

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

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Background: During acute HIV infection, HIV actively replicates but seroconversion has not yet occurred. Primary HIV infection (PHI) is characterized by a transient nonspecific 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 before completion of a full humoral response.

Methods: A symptom-based screening was used to identify acute HIV infection in the Manhica District Hospital in Mozambique. Plasma levels of biomarkers were determined by Luminex and enzyme-linked immunosorbent assay. Anti-HIV

antibodies were analyzed 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 B-cell activating factor, monocyte chemoattractant protein-1, sCD163, and monokine induced by interferon (IFN- γ). This cytokine signature classified patients in the preseroconversion phase with a sensitivity of 92.5% and a specificity of 81.2%

Conclusions: Identification of a cytokine signature specific for the preseroconversion 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.

Key Words: acute HIV infection, sub-Saharan Africa, cytokines, HIV pathogenesis, Fiebig, antibodies

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D.N. and J.B. study design. L.P., E.P., L.F.-S., and D.R. recruited subjects and collected clinical data. L.P., E.P., L.F.-S., L.L., and C.J. performed laboratory analysis at the field. L.P. and J.C. performed biomarker quantification at the laboratory and validation of the data. L.P., E.P., and V.U. performed statistical analyses. L.P., E.P., J.C., J.B., and D.N. interpreted the data. L.P. and E.P. drafted the article. D.N., J.B., and I.M. coordinated the work and supervised manuscript writing. All authors read and approved the final version of the manuscript.

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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 characterized by high HIV viral replication, massive gut-associated lymphoid tissue CD4⁺ T-cell destruction, immune activation, and establishment of viral reservoirs.³ As a result of high viremia in bodily fluids and high levels of genital viral shedding, individuals are considered hyperinfectious 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 nonspecific febrile illness; a diagnostic dilemma compounded in the African setting by a significant symptom overlap with malaria.^{3,9} 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.³ Thus, current gold standard test for confirming viremia is reverse transcriptase–polymerase chain reaction for plasma HIV RNA,¹⁰ whereas the most widely accepted assay for confirmation of HIV antibody testing is Western blot (WB) or indirect immunofluorescence assay.¹¹

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 categorized into “Fiebig stages”¹² that are useful in approximating the infection date with relative accuracy.¹³

As HIV viremia 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 before the generation of the humoral response.^{14–18} We hypothesize that the expression patterns of these early response cytokines may be different between preseroconversion and postseroconversion 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 Manhica District Hospital (MDH) in the district of Manhica, Southern Mozambique. The present analysis is a substudy of a prospective cohort of primary HIV-infected adults enrolled and followed up for 12 months in the study Gastrointestinal inflammation biomarkers in acute HIV-infected Mozambican adults (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 before participation.

Adults older than 18 years, who were residents of the established District Surveillance System study area, and who presented to the outpatient clinic of the MDH for nonspecific febrile symptoms or voluntary HIV counseling and testing were invited to participate in the study.

HIV Diagnosis

Blood was collected by 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 Uni-Gold 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 enrollment into the study. PHI 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 reverse transcriptase–polymerase chain reaction testing on frozen plasma using Abbott Real-Time HIV-1 assay according to the manufacturer’s instructions, with a sensitivity of detection of 150 copies/mL. HIV RNA was quantified by applying a multilevel pooling scheme of 10 samples/pool as described and validated in previous works.¹⁹ A subgroup of HIV-negative time-matched controls was selected by computer randomization.

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 article, the 1-month visit sample was only used to establish the reference level for the biomarkers in a nonfebrile non-HIV-infected group and the coinfections and CD4 and CD8 T-cell counts in both study groups. Demographic and clinical data were collected in a specific questionnaire. Medical consultation and HIV counseling were provided. CD4 and CD8 T-cell counts were determined using CD3, CD8, CD4, and CD45 fluorochrome-labeled antibodies on fresh whole blood in a single platform system using Trucount tubes (BD Biosciences, Franklin Lakes, NJ). Clinical and microbiological evaluation was performed, testing for the most prevalent infections in the area.

