

Plasma Cytokine Profiles as Predictive Biomarkers of HIV and Aids Progression among HIV Patients Attending Nakuru Provincial General Hospital, Kenya

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Abstract Introduction: Cytokines are produced by many cell types, mostly cells of the immune system, and act on diverse targets, often the white blood cells. They play a central role in the pathogenesis of many diseases including Acquired Immunodeficiency Disease Syndrome (AIDS). They reflect the local or systemic inflammatory setting, and could serve as predictive biomarkers in Human Immunodeficiency Virus (HIV) disease progression.

Objective: The aim of this study was to identify cytokines measurable in blood plasma in recently diagnosed HIV individuals before the commencement of antiretroviral therapy. **Study Population:** Eighty individuals, both males and females, were recruited for this study that comprised of forty newly diagnosed with HIV-1; twenty HIV negative individuals; and twenty HIV positive individuals currently on highly active antiretroviral therapy (HAART).

Method: Cytokines were measured using multiplex cytokine immunoassay. Five types of cytokines were detected. Data analyses were performed using Graph Pad Prism 6. Independent sample T tests were used to compare the cytokine means while Spearman Rank tests were used to test for correlations. Statistical analysis were done using SPSS version 17. **Results:** The study showed significantly ($p < 0.001$) higher levels of IL-12p70, TNF, IL-10, IL-6 and IL-1 β among the newly diagnosed HIV patients compared to those on highly antiretroviral therapy and HIV negative patients. **Conclusion:** Identification of plasma cytokines could be useful predictive biomarkers of HIV disease progression.

Keywords: plasma cytokines, human immunodeficiency virus, acute HIV infection, predictive biomarkers, HIV disease progression

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1. Introduction

Human immunodeficiency virus, the virus that causes acquired immunodeficiency syndrome has become one of the world's most serious health and development challenges [9]. Since the start of the epidemic, around 78 million people have become infected with HIV and 39 million people have died of AIDS-related illnesses. In 2013, there were 35 million people globally living with HIV of which 24.7 million were from sub-Saharan Africa [10]. Worldwide, 2.1 million people became newly infected with HIV in 2013. There were an estimated 1.5 million new HIV infections in sub-Saharan Africa, accounting for almost 70% of the global total of new HIV infections [10]. The total number of people living with HIV in Kenya was estimated to be 1.6 million in 2013. Children under 15 years of age account for 12% of all people living with HIV. An estimated 58% (815,630) of all adults aged 15+ living with HIV in Kenya are women

[12]. Worldwide, 2.1 million people became newly infected with HIV in 2013. There were an estimated 1.5 million new HIV infections in sub-Saharan Africa, accounting for almost 70% of the global total of new HIV infections [10], while in Kenya there were approximately 88,620 new HIV infections that occurred among adults and 12,940 among children in 2013 [12].

The Human Immunodeficiency Virus (HIV) targets the immune system and weakens the surveillance and defense system of the body against infections, resulting in HIV infected individuals becoming more susceptible to a wide range of infections normally cleared by the immune system of a healthy individual [26]. There is need to monitor HIV disease progression during the early stages of infection in order to advise and counsel individuals affected and not accepting their status, and consequently to avoid further spread and also it could guide future treatment of the disease.

Cytokines are soluble proteins that play an important role in immunity, inflammation, and hematopoiesis. They are rapidly produced by a variety of cell types and

secreted in response to specific and non-specific stimuli [13]. They are effector molecules that can instantly alter the quality of the immune response. The effect of a particular cytokine on a given cell depends on the cytokine, its extra cellular abundance, the presence (or absence) of the complementary receptor on the cell surface, and downstream signals activated by receptor binding [19].

