6264) and by the Ministry of Health of Mozambique (461/CNBS/12). All methods were carried out in accordance with the relevant guidelines and regulations. Written informed consent was obtained from patients prior to participation.

HIV diagnosis and clinical follow up

All study participants were over 18 years of age and residents of the established District Surveillance System (DSS) study area. During the screening, subjects presenting to the outpatient clinic of MDH for non-specific febrile symptoms or voluntary HIV counseling and testing (VCT) were included in the PHI group if they were negative or indeterminate for rapid test serology and HIV-RNA positive for pooled-VL testing (n=85). A control population was established by random selection among HIV-uninfected and individuals were invited to attend a study visit 1 month after the screening date (n=58). PHI individuals were followed up at seven consecutive visits 1, 2, 3, 4, 6, 9 and 12 months after the screening visit. Technical information and procedures regarding HIV diagnosis and monitoring, as well as the screening profile have been previously described [35]. Additionally, adults with documented HIV diagnosis ≥12 months earlier attending routine scheduled outpatient visits for clinical management of HIV/AIDS at the MDH, were enrolled as chronic HIV-infected (CHI) patients. CHI patients were included in the CHI-naïve or the CHI-ART, depending whether they had previously initiated treatment according to the current national guidelines (patients with a CD4 T-cell count ≤350 cells/mm^3 or presenting and AIDS-associated disease).

After screening, demographic and clinical data was collected, medical consultation and HIV counseling was provided and blood and stool samples were collected at all the study visits. CD4/CD8 T-cell counts was performed on fresh whole blood in a single platform system using Trucount tubes and LSRII cytometer. PBMCs were isolated by Ficoll density gradients and immediately stored in liquid nitrogen. VL determination was performed in plasma samples as previously described [35]. Microbiological evaluation was performed in plasma and stool samples, testing for the most prevalent infections in the area including malaria, hepatitis B virus (HBV), syphilis and gastro-intestinal protozoa, bacteria and parasites (S1 File).

Definition of primary HIV infection phases and quantification of biomarkers

HIV-specific serology was subsequently performed on frozen plasma samples by Western Blot. VL and WB results at screening visit were employed to categorize individuals into Fiebig stages I-III (VL positive, WB negative), IV (VL positive, WB indeterminate), V (VL positive, WB positive with p31 band negative), VI (VL positive, WB positive with p31 band positive) as described in previous work [35,49]. In order to approximate similar time since infection for the PHI individuals at every study visit, visits from individuals categorized in Fiebig stage V and VI at screening were moved 1 and 2 months forward, respectively, according to estimated days-post infection previously described [49–51]. After adjustment by Fiebig, new visits were grouped into M1, M2, M3, M4, M5, M6, M7-8, M9-11, and M12-15 according to estimated months since infection (S1 Fig). PBMCs or clinical data were not collected at the screening visit, so all the individuals included in M1 will not have this information, as well those individuals categorized in Fiebig stage V and VI at M2 and M3, respectively.

Multiplex biomarker profiling was performed for a total of 61 immune response biomarkers in plasma samples. Determinations were performed by ELISA commercial assays or Luminex technology as previously described [35,36]. From the resulting 49 quantifiable biomarkers, the difference in the median levels between PHI and non-HIV-infected individuals with reported fever was highly significant for 13 cytokines ($P<0.001$)[36]. In order to represent the dynamics along the first year of infection, these 13 cytokines were grouped into 2 main pathways depending on their main function: 1) Lymphocyte and monocyte function; 2) Inflammation, intestinal damage, cell death and proliferation.

CD4 and CD8 T-cell immunephenotyping

Cryopreserved PBMCs were thawed at 37°C, washed in RPMI/60% and RPMI/20% of fetal bovine serum (FBS) and incubated for 1 hour at 37°C in RPMI/10%FBS. PBMCs were then stained with the Fixabel Viability Stain-FVS780r (APC-H7 detect, BD Biosciences) for 15mins. After PBMCs washing in PBS/1%FBS, cells were plotted to a U-bottom 96-well plate at a density of 1.5 Millions/well and stained with selected 14-colour panel including CD3-BV605

(Clone SK7), CD4-FITC (Clone RPA-T4), CD8-V500 (Clone SK1), CD45RA-Alexa Fluor®700 (Clone HI100), CD197-PE-CF594 (Clone 150503), CD57-APC (Clone NK-1), CD279-BV421 (Clone EH12.1), HLA-DR-BV650 (Clone G46-6), CD38-PerCp-Cy5.5 (Clone HIT2), CD25-PE (Clone M-A251), CD127-BV786 (Clone HIL-7R-M21), CD196-BV711 (Clone 11A9), CD183-PE-Cy7 (Clone 1C6/CXCR3) (from BD Biosciences) for 15mins. After washing twice in PBS/1%FBS, cells were fixed in PBS/1% formaldehyde, acquired in a BD LSRFortessa cytometer using a plate HTS loader (BD Biosciences) and analyzed with FlowJo software (Tree Star). Gating strategy is described in S2 Fig. Lymphocyte gate was defined manually by morphological parameters excluding nonviable cells and singlets. Median of viability+ lymphocytes was 2.5% [IQR 1.6–4.1], as an estimation of death cells per sample. Subsets were identified as CD3+ cells and gated as CD4+CD8- or CD8+CD4-, while double positive cells and double negative cells were excluded from the analysis. T-cell maturation stage was analyzed automatically using R software for CD45RA and CD197/CCR7 expression to define naive (TN, CD45RA+CCR7+), central memory (TCM, CD45RA−CCR7+), effector memory (TEM, CD45RA−CCR7−) and effector memory RA+ cells (TEMRA, CD45RA+CCR7-). T-cell subsets were also analyzed automatically for the expression of HLA-DR and CD38 to define activated cells (HLA-DR+ and CD38+), CD279/PD-1 to define exhausted cells (CD279+) and CD57 to define senescent cells (CD57+++). CD4+ T-cells were subsequently analyzed manually for the expression of CD25 and CD27 to define Tregs subset (CD25+++CD27-) and automatically for the expression of CD183 and CD196 to define Th1/Th17 cells (CD183+CD196-).

Statistical analysis

Group comparisons were performed using the Fisher exact test for categorical variables and the nonparametric Kruskal-Wallis test for continue variables. Spearman's correlation was used to assess the strength of relationship between continuous variables and multiple testing was further adjusted by False Discover Rate (FDR). Individual comparisons between the different groups were performed using posthoc pairwise comparisons with the Tukey and Kramer (Nemenyi) test. Relative changes (Z-score) with respect to the HIV-uninfected group (in the case of VL the Z-score was calculated relative to the CHI-naïve group) have been represented by a transformation of the fitted longitudinal models by subtracting the mean and dividing by the standard deviation of HIV-non infected distribution, after a logarithmic transformation in the cases where it was required for normal distribution. Longitudinal behavior for analytes and immunological variables were modeled by fitting smoothing-splines mixed-effects models using the "sme" package of R. To infer if there was a significant association of selected biomarkers with the time variable, polynomial time effects approximation until third degree were fitted using linear mixed-effects regression models. Best model was selected based on likelihood-ratio tests under maximum likelihood models estimations. A two-phase exponential decay regression model was employed in the case of VL modeling.

Statistical analyses were performed using R-3.3.1 and Stata14 software.

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Author's contributions:

DN and JB study design. LP, EP and LF recruited subjects and collected clinical data. LP, EP, LF and CJ performed laboratory analysis at the field. LP and JC performed plasma biomarker quantification and validation of the data. LP, VU and JB performed PBMCs phenotyping and validation of the data. LP and VU performed statistical analyses. LP, VU, DN and JB interpreted the data. LP, EP, LF, IM, DN and JB study management and coordination. LP drafted the paper. VU, DN and JB critical data review and revision of manuscript writing. All authors read and approved the final version of the manuscript.

Competing interests

We declare that there are no conflicts of interest in relation to the submitted work. The information of this manuscript has not previously been presented in any public meetings or conferences.

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FIGURES

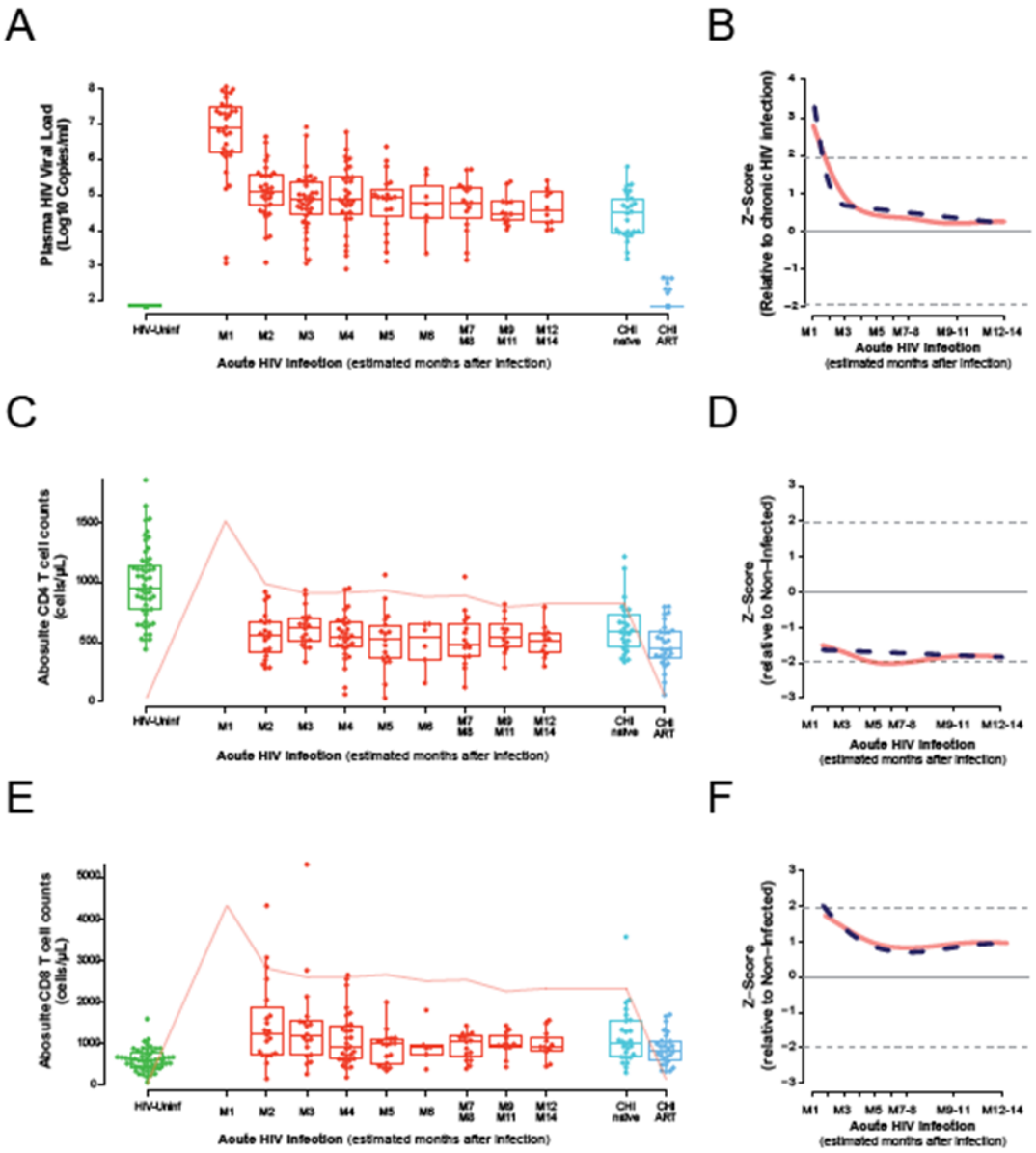

Figure 1. Virological and Immunological characteristics along HIV infection. Plasma viral load (VL) as RNA Log10 copies/mL (A), whole blood CD4 absolute count (C) and whole blood CD8 absolute count (E) across the different study groups and along time post-infection. M, months after infection. Box as IQR, middle line as median, whiskers as maximum and minimum and dots as individual observations in panels A, C and E. Dynamics of each parameter (panels B, D and F) are shown as Z-score values for PHI individuals over CHI-naïve (VL) and over HIV-uninfected individuals (CD4 and CD8 T-cell counts). Red lines show non-parametric models, while dotted blue lines indicate the best fitting for polynomial time effects regression approximation.

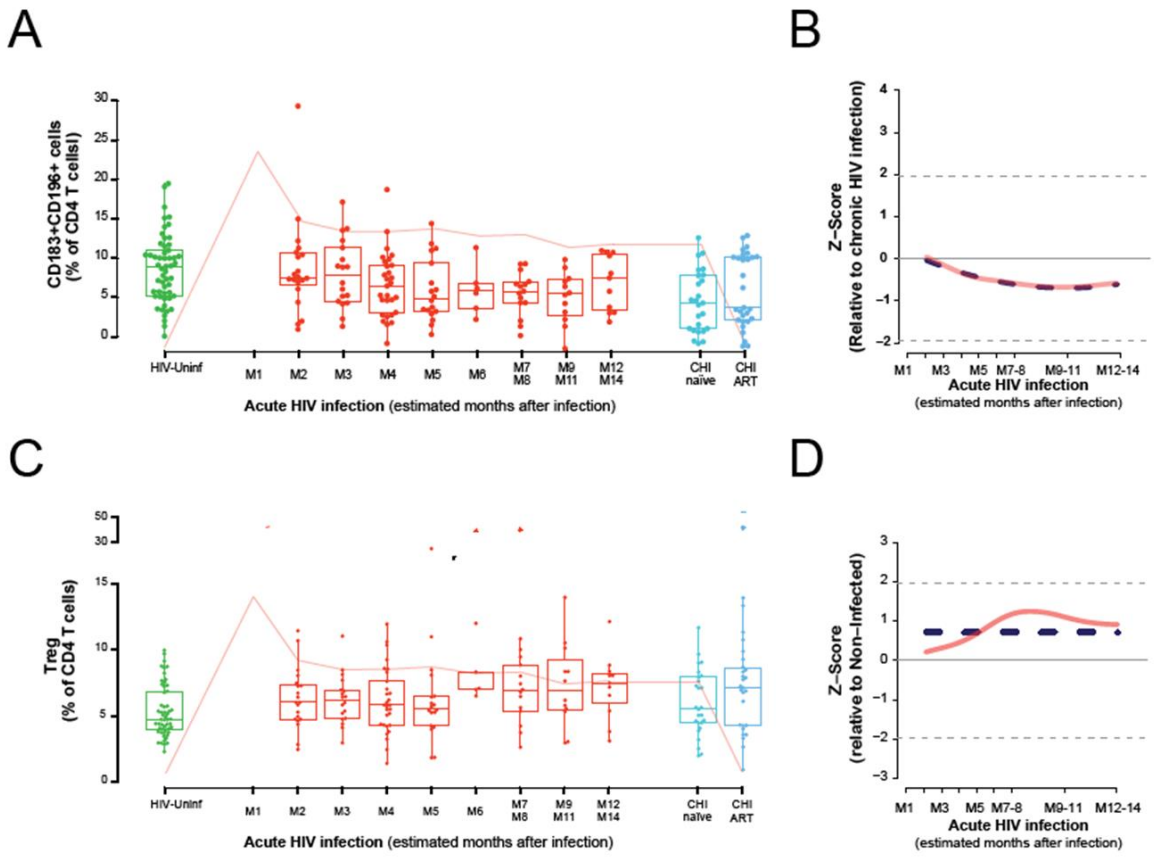

Figure 2. Dynamics of CD4 T-cell phenotypes along HIV infection. Characterization of Th1-Th17 (A), Tregs (C) across the different study groups and along time post-infection. M, months after infection. Box as IQR, middle line as median, whiskers as maximum and minimum and dots as individual observations. Dynamics of each parameter (panels B and D) are shown as Z-score values for acutely infected individuals. Red lines show non-parametric models, while dotted blue lines indicate the best fitting for polynomial time effects regression approximation.

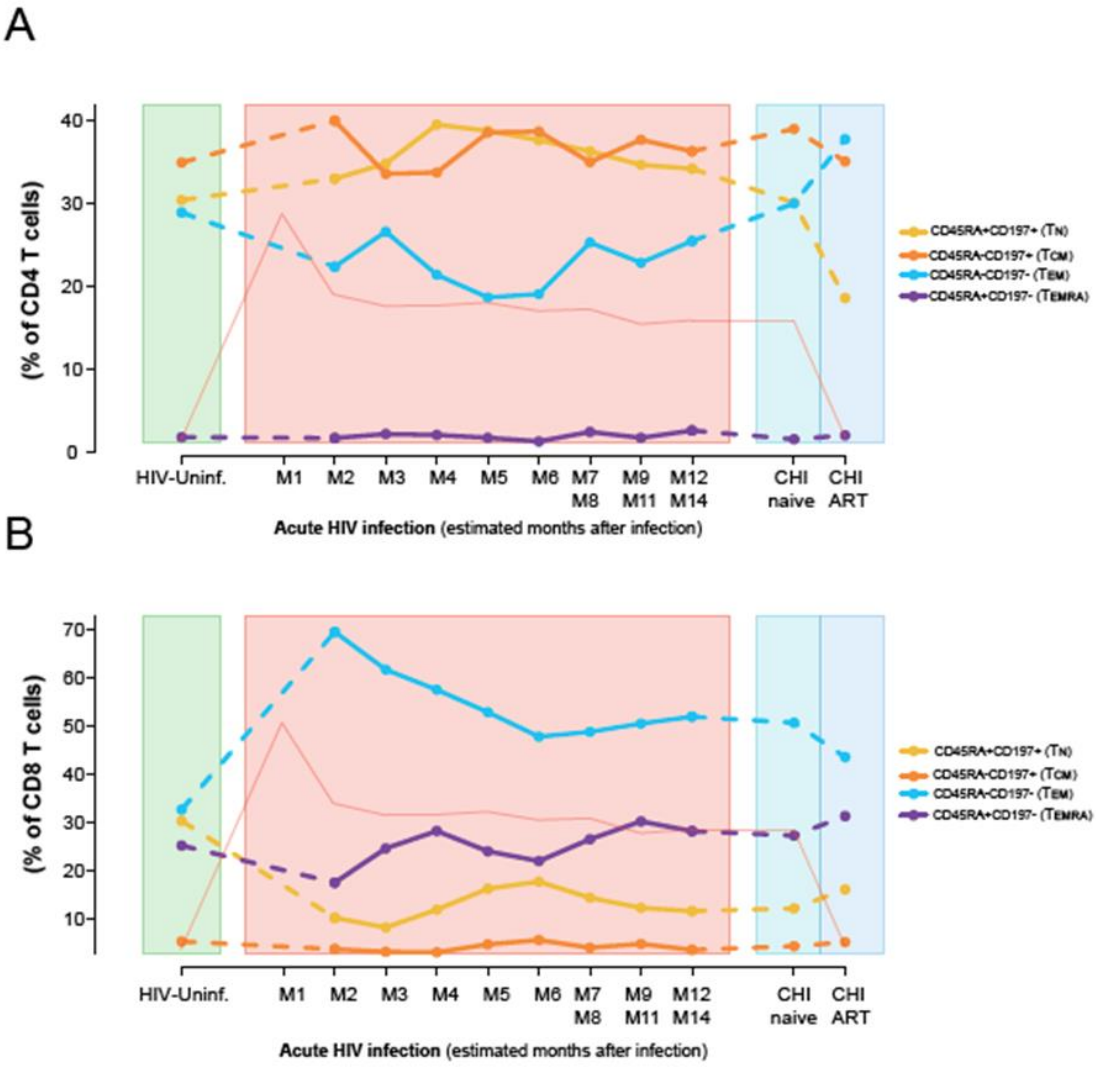

Figure 3. Dynamics of T-cell maturation phenotypes along HIV infection. Characterization of TN , TCM , TEM and TEMRA frequencies for CD4 (upper plot) and CD8 T cells (lower plot) across the different study groups and along time post-infection. M, months after infection. Dot as median.

