IMMUNOLOGICAL PROFILES IN HIV POSITIVE PATIENTS FOLLOWING HIGHLY ACTIVE ANTIRETROVIRAL TREATMENT INITIATION IN KIGALI-RWANDA

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Thesis Submitted in Partial Fulfilment for the Degree of Masters of Science in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature	Date

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This thesis has been submitted for examination with our approval as University Supervisors:

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DEDICATION

This work is dedicated to all HIV positive children.

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ABBREVIATIONS/ACRONYMS

AIDS:	Acquired Immune Deficiency Syndrome				
APC:	Antigen Presenting Cell				
ART:	Antiretroviral Therapy				
BecA-ILRI:	Bioscience East and Central Africa-International Livestock Research Institute				
CCL:	Chemokine Ligand				
CCR5:	Chemokine Receptor-5				
CXCR4:	C-X-C Chemokine Receptor Type 4				
CD:	Cluster of Differentiation				
CDC:	Centers for Disease Control and Prevention				
cDNA:	Complementary DNA				
COBAS:	Complete BioAnalytical System				
Cys:	Cysteine				
GM-CSF:	Granulocyte-Macrophage Colony-Stimulating Factor				
HAART:	Highly Active Antiretroviral Treatment				
HIV:	Human Immune Deficiency Virus				
IFN-γ:	Gamma Interferon				
IL:	Interleukin				
IL-10R:	Interleukin-10 Receptor				

JKUAT:	Jomo Kenyatta University of Agriculture and Technology				
mRNA:	messenger Ribonucleic Acid				
NISR:	National Institute of Statistics of Rwanda				
NK:	Natural Killers				
PD-1 :	Programmed Death 1 Receptor				
RBC:	Rwanda Biomedical Center				
RDHS:	Rwanda Demographic and Health Survey				
RNA:	Ribonucleic Acid				
RNEC:	Rwanda National Ethics Committee				
Th:	T helper				
TNF-α:	Tumor Necrosing Factor alpha				
TGF-b:	Transforming Growth Factor beta				
UNAIDS:	United Nations Programme on HIV/AIDS				
UR:	University of Rwanda				

ABSTRACT

Changes in the cytokine networks impact HIV pathogenesis, latency and persistence. Although several studies have been done to explain these interactions, they are yet to be fully understood. In this study, levels of Interleukin-10, ILnterleukin-2, Interferon-gamma and the current markers of HIV disease progression namely CD4 count and viral load; were assessed and correlated before HAART and at six months of treatment in 33 HIV⁺ patients. The aim of the study was to assess the shift from Th1 to Th2 profile in the course of HIV disease particularly in relation to HAART in Kigali. Viral load was measured using the COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test while CD4 count and cytokines measurement were done by flow cytometry and ELISA respectively. Following HAART, there was a drop in viral load (though only a small number of patients achieved an undetectable viral load); recovery of CD4⁺ cells, a decrease in IL-10 (but remained high for many patients); and an increase in IL-2 and IFN-y. CD4 count correlated negatively with viral load and IL-10 (but r < -0.5). IL-10 showed significant positive correlation with viral load at both time points (r > 0.5, p values <0.05). CD4 count did not show a statistically significant correlation with IL-2 and IFN- γ (p values >0.05). Results from this study demonstrated the down-regulatory effect of IL-10 on Th1 cytokines and that a shift from Th1 to Th2 cytokine profile is associated with progression of HIV disease. Successful HAART results in drop in viraemia and IL-10 with up-regulation of Th1 cytokines and CD4+ cells recovery. The findings from this study indicate potential usefulness of IL-10 as a marker of HIV disease progression. From the findings of the present study, it is recommended that further studies should be done to support the findings. Moreover, reference values for cytokines need to be determined.

CHAPTER ONE

1.0 INTRODUCTION

1.1. Background information

Global statistics from UNAIDS (UNAIDS, 2014) indicate that at the end of 2013, 35 million people were living with human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS). In the same year, new infections and deaths from the HIV/AIDS were 2.1 million and 1.5 million people respectively. Work undertaken by the National Institute of statistics of Rwanda (NISR), in the 2010 RDHS, indicated that in Rwanda, HIV prevalence was 3.7% and 2.2% among women and men, respectively (NISR, 2011). The molecular mechanisms causing the pathogenesis of HIV infection are still not well understood and are probably a composite of multiple factors (Catalfamo *et al.*, 2012; Mir *et al.*, 2012).