Cytokines are regulators of host responses to infection, immune responses, inflammation, and trauma. Some cytokines act to make disease worse (pro-inflammatory cytokines), whereas others serve to reduce inflammation and promote healing (anti-inflammatory cytokines). CD4+ T helper cells play a vital role in the immune system by secreting cytokines, which regulate the immune response. Cytokines are secreted by T cells when an intracellular infection is detected as in case of HIV infection. HIV is known to infect T cells that have CD4+ receptors present on their surface. These cells, among others in the immune system, secrete cytokines that are crucial for fighting off infections and in other immune responses. However, they can become dysregulated and pathological in inflammation, trauma, and sepsis [3].

Cytokine profiles are highly relevant parameters of an immune response. Different cytokines possess biologically overlapping functions, and they have the ability to regulate production of other cytokines. Therefore, analysis of production and function of the complete set of cytokines expressed during acute HIV infection may be valuable in predicting disease progression. Quantification of cytokine produced is a valuable adjunct in standard immunological assays for defining several pathologic processes. There may be alterations in the synthesis of host cytokines following HIV infection, thereby favoring successful survival of the virus inside the host and enhancing the susceptibility of the host to opportunistic infections. Most cytokines exert pleiotropic and sometimes contrasting effects on the immune response [11]. The concentration of cytokines in blood plasma can provide valuable information about the immune status of infected individuals. Measurement of cytokine levels may also be of use in the monitoring of disease progression and / or inflammation; this type of study has not been done in this region.

2. Materials and Methods

2.1. Study Site and Study Population

The study was carried out in the Nakuru Provincial General Hospital (PGH), Nakuru County, Kenya, after the approval by the hospital's administration. Consent was sought from the study group by the researcher with the assistance and guidance of the staff of the Centre for Comprehensive Care (CCC) before commencement of the study. Only those who gave consent were enrolled in the study and they completed a written informed consent form in accordance with the Helsinki Declaration [27].

2.2. Study Design and Sampling

This was a prospective cross sectional study that involved selecting consenting individuals who attended

the Voluntary counseling and Testing centre (VCT) and the Centre for Comprehensive Care at the hospital. Eighty consenting male and female of different ages were sampled according to Yamane [28] to participate in the study and were later subdivided into three groups as recommended by Sudman [23]. Forty patients that included sixteen males and twenty four females were sampled from those recently diagnosed with HIV before they started antiretroviral treatment (treatment naïve HIV patients); twenty patients that included four males and sixteen females were sampled from HIV positive patients that were receiving treatment with highly antiretroviral therapy (HAART) while twenty participants that included nine males and eleven females were sampled from HIV negative individuals.

2.3. Plasma Collection

Whole blood was collected into EDTA treated tubes, stored at room temperature and then centrifuged for 10 minutes at 3000 revolution per minute (r.p.m). Plasma was pipetted and aliquoted into cryovials that were labeled with appropriate information of the individuals in the study group. The plasma was refrigerated at -20°C until when the entire cohort had been collected in order to analyze all the samples simultaneously.

2.4. Plasma Cytokines Determination

All the eighty labeled plasma samples were thawed before flow cytometry for cytokine detection. The types and quantities of cytokines were detected by flow cytometry using a multiplex assay system that included Becton and Dickinson Cytometric Bead Array (BD CBA), Human Inflammatory Cytokine kit and Becton and Dickinson Fluorescence Activated Cell Sorter (FACSCalibur) flow cytometer (BD Biosciences, U.S.A). The workflow consisted of the following steps according to the recommended procedure (BD Biosciences, U.S.A):