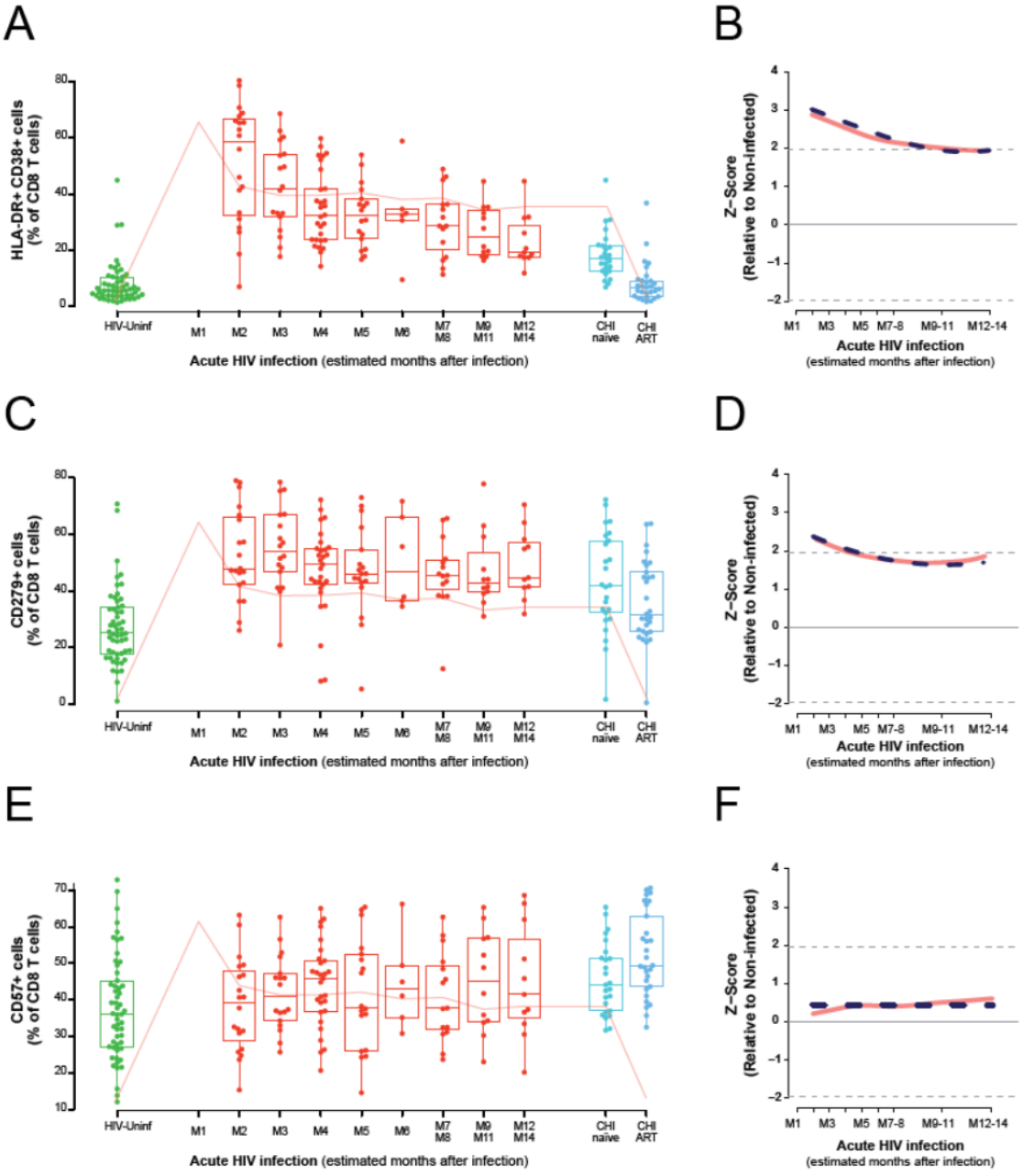

Figure 4. CD8 T-cell activation, exhaustion and immunosenescence along HIV infection. Characterization of activated (A), exhausted (C), and senescent CD8 T-cells (E) across the different study groups and along time post-infection. M, months after infection. Box as IQR, middle line as median, whiskers as maximum and minimum and dots as individual observations. Dynamics of each parameter (panels B, D and F) are shown as Z-score values for acutely infected individuals. Red lines show non-parametric models, while dotted blue lines indicate the best fitting for polynomial time effects regression approximation.

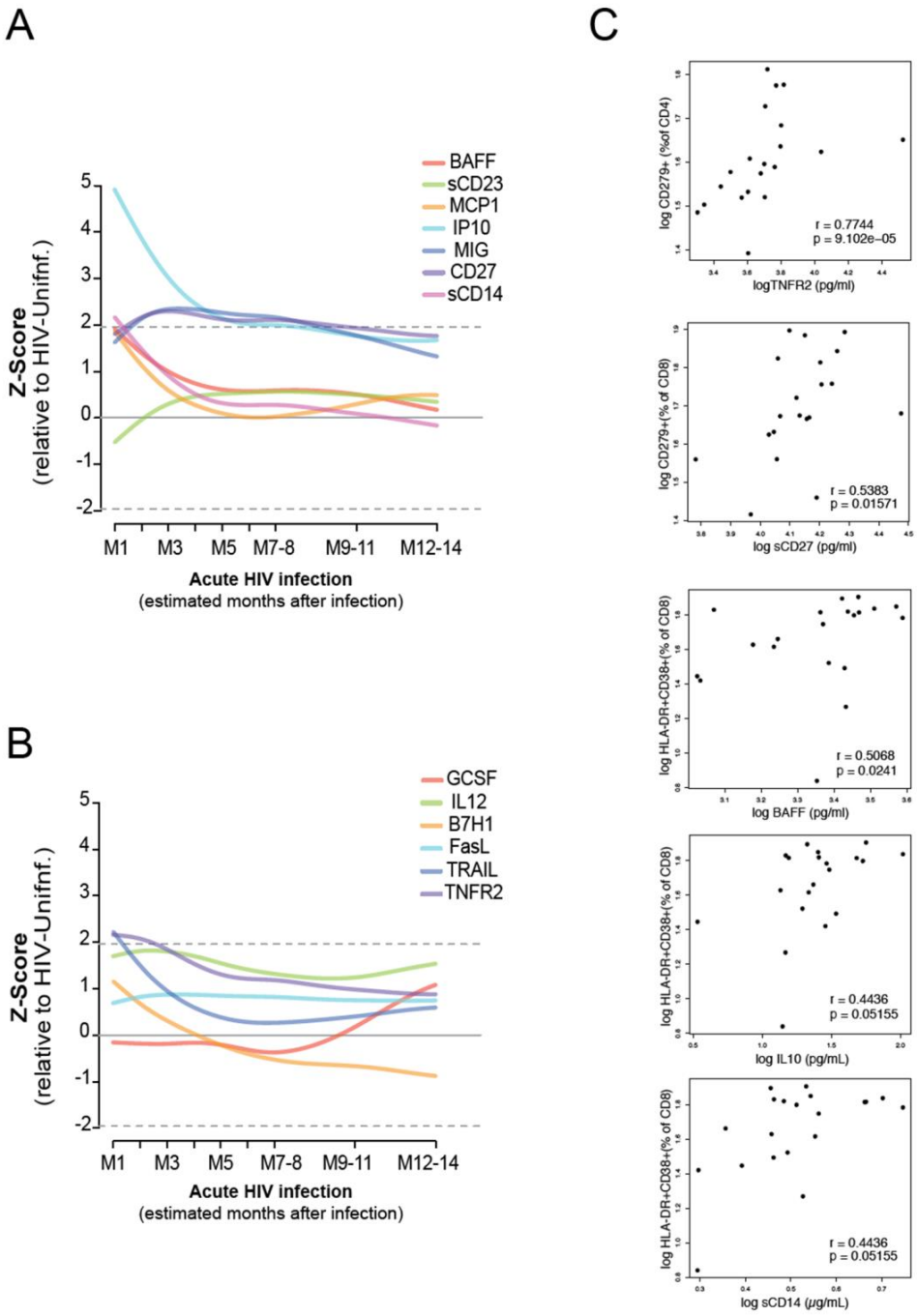

Figure 5. Association of plasma cytokine levels and T-cell phenotypes during early HIV infection. Cytokine normalized expression levels (Z-socre relative to HIV-uninfected controls) along the first year post-infection (Panels A and B). Cytokines showing significant correlation at one month post-infection with the subsequent exhausted or activated T-cell phenotypes at 2 months post-infection are shown in panel C. Spearman rho correlation and p-value are shown for each plot

SUPPLEMENTARY MATERIAL

SECTION 1. Methodology employed for the screening of the most prevalent co-infections:

- Plasmodium falciparum malaria: Whole blood was used to screen for *Plasmodium falciparum* malaria using a glass slide for optic microscopic determination.

- Hepatitis B Virus (HBV): Rapid testing for HBV surface antigen was performed in whole blood by using the *Determine assay (Inverness Laboratories)* according to manufacturer's instructions.

- Treponema pallidum: Screening for syphilis was performed in plasma samples using the Rapid plasma reagin (RPR) according to manufacturer's instructions (*Human Diagnostics*). All rapid plasma reagin–positive samples were confirmed by a using a Treponema pallidum Hemagglutination (TPHA) assay (*Human Diagnostics*).

- Gastro-intestinal infections were evaluated in stool samples as:

    - *Giardia Lamblia, Cryptosporidium sp.* and *Entamoeba Histolytica* were assessed by ELISA immune sorbent assay.

    - *Salmonella, Shigella spp, Vibrio cholerae, Aeromona, Yersinia* were cultured and identified by selective mediums.

    - Parasitological infections were examined by optic microscopy.

    - *Cryptosporidium difficile* infection was detected using a commercial immunoassay which detects oocyte antigen and according to manufacturer's instructions *(C. diff quik chek c omplete test, Alere, TechLab).*

SECTION 2: Supplementary Figures

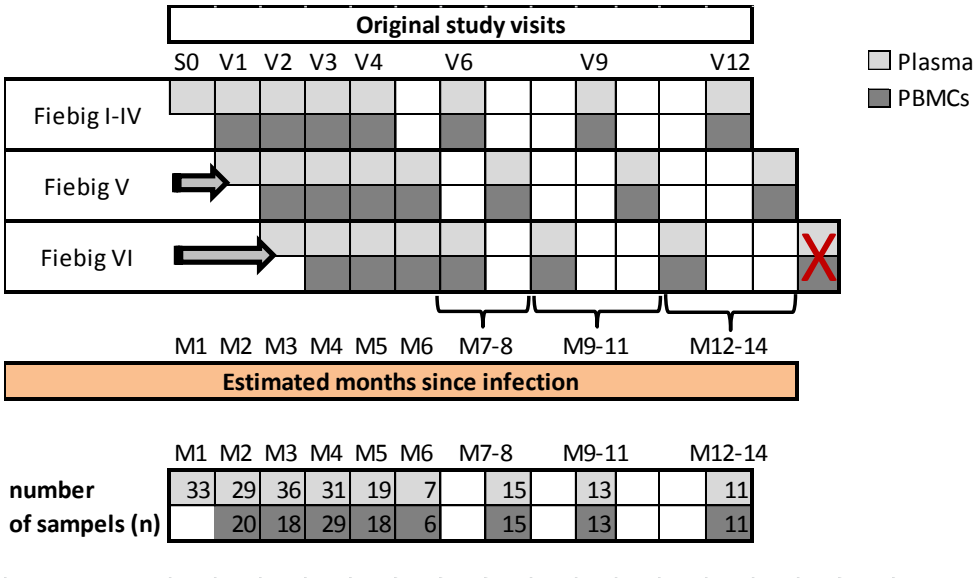

Figure S1. Study time points after adjustment by Fiebig. According to estimated months since infection[48–50], new visits were grouped into M1, M2, M3, M4, M5, M6, M7-8, M9-11, and M12-14. S0, screening visit; V, visit; M, month; "X", excluded visits.

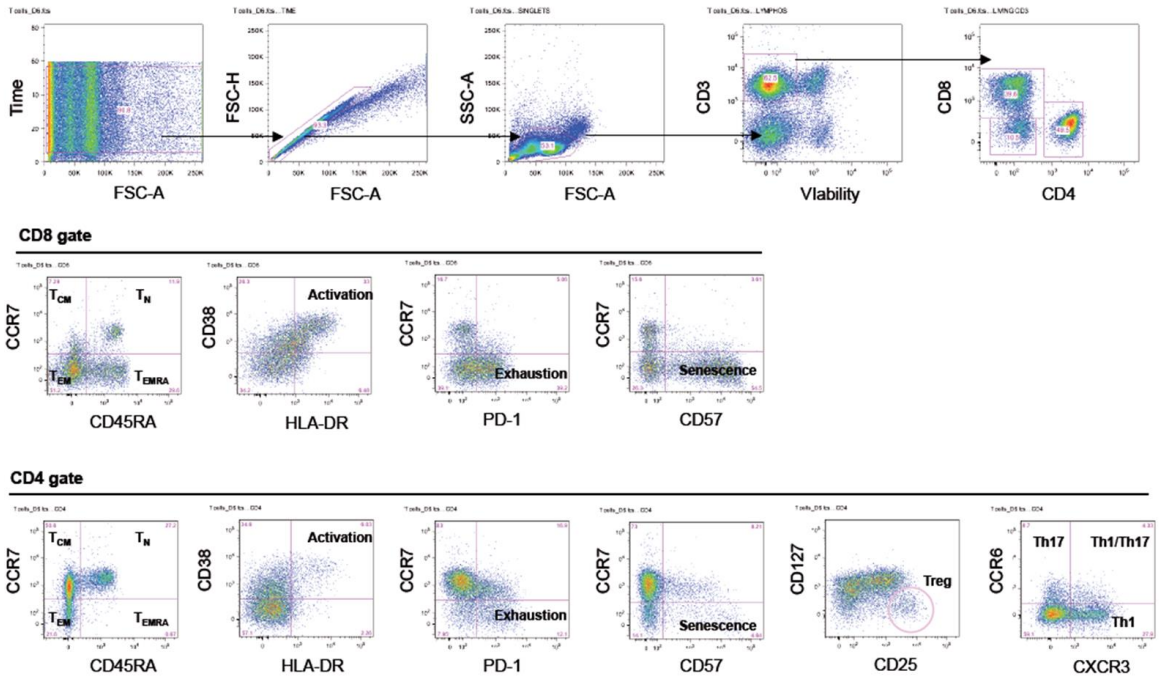

Figure S2. Gating strategy for T-cell analysis.

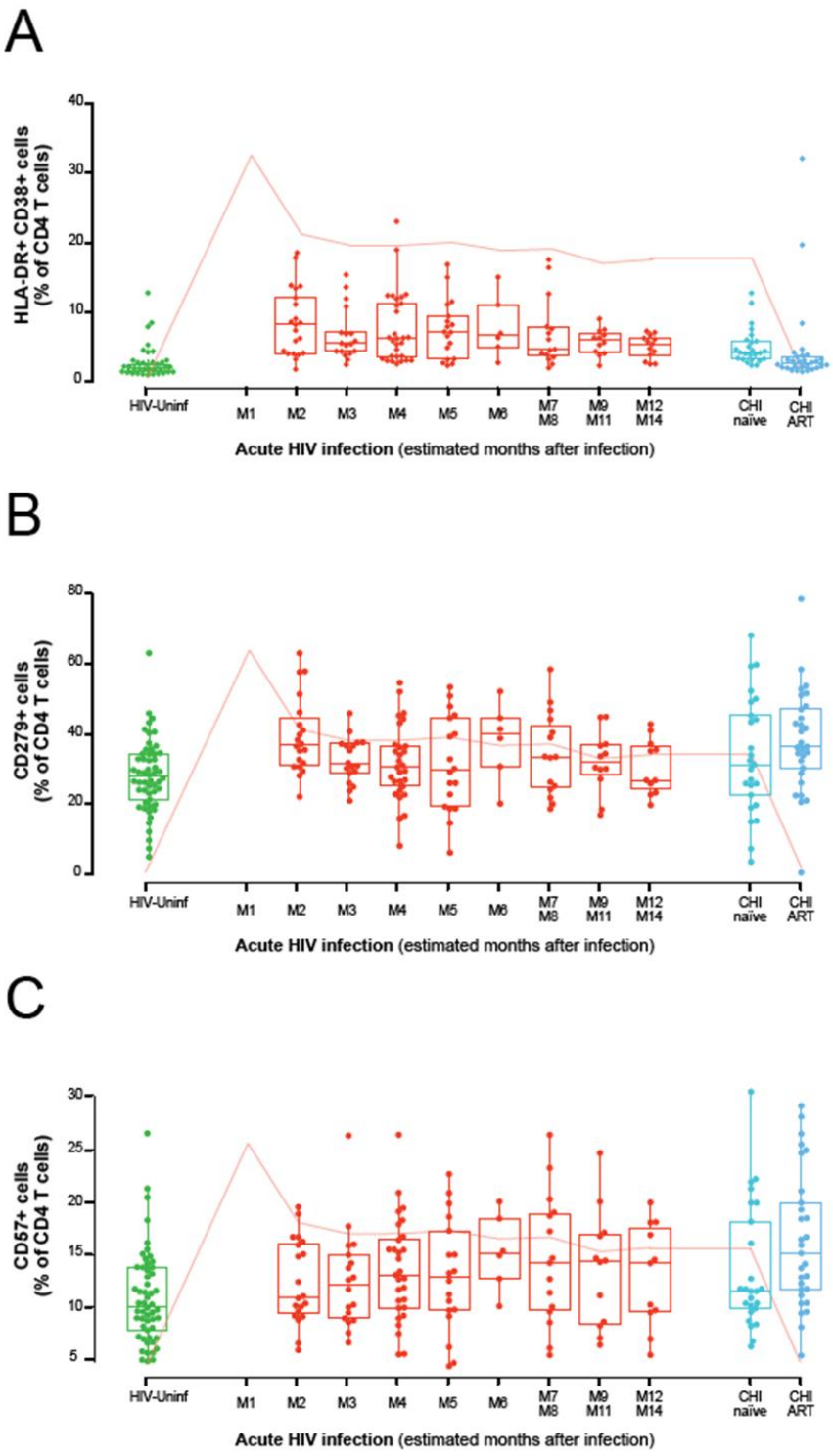

Figure S3. CD4 T-cell activation along HIV infection.