Sub-Saharan Africa has the most serious HIV and AIDS epidemic in the world. In 2012, roughly 25 million people were living with HIV, accounting for nearly 70% of the global total. In the same year, there were an estimated 1.6 million new HIV infections and 1.2 million AIDS-related deaths (UNAIDS, 2013). As a result, the epidemic has had widespread social and economic consequences, not only in the health sector but also in education, industry and the wider economy.

In 2013, there were 111,442 people on HAART in Rwanda (PEPFAR, 2014). Despite effective ART, HIV positive patients have higher morbidity and mortality rates attributed to chronic inflammation and immune system dysfunction (Reus *et al.*, 2013). Studies show that patients who test positive for HIV should undergo a biological follow up to assess the infection progression as well as response to antiretroviral therapy (ART), using periodic CD4 count and the plasma viral load (Viana *et al.*, 2011). In some patients, CD4 count does not represent a sensitive marker as it doesn't correlate with viral load which is also expensive to determine (Prabhakar *et al.*, 2011). Therefore, there is need to investigate and identify alternative markers easily affordable and that give a better correlation with disease

progression. Recent studies suggest a powerful prognostic value for blood cytokine levels in different diseases (Bortolin *et al.*, 2012).

Tumor necrosis factor alpha (TNF- α), interferon gamma (IFN- γ), Intereukin-1 (IL-1), Interleukin-2 (IL-2), Interleukin-6 (IL-6) and Interleukin (IL-10) are some of the cytokines affected during HIV infection. Analysis of these cytokines may give an indication of the degree of immune activation, the extent of the immune response and disease progression (Williams *et al.*, 2013). The ability of several cytokines to interfere with the molecular mechanisms responsible for HIV latency makes them attractive candidates for therapeutic strategies aimed at reducing the pool of latently infected cells (Vandergeeten *et al.*, 2012). Understanding the molecular mechanisms of the disruption of this network of cytokines may prove critical in devising novel therapeutic strategies for the HIV treatment and restoring immune responsiveness. Such efforts are desperately needed since current HAART regimens are inadequate to clear HIV from the body, and have debilitating side effects over the long-term (Imami *et al.*, 1999; Sirskyj *et al.*, 2008).

1.2. Problem statement

The plasma viral load and CD4 count are the current most relied on markers to monitor HIV disease progression. The viral load gives more direct and immediate picture of the response to antiretroviral treatment compared to CD4 count and in some patients CD4 count does not correlate with the disease progression. The implementation of these tests especially the viral load in a sufficient number of health facilities is still a challenge. For instance, the viral load measurement services are only offered from a limited number of hospitals due to the test laboriousness, requirement for highly skilled personnel as well as advanced and expensive equipment. In 2013, there were only two sites in Rwanda namely The National Reference Laboratory and the Centre Hospitalier Universitaire de Butare where HIV plasma RNA could be quantified. There is need for other easily implementable and affordable alternative markers to assess the progression of HIV disease. Cytokines are involved in regulating immune response to HIV as well as HIV replication. Their levels may give a picture of the rate of viral replication as well as the damage or restoration of immune system.

Human immunodeficiency virus (HIV)/AIDS DALYs for countries where HIV/AIDS is the leading cause or in the top five leading causes of burden accounts for 44.6 and 75.2% of global HIV/AIDS DALYs, respectively in 2010. Sub-Saharan African countries, in particular, dominate the proportion of global HIV/AIDS DALYs; in 2010, the 47 countries in this region contributed to 70.9% of global DALYs attributable to HIV/AIDS (Ortblad *et al.*, 2013).

1.3. Justification of the study

Currently, viral load and CD4 count are the only markers employed for HIV disease progression assessment. Though less expensive compared to viral load, CD4 cells count does not always correlate with the viral load and disease progression and this brings need for affordable alternative markers to assess the effects of treatment as well as disease progression. Although several studies have been done to detail cytokine interactions in relation to HIV disease, these interactions are yet to be fully understood. In this study, the levels of CD4⁺ cells, IL-2, IL-10, and IFN- γ were assessed and correlated with viral load at the start of HAART and six months after HAART initiation among HIV positive patients in Kigali/Rwanda, where no such studies have been carried out. This study will contribute to a better understanding of the changes and interactions between various cytokines in the course of HIV infection; in relation to treatment. This study may also lead to identification of additional and hopefully more affordable markers for assessing HIV disease prognosis in relation to treatment.

1.4. Research questions

- How does the viral load change following HAART among HIV infected patients in Kigali?
- 2. How does CD4 count change following HAART among HIV infected patients in Kigali?
- How do IL-2, IL-10 and IFN-γ change following HAART among HIV infected patients in Kigali?
- 4. What is the correlation between the CD4 count, IL-2, IL-10, IFN-γ and the viral load before and six months after HAART initiation among HIV infected patients in Kigali?