2.4.1. Preparing Human Inflammatory Cytokines Standards

A vial of lyophilized Human Inflammatory Cytokine Standard was opened and the standard spheres transferred to a 1 ml polypropylene tube. The tube was labeled "Top Standard" (Tube 10). The standards were reconstituted with 2 ml of assay diluent and allowed to equilibrate for 15 minutes at room temperature. The reconstituted protein was gently mixed by pipette. Eight 12x75 mm tubes were labeled and arranged in the following order: 1:2 (Tube 9; 2,500 pg/mL), 1:4 (Tube 8; 1,250 pg/mL), 1:8 (Tube 7; 625 pg/mL), 1:16 (Tube 6; 312.5 pg/mL), 1:32 (Tube 5; 156 pg/mL), 1:64 (Tube 4; 80 pg/mL), 1:128 (Tube 3; 40 pg/mL) and 1:256 (Tube 2; 20 pg/mL). 300 µl of assay diluent was pipetted in each of the tubes. Serial dilution was performed by transferring 300µl from the Top Standard to the 1:2 dilution tube and mixed thoroughly by pipette, and continued by transferring 300µl from 1:2 tube to the 1:4 tube until to the 1:256 tube. One tube was prepared containing only assay diluent to serve as the 0 pg/mL negative control. This was labeled as Tube 1 and had no standard dilution. The standard curve for each protein covers a defined set of concentrations from 20 to 5,000 pg/mL (BD Biosciences, U.S.A).

2.4.2. Mixing Human Inflammatory Cytokine Capture Beads

Six Capture Beads that had been bottled individually were pooled before using them in the assay. Each capture bead suspension was vortexed before mixing. 10 μ l aliquot of each capture bead, for each assay tube to be analyzed, was added into a single tube labeled "Mixed Capture Beads". Eighty assay tubes were to be analyzed and so the mixture comprised of the following capture beads: 800 μ l (10 x 80) of interleukin 8 (IL-8), 800 μ l of interleukin 1 β (IL-1 β), 800 μ l of interleukin 6 (IL-6), 800 μ l of interleukin 10 (IL-10), 800 μ l of tumor necrosis factor (TNF) and 800 μ l of interleukin 12p-70 (IL-12p70). The mixture of capture beads was vortexed thoroughly and was ready for transfer to the assay tubes.

2.4.3. Performing the Human Inflammatory Cytokine Assay

After preparing the standards and mixing the capture beads, the next step was to perform the assay. The mixed capture beads were vortexed and 50 μ l added to all assay tubes. 50 μ l of the sample was added to eighty labeled tubes each containing about 12.5 μ l of plasma that included forty from HIV positive patients, twenty from HIV negative and twenty from HIV positive patients on HAART. The assay tubes were incubated for 1.5 hours at room temperature. After the incubation period, 1 ml of wash buffer was added to each assay tube and centrifuge at 200g for 5 minutes; the supernatant was aspirated and discarded. 50 μ l of the human inflammatory cytokine phycoerythrin (PE) detection reagent was added to all assay tubes and incubated for 1.5 hours at room temperature. After this second incubation period, again 1 ml of wash buffer was added to each assay tube and centrifuged at 200g for 5 minutes; the supernatant was aspirated and discarded from the assay tubes and finally 300 μ l of wash buffer was added to each assay tube to resuspend the bead pellet.

2.4.4. Sample Acquisition and Analysis of Data

The samples were acquired on the flow cytometer. The assay setup procedure was followed according to the protocol by the manufacturers (BD Biosciences, U.S.A) and the appropriate acquisition template was available. Each sample was vortexed for 3-5 seconds immediately

before acquiring on the flow cytometer. Tube 1 (0 standard) was vortexed and ran in setup mode according to the protocol. Sample acquisition continued by running Tube 2 (20 pg/mL), followed by Tube 3 (40 pg/mL), and so on throughout Tube 10 (Top Standard). The test samples were run after the standards. Data for the detection of individual proteins was analyzed using BD Cell Quest software, according to the recommended protocol (BD Biosciences, U.S.A).

2.4.5. Data Analysis

Descriptive statistics were applied. Data analyses were performed using Graph Pad Prism 6. Independent sample T tests were used to compare the cytokine means while Spearman Rank tests were used to test for correlations. Statistical analysis were done using SPSS version 17.