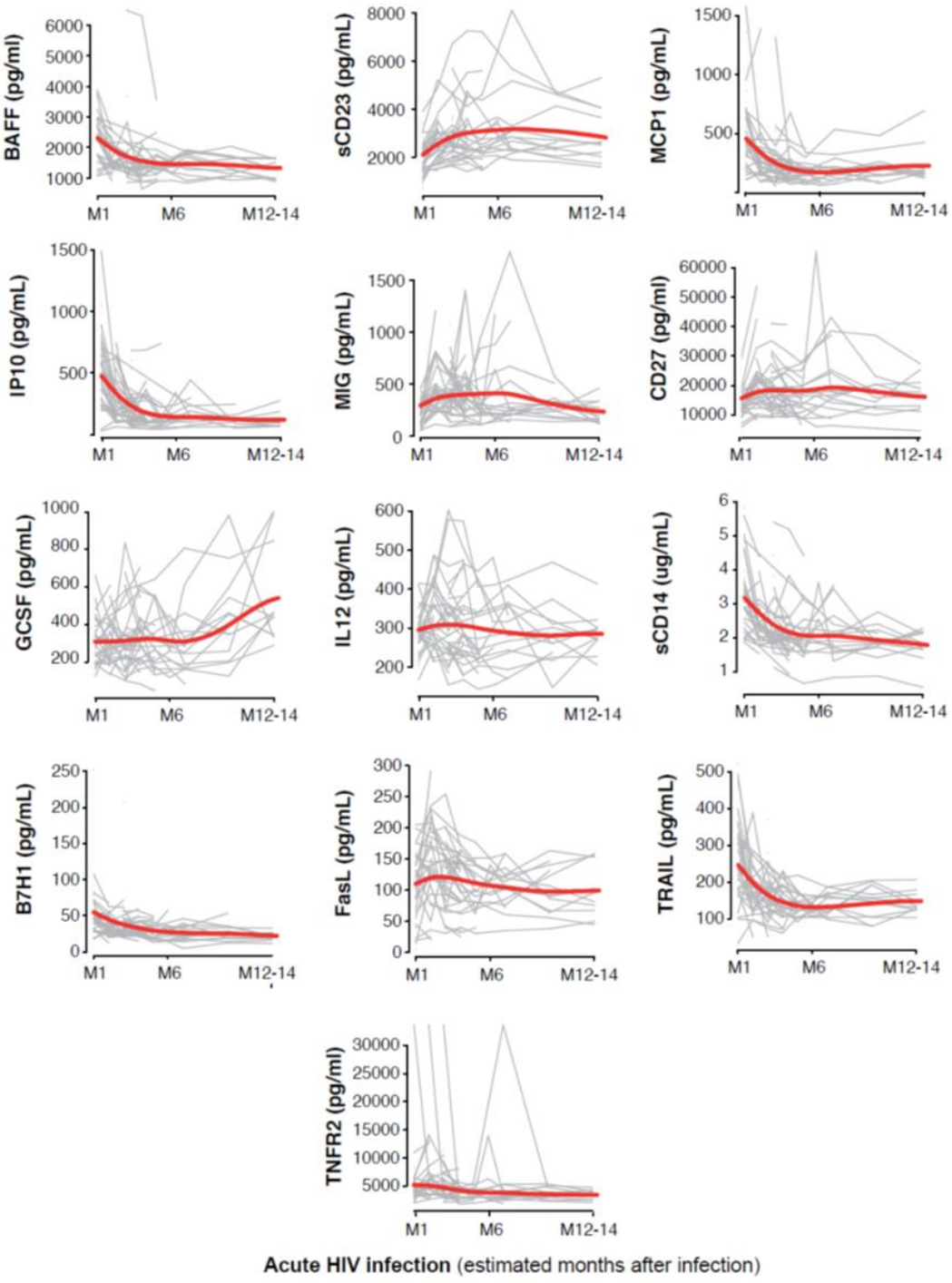

Figure S4. Cytokine dynamics along primary HIV infection: cytokine individual profiles.

Table S1. Summary of the polynomial models parameters.

		time		time^2		time^3		
	Intercept	**coef.**	**pval**	**coef.**	**pval**	**coef.**	**pval**	**LR test**
CD4cnt	562,38	-5,36	0,0349					0,0328
ln(CD8cnt)	7,42	-0,29	0,0019	0,04	0,0095	0,00	0,0231	0.0019
CD4..CD183+CD196+	10,37	-0,73	0,0116	0,04	0,0373			0,0127
CD4..Treg	7,16							
CD8..CD45RA+CD197+	11,21	1,73	0,0021	-0,11	0,0025			0,0089
CD8..CD45RA-CD197+	4,03							
CD8..CD45RA-CD197-	65,07	-3,42	0,0005	0,19	0,0042			0,0003
CD8..CD45RA+CD197-	21,95	0,66	0,0005					0,0005
ln(CD8..HLADR+CD38+)	3,94	-0,15	< 0.0001	0,01	0,0042			< 0.0001
ln(CD4..HLADR+CD38+)	2,08	-0,11	0,0044	0,01	0,0506			< 0.0001
CD8..CD279+	54,26	-2,25	0,0094	0,12	0,0402			0,0055
CD8..CD57+	42,13							
CD4..CD279+	37,97	-0,51	0,0001					0,0001
CD4..CD57+	10,48							
CD4..CD45RA+CD197+	36,08							
CD4..CD45RA-CD197+	34,75							
CD4..CD45RA-CD197-	26,57							
CD4..CD45RA+CD197-	2,75							

ARTICLE 3

P-10 Levels as an Accurate Screening Tool to Detect Acute HIV Infection in Resource-Limited Settings.

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IP-10 Levels as an Accurate Screening Tool to Detect Acute HIV Infection in Resource-Limited Settings

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Running title: IP-10 for acute-HIV-infection detection

Keywords: AIDS; resource-limited settings; acute-HIV-infection diagnosis; HIV-treatment as prevention; operational research; early ART-initiation; undifferentiated-fever; sub-Saharan Africa.

ABSTRACT

Acute HIV infection (AHI) is the period prior to seroconversion characterized by high viral replication, hyper-transmission potential and commonly, non-specific febrile illness. AHI detection requires HIV-RNA viral load (VL) determination, which has very limited access in low-income countries due to restrictive costs and implementation constraints. We sought to identify a biomarker that could enable AHI diagnosis in scarce-resource settings, and to evaluate the feasibility of its implementation. HIV-seronegative adults presenting at the Manhiça District Hospital, Mozambique, with reported-fever were tested for VL. Plasma levels of 49 inflammatory biomarkers from AHI (n=61) and non-HIV infected outpatients (n=65) were determined by Luminex and ELISA. IP-10 demonstrated the best predictive power for AHI detection (AUC=0.88 [95%CI 0.80-0.96]). A cut-off value of IP-10≥161.6pg/mL provided a sensitivity of 95.5% (95%CI 85.5-99.5) and a specificity of 76.5% (95%CI 62.5-87.2). The implementation of an IP-10 screening test could avert from 21 to 84 new infections and save from US$176,609 to US$533,467 to the health system per 1,000 tested patients. We conclude that IP-10 is an accurate biomarker to screen febrile HIV-seronegative individuals for subsequent AHI diagnosis with VL. Such an algorithm is a cost-effective strategy to prevent disease progression and a substantial number of further HIV infections.

INTRODUCTION

Acute HIV infection (AHI) is the period between the acquisition of human immunodeficiency virus (HIV) and the development of HIV-specific antibodies that define seroconversion[1]. AHI is characterised by high HIV viral replication and, in most cases, a transient non-specific febrile illness that typically occurs around 2 weeks after HIV acquisition[1,2]. As a result of high viraemia in bodily fluids and high levels of genital shedding of the virus, individuals are considered hyper-infectious during AHI[3,4]. In areas of high HIV incidence, this phenomenon could contribute greatly to fuelling the worldwide HIV pandemic[5].

Despite the importance of early diagnosis and treatment to reduce onward transmissions[6,7] and prevent substantial irreversible immunological damage in gut associated lymphoid tissue[8,9], AHI represents a 'window period' during which persons infected with HIV are commonly undiagnosed[1,10]. Routine second generation HIV-rapid test algorithms provide negative or indeterminate results for up to 6-8 weeks after infection[11]. During this time, HIV can only be diagnosed by detecting the presence of the virus itself[12]. The current gold-standard test for confirming viraemia is RT-PCR for plasma HIV-RNA[11]. However, technical and financial constraints make this technique very limited in low-income areas[1] such as Sub-Saharan Africa (SSA), where the prevalence of AHI among febrile patients may reach 3%[13–15].

As viraemia increases during AHI, there is a striking cascade response of inflammatory cytokines[2]. Significant efforts have been made to characterise host and viral proteins present during AHI aiming to identify biomarkers of progression or key pathological pathways that could be targeted to minimize HIV-induced immune damage over the course of infection[2,16–19]. In the present study we sought to determine whether a single or a combination of biomarkers could differentiate individuals in AHI from non-HIV infected individuals within a population of patients reporting febrile symptoms at a rural hospital in Southern Mozambique. Once plasma level of IP-10 was identified as candidate biomarker, we assessed the cost-effectiveness of implementing it for AHI diagnosis in low-income settings.

RESULTS

Characteristics of the study population

From the 2748 rapid-test seronegative patients who presented at Manhiça District Hospital (MDH) with reported fever during the screening period of the study, 61 (2.2%) were identified as AHI. Of the remaining HIV-RNA negative individuals, 65 (2.4%) were randomly selected as non-HIV-infected individuals with reported fever (NIF). As described in methods, of the 126 AHI and NIF controls, 95 individuals representing 75% of the study population were used for the model cohort. Median age of the model cohort was 23 years

(IQR 20-30) and 68% were female. Eleven individuals (12%) had malaria at the moment of screening. Median HIV-RNA level in the AHI group was Log_{10} 6.8copies/mL (IQR 6.1–7.4) and 70.4% patients showed a negative Western Blot for HIV-specific antibodies. There were no significant differences in age, gender or malaria status by study group (p>0.1).

Comparison of plasma inflammatory biomarker profile between AHI and non-HIV infected febrile controls

From a total of 49 quantifiable biomarkers, the median levels of 24 biomarkers were found to significantly differ between AHI and NIF individuals (p-value<0.05). When comparing normalized biomarker levels between AHI and NIF individuals, the AHI group had a higher fold change for all the 24 biomarkers except CRP, GCSF, MIP-1alpha, sCD23 and EGF (Figure 1a). IP-10 and CRP showed the most significant increase in the AHI and the NIF groups, respectively (p-value<0.05). The distribution of the normalized biomarker expression by study group revealed overlap of interquartile range (IQR) values between AHI and NIF groups for most biomarkers; the exceptions being IP-10, MCP-1 and TRAIL (Figure 1b). When assessing the correlation of the quantifiable biomarkers with the VL levels in the AHI individuals, IP-10 showed the strongest association (rho=0.6, p<0.0001).

Designing a biomarker-based predictive model for AHI detection

Univariate analysis showed that 29 of the 49 biomarkers were associated with AHI (p-value<0.2) and were included in the multivariate analysis. A total of 6 cytokines with a p-value<0.05 were retained in the multivariate model (Table 1). IP-10, sCD14 and GMCSF were positively associated with AHI whereas CRP, MIP-1alpha and IL-10 were negatively associated; among these, IP-10 showed the strongest association with AHI (p-value=0.0008).

Of the 29 biomarkers associated with AHI in univariate models, IP-10, TRAIL, BAFF and IL-12 cytokines showed the highest predictive power (Area under the curve (AUC)>0.8) (Figure 2a), where IP-10 demonstrated best accuracy as a single biomarker (AUC=0.88 [95%CI 0.80-0.96]). Multivariate model adjusted by age, sex and malaria infection of the above-mentioned 6 cytokines further increased the classification performance (AUC=0.98 [95%CI 0.96-1.00]) (Figure 2b). Thus, IP-10 alone and the multivariate 6-cytokine model were identified as the best classification methods for AHI detection.

Receiver-operating characteristic (ROC) curves of the selected models were used to evaluate several cut-off values prioritizing the highest sensitivity (Figure 2c and 2d). A cut-off of ≥161.6pg/mL for the univariate IP-10 model provided a sensitivity of 95.5% (95%CI 85.5-99.5) and a specificity of 76.5% (95%CI 62.5-87.2) (Figure 2c) for predicting AHI. A cut-off score of ≥-0.89 for the multivariate model provided a sensitivity of 97.7% (95%CI 88.0-99.9) and a specificity of 82% (95%CI 69.1-91.6) (Figure 2d). Classification performance between the univariate and multivariate methods showed a substantial level of agreement for AHI detection with kappa-statistic of 0.66 (95%CI 0.51-0.81).

Table 1. Adjusted multivariate logistic regression of biomarkers associated with AHI. Coefficients per log_{10} cytokine unit increment, 95% confidence interval and p-value of the cytokines and confounders that entered into the model ($p<0.05$) as described in methods.

Variable		Coef.	(95% Conf. Interval)	p-value
Malaria status		3.21	(-0.81; 7.23)	0.1172
Sex	F	ref		0.7007
	M	0.40	(-1.66; 2.47)	
Age		-0.03	(-0.12; 0.06)	0.5253
LogIP10 (pg/mL)		5.68	(2.34; 9.01)	**0.0008**
LogCRP (ug/mL)		-1.99	(-3.68; -0.30)	**0.0209**
LogsCD14 (ug/mL)		14.20	(3.34; 25.07)	**0.0104**
LogGMCSF (pg/mL)		6.01	(1.44; 10.59)	**0.0100**
LogMIP1A (pg/mL)		-4.59	(-9.12; -0.06)	**0.0469**
LogIL10 (pg/mL)		-5.09	(-9.73; -0.45)	**0.0315**
Intercept		*-5.54*	*(-13.53; 2.45)*	*0.1743*

Performance and validation of IP-10 as a single biomarker for AHI identification

Given the greater simplicity of implementing a single cytokine assay, the univariate IP-10 model with a cut-off of ≥161.6pg/mL was assessed for the AHI prevalence observed in the study cohort. We calculated positive and negative predictive values (PPV and NPV) using model sensitivity and specificity and their respective 95%CIs limits (Figure 3). For an AHI prevalence of 2.2% in HIV-seronegative patients reporting fever, at a specificity of 76.5%, the PPV was 8.38% (Figure 3a), and at a sensitivity of 95.5%, the NPV was 99.87% (Figure 3b).

Using this IP-10 predictive model, 88% of the 17 AHI individuals and 86% of the 14 NIF controls in the validation sample set were correctly classified. These values fell within the 95%CI predicted for the sensitivity and specificity of IP-10, thus validating the model prediction power.

Cost-effectiveness analysis of implementing an IP-10 rapid test to screen AHI cases in low-income settings

Projecting the observed AHI prevalence of 2.2% in a simulated cohort of 1,000 seronegative febrile individuals, 22 individuals would be expected to have AHI. Assuming a high and low

transmission rate of 1:4 and 1:1, we would expect the 22 AHI to give rise to 88 or to 22 new infections, respectively. In a high transmission scenario, lifetime costs of the 110 HIV infections would total US$623,085 (Figure 4a). In comparison, at a low transmission scenario, the mean cost for management of 44 HIV infections would be US$249,234.

The introduction of an IP-10 AHI pre-screening test would include additional costs for IP-10 tests and confirmatory VL determinations. With a sensitivity of 95.5%, IP-10 would identify 21 of the 22 AHI with the assumption that they would receive ART and thus not transmit the infection. However, 1 AHI would not be identified (false negative) and would generate 4 or 1 new infections depending on the transmission rate. For a high transmission scenario, the mean cost to the health system for a total of 26 HIV infected individuals would be US$89,618 (Figure 4b) whereas for a low transmission scenario, the mean cost for 23 HIV infected individuals would be US$72,625. Thus, the cost savings are estimated to be US$6,351-8,410 per infection averted. Therefore, we estimate that the introduction of an IP-10 pre-screening test would save between US$176,609 and $533,467 and avert 21-84 new HIV infections per 1000 febrile seronegative outpatients.

DISCUSSION

We have demonstrated that among 49 inflammatory biomarkers assessed, the IP-10 cytokine has the highest accuracy in identifying individuals with AHI in a cohort of febrile HIV-seronegative individuals. A cut-off value for an IP-10 level of 161.6pg/mL gave a sensitivity of 95.5% (95%CI 85.5-99.5) and a specificity of 76.5% (95%CI 62.5-87.2) in identifying AHI, which was confirmed in the validation sample set. The combination of IP-10 with other biomarkers showed a slightly higher prediction power for AHI detection but a substantial agreement was observed for classification performance between IP-10 and the multi-biomarker models. This suggests that IP-10 alone may provide a simple and precise way to screen febrile seronegative individuals for subsequent AHI diagnosis.