1.5. Main Objective

To determine the immunological profiles of HIV positive patients following initiation of HAART in Kigali/Rwanda

1.6 Specific objectives

- 1. To determine the viral load in HIV positive patients before HAART and at six months after HAART initiation in Kigali/Rwanda.
- 2. To determine the levels of CD4⁺ in HIV positive patients before and at six months of HAART initiation.
- 3. To determine the levels of IL-2, IL-10, IFN-γ cytokines in HIV positive patients before and at six months of HAART initiation.
- 4. To determine the correlation between viral load levels, $CD4^+$ T cells, levels of IL-2, IL-10, IFN- γ cytokines before and at six months of HAART initiation.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Human Immunodeficiency virus

Human Immunodeficiency virus evolved as a human pathogen very recently relative to most other known human pathogens. However, the degree of morbidity and mortality caused by HIV and the global impact of HIV infection on health care resources and economics are already enormous and continue to grow. Approximately 70% of all people infected by HIV are in Africa and 20% in Asia, and almost 3 million die of the disease every year. It is estimated that there are approximately 14,000 new infections every day (Abul *et al.*, 2007).

Human Immunodeficiency Virus is a lentivirus belonging to the family of retroviridae and is classified into two types, HIV-1 and HIV-2. Human Immunodeficiency Virus-1 comprises of groups M (major), N (non-M non-O), and O (outlier). Group M of HIV-1 accounts for the vast majority of HIV infections worldwide. Due to a high level of genetic diversity, group M is further sub-classified into subtypes (A to D; F to H; J, and circulating recombinant forms (Catherine, 2007; Fischetti *et al.*, 2004). While HIV-2 remains essentially confined to West Africa, HIV-1 spreads around the world (Fischetti *et al.*, 2004).

The HIV-1 virion is an enveloped structure containing 72 external spikes formed two major viral envelope proteins, gp120 and gp41. The HIV-1 lipid bilayer is also studded with various host proteins, including class I and class II major histocompatibility complex molecules, acquired during virion budding (Charles *et al.*, 2001; Brian *et al.*, 2008). The cone-shaped core of HIV-1 contains four nucleocapsid proteins which are p25, p17, p9, p7. The phosphorylated p25 polypeptide forms the chief component of the inner shelf of the nucleocapsid, whereas the p17 protein is associated with the inner surface of the lipid bilayer and stabilizes the exterior and interior components of the virion. The p7 protein binds directly to the genomic RNA through a zinc-finger structural motif and together with p9 forms the nucleoid core (Jay, 1993). The retroviral core contains two copies of linear, positive-sense single-stranded HIV-1 genomic RNA that is associated with the various

preformed viral enzymes, including the reverse transcriptase, integrase, ribonuclease, and protease (Prescott *et al.*, 2002).



Figure 2.1: HIV structure: The structure of HIV shows an enveloped virus with the two major envelope glycoproteins (gp120 and gp 41), two segments of genomic RNA and associated enzymes as well as other important viral proteins (adapted from Prescott *et al.*, 2002).

Human Immunodeficiency virus can be transmitted by sexual contact, exposure to infected blood or blood products, or by an infected mother to her fetus (Jay, 1993). Diagnosis of the infection is based on detection of antibodies against the virus or detection of viral proteins and nucleic acid (Benjamini & Leslowitz, 1991).

Entry into the host cell begins when the envelope fuses with the plasma membrane of a CD4 positive cell through interaction between viral gp120, gp41 and the host cell CD4 (including CD4⁺ helper T cells, macrophages, and dendritic cells) receptor-CCR5/CXCR4 co-receptors, which is followed by release of viral core and two RNA strands into the cytoplasm of the target cell (Siegert *et al.*, 2005).



Figure 2.2: Interaction between HIV envelope glycoproteins and target cell membrane. Human Immunodeficiency virus envelope glycoprotein 120 and glycoprotein 41 and cell surface receptors (CD4 marker)/co-receptors (CCR5 or CXCR4) before entering a CD4⁺ T lymphocyte (adapted from Abul *et al.,* 2007).