3. Results

3.1. Types and Quantities of Plasma Cytokines

Five types of plasma cytokines: Interleukin 12p70 (IL-12p70), Tumor Necrosis Factor (TNF), Interleukin 10 (IL-10), Interleukin 6 (IL-6) and Interleukin 1 β (IL1 β) were detectable in the study population. IL-12p70, TNF, IL-10 and IL-6 were detected in treatment naïve HIV patients; in HIV positive patients on HAART and in HIV negative patients. IL-1 β was detected in treatment naïve HIV patients and in HIV positive patients on HAART but none was detectable in HIV negative patients (Table 1).

The mean concentrations of IL-12p70 (3.317 \pm 4.441 pg/mL), TNF (7.707 \pm 13.40 pg/mL), IL-10 (2.794 \pm 4.437 pg/mL) and IL-6 (6.629 pg/mL) were higher in the treatment naïve HIV patients compared to the HIV positive patients on HAART (0.5593 \pm 1.397 pg/mL, 1.975 \pm 4.980 pg/mL, 0.6918 \pm 1.628 pg/mL and 2.602 \pm 4.620 pg/mL and the HIV negative patients (0.2815 \pm 0.5145 pg/mL, 0.7895 \pm 2.247 pg/mL, 0.1630 \pm 0.5032 pg/mL, 0.5135 \pm 1.206 pg/mL) respectively. The mean concentration of IL-1 β (5.401 \pm 10.14 pg/mL) was higher in the treatment naïve HIV patients compared to HIV positive patients on HAART (0.7028 \pm 4.445 pg/mL). There was no detectable IL-1 β in HIV negative patients (Table 1).

Table 1. Plasma Cytokine Profiles of the Study Population

	Treatment naïve HIV patients (N=40; 50%)	HIV Positive patients on HAART (N=20; 25%)	HIV Negative patients (N=20; 25%)
	Quantity (Mean \pm SD)	Quantity (Mean \pm SD)	Quantity (Mean \pm SD)
IL12p70 pg/mL	3.317 \pm 4.441	0.5593 \pm 1.397	0.2815 \pm 0.5145
TNF pg/mL	7.707 \pm 13.40	1.9750 \pm 4.980	0.7895 \pm 2.247
IL-10 pg/mL	2.794 \pm 4.437	0.6918 \pm 1.628	0.1630 \pm 0.5032
IL-6 pg/mL	6.629 \pm 8.621	2.6020 \pm 4.620	0.5135 \pm 1.206
IL-1 β pg/mL	5.401 \pm 10.14	0.7028 \pm 4.445	0.0000

3.2. Comparison between Plasma Cytokines Levels among the Study Population

The means of plasma cytokines of treatment naïve HIV positive patients and HIV negative patients were compared. The treatment naïve HIV patients had significantly ($p < 0.001$) high means of IL-12p70, TNF, IL-10, IL-6 and IL-1 β compared to HIV negative patients (Table 2).

Table 2. Means of blood cytokines of HIV patients and HIV negative patients and associated p-values

	Treatment naïve HIV patients (Mean ± SD)	HIV Negative patients (Mean ± SD)	P-value
IL12p70pg/mL	3.317± 4.441	0.2815± 0.5145	p<0.001
TNF pg/mL	7.707± 13.40	0.7895± 2.247	p<0.001
IL-10 pg/mL	2.794± 4.437	0.1630± 0.503	p<0.001
IL-6 pg/mL	6.629± 8.621	0.5135± 1.206	p<0.001
IL-1βpg/mL	5.401± 10.14	0.0000	p<0.001

The means of plasma cytokines of treatment naïve HIV patients and HIV patients on HAART were also compared. The treatment naïve HIV patients had significantly

(<0.001) high means of 12p70, TNF, IL-10, IL-6 and IL-1β compared to HIV patients on HAART (Table 3).