IP-10, also known as CXCL10, is a small cytokine belonging to the CXC chemokine family. IP-10 is produced as part of the innate immune response to viruses, bacteria, fungi and parasites[20]. At the initial stages of HIV infection, IP-10 has been shown to greatly increase prior to the development of clinical symptoms paralleling HIV-VL[19]. Previous data suggested that IP-10 levels were associated with immune activation[21] and predictive of rapid disease progression[18,22], representing an earlier biomarker than CD4 T-cell counts or viraemia levels[18]. However, to our knowledge, the strong association between IP-10 and VL levels has not been assessed for its ability to identify AHI among seronegative individuals. Our results show that IP-10 can indeed be used to screen HIV-seronegative individuals with high predictive ability to differentiate AHI from other patients with undifferentiated fever.

IP-10 has been explored for its use as a prognostic or diagnostic marker for other infectious diseases such as malaria, where plasma IP-10 level is associated with fatal cerebral

malaria[20]; hepatitis C, where plasma IP-10 has the power to predict liver damage[23], or tuberculosis, where IP-10 levels differentiate between active and latent tuberculosis irrespective of HIV infection[24]. Interestingly, high IP-10 levels during HIV infection have been associated with increased susceptibility to malaria infection[25], however this was not observed in our cohort, and our results have not shown an interference of malaria in AHI identification.

Different infections may be distinguished by distinct IP-10 thresholds, but additional research is necessary to explore the impact of malaria, tuberculosis and hepatitis C, common HIV comorbidities, on AHI-induced levels of IP-10. Parameters such as sensitivity, specificity and cut-off value also need to be optimized expanding to other populations affected by different HIV-subtypes or other prevalent co-infections, as well as to non-febrile seronegative individuals and pregnant women. Thus, although the IP-10 cut-off value of 161.6pg/mL provided the highest sensitivity in our cohort, it may differ according to context. Importantly, if IP-10 accuracy for AHI detection is validated in non-febrile seronegative individuals, this biomarker could also be valuable for screening blood donors in low-income countries.

IP-10 is not a disease-specific biomarker[20], and its potential use to identify AHI would require a second step of confirmation through point of care (POC) VL detection or other HIV-specific detection method. Here we suggest the use of IP-10, potentially developed as rapid test, as a pre-screening tool to discriminate AHI from other febrile seronegative outpatients in settings of high HIV incidence followed by a POC-VL. IP-10 showed a specificity of 76.5%, which would not allow IP-10 to be used as a standalone diagnostic test but represents a powerful screening tool to exclude non-HIV infected individuals. Furthermore, at estimated AHI prevalences below 5%, the NPV for the IP-10 remains above 99%, thus ensuring that a low number of AHI cases would go undiagnosed. In low-income countries, where the cost of individual as well as pooled HIV-VL determinations or similar antigen-based assays is prohibitive and the logistics for VL implementation remain highly complex, this powerful tool would make AHI screening affordable and feasible, allowing early ART in these individuals and preventing a substantial numbers of new HIV infections.

Current practices in Mozambique and similar SSA settings use HIV rapid test serology, which detects infection from 6 to 8 weeks post-transmission onward, and AHI screening is not conducted. The implementation of an IP-10 test to pre-screen febrile seronegative patients could save from 176,609 to 533,467 US$ in treatment and diagnostic costs to the health system per 1,000 patients tested, while averting up to 84 new HIV infections. Hence, despite the necessity of confirming positive diagnosis with a POC-VL or similar, the introduction of a potential IP-10 rapid test as a pre-screening tool would be an important cost-saving strategy compared to leaving AHI undiagnosed. We used a public negotiated cost of $16.80 as a POC-VL instead of employing a pooling or individual VL strategy because same day return of results has been shown to accelerate ART initiation[26]. However, for

current laboratory-based HIV-VL there are major pricing discrepancies across countries[27,28]. Recently, MSF reported that comprehensive costs including human resources, sample collection, reagents and consumables ranged from US$24.90 to 43.42 in the five Sub-Saharan Africa countries surveyed in 2013 (Kenya, Lesotho, Malawi, Swaziland, and Zimbabwe)[29]. We assumed a best case scenario but using these values would increase the cost savings. We assumed a cost of an IP-10 rapid test of $1.50 based on commercially available costs for CRP rapid test employed in the management of undifferentiated fever[30]. Both CRP and IP-10 are inflammatory proteins with similar costs for detection by ELISA assays purchased at market price. However, the cost-effectiveness model was neither probabilistic nor dynamic. Importantly, we disregarded the cost of HIV-associated comorbidities in the simulated cohort which, if included, would most likely increase the cost-effectiveness of the IP-10 followed by POC-VL intervention.

Finally, as it has been amply demonstrated, early ART stops progression to AIDS[31,32], diminishes the viral reservoir[33] and early immunological damage[9], as well as reducing further transmissions[7]. Since the World Health Organization updated the HIV recommendations in 2015[34], many SSA countries are rolling out "test and start" programs to initiate ART regardless of CD4 counts. This implies expansion of serological HIV testing strategies but also places AHI detection in the spotlight. Although the development of POC-VL systems has rapidly advanced, implementation costs remain elevated. Confronted with the sheer volume of patients requiring VL determinations for ART monitoring in many SSA countries, health services will not be able to implement individual or pooled VL for AHI diagnosis among febrile seronegative outpatients in the near future[35]. A new approach for AHI detection using an affordable IP-10 pre-screening tool to reduce the number of VL determinations necessary by 75%, may be an opportunity to bring AHI diagnosis to low-resource high HIV-burden settings.

In conclusion, we demonstrated that a screening based on IP-10 levels is an accurate and cost-effective strategy to detect AHI in HIV-seronegative patients with undifferentiated fever. This algorithm renders AHI diagnosis feasible in low-income settings and by treating these individuals a substantial number of progressions to AIDS and further HIV transmissions could be averted.

METHODS

Study population

The study population was enrolled between 2013 and 2014 at the MDH in Mozambique. The present analysis is a sub-study of a prospective cohort of primary HIV-infected adults enrolled[15] and followed up for 12 months in the Gastro-intestinal biomarkers in AHI Mozambican Adults study (GAMA). This study was approved by local institutional review boards at Barcelona Clinic Hospital (2011/6264) and by the Ministry of Health of

Mozambique (461/CNBS/12). All methods were carried out in accordance with the relevant guidelines and regulations. Written informed consent was obtained from patients prior to participation.

HIV diagnosis and biomarker quantification

Technical information and procedures regarding HIV diagnosis, clinical follow up, HIV-specific antibody determination and biomarker quantification have been previously described[15]. Individuals presenting to the outpatient clinic of MDH with reported fever, flu-like symptoms or malaria suspicion were included in the AHI group of this sub-study if they were HIV-rapid test seronegative and HIV-RNA positive. A control population was established by random selection among NIF and their screening samples were employed to compare cytokine expression levels with the AHI group. At an additional visit, non-febrile HIV-uninfected controls (n=58) provided samples that were used to establish biomarker reference levels in the absence of fever illness[15]. HIV-specific serology was performed by Western Blot. Plasma levels of non-specific antibody isotypes were quantified by an in house ELISA. Multiplex cytokine profiling was performed in plasma samples with particular interest in inflammatory cytokines, chemokines, hematopoietic factors and biomarkers of intestinal damage. Determinations were performed by ELISA commercial assays or Luminex[15].

Statistical analysis

Of the 126 individuals included in this cohort, 75% were used for the data analysis and training set (n=95) and 25% for model validation (n=31). Proportions were compared using chi-square test. Biomarker values were log transformed for a better adjustment of skewed data. In order to normalize biomarker levels, fold change was calculated as the ratio of the biomarker level in individuals reporting fever over the median value of the non-febrile HIV-uninfected reference group. Distributions of biomarkers in AHI and NIF groups were compared by Mann Whitney U-test. Spearman's correlation was used to assess the strength of relationship between continuous variables.

In order to assess the predictive ability of differentially expressed biomarkers to discriminate between AHI and NIF individuals, logistic regression with penalized likelihood was performed[36]. A multivariate model was constructed using backward step-wise elimination with an inclusion criterion of p-value<0.2 in univariate analysis. Age, gender and malaria infection were retained as potential confounders in the multivariate model because of their previous association with alterations in the cytokine expression levels[37–39]. ROC curves from univariate and adjusted-multivariate models were compared for the best prediction[40]. The level of agreement between the classification methods was assessed by the κ-statistic[41].Data were analysed using R-3.2.4 software (R Core Team 2016) and Stata Statistical Software: Release 14 (StataCorp 2015. College Station, TX).

Cost-effectiveness analysis

A deterministic decision analysis was used to estimate the cost-effectiveness of introducing a potential IP-10 rapid test to screen febrile HIV-seronegative patients attending a health facility compared to current practices in Mozambique, which at present include only HIV antibody-based rapid testing, and thus no AHI identification. Health effects and costs to the health care provider associated with HIV/AIDS were estimated for a simulated cohort of 1,000 febrile seronegative patients 20 years of age. Economic model outcomes were determined using the AHI prevalence observed in this study and the sensitivity and specificity of IP-10 obtained in the univariate model. Health effects were expressed as the number of averted infections. Costs per person-year to the health system included: (1) first-line ART drugs and their delivery (assuming complete linkage-to-care); (2) laboratory tests and patient surveillance; and (3) treatment of opportunistic infections including tuberculosis prophylaxis[42]. A unit cost of US$1.50 for a hypothetical IP-10 rapid test was based on costs for the CRP rapid test, similarly employed to assist diagnosis among patients with undifferentiated fever[30]. A cartridge-based nucleic acid amplification test (GeneXpert; Cepheid, Sunnyvale, CA, USA) was included as a POC-VL confirmation assay with a unit cost of US$16.80, according to FIND negotiated prices for eligible countries[43]. Costs per HIV-infected individual were different between early (CD4 T-cells count>200) and late presenters (CD4 T-cells count=<200) and both were multiplied by their estimated life expectancy to model life-long treatment costs[44]. Costs were expressed in US$, inflation-adjusted (3%) and discounted (3%). Costs and effects were estimated for two different HIV transmission scenarios[45]. This economic analysis represents a static model where transmission only occurs from our cohort of 1,000 individuals to a number (n) of other individuals, where n can assume the values of 1 or 4 (low and high transmission scenarios, respectively).

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

DN, JB and LP conceptualization and study design. LP, EP and LF recruited subjects, collected and validated clinical data. LP, CJ, EP and LF performed laboratory analysis and validation of the immunological data at the field. LP and JC performed biomarker quantification at the laboratory and validation of the data. LP and AC performed statistical analyses. SA performed the economic analysis. LP, AC, SA, JB and DN interpreted the data. DN, JB, IM, LP, EP and LF study management and coordination. LP drafted the paper. DN and JB critical data review and revision of manuscript writing. All authors read and approved the final version of the manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests in relation to the submitted work.

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FIGURES

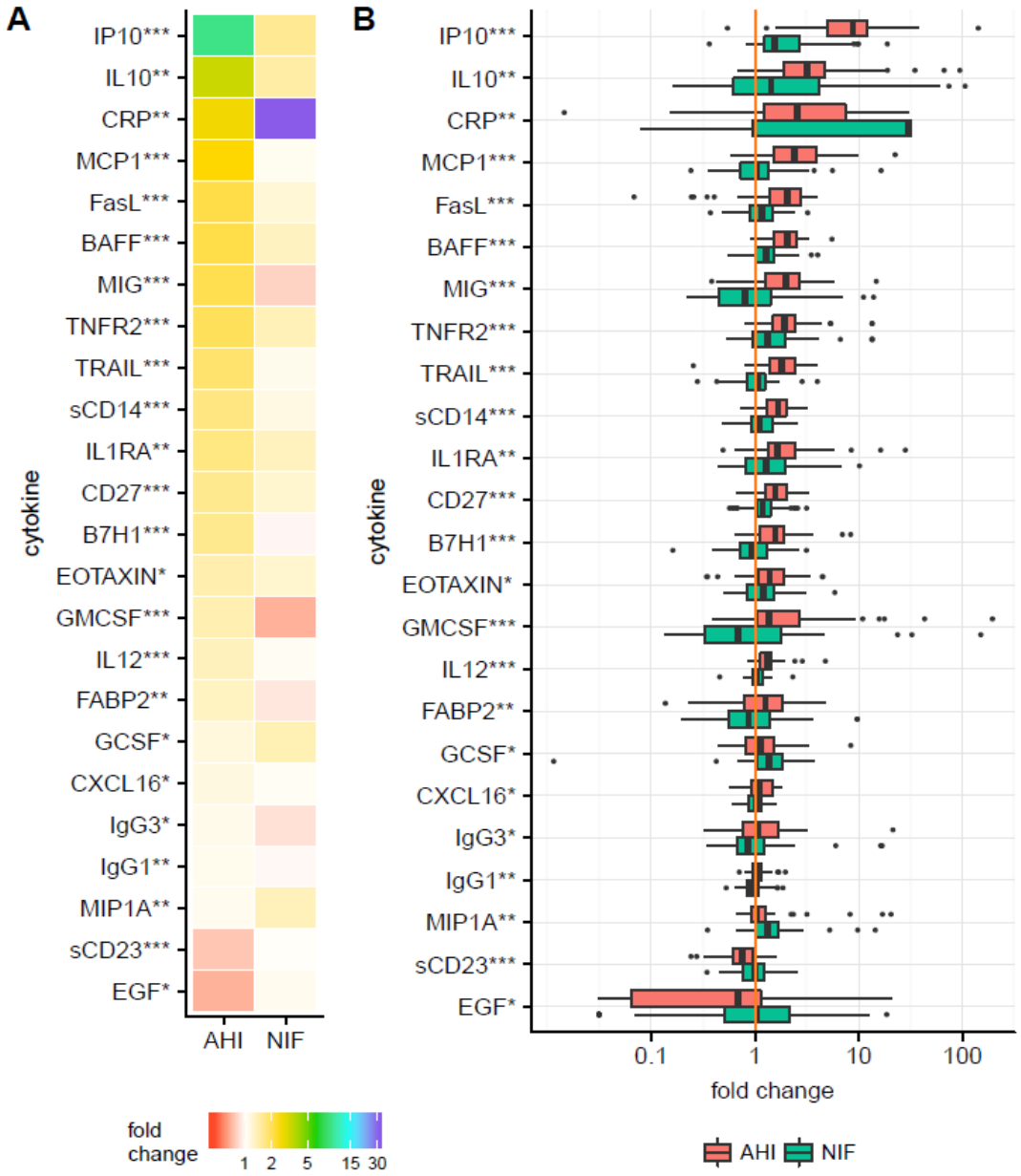
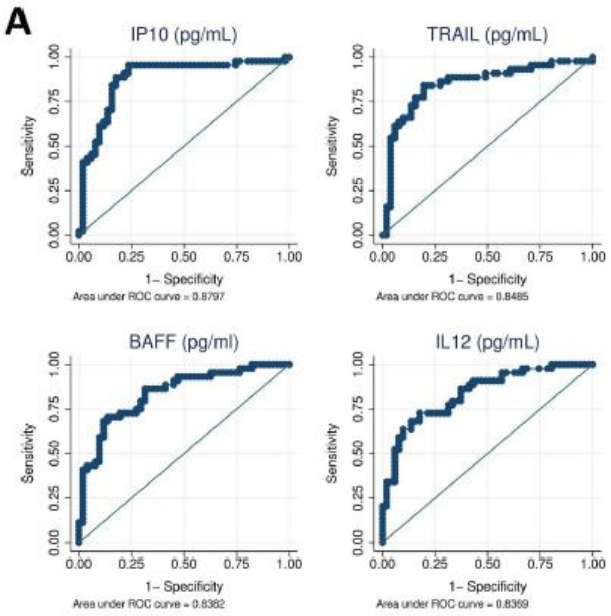
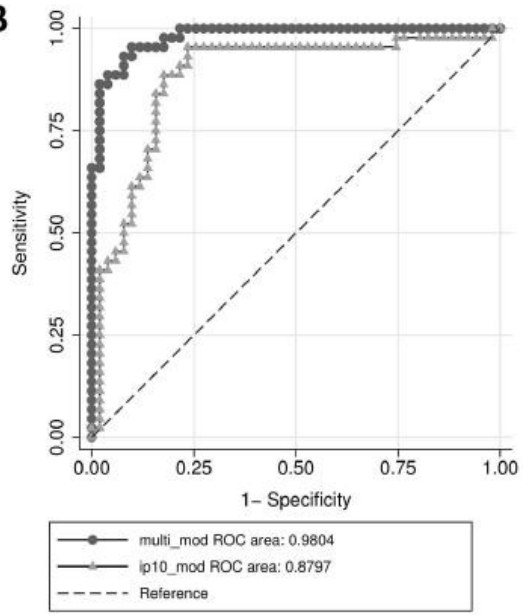


Figure 1. Normalized biomarker levels in AHI and NIF controls. a. The levels of 24 plasma biomarkers are expressed as fold change compared to a reference level (defined in methods) for both AHI and NIF groups. Intensity of colour represents biomarker fold change and biomarkers are sorted by fold change value in the AHI group. Non-parametric significance of biomarker expression by study group is indicated as *** if P < 0.001, ** if P < 0.01, and * if P < 0.05. b. Distribution of the normalized biomarker levels by study group. Results are expressed as fold change. Box as IQR, middle line as median, whiskers as Tukey values (1.5*IQR). The orange line corresponds to the null change compared to the reference level.





C IP-10 univariate model cut-off values

Cut-off value	Sensitivity (%)	Specificity (%)
≥ 238.4	84.1	84.3
≥ 229.7	86.4	82.4
≥ 227.9	88.6	82.4
≥ 212.5	90.9	78.4
≥ 192.2	93.2	76.5
≥ 161.6	95.5	76.5

D Multivariate model score cut-off values

Cut-off value	Sensitivity (%)	Specificity (%)
≥ 0.12	90.9	92.2
≥ 0.09	93.2	92.2
≥ -0.32	95.5	90.2
≥ -0.89	97.7	82.4
≥ -1.27	100.0	78.4

Figure 2. Performance of univariate and multivariate cytokine models in predicting acute HIV infection. a. ROC curves for individual biomarkers with best AHI predictive accuracy (AUC>0.8). b. Comparison between ROC curves for univariate IP-10 model (AUC=0.88) and adjusted multivariate biomarker model (AUC=0.98). (C) IP-10 univariate model cut-off points (pg/mL) and (D) multivariate model score cut points with their respective sensitivity and specificity values.