For quantification of immunoglobulins and definition of PHI phases, see Detailed Methodology section in the Supplemental Digital Content, <http://links.lww.com/QAI/A958>. HIV-specific antibodies present in plasma screening samples were assessed by WB assay for sg-p120, gp41, p31, p24, and 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 preseroconversion acute stage, and patients with a positive WB result were considered to be in the postseroconversion 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 the screening visit were used 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), and VI (VL positive, WB positive with p31 band positive) according to previous work.¹²

Seven plasma antibody isotypes (IgA, IgM, IgG1, IgG2, IgG3, IgG4, and total IgG) were quantified in the

screening samples by an in-house enzyme-linked immunosorbent assay. HIV-specific antibodies of different subtypes (IgG1, IgG2, IgG3, and IgG4) for HIV antigens (gp41-Envelope, p24-Gag, and p31-integrase) were performed by multiparametric flow cytometry.

For quantification of plasma cytokines, see Detailed Methodology section in the Supplemental Digital Content (<http://links.lww.com/QAI/A958>), 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) (see Table 1, Supplemental Digital Content, <http://links.lww.com/QAI/A958>). Determinations were performed by enzyme-linked immunosorbent assay or Luminex multianalyte profiling technology.

Statistical Analysis

Group comparisons by Fiebig stage were performed using the nonparametric Kruskal–Wallis test and χ^2 test for categorical variables. Multiple tests were adjusted using the Bonferroni correction. Post hoc pairwise comparisons between Fiebig stages were performed by Dunn test with Bonferroni adjustment. Comparisons between PHI and non-HIV-infected controls were based on the nonparametric Mann–Whitney *U* test. Identification of cytokines with the best performance in distinguishing between preseroconversion and postseroconversion stages was performed using random forest analysis. Random forest is a supervised learning method based on the ensemble of multiple classification trees.²⁰ Relation between early and late Fiebig stages with respect to the selected cytokines were explored by principal component analysis (PCA). Patterns of the selected cytokines by PHI phases were assessed by multivariate

logistic regression. Outcome was a binary variable where 1 represents preseroconversion AHI group (Fiebig I–IV) and 0 represents recent HIV-infected group (Fiebig V–VI). In both PCA and regression model, monokine induced by IFN- γ (MIG), monocyte chemotactic protein-1 (MCP-1), and B-cell activating factor (BAFF) values were log transformed for a better adjustment of the data. Clinical variables (age, sex, VL, and other coinfections) were tested for inclusion in the model by backward stepwise 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 ninety-nine (20%) were seropositive on rapid testing, and a further 92 (2.3%) were excluded before serological testing, mainly because of residence outside the study area (Fig. 1). A majority (64.4%) of screened patients were younger than 30 years, and most (62.8%) were female. These age and sex tendencies were similar between seropositive patients and those subjects who met the 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 nonspecific 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