Table 3. Means of plasma cytokines of treatment naïve HIV patients and HIV patients on haart and associated p-values

	Treatment naïve HIV patients Mean ±SD	HIV patients on HAART Mean ±SD	P-value
IL12p70pg/mL	3.317± 4.441	0.5593±1.397	p<0.001
TNF pg/mL	7.707± 13.40	1.9750±4.980	p<0.001
IL-10 pg/mL	2.794± 4.437	0.6918±1.628	p<0.001
IL-6 pg/mL	6.629± 8.621	2.6020±4.620	p<0.001
IL-1βpg/mL	5.401± 10.14	0.7028±4.445	p<0.001

Finally, the means of plasma cytokines of HIV negative patients and HIV patients on HAART were compared. HIV patients on HAART had significant high means of TNF (<0.05) and IL-6 compared to HIV negative patients

(<0.05; Table 4). IL-12p70 (p=0.079) and IL-10 (p=0.008) were significant in HIV patients on HAART; IL-β (p=0.154) was the least significant in this group compared to HIV negative patients (Table 4).

Table 4. Means of plasma cytokines of HIV negative patients and Hiv patients on haart and associated p-values

	HIV negative patients Mean ±SD	HIV patients on HAART Mean ±SD	P-value
IL12p70pg/mL	0.2815±0.515	0.5593±1.397	p=0.079
TNF pg/mL	0.7895±2.247	1.9750±4.980	p=0.079
IL-10 pg/mL	0.1630±0.5032	0.6918±1.628	p=0.008
IL-6 pg/mL	0.5135±1.26	2.6020±4.620	p=0.001
IL-1βpg/mL	0.0000	0.7028±4.445	p=0.154

4. Discussion

Plasma interleukin 12p70 (IL-12p70), tumor necrosis factor (TNF), interleukin 10 (IL-10), interleukin 1β (IL-β), interleukin 6 (IL-6), and interleukin 1β (IL-β) were detected in the study population. The treatment naïve HIV patients had higher concentration levels of all cytokines detected. Reduced concentration levels of the cytokines detected in this study was observed in patients on antiretroviral therapy, an indication that treatment of HIV with antiretroviral therapy may have had some effects on cytokine production. The HIV negative patients had the lowest levels of detectable cytokines. There was no detectable IL-1β in these patients. These individuals may have been suffering from other diseases other than HIV that made them seek medical attention. This finding indicate varied immune responses among the study population and agrees with a study that suggested that cytokine levels are indicative of the nature of the immune response in a particular individual [3].

The study showed that the treatment naïve HIV patients had significantly higher (p=<0.001) concentration levels of IL-12p70 compared with HIV negative individuals and with HIV patients on HAART (p=0.001). The mean concentration levels of IL-12p70 of HIV patients on HAART was higher than in the HIV negative

patients though not significant (p=0.079). This observation differs from a study by Tudela *et al.*, [24] who found lowered concentrations of IL-12p70 in HIV positive participants but agrees with a study by Lindi *et al.*, [15] who found higher levels of IL-12p70 during acute HIV infection.

This study showed that treatment naïve HIV patients had significantly high concentration levels of IL-10 (p=<0.001) compared to HIV negative patients. The treatment naïve HIV patients also had significantly high concentration levels of IL-10 (p=0.001) compared to HIV patients on HAART. This observation is in agreement with a study by Tudela *et al.*, [24] who observed significant increase in IL-10 in participants positive for HIV infection. Lindi *et al.*, [15] also observed and increase in IL-10 concentration levels during acute HIV infection. Stylianou *et al.*, [22] also demonstrated that HIV-infected patients had significantly higher circulating IL-10 levels; the same study found a significant fall in IL-10 levels during HAART and observed that HAART had an effect on IL-10 levels. In this study, the HIV patients on HAART had slightly higher concentration levels of IL-10 (p=0.008) compared to HIV negative patient. This is in agreement with a study by Haissman *et al.*, [8] who found only slightly lower levels of cytokines in HIV uninfected compared to individuals on HAART. While IL-10 is a potent down-regulator of TNF production, TNF is in itself a stimulus for IL-10 release from various cells. The

balance between these two cytokines has been suggested to be of importance in the pathogenesis of HIV infection.