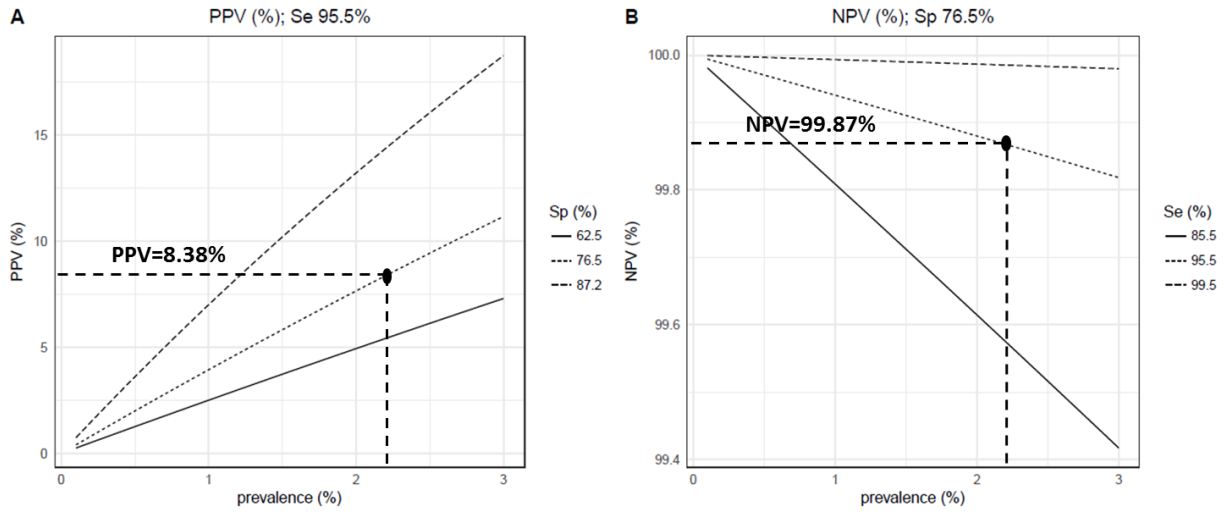


Figure 3. AHI Predictive power of the IP-10 model according to prevalence of AHI. a. Positive predictive value (PPV) for varying AHI prevalence estimated for sensitivity=95.5% and 3 different specificity scenarios according to the estimated confidence interval (Sp=76.5% [95%CI 62.5-87.2]). b. Negative predictive value (NPV) for varying AHI prevalence as estimated for specificity=76.5% and 3 different sensitivity scenarios according to the estimated confidence interval (Se=95.5% [95%CI 85.5-99.5]).

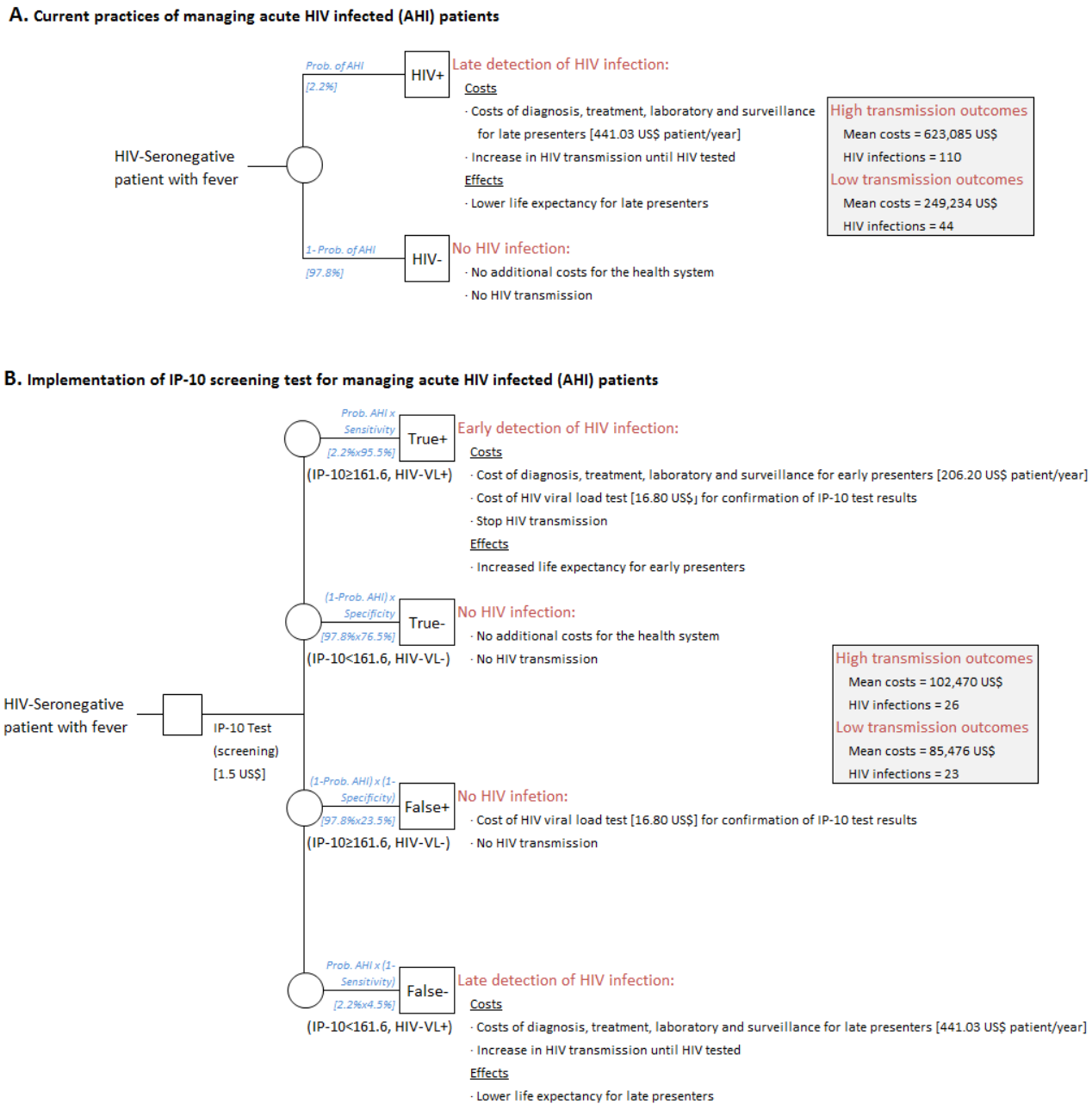


Figure 4. Graphic modelling of the cost-effectiveness analysis. Cost comparison between current practices which do not identify AHI (1a) and the implementation of a potential IP-10 rapid pre-screening test (1b) in seronegative febrile outpatients for AHI detection in a Sub-Saharan setting.

ARTICLE 4

IP-10 as a Screening Tool to Optimize HIV Viral Load Monitoring in Resource-limited Settings.

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IP-10 as a Screening Tool to Optimize HIV Viral Load Monitoring in Resource-limited Settings

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Running tittle: IP-10 as a surrogate of ART failure

Keywords: global health; cytokines; implementation research; scale up VL; sub-Saharan Africa.

Key points: Interferon-ɣ-inducible protein-10 (IP-10) is an affordable and easily-quantifiable biomarker that can be used to accurately screen individuals on anti-retroviral treatment (ART) for detectable viremia, optimizing the use of costly viral load (VL) determinations required to monitor ART in low-income countries.

ABSTRACT

Background: Achieving effective anti-retroviral treatment (ART) monitoring is a key determinant to ensure viral suppression and reach the UNAIDS 90-90-90 targets. The gold standard for detecting virological failure is plasma HIV-RNA viral load (VL) testing; however its availability is very limited in low-income countries due to cost and operational constraints.

Methods: HIV-1-infected adults on first-line ART attending routine visits at the Manhiça District Hospital, Mozambique, were previously evaluated for virological failure. Plasma levels of interferon-□-inducible protein-10 (IP-10) were quantified by ELISA. Logistic regression was used to build an IP-10-based model able to identify individuals with VL>150 copies/mL. From the 316 individuals analyzed, 253 (80%) were used for model training and 63 (20%) for validation. Receiver operating curves were employed to evaluate model prediction.

Results: From the individuals included in the training-set, 34% had detectable VL. Mean age was 41 years, 70% were females and median time on ART was 3.4 years. IP-10 levels were significantly higher in subjects with detectable VL (108.2 pg/mL) as compared to those with undetectable VL (38.0 pg/mL) (U-test p<0.0001). IP-10 univariate model demonstrated high

classification performance (AUC=0.85 [95% Confidence Interval (CI) 0.80-0.90]). Using a cut-off value of IP-10≥44.2 pg/mL, model identified detectable VL with 91.9% sensitivity (95% CI 83.9-96.7) and 59.9% specificity (95% CI 52.0-67.4), values confirmed in the validation set.

Conclusions: IP-10 is an accurate biomarker to screen individuals on ART for detectable viremia. Further studies should evaluate the benefits of IP-10 as a triage approach to monitor ART in resource-limited settings.

INTRODUCTION

In 2014, Joint United Nations Programme on HIV/AIDS (UNAIDS) announced bold new targets for the global response to HIV, commonly known as the 90-90-90 strategy, consisting of 90% of people living with HIV aware of their status, 90% of people HIV-diagnosed on treatment and 90% of people on treatment attaining virological suppression, by 2020 [1]. Although considerable international efforts have resulted in a dramatic increase in antiretroviral therapy (ART) coverage in the last years, relatively little progress has been achieved in the development of simple, accurate and affordable tools that allow proper surveillance of ART efficacy [2,3]. In 2013, World Health Organization (WHO) recommended routine HIV-RNA viral load (VL) testing at 6 months after ART initiation and every 12 months as the preferred monitoring approach to supervise treatment adherence and minimize failure [4]. According to last WHO guidelines, virological failure (VF) is defined by a persistently detectable VL exceeding 1000 copies/mL after at least six months of starting a new ART regimen [5]. VL monitoring is important for timely diagnosis of VF to allow early adherence interventions, prevent further transmissions and avoid delays in regimen switches that could lead to disease progression or emergence of drug resistances [6]. However, despite the international recommendations, VL testing is still not widely available in many low and middle-income countries (LMIC) due to high cost and implementation constraints [3,7]. The alternative to VL has often been clinical and/or immunological monitoring, which frequently results in patients remaining on failing ART as well as unnecessary regimen switches [8,9].

Expression of several inflammatory and immune response cytokines is increased during HIV replication [10]. Previous studies have shown that plasma levels of interferon-□-inducible protein-10 (IP-10) correlated with VL [11,12] and VL set-point [13] during untreated primary HIV infection and decline after ART initiation both in early [14] and chronically HIV-infected individuals [15]. Similarly, we have seen that from a total of 42 inflammatory biomarkers, IP-10 shows the strongest association with VL and the best predictive power to identify acute HIV infection among febrile seronegative patients (L Pastor, A Casellas, J Carrillo et al. manuscript in preparation). We hypothesized that because of its strong association with VL, plasma IP-10 levels could be a surrogate marker of detectable viremia in ART-treated individuals, providing a simple and affordable screening tool to detect individuals with VF in LMIC.

METHODS

Study population

The present analysis is a sub-study of a cross-sectional cohort for detecting drug resistances in ART-treated adults enrolled between February and March 2013 at the Manhiça District Hospital (MDH), Maputo, Southern Mozambique [16]. At the time of the study, current HIV

national guidelines recommended ART initiation in patients with a CD4 T-cell count ≤350 cells/mm^3 and no routine VL monitoring was provided after ART initiation. The study protocol was approved by the institutional review boards and ethics committees of the Barcelona Clinic Hospital, Badalona Germans Trias i Pujol Hospital (Spain) and the National Committee on Health Bioethics, Mozambique. All study participants provided signed informed consent.

Briefly, adults over 18 years of age attending routine scheduled outpatient visits for clinical management of HIV/AIDS at the MDH were enrolled in the study. All patients had documented HIV infection, documented ART initiation ≥12 months earlier and provided a signed informed consent. The sample and data collection procedures have been previously described [16] and include a single blood sample and socio-demographic and clinical data collected in a specific questionnaire.

Laboratory procedures

HIV-RNA levels were determined in plasma samples by RT-PCR (Abbott m2000 RealTime System with a detection limit of 150 copies/mL) and CD4+ T cell counts were determined in whole blood by flow-cytometry using FACSCalibur (BD Biosciences) as previously described [16]. IP-10 level was measured in plasma samples by enzyme-linked immunosorbent assay (Human Duo-Set ELISA, R&D Systems, Minneapolis, USA) according to manufacturer's instructions. 0.05% Tween®20 (Sigma Aldrich, Saint Louis, USA) 1% BSA (Sigma Aldrich, Saint Louis, USA) in PBS was used as blocking solution, TMB (Sigma Aldrich, Missouri, USA) as substrate and 4N H_2SO_4 as stop solution. Optical density was measured at 492 and 620nm. Values assigned to data falling outside quantification limits were the double and the half of the upper and lower quantification limits, respectively.

Statistical analysis

Data were double entered using Fox Pro version 2.6 (Microsoft Corporation, Redmond, WA, USA) and analyzed using R-3.2.2 software and STATA version 14 (Stata Corporation, College Station, TX, USA).

Proportions and continuous variables were compared using chi-square and non-parametric U-Mann Whitney test, respectively. IP-10 values were log transformed for a better adjustment of skewed data. Spearman's test was used to assess correlation coefficients for continuous variables.

In order to assess the capacity of IP-10 levels and clinical variables to correctly identify the cases, logistic regression with penalized likelihood was performed [17]. According to random selection of the 316 individuals included in this analysis, 80% were used for data analysis and model construction (n=253) and 20% for model validation (n=63). A multivariate logistic regression model was built applying a stepwise selection to the set of variables: IP-10, gender, age, CD4 T-cell count, body mass index (BMI), days on ART and presence of symptoms. In the selection, variables with p-values <0.05 could enter into the

model while required a p-value<0.10 to be retained. Outcomes tested were detectable VL defined as VL>150 copies/mL and VF defined as VL>1000copies/mL. Diagnostic capacity was determined using receiver-operating characteristic (ROC) analyses. ROC curves from univariate and multivariate models were compared for the best prediction.

RESULTS

Population characteristics

Of the 332 individuals included in the cross-sectional analysis for drug resistances [16], IP-10 was determined in 316 (95.2%). We thus trained our model on 253 (80%) of the individuals with available IP-10 data. The mean age of the 253 individuals included in the training set was 41 years (SD 10 years) and 70% were females. Median time on ART was 3.4 years (IQR 2.1–5.3 years) and 89% were receiving zidovudine/lamivudine/nevirapine at the time of the survey. Mean BMI was 23.2 kg/m^2 (SD 3.9 kg/m^2), median CD4 T-cell count was 439 cells/mm^3 (IQR 273-593 cells/mm^3) and 38% presented any type of symptoms at the time of the survey. Thirty-four percent had detectable VL (86/253) and 25% (64/253) met criteria for the standard definition of VF (VL>1000copies/mL) [4]. In contrast, when clinical and immunological criteria were used, only 12% (29/245) of subjects were suspected to have ART failure. Population characteristics did not significantly differ from those of individuals included in the cross-sectional analysis of resistance [16] nor of those included in the validation set (P>0.1).

Evaluation of an IP-10-based model to identify detectable viremia

Median IP-10 levels were significantly higher among individuals with detectable VL compared to those with undetectable VL (108.2pg/mL vs. 38.0pg/mL respectively; U-test P<0.0001; Figure 1). IP-10 levels did not significantly differ from those of individuals included in the validation set (P>0.1). Among those individuals with detectable VL, IP-10 levels were significantly correlated with VL (rho=0.33, p=0.002).

Univariate analysis showed that IP-10 was significantly and positively associated with detectable VL (OR=1.47 per 10% IP-10 pg/mL increase; P<0.0001). ROC curve demonstrated high predictive power for classification of individuals with detectable VL with an area under the curve (AUC) =0.85 [95% confidence interval (CI) 0.80, 0.90]. Gender, age, BMI, T-CD4 cells count, days on ART and presence of any symptoms at the visit day were considered for inclusion in a multivariate analysis together with IP-10. However, only IP-10 and CD4 T-cell count were retained in the model and the resulting multivariate model did not increase the classification performance (AUC=0.85 [95%CI 0.80, 0.90]; Figure 2).

Then, ROC curve for the univariate IP-10 model was used to evaluate several cut-off values prioritizing the highest sensitivity. A cut-off of IP-10≥44.2pg/mL was selected, providing a

sensitivity of 91.9% (95%CI 83.9, 96.7) and a specificity of 59.9% (95%CI 52.0, 67.4) for predicting detectable viremia (Figure 2B).

The IP-10 model with a cut-off of ≥44.2pg/mL was assessed for predictive accuracy to identify individuals with detectable VL. We calculated positive and negative predictive values (PPV and NPV respectively) using model sensitivity and specificity and their respective 95% CIs limits (Figure 3). Applying the prevalence of detectable VL of 36% observed in the cross-sectional resistance study [16] and the IP10 model sensitivity and specificity, the PPV would be 56.3% (Figure 3A) and the NPV would be 92.9% (Figure 3B).

Validation of the model accuracy

When we applied the IP-10 model to the validation panel of samples, 80% of the 20 individuals with detectable VL and 58% of the 43 individuals with undetectable VL were correctly classified. The sensitivity and specificity derived from the validation panel were not statistically different from those obtained with the training set (equality of proportions test p-values 0.115 and 0.953, respectively), thus confirming the predictive power of the univariate IP-10 model for identification of ART-treated individuals with detectable VL.

The prevalence of standard VF (VL>1000copies/mL) observed in the study population was 25% (64/253). Both univariate and multivariate models were also tested for their ability to detect individuals with standard VF, showing lower specificity for a given sensitivity than the model designed to identify individuals with detectable VL (VL>150copies/mL; data not shown).