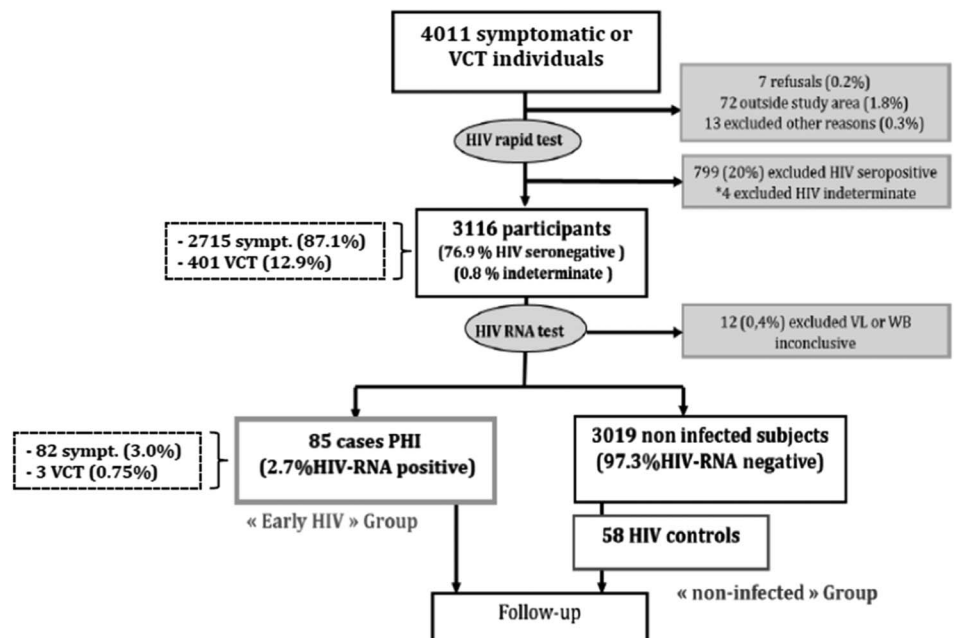


FIGURE 1. Screening profile: Symp, symptomatic patients; VCT, patients presented for voluntary counseling and testing; VL, viral load; WB, Western blot test. *Study protocol was changed to include rapid test indeterminate patients; however, 4 individuals were excluded before this change.

positive for antibodies against all the HIV antigens by subsequent WB testing and excluded (Fig. 1). Eighty-five individuals thus fulfilled criteria for PHI; a prevalence of 2.73% (95% confidence interval [CI] 2.18 to 3.36) among all enrolled patients, and 3.02% (95% CI 2.41 to 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 (interquartile range [IQR] 20–30), 58% were female, and most (96%) presented with a nonspecific febrile syndrome to the outpatient ward including reported fever, flu, or headache (Table 1). There was no significant difference in age, sex balance, or malaria coinfection between the 85 PHI cases and the 3019 HIV-negative subjects. PHI cases reported a significantly greater total number of symptoms than the HIV-negative patients and were significantly more likely to report fever in the last 24 hours (*P*-value = 0.024) and intestinal complaint (diarrhea 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, sex, or HIV RNA between patients with PHI who returned and those who were lost to follow-up (see Table 2, Supplemental Digital Content, <http://links.lww.com/QAI/A958>). After 1 month of follow-up, median CD4 T-cell count in individuals with PHI was significantly lower than those in the non-HIV-infected group, 585 (IQR 460–682) vs. 955 (IQR 773–1149) cells/ μ L, respectively (*P*-value < 0.0001), whereas median CD8 T-cell

count was significantly higher, 1119 (IQR 741–1650) vs. 591 cells/ μ L (IQR 417–746), respectively (*P*-value < 0.0001).

Fiebig Staging of Patients With PHI

Most PHI cases (74.1%) were seronegative at presentation (Table 1), with the remainder indeterminate on rapid tests. Patient HIV-RNA levels at presentation were high, with a median of Log₁₀ 6.3copies/mL (IQR 5.2–7.3) (Table 1). Classification according to Fiebig¹² revealed that most 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, sex 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 a 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.

Kinetics of Subclass HIV-Specific Humoral Responses During PHI

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

TABLE 1. Clinic and Demographic Characteristics of Study Population According to HIV Status and Fiebig Stage

	HIV Neg at Screening (n = 3019)	PHI at Screening (n = 85)	<i>P</i> (1)	Fiebig I–III (n = 45)	Fiebig IV (n = 8)	Fiebig V (n = 12)	Fiebig VI (n = 20)	<i>P</i> (2)
Age, yrs, median (IQR)	26 (20–35)	24 (20–30)	0.083*	23 (19–32)	25 (20–35)	23 (19–31)	26 (20–28)	0.8994†
Sex, F (%)	1900 (63)	50 (58)	0.441‡	28 (62)	3 (37)	8 (67)	11 (55)	0.543‡
Initial complaint, n (%)								
Headache	80 (3)	1 (1)	0.401‡	1 (2)	0 (0)	0 (0)	0 (0)	0.395‡
Febrile syndrome	2385 (79)	75 (88)	0.039‡	41 (91)	8 (100)	11 (92)	15 (75)	
Flu symptoms	153 (5)	6 (7)	0.412‡	2 (4)	0 (0)	0 (0)	4 (20)	
Voluntary testing	395 (13)	3 (4)	0.010‡	1 (2)	0 (0)	1 (8)	1 (5)	
Others	6 (0)	0 (0)	1‡	0 (0)	0 (0)	0 (0)	0 (0)	
Fever last 24 h, n (%)	2503 (83)	80 (94)	0.024‡	43 (96)	8 (100)	11 (91)	18 (90)	0.703‡
Malaria, n (%)	337 (17)	7 (11)	0.234‡	1 (3)	0 (0)	3 (33)	3 (17)	0.049‡
Intestinal complaint last week, n (%)	84 (3)	9 (11)	<0.001‡	1 (2)	1 (12)	1 (8)	6 (30)	0.010‡
Rapid test Serostatus n (%)								
Negative	—	63 (74)	—	44 (98)	8 (100)	8 (67)	3 (15)	<0.001‡
Indeterminate	—	22 (26)	—	1 (2)	0 (0)	4 (33)	17 (85)	
VL (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†

P-value obtained from (1) comparison by study group or (2) comparison by Fiebig group.