Inside the infected cell, the core protein remains associated with the RNA as it is copied into a single strand of DNA by the RNA/DNA-dependent DNA polymerase activity of the reverse transcriptase enzyme. The RNA is next degraded by another reverse transcriptase component, ribonuclease H, and the DNA strand is duplicated to form a double-stranded DNA copy of the original RNA genome. A complex of the double-stranded DNA (the provirus) and the integrase enzyme moves into the nucleus (Brian *et al.*, 2008). Then the proviral DNA is integrated into the cell's DNA through a complex sequence of reactions catalyzed by the integrase and this integrated provirus can remain latent, giving no sign of its presence or alternatively it can force the cell to synthesize viral mRNA for viral genome and production of viral proteins by the cell's own ribosomes. Viral proteins and the complete HIV-1 RNA genome are then assembled into new virions that bud from the infected host cell that eventually lyses (Hogg, 2005).



Figure 2.3: HIV replication cycle. Human Immunodeficiency virus replication cycle showing viral attachment to a $CD4^+$ cell, entry in the cell, reverse transcription of genomic RNA by reverse transcriptase, integration of proviral DNA into host cell genome, synthesis of viral proteins as well as viral genomic RNA, assembly of viral components and release of new virions by infected cell. For the sake of clarity, the production and release of only one new virion are shown (adapted from Abul *et al.*, 2007).

Human Immunodeficiency virus infection impairs function of both the adaptive and innate immune systems by direct cytopathic effects of the virus and indirect effects particularly on $CD4^+$ cells that lead to the depletion and loss of function of these cells (Gougeon *et al.,* 1993). Cytopathic effects include syncytia formation, reduced cellular protein synthesis due to interfering viral replication that leads to cell death, and expression of gp41 in the plasma membrane and budding of viral particles, may lead to increased plasma membrane permeability and the influx of lethal amounts of calcium, which induces apoptosis, or osmotic lysis of the cell caused by the influx of water (Abul *et al.,* 2007).

2.2. Human Immunodeficiency virus infection and Cytokines

Cytokines are polypeptides produced by many different cell types mostly after their activation, and then affect cells that bear the matching receptors (Sabat, 2010) to mediate

inflammatory and immune reactions and are principal mediators of communication between cells of the immune system (Abul *et al.*, 2007).

Structural studies have shown that cytokines belong to one of the four groups: the hematopoietin family (participate to Haemopoiesis. e.g: IL-2), the interferon family (one of their functions is to interfere with viral replication within a host cell. e.g: IFN- γ), the chemokine family (Chemotactic molecules e.g: CCL14), or the tumor necrosis factor family (can induce cell death among other many functions. e.g: TNF- α) (Riott *et al.*, 2001; Judith *et al.*, 2013).

In the course of HIV infection; proliferation, differentiation, function and maintenance of T cells are controlled in large part by cytokines and HIV-induced T lymphocyte dysfunction may be related to defects in the cytokine network, as manifested by altered cytokine secretion and responsiveness to these cytokines (Sirskyj *et al.*, 2008; Reuter *et al.*, 2012).

Cytokines play a major role during HIV pathogenesis by regulating viral replication as well as innate and adaptive immune responses. Their role during chronic untreated HIV infection has been extensively studied and a large variety of cytokine deregulations contributing to HIV disease progression have been identified during the past 30 years (Vandergeeten *et al.*, 2012). Numerous studies show that HIV can alter cytokine production and responsiveness, particularly in T cells; which serves to increase virus production and hinder the immune response. Based on cytokines production, CD4+ T cell lineages; the targets of HIV are classically divided into either Th1 cells, which initiate a cellular immune response, or Th2 cells, which initiate a humoral immune response (Reuter *et al.*, 2012). However, CD4+ T cells can also become IL-17 producing Th17 cells, immunosuppressive T regulatory cells, Th22 or T follicular helper cells (Roitt *et al* 2001; Reuter *et al.*, 2012). T Helper cells 1 (Th1) produce mainly IL-1, IL-2, IL-12, IL-15, IL-18, IFN- γ and TNF- α while Th2 cells produce a number of cytokines, including IL-4, IL-5, IL-6, IL-10 and IL-13 (Jacek, 2005).

Human Immunodeficiency Virus infection has been associated with reduced production of the Th1 cytokines, especially IL-2. Increases in Th2 cytokine production particularly IL-4 and IL-10, restrict Th1 activity and are associated with HIV infection progression, as is a

skewing of the CD4+ T cell population toward a Th2 phenotype (Reuter et al., 2012).