Comparison of plasma IP-10 vs. HIV-RNA quantification to predict detectable VL

We then combined the training and validation panels to estimate the number of VL assays required for ART monitoring in this population using either the IP-10-based algorithm or standard VL testing (Table 1). Whereas using VL monitoring alone would require 316 VL determinations to detect 106 individuals harboring detectable VL, the use of an IP-10 screening test followed by VL confirmation would only require 180 VL determinations. The IP-10 screening test would thus require 43% fewer VL determinations and identify 89.6% of those with detectable VL in this sample population.

An analysis from the Global Fund's Price and Quality Reporting Tool found that VL reagent costs alone varied from $13.13 to $43.34 between countries [3,18]. Considering US$28 as the average reagent cost for HIV-VL testing, translated to dollar values, the VL testing of these 316 treated individuals would imply around US$8850 per year to the health system. On the other hand, considering a unit cost of US$1.50 based on costs for other similar cytokines [19], the IP-10 screening together with the 180 VL determinations required in this cohort, would imply a cost of around US$5510 per year. This means that introducing an IP-10-based screening for ART monitoring would save US$3340 a year for this cross-sectional study cohort, resulting in a saving of 38% in VL-associated cost.

DISCUSSION

We have demonstrated that a cut-off value for IP-10≥44.2pg/mL gave a sensitivity of 91.9% (95%CI 83.9, 96.7) and a specificity of 59.9% (95%CI 52.0, 67.4) for predicting detectable viremia in individuals on ART for more than a year. Thus, we have shown that IP-10 is simple biomarker that can be used to screen individuals on ART for VF, reducing the number of costly VL determinations required to monitor ART in LMIC.

The most recent WHO guidelines recommend routine tracking of ART effectiveness using VL testing at 6 and 12 months after treatment initiation and every 12 months in stable patients in order to minimize treatment failure [5]. However, the high cost and technical complexity of VL testing has hampered scale-up in resource-limited settings. In absence of routine VL testing, the use of clinical and CD4 monitoring in many Sub-Saharan African countries has been shown to favor the emergence of VF and drug resistances [8,16]. In Mozambique, recent cross-sectional surveys reported that 23% [7] and 36% [16]of individuals on ART, in Maputo and Manhiça District respectively, had detectable HIV viremia.

IP-10 is an inflammatory cytokine produced as part of the innate immune response to different pathogens [20]. IP-10 has been explored for its use as both a diagnostic and prognostic marker for several infectious diseases, such as malaria, hepatitis C or tuberculosis [20–22]. In the case of HIV infection, previous data suggested that IP-10 levels were predictive of disease progression [11,23] and significantly decreased between 6 months [14] and 2 years [15] after ART initiation; however, to our knowledge, the use of IP-10 as a biomarker for VL levels has not been assessed for its accuracy to detect VF in individuals on ART.

Our results show that IP-10 can indeed be used as a surrogate marker of VL with high accuracy to screen ART-treated individuals and identify patients most likely to have VL levels over 150 copies/mL. This is particularly relevant in countries with scarce resources where the scale-up of ART often leads to delays and even failure to return VL results due to congested and/or centralized health facilities, which compromises both quality health services and patient retention in care [3]. Health and laboratory system strengthening together with reductions in costs and decentralization of services are required to implement the effective VL monitoring necessary to reach UNAIDS 90-90-90 targets in low-income settings [3]. Here we suggest a new algorithm to reduce the cost of ART monitoring by using IP-10 as a screening tool to target the individuals on ART most likely to require VL testing. Thus, allocation of resources to VL would be prioritized without jeopardizing quality of care. In a limited resource setting, an IP-10 assay would halve the number of VL required to monitor the same number of patients and could be combined with pooled VL testing to further reduce the number of VL assays. [7]. Despite the difficulty of adding an extra test to a clinical algorithm, IP-10 can be quantified by an inexpensive commercially available enzyme immune-assay (EIA) with a four hour turnaround time and could be developed into

a point of care test as have other similar cytokines [19]. Therefore, WHO recommendations would be followed but VL testing would be requested only if IP-10 screening indicates potential VF. Standard recommendations for counseling, adherence support and repeating VL within a 3-month interval would continue to be fulfilled in order to determine VF and subsequent antiretroviral regimen switching following WHO guidelines [5]. Pilot studies could be designed to evaluate the feasibility of integrating this test into the existing laboratory facilities.

IP-10 is an affordable and easily quantifiable biomarker, however its expression level is affected by a number of inflammatory responses [20]. Hence, its potential use to identify VF would require a second step of confirmation through VL. In this sub-analysis of the cross-sectional study carried out in adults on ART at the MDH in Southern Mozambique [16], in order to detect the 34% of individuals with detectable VL, 316 VL tests were necessary. However, using IP-10 to screen out those individuals not likely to have VF, only 180 VL tests would have had to be conducted, thus reducing by 43% the VL determinations required for ART monitoring in these settings. Although there are major pricing discrepancies across countries and policies globally [3] and disregarding costs of instrument maintenance, human resources, sample transportation and indirect cost associated to late VF detection, according to data from our cohort, this approach could potentially save 38% of VL-derived costs to the health system. Nevertheless, this IP-10-based screening is not only presented as a cost-saving strategy, but also, and maybe more importantly, as a way to decongest the overloaded core health and laboratory facilities. Even though most of the cases with detectable viremia were identified by employing IP-10 testing, up to 10.4% were misclassified as not requiring VL confirmation. This represents an important population whose VF would remain undiagnosed. However, depending on the setting in LMIC, the consequences of this misclassification need to be weighed against the risks of a higher proportion of individuals not receiving VL monitoring due to overburdened or isolated health services.

Since IP-10 plasma level is influenced by immune recovery [24], we assessed a multivariate model including clinical variables; however predictive power was not significantly increased. Further studies should evaluate whether a combination of IP-10 with another inflammatory cytokine involved in virological responses or any other surrogate of immune activation could improve the accuracy for predicting detectable viremia and reduce misclassification. Moreover, optimization of model parameters across HIV-subtypes and comorbidities present in different populations as well as sample types including dried blood spots (DBS) could render this approach generalizable. Indeed, recent studies have shown accurate VL measurements by using DBS in order to monitor patients on ART in low-income settings [25]. IP-10 levels have also been demonstrated to be easily and precisely quantifiable in DBS [26] and this approach has been recently validated for the diagnosis of tuberculosis [27] and monitoring anti-tuberculosis treatment response [28]. Further studies should evaluate the predictive power of IP-10 levels quantified in DBS to identified cases

with detectable viremia among ART-treated individuals. This strategy would offer decentralized VF diagnosis, facilitates sample storage and transportation and reduces test unit cost [29].

We thus propose a novel IP-10 screening algorithm for detection of VF. At a time when UNAIDS estimated that 18.2 million people were receiving ART in 2016 and nearly 12.7 million people will initiate ART in the next few years [30], further studies are warranted to assess the impact of implementing this simple triage approach to reduce the volume of VL determinations required for monitoring viral suppression.

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Competing interests

We declare that there are no conflicts of interest in relation to the submitted work.
The information of this manuscript has not previously been presented in any public meetings or conferences.

Author's contributions

LP, JC, RP, JB and DN conceptualization and study design. MR and SM recruited subjects, collected and validated clinical data. MR and CJ coordinated sample collection and processing at the field. LP performed biomarker quantification at the laboratory and validation of the data. LP and AC performed statistical analyses. LP, AC, JB and DN

interpreted the data. LP drafted the paper. JB and DN critical data review and revision of manuscript writing. All authors read and approved the final version of the manuscript.

Abbreviations

95% CI, 95% confidence interval; ART, antiretroviral therapy; AUC, area under the curve; BMI, body mass index; DBS, dried blood spots; interferon-γ-inducible protein-10 (IP-10); IQR, interquartile range; LMIC, low and middle-income countries; MDH, Manhiça District Hospital; NPV, negative predictive value; PPV, positive predictive value; ROC, receiver-operating characteristic; VF, virological failure; VL, HIV-RNA viral load; WHO, World Health Organization.

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FIGURES

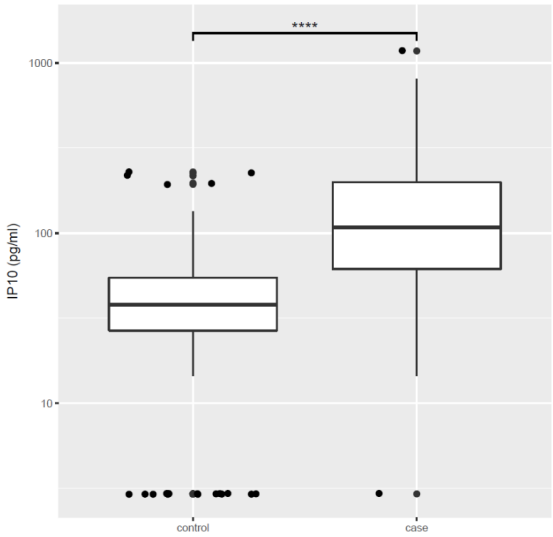
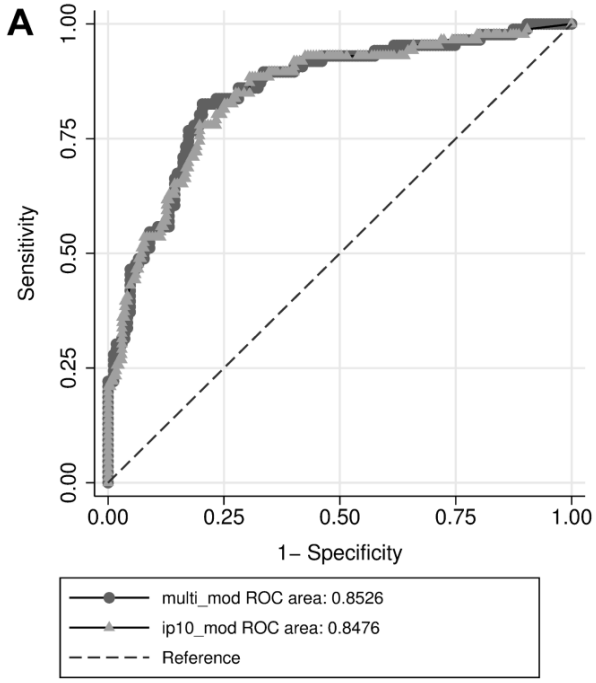


Figure 1. Comparison of IP-10 levels in ART-treated individuals with and without detectable VL (viral load>150copies/mL). Box as IQR, middle line as median, whiskers as Tukey values (1.5*IQR) and dots as outliers. Non-parametric U-test significance is indicated.



B **IP-10 univariate model cut-off values**

Cut-off value	Sensitivity (%)	Specificity (%)
53.3	83.7	73.1
52.2	84.9	72.5
51	87.2	69.5
49.8	88.4	69.5
48.1	89.5	65.3
44.2	91.9	59.9

C **IP-10 multivariate model cut-off values**

Cut-off value	Sensitivity (%)	Specificity (%)
-.94	83.7	76.0
-1.04	86.0	71.9
-1.11	87.2	67.1
-1.12	88.4	67.1
-1.14	89.5	66.5
-1.28	90.7	59.3
-1.35	91.9	57.5

Figure 2. Performance of univariate and multivariate IP-10 models in predicting detectable VL. **A.** Comparison between ROC curves for univariate IP-10 model (line with circles) and multivariate model (line with triangles) including IP-10 and CD4 T-cell count. **B.** IP-10 univariate model cut-off points (pg/mL) and (C) multivariate model score cut-off points with their respective sensitivity and specificity values.

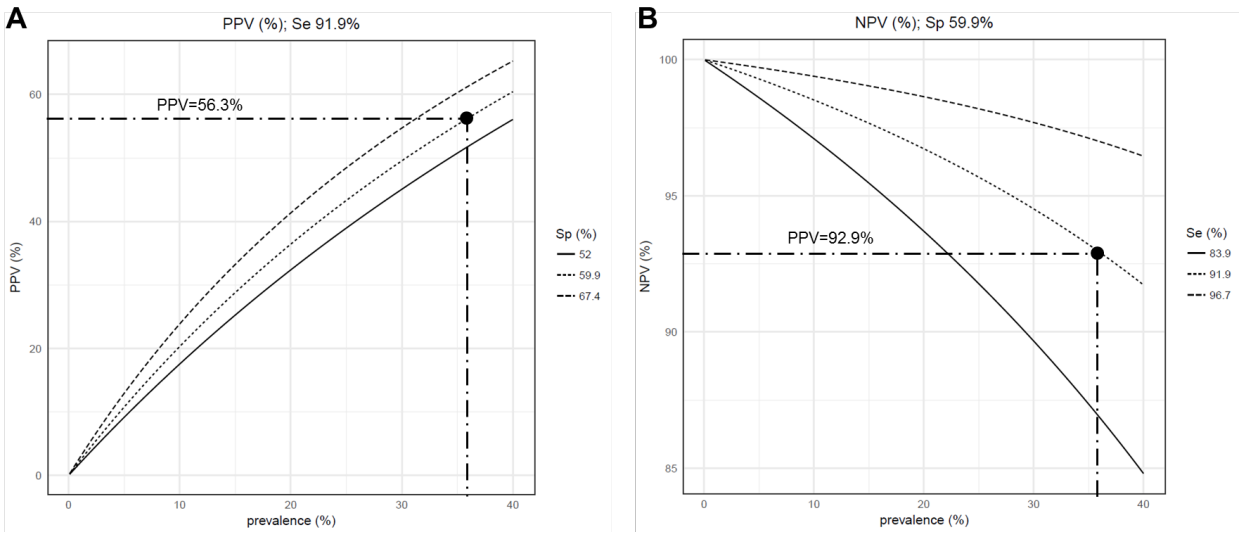


Figure 3. Accuracy of the IP-10 model for identifying patients with detectable VL according to the observed prevalence of individuals on ART with detectable viremia. A. Positive predictive value (PPV) estimated for sensitivity=91.9% and 3 different specificity scenarios according to the estimated confidence interval (Sp=59.9% [95%CI 52.0-67.4]). **B.** Negative predictive value (NPV) estimated for specificity=59.9% and 3 different sensitivity scenarios according to the estimated confidence interval (Se=91.9% [95%CI 83.9-96.7]).
" ▬ · · ▬ " indicates PPV and NPV at the prevalence of detectable VL observed in the cross-sectional resistance study [16].

SUMMARY OF RESULTS AND DISCUSSION

E. SUMMARY OF THE RESULTS AND DISCUSSION

The findings from the studies that constitute this thesis provide a detailed characterization of the dynamics for immune response biomarkers over the different stages of HIV infection among adults in Mozambique. The biomarkers described are soluble plasma cytokines involved in inflammation, cell activation, intestinal damage and other immunological responses; unspecific and HIV-specific antibody subtypes; and cellular markers of different T-cell phenotypes. The comparison of these biomarkers levels between PHI, chronically HIV infected and HIV-uninfected individuals shed light into the early pathogenic events following HIV acquisition that could guide vaccine or immunomodulatory approaches aimed at reducing the irreversible immune damage inflicted during PHI. This immunological description allowed us to identify an accurate biomarker that can be used as a surrogate of viremia levels in order to screen individuals for AHI or virological failure in LMIC. Importantly, modeling the implementation of this biomarker as a screening tool showed it to be a cost-saving strategy that could facilitate early HIV diagnosis and treatment monitoring in scarce-resource settings.

I. Characterization of soluble and cellular biomarkers during PHI

The challenges of identifying AHI in low-income countries have resulted in a lack of critical information regarding the immune response and pathogenic events that constrains the development of early therapeutic interventions in these areas[77,222]. Several studies have characterized the kinetics of soluble biomarkers[17,123,125–128,130] or T-cell responses[83,84,100,108,156,222] during PHI. However, most of these studies have been performed out of the Sub-Saharan setting where males represent most of the study population. Herewith, we describe the clinical and immunologic responses in a longitudinal cohort of PHI Mozambican adults followed over one year after infection and compare them with individuals CHI recruited at a rural hospital in southern Mozambique.

A total of 85 PHI individuals were identified in our cohort as seronegative or indeterminate for rapid test and as positive for viremia by HIV-RNA detection among Mozambican adults seeking health care at the MDH. This represents an AHI prevalence of 2.7% (95% CI 2.2-3.4) among all screened individuals and 3.0% (95% CI 2.4-3.7) among those who were reporting febrile illness. Despite the roll out of ART and HIV prevention activities, the 3.0% prevalence of PHI among people presenting with fever-like symptoms at the outpatient ward of this rural area of Mozambique has remained unchanged as compared to a study performed 5 years earlier which showed an PHI prevalence of 3.3% (95% CI 1.3–6.7)[120]. Given the high risk of transmission during this phase, these high levels represent an important threat to public health. Periodic cross-sectional PHI screening of people presenting with fever like symptoms in malaria-endemic high HIV incidence settings could act as an indirect surrogate to monitor evolution of HIV epidemic and compare tendencies between different communities or assessing the impact of prevention campaigns.

We defined seroconversion in our cohort as WB positive and we hypothesized that the expression patterns of innate response cytokines may be different in pre and post-seroconversion stages of the PHI phase. In order to approximate time since infection, PHI individuals were categorized into Fiebig stages I-III (n=45), IV (n=8), V (n=12) and IV (n=20) as previously described in introduction and methods sections[85,223,224]. Due to the limited sample size, participants were not further differentiated into Fiebig stages I, II and III, which might have brought specificity to cytokine associations with a narrower time frame. Soluble biomarkers of innate and adaptive responses were described over the different Fiebig categories, together with clinical and immunological characteristics. The expression of 9 biomarkers was significantly different across Fiebig categories including total plasma IgG, IgG1, sCD163, MCP-1, sCD23, BAFF, MIG, TRAIL, and IFNα. HIV-specific antibody levels significantly increased in Fiebig V and Fiebig VI as compared to Fiebig I–III and IV. As Fiebig stages are an estimate of days since HIV-infection, the quantification of these biomarkers by Fiebig category represents a descriptive dynamic of the innate and adaptive immune responses during the early HIV infection events. We compared cytokine levels in PHI

between individuals at pre- (Fiebig I-IV) and post-seroconversion (Fiebig V-VI) at the screening visit. Thus, we identified a signature of four cytokines composed of BAFF, MCP-1, sCD163 and MIG that is highly associated with PHI prior to development of the HIV-specific humoral response as determined by WB serology. These findings may help to understand the earliest HIV pathogenic events and identify new potential targets for immunotherapy aimed at modulating the cytokine response to HIV infection.