*Continuous variables by Mann–Whitney *U* test.

†For the 2 groups, comparison and global comparison by Kruskal–Wallis test.

‡Comparisons for proportions were performed by χ^2 test.

Fiebig V and Fiebig VI as compared to Fiebig I–III and IV (Fig. 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 a total of 61 biomarkers assessed, 49 were quantifiable in more than 75% of the samples and were included for analysis (see Table 1, Supplemental Digital Content, <http://links.lww.com/QAI/A958>). Besides VL, 9 biomarkers were differentially expressed across Fiebig groups including total plasma IgG, IgG1, sCD163, MCP-1, sCD23, BAFF, MIG, TNF-related apoptosis-inducing ligand (TRAIL), and $IFN\alpha$ (P -value < 0.1, Fig. 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 VL ($\rho < -0.4$). In contrast, MCP-1, BAFF, TRAIL, and $IFN\alpha$ 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 $IFN\alpha$ $\rho = 0.47$). 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

To determine whether the subgroup of 8 biomarkers was associated with the acute preseroconversion stage of the PHI, cases were grouped into 2 categories: (1) the preseroconversion acute group roughly covering the first month of PHI (Fiebig I–IV) and (2) the postseroconversion group referring to recent PHI (Fiebig V–VI) when HIV-specific antibodies are detectable by WB.

Random forest analysis models consistently selected BAFF, MCP-1, sCD163, and MIG as having the best power to discriminate between the preseroconversion and postseroconversion phases. In addition, a biplot representation generated from PCA showed differential expression patterns for the selected biomarkers according to the grouping (Fig. 4A). Multivariate regression was performed to assess the power of the 4 cytokines to distinguish