In this study, the treatment naïve HIV patients were found to have significantly higher concentration of TNF ($p < 0.001$) compared to HIV negative patients. The HIV negative patients had a significant lower concentration of TNF ($p = < 0.05$) compared with HIV patients on HAART. This observation agrees with a study by Lindi *et al.*, [15] who found increased concentration of TNF in recently HIV infected individuals compared with HIV uninfected. Haissman *et al.*, [8] also found elevated levels of TNF in HIV infected individuals. In this study, the treatment naïve HIV patients were also found to have significantly high concentration of TNF (< 0.001) compared to HIV patients on HAART. This is in agreement with Haissman *et al.*, [8] who observed a decrease in TNF levels in HIV patients upon administration of antiretroviral therapy.

Interleukin 6 (IL-6) has been found to serve as a growth factor for the HIV [24]. High plasma interleukin-6 levels are associated with morbidity and mortality in HIV patients [4, 14, 6]. In this study, the treatment naïve HIV patients were found to have significantly higher levels of IL-6 ($p = < 0.001$) compared with HIV negative patients and also compared with HIV patients on HAART ($p = < 0.001$). The HIV negative patients were found to have significantly low concentration levels of IL-6 ($p = < 0.001$) compared with HIV patients on HAART. This observation agrees with Tudela *et al.*, [24] who found significant increase of IL-6 in participants positive for HIV infection and another one by Haissman *et al.*, [8] who also observed significantly higher levels of IL-6 in HIV infected individuals compared with HIV-uninfected. Haissman *et al.*, [8] also observed that IL6 decreased after administration of antiretroviral therapy. Plasma levels of IL-6 were also observed to be higher in HIV infected individuals in a study by Martyn *et al.*, [17].

IL-1 β plays a crucial role in triggering the immune response during various diseases. It has been shown that IL-1 β is an important mediator during systemic [21, 20] as well as local inflammation [2], and it may also be involved in the development of autoimmune diseases [1]. This cytokine is thought to have an inducing effect on Kaposi's sarcoma in association with acquired immunodeficiency syndrome [16] and possibly contributes to several other secondary complications during the course of HIV disease, such as neurological [25] and haematological disorders [18]. Additionally, it has been shown that IL-1 can induce HIV viral replication [5,7]. In this study, the detectable IL-1 β concentration levels in treatment naïve HIV patients was significantly higher ($p = 0.001$) than in the HIV negative patients and in HIV patients on HAART ($p = < 0.001$). The HIV patients on HAART had higher IL-1 β levels ($p = 0.154$) compared with HIV negative patients. These findings agree with Lindi *et al.*, [15] who found increased levels of IL-1 β during acute HIV infection compared with pre-infection samples but differ with an observation by Martyn *et al.*, [17] who found lower levels of IL-1 β in HIV infected individuals compared with controls.

5. Conclusions

Higher levels of plasma cytokines characterized the period of early human immunodeficiency virus infection,

an indication of the hosts' response to the disease. Human immunodeficiency virus (HIV) promotes plasma cytokine production that could be used to predict subsequent disease progression. Tumor necrosis factor (TNF) and interleukin - 6 (IL-6) are more informative biomarkers of HIV disease progression. Higher plasma cytokine levels were observed among the younger treatment naïve HIV patients and there was no much difference in cytokine production within the gender. Concentration levels of plasma cytokines produced were altered by administration of antiretroviral therapy.

6. Recommendations

Tumor necrosis factor (TNF), Interleukin 6 (IL-6) and Interleukin 1 β (IL-1 β) can be useful biomarkers in HIV disease progression.

Further Research

Investigating other biomarkers that can be detectable on other samples, other than blood, that could be useful in HIV disease progression, particularly in resource limited set ups.

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Conflicting Interest

The authors declare that there is no existing conflicting interest.

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