Throughout longitudinal follow-up of the PHI individuals identified we found that most inflammatory soluble biomarkers, CD4 counts and VL stabilized between 3 and 5 months after HIV infection. However, the stabilization in certain T-cell subsets took longer to be reached. In our study we observed that both activated and effector memory CD8 T-cells reach the stable levels several months after viremia control. Thus, we suggest that most alterations observed in the CD8 T-cell compartment during HIV-infection may not be exclusively viremia driven. Additionally we found that the frequency of systemic Th1Th17 cells in T cells continued decreasing until at least 7 months after infection and that this cell-subset showed an activated phenotype early after infection. Conversely, no signs of increased expression of CD57 in CD4 and CD8 T cells were observed over the first year of HIV infection, suggesting immunosenescence to be a later effect of HIV infection. The analysis of the cytokine dynamics provided us with further approximation to the innate and adaptive response changes over the initial months post-infection. We observed that levels of IP-10, MCP-1, BAFF, sCD14, TNFR2 and TRAIL were significantly overexpressed at the first month of infection and underwent a rapid decrease in the subsequent months, while MIG and CD27 levels started to increase 1 month after infection and remained over-expressed for almost one year post-infection. TNFR2 and sCD27 levels at 1 month post-infection were found to be associated with subsequent exhausted T-cell phenotypes, while BAFF and sCD14 were associated with CD8 T-cells activation. Tumor necrosis factor receptor-2 (TNFR2) is involved in cell survival that can result in cell proliferation, while CD27 participates in generation and long-term maintenance of T-cell immunity. Thus, the association of these two cytokines with exhausted T-cell subtype levels could have a functional meaning that needs further exploration. B-cell activating factor (BAFF) and sCD14 are both produced by monocytes and macrophages after infection or tissue inflammation and contribute to ensuring a proper immune response [225,226]. Thus, their association with the subsequent CD8 T-cell activation could indicate a mechanism of controlling the cellular response to the HIV-infection. Despite rapid immune control over virus replication in our cohort, the stabilization of immunological changes in the T-cell compartment occurred months after viremia or CD4 count stabilized, suggesting persistent immune dysfunction at PHI in T cell compartments other than those due to CD4 depletion after suppressing viremia. These results add evidence to the arguments for treatment initiation regardless of CD4 counts or viremia levels. They also point to a potential need for exploration of the clinical impact of this prolonged dysfunction in various T-cell

compartments. Development of adjunct therapies to ART may be necessary in order to enhance immune recovery and diminish immune activation during early HIV infection.

Although we described early changes in T-phenotypes, we were not able to study the T-cell profiles during the first month after infection due to the study design, which did not collect PBMC at the first visit. This would have allowed a characterization of the earliest responses in the T-cell compartment and would have provided additional data to better delineate cell dynamics, exhaustion, senescent and maturation stage or other alteration in T-cell phenotypes. Another study limitation is the lost to follow-up along the longitudinal visits which may have resulted in having insufficient power to detect additional significant differences and it has also hampered the possibility to study the associations between soluble and cellular makers with clinical disease progression in our cohort. This high attrition rate is common in the Sub-Saharan setting[227]. Attendance of scheduled visits is complicated by high rates of migration, long distances to health centers and difficulties missing work which threat continuity of care. Additionally, the high burden of diseases prevalent in the study area could have impacted the T-cell phenotypic characteristics and cytokine expression levels. Individuals in our cohort were tested for the most prevalent infections described in the area, including malaria, hepatitis B, syphilis, different protozoa and intestinal parasites and we did not observe any significant difference according to the co-infection status in the stage of CD8 activation. However, additional studies should evaluate the dynamics of plasma cytokines and these T-cell lineages in bigger cohorts and further assess the association of these biomarkers with potential rapid and slow disease progression.

II. Soluble biomarkers as a useful tool for early HIV detection and viral suppression monitoring

The AHI period represents a 'window of opportunity' for treatment and prevention strategies which can bring tremendous benefit both at the public health and individual levels. As a result of extremely high viremia levels in bodily fluids and intensive viral genital shedding, individuals are considered hyper-infectious during AHI[112,228]. In areas of high HIV incidence, this phenomenon could contribute greatly to fuelling the worldwide HIV pandemic[115] and ART initiation at this stage could importantly reduce further transmissions[53]. Regarding the benefits for the individual concerned, ART initiation during AHI has been documented to diminish the size of viral reservoir[110] and to prevent irreversible intestinal damage[59].

Based on conclusive clinical trials demonstrating that early ART stops progression to AIDS[54,55], WHO updated the HIV recommendations and released a comprehensive-revised guidelines in 2015[61,62], recommending ART initiation in everyone living with HIV regardless

of WHO clinical stage or CD4 T-cell count. Thus, many SSA countries started rolling out 'test and treat' programs to initiate ART in all the HIV-diagnosed individuals. This implies expansion of serological HIV testing strategies but also places AHI detection in the spotlight. The difficulty lays on the identification of AHI individuals for early treatment initiation. Individuals on AHI are often asymptomatic, or experience a non-specific febrile illness; a diagnostic dilemma compounded in the African setting by a significant symptom overlap with malaria and other infectious diseases [77,119]. During AHI, HIV-specific antibodies are not yet developed and HIV-diagnosis must be performed by the detection of viral nucleic acids or proteins[229]. The current gold-standard test for confirming viremia is RT-PCR for plasma HIV-RNA[121], commonly referred as VL testing. However, technical and financial constraints make this technique very limited in LMIC[77]. Although the development of POC-VL systems and similar antigen-based assays has rapidly advanced, implementation costs remain elevated[230,231]. Confronted with the sheer volume of patients requiring VL determinations for ART monitoring in many SSA countries, health services will not be able to implement individual or pooled VL for AHI diagnosis among febrile seronegative outpatients in the near future[232]. A more affordable attempt to diagnose AHI in scarce-resource settings is through 4th generation RDTs, which detect p24 HIV-antigen. However, recent studies performed in Australia, Swaziland, the United Kingdom and Malawi reported 0% sensitivity of the p24 component[122,233], thus invalidating this technique for AHI diagnosis. As a consequence of a lack of affordable and easy to implement tools, AHI is mostly not detected in LMICs. Even though the prevalence of AHI among febrile patients may reach 3% in high-incidence SSA populations[119,120,234], HIV-infected individuals are unnoticed at the health facilities, representing a public health threat and Individuals who could potentially become late presenters.

The UNAIDS 90-90-90 targets announced in 2014, call for 90% of people living with HIV aware of their status, 90% of people HIV-diagnosed on ART and 90% of people on ART attaining virological suppression, by 2020[235]. In order to monitor virological suppression and detect treatment failure, WHO recommends routine VL testing at 6 months after ART initiation and every 12 months[192]. Frequent monitoring enables the diagnosis of virological failure before the development of drug resistance mutations, which would ultimately lead to treatment failure and allow for possible further transmissions[236]. Despite the international recommendations, VL testing is still not widely available in many LMICs[205,206,237] with some countries still not recommending routine VL testing at all in their national guidelines[231]. In contrast to the widespread and routine use of VL testing in wealthy countries, its high cost, complexity and implementation constraints have presented major barriers to its scale-up and use in LMICs as Sub-Saharan Africa[77,207,232]. Indeed, implementation of VL detection is very challenging in resource-limited settings, specifically due to the required level of laboratory infrastructure, the need for trained staff and the sample transportation difficulties associated with centralized-services[205,207]. Moreover, there are major pricing discrepancies across countries for current laboratory-

based HIV-VL[205,231]. Recently, MSF reported that comprehensive costs including human resources, sample collection, reagents and consumables ranged from US$24.90 to 43.42 in the five Sub-Saharan Africa countries surveyed in 2013 (Kenya, Lesotho, Malawi, Swaziland, and Zimbabwe)[230]. There have been rapid developments in implementation of DBSs to facilitate sample transportation and POC technologies that are easy to use and can be performed by any healthcare worker, thus decentralizing VL testing[237]. Even though, implementation costs remain elevated for LMICs[77]. The alternative has often been clinical and/or immunological monitoring, which frequently results in patients remaining on failing ART as well as unnecessary regimen switches[208,238]. In Mozambique, recent cross-sectional surveys reported that 23%[206], 28%[236] and 36%[220] of individuals on ART, in Maputo and Manhiça District, had detectable HIV viremia. Health and laboratory system strengthening together with reductions in costs and decentralization of services are required to implement the effective VL monitoring necessary to reach UNAIDS 90-90-90 targets in LMICs[205].

In order to identify an affordable and simple alternative tool to detect both AHI and treatment failure, we evaluated a total of 49 soluble host biomarkers for their accuracy to identify patients with detectable viremia. Firstly, we compared the biomarker expression level in plasma between seronegative PHI and non-HIV-infected individuals presented with reported fever at the MDH (Mozambique). Among the different soluble biomarkers assessed, IP-10 alone showed the strongest predictive power to identify PHI individuals, providing a sensitivity of 95.5% (95%CI 85.5-99.5) and a specificity of 76.5% (95%CI 62.5-87.2) in our cohort. Since IP-10 is not an HIV-specific biomarker[239] its potential use to diagnose AHI would require a second step of confirmation through an HIV-specific detection method. We suggest an algorithm employing IP-10 as an approach to screen febrile seronegative individuals for subsequent AHI diagnosis with VL (Figure 21A). This IP-10-based approach could render AHI diagnosis and treatment feasible in low income settings by reducing the number of VL determinations necessary for AHI detection by 75%. However, in this study we compared febrile seronegative individuals in PHI with a random selection of non-HIV-infected individuals at approximately a 1:1 proportion. Although we modelled the IP-10 predictive power at different AHI prevalences commonly occurring in LMIC, further studies should assess performance onsite in settings with varying prevalences to evaluate whether sensitivity and specificity values could be affected. Thus our findings indicate that IP-10 is a powerful and feasible tool to detect AHI in resource-limited areas, allowing early ART initiation in these individuals and preventing a substantial numbers of new HIV infections.

In order to complement the proof of concept of IP-10 as an AHI screening tool, we performed a cost-effectiveness analysis comparing the suggested IP-10-based algorithm to diagnose and treat AHI with current practices in Mozambique, that imply leaving AHI undiagnosed. We found that the implementation of an IP-10 screening test could avert

from 21 to 84 new infections and save from US$176,609 to US$533,467 to the health system per 1,000 tested patients. Hence, we provide an affordable alternative to facilitate early diagnosis and treatment in low-resource high HIV-burden settings. In this analysis, we used a public negotiated cost of $16.80 as a POC-VL instead of employing a pooling or individual VL strategy because same day return of results has been shown to accelerate ART initiation[240]. However, for current laboratory-based HIV-VL there are major pricing discrepancies across countries[205,231]. Recently, MSF reported that comprehensive costs including human resources, sample collection, reagents and consumables ranged from US$24.90 to 43.42 in the five Sub-Saharan Africa countries surveyed in 2013 (Kenya, Lesotho, Malawi, Swaziland, and Zimbabwe)[230]. We considered a best case scenario for inexpensive VL, but using other values would increase the cost savings. We also assumed a cost of an IP-10 rapid test of $1.50 based on commercially available costs for CRP rapid test employed in the management of undifferentiated fever[241]. Both CRP and IP-10 are inflammatory proteins with similar costs for detection by ELISA assays purchased at market price. Moreover, we did not include the cost of HIV-associated comorbidities in the simulated cohort which, if included, would most likely increase the cost-effectiveness of the IP-10 followed by POC-VL intervention.

Interferon-□-inducible protein-10 (IP-10) is produced as part of the innate immune response to viruses, bacteria, fungi and parasites[239]. At the initial stages of HIV infection, IP-10 has been shown to greatly increase prior to the development of clinical symptoms paralleling HIV-VL[123]. Previous data suggested that IP-10 levels during PHI were correlated with cell-associated HIV-DNA[242], immune activation[127] and predictive of rapid disease progression[127,243], representing an earlier biomarker than CD4 T-cell counts or viremia levels[127]. IP-10 is a pro-inflammatory chemokine and a ligand of CXCR3. As CXCR3+ CD4 T-cells are the main cellular targets of HIV[244,245], it is conceivable that IP-10 contributes to the observed changes in CXCR3 (CD183)+ CD4 T cells since it regulates the trafficking of HIV target cells to lymphoid tissues, thereby promoting new rounds of infection and helping to establish viral reservoirs[246]. This recruitment might enhance the sequestration of T cells in infected lymphoid organs and the spread of infection between cells, contributing to the immunopathology of AIDS. Indeed, elevated systemic IP-10 levels before HIV infection were found to be associated with a more rapid disease progression and studies of aviremic macaques suggest that high IP-10 is indicative of viral replication in lymphoid tissues[242]. However, to our knowledge, the strong association between IP-10 and VL levels has not been assessed for its ability to identify AHI among seronegative individuals. Our results show that IP-10 can indeed be used to screen HIV-seronegative individuals with high predictive ability to differentiate AHI from other patients individuals with undifferentiated fever. Additionally, IP-10 has been previously explored for its use as a prognostic or diagnostic marker for other infectious diseases such as malaria[239]; hepatitis C[247] or tuberculosis[248,249]. Therefore, further research is warranted in order to explore the impact of other common HIV comorbidities on HIV-induced levels of IP-10, validate the IP-10

predictive power and optimize model cut-off values in other populations. Importantly, if IP-10 accuracy for AHI detection were to be validated in non-febrile seronegative individuals, this biomarker could also be valuable for screening blood donors or pregnant women at the antenatal care units in LMIC. This approach could thus bring AHI diagnosis to targeted vulnerable populations which otherwise would go undiagnosed and represent an important threat for public health.

As a result of the high predictive power of IP- 10 levels to identify PHI among febrile seronegative patients and its strong association with VL reported by different studies[123,127,250,243], we hypothesized that plasma IP-10 levels could also be employed as a surrogate marker of detectable viremia in ART-treated individuals. Consequently, we assessed the plasma levels of IP-10 in 316 individuals on ART for more than a year that were attending routine outpatient visits at the MDH. Thus, IP-10 demonstrated a sensitivity of 91.9% (95%CI 83.9, 96.7) and a specificity of 59.9% (95%CI 52.0, 67.4) for predicting detectable viremia (n=106) in this cohort. This showed that IP-10 could not only predict high viremia observed in AHI, but also levels of viremia in ART treated patients which were roughly one-thousand-fold lower than in AHI. We suggest a novel algorithm to reduce the cost and complexity of ART monitoring by using IP-10 as a screening tool to target the individuals on ART most likely to require VL testing (Figure 21B). Comparing to the predictive power for AHI detection, the specificity obtained for this approach is moderate probably due to the lower viremia levels in individuals on ART. This is also consistent with the lower model cut-off value IP-10≥44.2pg/mL for detecting viremic patients compared to the 161.6pg/mL that we used to identify AHI. Despite the lower specificity, considering a prevalence of 36% of detectable viremia in ART treated patients, an IP-10 screening would reduce by 43% the number of VL determinations required to monitor the same number of patients. Besides, IP-10 screening could be combined with pooled VL testing to further reduce the number of VL assays required. In addition, translating data from our cohort to US$ values, this approach could potentially save 38% of VL-derived costs to the health system. Considering US$28 as the average reagent cost for HIV-VL testing and assuming a unit cost of US$1.50 for IP-10 testing, the VL testing of the 316 treated individuals analysed, would imply around US$8850 per year to the health system. While, the IP-10 screening together with the 180 VL determinations required in this cohort, would imply a cost of around US$5510 per year. This means that introducing an IP-10-based screening for ART monitoring would save US$3340 a year for this cross-sectional study cohort. But also, and maybe more importantly, it could decongest the overloaded core health and laboratory facilities in LMICs which are currently challenged with sending samples to centralized laboratories. This often leads to months of delay and even failure to return VL results, which compromises both quality health services and patient retention in care[205]. At a time when UNAIDS estimated that 18.2 million people were receiving ART in 2016 and nearly 12.7 million people will initiate ART in the next few years[7], further studies are warranted to

assess the impact of simple and affordable triage approaches to reduce the volume of VL determinations required for monitoring viral suppression.

Parameters such as sensitivity, specificity and cut-off values for both strategies would need to be optimized by extending our approach to other populations affected by different HIV-subtypes or other prevalent co-infections. Here we suggest the use of IP-10, potentially developed as POC-RDT, as a pre-screening tool to discriminate AHI from other febrile individuals or to detect VF in patients on ART. Because IP-10 expression level is not HIV-specific, both approaches would require a confirmation step with VL-testing, which ideally would be POC-VL to facilitate same-day return of results and rapid clinical decision-making. An IP-10-based test could be developed as a RDT as achieved for other similar cytokines[251]. In order to target AHI and VF detection, an RDT could be designed according to a low and a high IP-10 threshold, so both applications could be implemented at the POC. Interestingly, recent studies have shown that IP-10 can be successfully employed for the diagnosis of tuberculosis[252,253], to discriminate between active and latent disease[248] and to assess response to treatment[248,249]. Thus, the development of an IP-10 RDT useful for HIV and tuberculosis monitoring might garner increased market interest. Since HIV and tuberculosis are common comorbidities in LMIC[254], the validation of IP-10 for the enumerated applications could be tested in the same target population. Hence, different IP-10 thresholds could indicate different clinical outcomes for these two associated infectious.