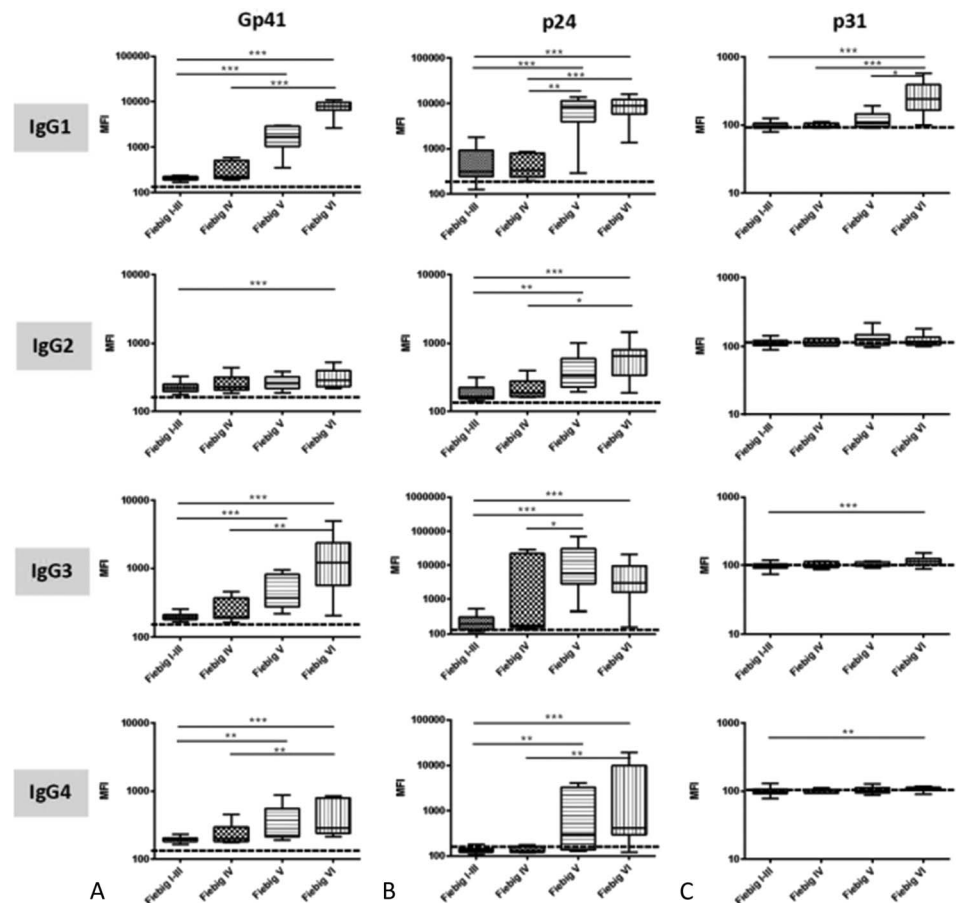
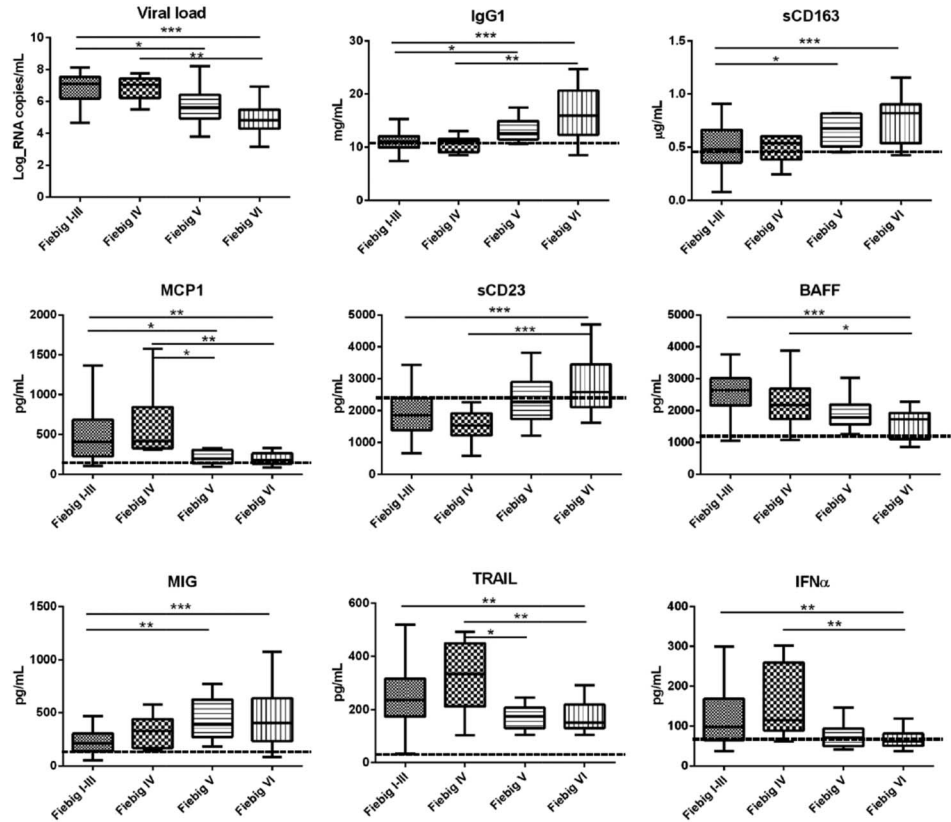


FIGURE 2. HIV-specific antibody expression according to Fiebig stage. The median MFI (mean fluorescence intensity) is shown for antibodies specific for (A) gp41, (B) p24, and (C) p31. Box as IQR, middle line as median, and whiskers as Tukey values (1.5 IQR). Values are Log_{10} scale for a better adjustment of the data. Nonparametric pairwise multiple comparisons were performed using Dunn test. Bonferroni-adjusted significance is indicated as *** if $P < 0.001$, ** if $P < 0.01$, and * if $P < 0.05$. Median values for the nonfebrile 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, MCP-1, sCD23, BAFF, MIG, TRAIL, and IFN α (P -value < 0.1), as was VL (P -value < 0.001) by Kruskal–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 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 nonfebrile non-HIV-infected reference group are indicated as dotted lines.



between the 2 categories (Fig. 4B). Based on regression diagnostics measures, 5 outlier observations were excluded from model fitting. Clinical variables (age, sex, VL, or other coinfections) 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 preseroconversion group, whereas sCD163 and MIG were negatively associated (Fig. 4B). The multivariate model was capable of correctly identifying 49 of the 53 individuals in the preseroconversion phase and 26 of the 32 in the postseroconversion phase. This goodness of fit allowed the model to identify patients in the

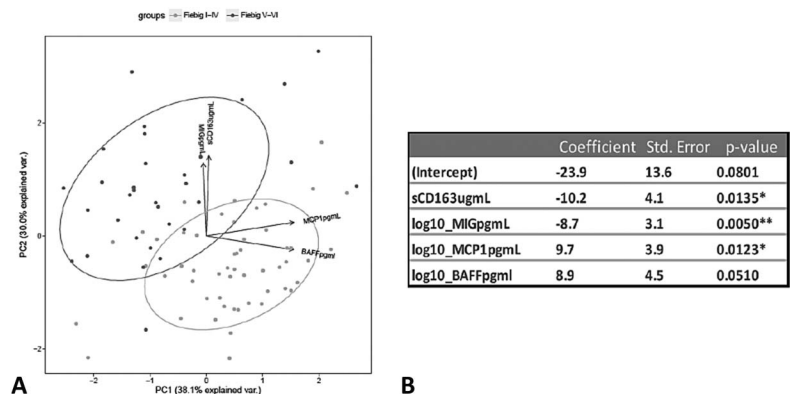
preseroconversion phase with a sensitivity of 92.5% and a specificity of 81.2%.

DISCUSSION

This study has identified a signature of 4 cytokines composed of BAFF, MCP-1, sCD163, and MIG that is highly associated with PHI before development of the HIV-specific humoral response as determined by WB.^{12,13,21} 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 antiretroviral therapy (ART) and HIV prevention activities, the 3.0% (95% CI: 2.4 to 3.7)

FIGURE 4. Cytokines with best discriminatory power to differentiate between early and late Fiebig stages. A, Component analysis of cytokine levels. PCA of BAFF, MCP-1, MIG, and sCD163 levels in patients in preseroconversion or post-seroconversion stages (Fiebig I–IV, Fiebig V–VI, respectively). B, Multivariate logistic regression to identify cytokines associated with preseroconversion 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 models, MIG, MCP-1, and BAFF values were log transformed for a better adjustment of the data.



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 with a study performed 5 years earlier which showed an PHI prevalence of 3.3% (95% CI: 1.3 to 6.7).¹⁹ 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-Envelope HIV-1 plasma antibodies are predominantly IgG1 subclass, followed by anti-Envelope IgG3 subclass during PHI.²² 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^{22,23} where they found anti-gp41 IgG3 to be positive for all individuals with PHI. 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 VL, 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 before seroconversion.^{14,16–18,24} 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, 4 of these biomarkers, BAFF, MCP-1, sCD163, and MIG, exhibited the best predictive power to distinguish between individuals in preseroconversion and postseroconversion stages of PHI. The expression profile of these 4 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. Although 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 coinfections were not found to be associated with stages 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 preseroconversion phase of PHI. In our cohort, increased preseroconversion 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. MCP-1 and BAFF are both produced by monocytes, macrophages, and dendritic cells after infection or tissue inflammation to assure a proper immune response and maintenance of normal immunity.^{25–27} In contrast, macrophage low-grade inflammation marker (sCD163) and MIG are cytokines involved in T-cell trafficking to inflammatory sites and control of T-cell proliferation.^{28–30} Because 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 nonhuman primates may show whether BAFF and MCP-1 expression are associated with viral shedding or early HIV-induced gut-associated lymphoid tissue 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.^{7,31–33} Recent studies also point out that ART initiated during early AHI either prevents loss of (at Fiebig stage I/II) or restores (at Fiebig stage III) mucosal Th17 cells.³⁴ Consequently, very early ART is associated with normalization of local and systemic immune activation, reversing a hallmark of HIV pathogenesis.³⁴ 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.^{35,36}

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