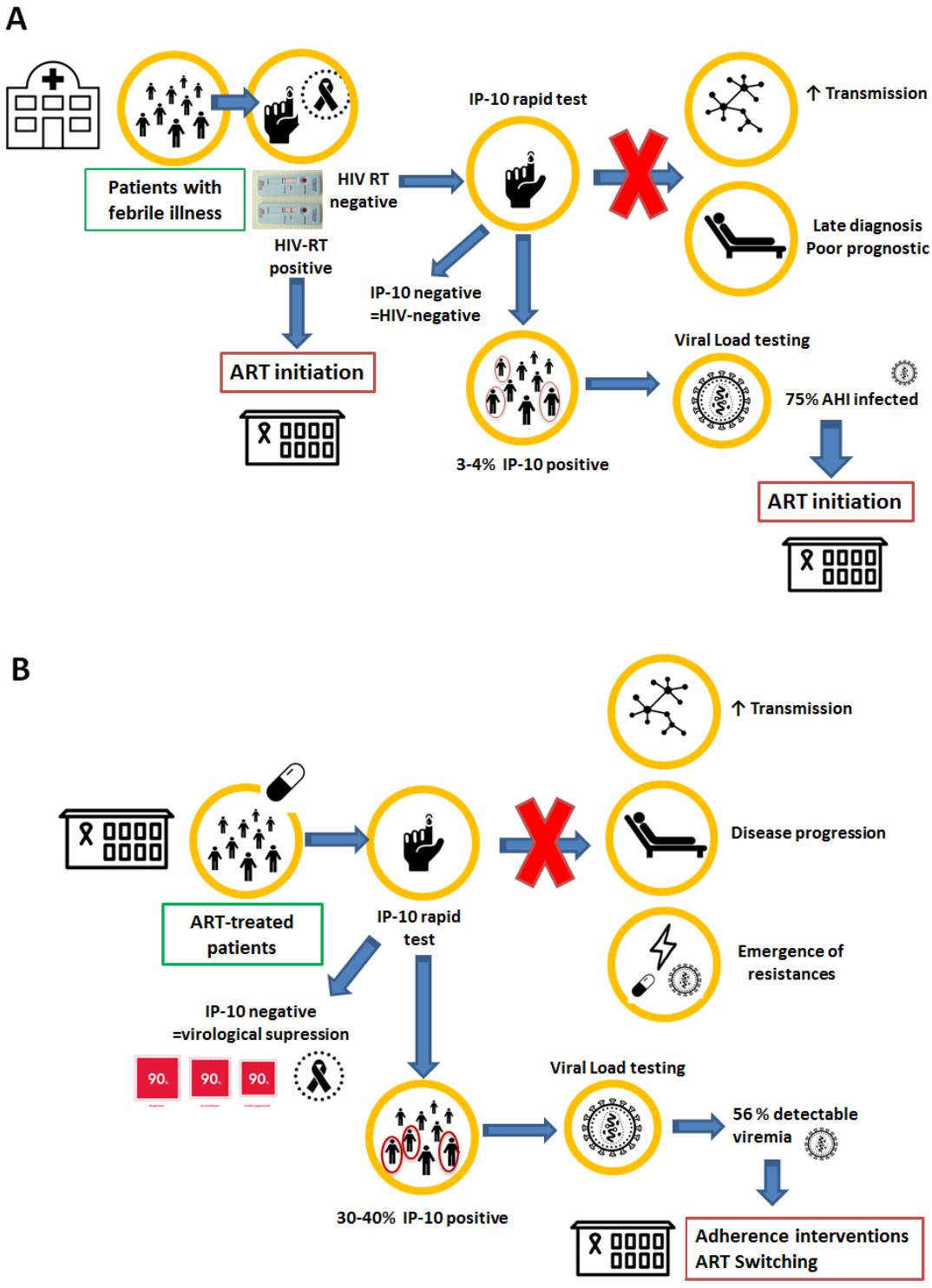


Figure 21. Suggested IP-10-based algorithms to detect AHI in febrile seronegative individuals (A) and VF in individuals on ART (B).

CONCLUSIONS

F. CONCLUSIONS

1. AHI prevalence in febrile seronegative adults presenting at the MDH was found to be 3% as reported in 2008, having thus remained unchanged in the last 5 years.

2. A signature of four cytokines composed of BAFF, MCP-1, sCD163 and MIG is highly associated with the PHI phase prior to development of HIV-specific humoral responses as determined by WB serology.

3. Activated and effector memory CD8 T-cells together with Th1Th17 cells reached stable levels at an estimated 9-11 months post-infection, suggesting persistent immune dysfunction in this cell compartment at PHI after viremia stabilization. However, no sign of immunosenescence was detected over the first year of HIV infection.

4. Early TNFR2 and sCD27 levels were found to be associated with subsequent exhausted T-cell phenotypes, while BAFF, IL10 and sCD14 were associated with CD8 T-cells activation.

5. From 49 soluble biomarkers assessed, IP-10 demonstrated the best predictive power for AHI detection among febrile seronegative individuals, providing a sensitivity of 95.5% and a specificity of 76.5%.

6. The implementation of an IP-10-based screening for subsequent AHI diagnosis with VL is a cost-effective strategy to prevent disease progression and a substantial number of further HIV infections. This algorithm could avert up to 84 new infections and save up to US$500.000 to the health system per 1,000 tested patients.

7. IP-10 is an accurate biomarker to screen individuals on ART for virological failure, identifying 91.9% of patients with detectable viremia with a specificity of 59.9%.

8. Employing IP-10 as a screening tool to target the individuals on ART most likely to require VL testing would reduce by 43% the number of VL determinations required to control viral suppression and it could save 38% of VL-derived costs to the health system.

FUTURE PERSPECTIVES

G. FUTURE PERSPECTIVES

There is a global health need for developing alternative HIV diagnostic and monitoring tools adapted to LMIC. This thesis provides a further description of immune-biomarker dynamics across the different stages of HIV infection and contributes to the identification of useful biomarkers for HIV monitoring in resource-limited settings.

Further investigations should evaluate whether a combination of IP-10 with other cytokine involved in virological responses or any other surrogate marker of immune activation could improve the accuracy for predicting detectable viremia and reduce misclassification. Moreover, optimization of model parameters across HIV-subtypes and comorbidities present in different populations as well as sample types could render this approach generalizable. Importantly, if accuracy for AHI detection is validated in non-febrile seronegative individuals, this biomarker could also be valuable for screening blood donors in low-income countries. Besides, if IP-10 model parameters are optimized in infants or pregnant women, AHI could be diagnosed in these vulnerable populations which would benefit significantly from prompt ART initiation. Recent studies have shown accurate VL measurements by using DBS in order to monitor patients on ART in LMICs[255]. IP-10 levels have also been demonstrated to be easily and precisely quantifiable in DBS[256] and this

approach has been recently validated for the diagnosis of tuberculosis[257] and monitoring anti-tuberculosis treatment response[258]. Future studies could assess the predictive power of IP-10 levels quantified in DBS to identified cases with detectable viremia among ART-treated individuals. This strategy would offer decentralized VF diagnosis, facilitate sample collection, storage and transportation and would reduce test unit cost[204].

After further validation of IP-10 performance for AHI and VF detection in other populations, pilot studies could be designed to evaluate the feasibility of integrating this test into the existing laboratory facilities. We suggest two potential scenarios for integrating these screening algorithms into the health system: 1) blood collection followed by ELISA quantification with minimal laboratory processing and confirmation with plasma VL or pooled-VL; and 2) development of POC IP-10-based RDTs for subsequent confirmation with POC-VL. POC strategy represents the best option for clinic monitoring since it would offer same-day results without depending on laboratory facilities. Such approach could assure rapid AHI or VF detection that would enhance same-day ART initiation or adherence intervention/switch to second line regimens, as appropriate. However further cost-effectiveness analysis should be performed to evaluate the potential benefits of each scenario.

In order to the complete the main objectives of the GAMA study, these characterized inflammatory and immune response biomarkers are being further assessed for their ability to discriminate between recent and longstanding HIV-infections in the described cohort. Methods for measuring HIV incidence are a key element for tracking the progress in containing the HIV pandemic. A cost-effective, rapid, reliable HIV incidence assay would contribute tremendously to the evaluation of ongoing prevention programs and development of new prevention interventions. However, existing assays have a short duration of expression and a high false recent rate and therefore do not offer reliable results to estimate population incidence rates[259–261]. The tremendous damage inflicted on the GALT during early HIV infection may provide an additional opportunity to identify promising biomarkers which present altered expression during the early phases of HIV infection as compared to later chronic infection. Because early HIV-induced GI pathophysiology shares many similarities with Intestinal Bowel Diseases (IBDs), in order to test this hypothesis stool biomarker employed in the diagnosis of IBDs were also assessed for this purpose. Understanding the dynamics of gastro-intestinal damage biomarkers and associating them with standard or newly developed serological responses in blood and stool during early HIV infection may reveal specific changes in expression patterns of biomarker which are able to distinguish recent from longstanding HIV infection, an urgent need to assess the efficacy of prevention campaigns, and may also provide valuable information for HIV progression monitoring.

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H. REFERENCES

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ANNEXES

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Case Report: Rapid HIV Progression During Acute HIV-1 Subtype C Infection in a Mozambican Patient with Atypical Seroconversion

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Abstract. Acute human immunodeficiency virus (HIV) infection (AHI) refers to the period between viral transmission and development of an adaptive immune response to HIV antigens (seroconversion) usually lasting 6–8 weeks. Rare cases have been described in which HIV-infected patients fail to seroconvert and instead, develop rapid HIV-mediated clinical decline. We report the case of a Mozambican woman with AHI and malaria coinfection who showed atypical seroconversion and experienced rapid deterioration and death within 14 weeks of diagnosis with AHI. Atypical seroconversion may be associated with rapid progression. Fourth generation rapid tests could lead to earlier identification and intervention for this vulnerable subgroup.

INTRODUCTION

Acute human immunodeficiency virus (HIV) infection (AHI) refers to the period between viral infection and the development of an adaptive immune response to HIV antigens (seroconversion) usually lasting 6–8 weeks.[1,2] This period is typically associated with high viral loads, a massive early innate immune response, and a rapid decrease in $CD4^+$ T-lymphocyte counts. In parallel, an HIV-specific humoral immune response is mounted, leading to seroconversion.[3,4] Rare cases have been described in which HIV-infected patients do not develop the classic antiviral antibody response and instead, develop a fast HIV-mediated immunodepression and rapid clinical progression.[5–7] We report the case of a 30-year-old black Mozambican woman with acute HIV infection and *Plasmodium falciparum* malaria coinfection who showed an atypical pattern of seroconversion and experienced rapid deterioration and death within 14 weeks of diagnosis with AHI.

CASE REPORT

The patient presented to the Manhiça District Hospital in Mozambique with a febrile syndrome, was referred, and gave informed consent to an ongoing study of acute HIV. The patient had no prior history of HIV testing and tested negative for an HIV antibody rapid test (Determine HIV 1/2; Abbott Laboratories, Chicago, IL), but plasma reverse transcription polymerase chain reaction (real-time-PCR) revealed a high HIV viral load ($\log_{10}$ 6.7 copies/mL) (all results are in Table 1), thus identifying a case of AHI. Both malaria slide and sputum smear for tuberculosis (TB) at this time were negative.

Subsequently, the patient was seen three times in 14 weeks of follow-up. HIV rapid testing on two occasions revealed indeterminate HIV serology (positive for Determine but negative for Unigold; Trinity Biotech Co., Wicklow, Ireland) (Table 1). At the 1-month follow-up visit (day 34), the patient had complaints of paroxysmal diarrhea, fever, night sweats, and productive cough with mucoid expectoration. Sputum samples continued to be negative for TB, and no pathological signs were observed on chest X-ray. Her hematology was normal; however, her CD4 counts were 64 cells/mm^3 (Table 1). She was diagnosed with *P. falciparum* malaria by blood film (Table 1) and treated with first-line antimalarial treatment (artemether and lumefantrine). At her 2-month follow-up visit (day 64), the patient reported migraine, dizziness, cough, and continued weight loss. She was prescribed metronidazole for treatment of *Clostridium difficile*, and cotrimoxazole prophylaxis was initiated. Her CD4 counts had fallen to 33 cells/mm^3, and HIV RNA levels remained above 6.0 $\log_{10}$ copies/mL (Table 1). Quantitative PCR for *P. falciparum* revealed low parasitemia (69 parasites/μL). Although antiretroviral treatment (ART) in the absence of confirmed seroconversion is not included in the Mozambican national guidelines, the patient was referred to start outpatient counseling for ART.

Five days later, the patient's condition had further deteriorated. She had notably rapid weight loss, and clinicians were suspicious of acquired immune deficiency syndrome (AIDS) wasting syndrome. Clinicians agreed that TB treatment followed by ART should be initiated according to national guidelines. During the next few weeks, the patient declined follow-up by field workers and did not return to the clinic. An additional field worker home visit reported that the patient died at home 14 weeks post-diagnosis.

Sequencing RT and Protease from plasma samples from three visits (days 0, 34, and 64) confirmed infection by HIV-1 subtype C and C-C chemokine receptor type 5 (CCR5) tropism. Furthermore, sequencing of the gp120 V3 region in all samples showed identical sequences over the 2-month period. P24 antigen was detected in plasma at 1 month by enzyme-linked immunosorbent assay (ELISA). Western blot (WB) analysis showed an atypical profile across time points with antibody positivity against gp41, gp120, and p31, while remaining negative for p24 and p17 gag-specific antibodies (Table 1). The pattern was atypical, because there was no detectable

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TABLE 1
Patient results at initial and follow-up visits

	Visit 1 (day 0)	Visit 2 (day 34)	Visit 3 (day 64)
HIV viral load (log_{10} copies/mL)	Log_{10}6.7	Log_{10}6.3	Log_{10}6.4
Determine HIV-1/2 rapid test	–	+	+
UniGold rapid test	ND	–	–
WB	gp41++	gp41++	gp41++
WB	gp120+/–	gp120+/–	gp120+
WB	p31+/++	p31+/++	p31+
WB	p24–	p24–	p24–
WB	p17–	p17–	p17–
p24 ELISA (pg/mL)	388	ND	ND
Lymphocytes (cells/μL)	ND	1,656	998
T cells (cells/μL)	ND	963	566
T cells (% of lymph)	ND	58	57
CD4 T-cell counts (cells/μL)	ND	64	33
CD4 T cell (% of lymph)	ND	4	3
CD8 T-cell counts (cells/μL)	ND	742	460
CD8 T cell (% of lymph)	ND	45	46
Ratio CD4/CD8	ND	0.09	0.07
Red blood cells (10^6 cells/μL)	ND	4.19	ND
Hemoglobin (g/dL)	ND	11.0	ND
Hematocrit (%)	ND	33.1	ND
MCV (fL)	ND	79.0	ND
White blood cells (10^3 cells/μL)	ND	4.20	ND
Neutrophils (10^3 cells/μL)	ND	2.09	ND
Lymphocytes (10^3 cells/μL)	ND	1.57	ND
Monocytes (10^3 cells/μL)	ND	0.46	ND
Eosinophils (10^3 cells/μL)	ND	0.04	ND
Platelets (10^3 cells/μL)	ND	191	ND
Malaria blood film*	–	3,240	ND
Syphilis	ND	–	ND
TB	–	–	ND
Hepatitis B	ND	–	ND

ND = not done.
*Blood film microscopy expressed in parasites per microliter.

antibody to gag proteins. Recognition of gp120 increased over time, reflecting a slow seroconversion process. Human Leukocyte Antigen (HLA) System typing showed homozygosis at all loci, except for HLA-A (HLA-A*02, A*26, B*58, –; C*07, –; DRB1*11, –; DQB1*03, –).

DISCUSSION

A substantial subset of HIV-1–infected individuals experience a sharp decline in CD4 T-cell counts quickly after primary infection, with progression to AIDS occurring within the first 1–3 years of infection.[8] These rapid progressors are estimated to comprise 10–15% of HIV-1 cases,[8] although exact figures are unknown, particularly in resource-poor settings. HLA homozygosis as well as certain HLA types are associated with rapid HIV progression.[9–11] Seronegative HIV infection is also described to be associated with high viral loads, rapid disease progression, and significant mortality.[12] Seronegative HIV infections are rare and have been hypothesized to occur by overwhelming a particularly susceptible host immune system with HIV antigen.[13] The clinical presentations of two known case reports of seronegative subtype C infection are very similar to that observed in our patient.[14,15]

In the Mozambican patient, seroconversion was slow in the presence of rapid disease progression. This patient was rapid test indeterminate on two occasions and showed increasingly positive WB results, suggesting that she developed an initial humoral response but that the response never fully matured. Furthermore, the patient's lack of gp120 V3 region sequence diversity was consistent with a poor antibody response, resulting in little immune selection pressure. Although the lack of anti-gag antibodies has been also reported in late-stage AIDS,[16,17] the shift from negative to positive Determine rapid test over time, the high viral load at diagnosis, which subsequently declined, the uniformity of the gp120 V3 region, and the HLA pre-disposition argue for severe primary infection.

The combination of coinfection with *P. falciparum* malaria and extreme homozygosis for all HLA loci may have contributed to this patient's atypical seroconversion and rapid HIV progression. The patient was diagnosed with *P. falciparum* malaria at day 34 by microscopy, but we cannot exclude that she had submicroscopic *P. falciparum* infection at her initial day 0 visit. She still had low parasitemia at day 64, despite antimalarial treatment. *P. falciparum* has been shown to lead to immune activation, increased HIV replication, and a decrease in CD4 counts during chronic HIV infection.[18–20] However, there is no information on *P. falciparum* infection during acute HIV infection. The heightened state of immune activation associated with *P. falciparum* coinfection may have led to early HIV-specific clonal depletion of CD4 T cells, resulting in impairment of both B- and T-cell responses as described for acute HIV infection in a psoriasis patient.[13] In addition, the extreme homozygosity of this patient may have led to a narrow or insufficient HIV-specific CD8 T-cell response, thus allowing for heightened HIV replication. Although the patient was negative for TB, we cannot exclude atypical TB presentations, which have been observed for HIV patients.[21,22] In Mozambique, as in much of sub-Saharan Africa, routine HIV diagnoses are based on rapid antibody testing algorithms, and HIV RNA detection is not readily available. Consequently, there are no provisions in national treatment guidelines for patients who have negative or indeterminate rapid HIV test results but progress to AIDS. Outside of the context of a specific study, it is unlikely that this patient would have been identified as HIV-positive, much less as rapidly progressing.

The frequency of inadequate serological immune responses to HIV subtype C infections resulting in poor outcomes is unknown and not likely to be high. It may be plausible to speculate that, in areas of high HIV incidence, widespread coinfection with malaria (and indeed, other organisms) could impair or skew antibody responses and more commonly, lead to rapidly progressing HIV infection in the absence of an HIV diagnosis. HLA combinations pre-disposing to rapid progression could contribute to undermining the HIV-specific immune response during acute HIV complicated by malaria. Under existing antibody-reliant diagnostic algorithms, these HIV infections would go unidentified. This might warrant the use of HIV diagnostic tests that are able to detect HIV at an earlier stage. Fourth generation rapid diagnostic tests including detection of p24 are becoming available, and their use could aid in the identification of HIV rapid test-negative or indeterminate acute infections in resource-poor settings and lead to earlier intervention for this vulnerable subgroup with high mortality rates.

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