In vitro studies as well as *in vivo* observations have identified cytokines as important factors regulating the immunological and anti-viral mechanisms involved in HIV persistence (Vandergeeten *et al.*, 2012). Cytokines may play a crucial role in the failure to eradicate HIV by promoting mechanisms of HIV persistence in HIV-infected subjects receiving suppressive HAART (Bortolin *et al.*, 2012). Cytokines involved in the maintenance of memory CD4+ T cells promote the persistence of these cells during HAART while proinflammatory cytokines may favor HIV persistence by exacerbating low levels of ongoing viral replication in lymphoid tissues even after prolonged therapy (Vandergeeten *et al.*, 2012).

Interleukin-10, IL-2 and IFN- γ are some of the crucial cytokines associated with HIV infection and pathogenesis (Williams *et al.*, 2012). Interleukin-2 permits rapid and selective expansion of effector T cell populations (both CD4⁺ and CD8⁺) activated by antigen (Judith *et al.*, 2013). It also exerts effects on cellular metabolism and glycolysis, necessary for long-term survival of T cells. Interleukin-2 is a growth factor for NK cells and promotes production of NK-derived cytokines such as TNF- α , IFN- γ and GM-CSF (Abul *et al.*, 2007; Sarah *et al.*, 2004). Furthermore, IL-2 and IL-12 act synergistically to enhance NK cytotoxic activity (Sarah *et al.*, 2004).

Gamma interferon; the primary macrophage activating cytokine, plays a critical role in resistance of the host to infection with intracellular pathogens (Zhao *et al.*, 2006). IFN- γ stimulates the development and function of immune effector cells. It also activates transcription of a large number of genes that play critical roles in antiviral activity, apoptosis, antigen processing, MHC protein expression, and type 1 T helper cells development. IFN- γ also activates macrophages to kill or restrict growth of microbial targets (Billiau *et al.*, 2009). IFN- γ mediates a range of immunomodulatory effects on both innate and acquired immunity and has been shown to up-regulate MHC I and MHC II expression, activate macrophages/microglia in an antigen-specific fashion, and induces several Interferon-inducible antiviral mechanisms. It primes mononuclear phagocytes for production of monokines; like TNF- α and IL-12. Additional IFN- γ is produced by activated

 $CD4^+$ and $CD8^+$ T cells in presence of IL-12 (Chesler & Reiss, 2002).

Interleukin-10 is an important cytokine with anti-inflammatory properties (Beebe *et al.*, 2002; Sabat *et al.*, 2010; Petit- Bertron *et al.*, 2005) besides TGF- β and IL-35. It acts through a transmembrane receptor complex, composed of IL-10R1 and IL-10R2, and regulates the functions of many different immune cells. The anti-inflammatory activity of IL-10 is due to its ability to block signaling through other cytokine receptors (Sabat *et al.*, 2010). II-10 suppresses all functions of monocytes-macrophages, and this impairs the role of these cells in both innate and adaptive immunity (Sabat, 2010; Sabat *et al.*, 2010). IL-10 inhibits Th1 cell cytokines synthesis including IFN- γ , IL-2, IL-3, LT and GM-CSF at mRNA and protein level. This cytokine also inhibits the expression of costimulators and class II MHC molecules on macrophages and dendritic cells. Because of these actions, IL-10 is able to inhibit T cell activation and terminate cell-mediated immune reactions (Abul *et al.*, 2007; Sabat, 2010; Sabat *et al.*, 2010).

Some studies done on the secretion of cytokines in the course of HIV infection, particularly following HAART initiation have shown that CD4 count and IL-2 correlate positively but negatively with HIV load and IL-10 (Watanabe *et al.*, 2010; Schulbin *et al.*, 2008; Trabattoni *et al.*, 2002; Sindhu *et al.*, 2006). While some studies suggested a positive correlation between CD4 count, IL-2 and IFN- γ ; others have claimed a negative correlation between this interferon and CD4⁺ cells count-IL-2 duo (Sachdeva *et al.*, 2010; Imani *et al.*, 1999; Watanabe *et al.*, 2010; Spitsin *et al.*, 2012; Trabattoni *et al.*, 2002).

2.3. Cytokine network in relation to HIV plasma RNA and CD4 cell count

Sachdeva *et al.* (2010) studied alterations in cytokine and chemokine levels both in peripheral blood and genital secretions among 40 HIV infected women on ART. Repeated sampling was performed at 24 weeks and cytokines and chemokines were measured. There was a decrease in viral load to undetectable levels in 29 patients in the blood and in 33 cases in the genital secretions, as well as a significant decrease in mean levels of TNF-alpha, interleukin-6, IL-1beta after therapy. While IL-2 levels increased significantly, IFN-gamma decreased in both compartments. Also, Mean levels of IL-4 and IL-10 decreased significantly in the serum. IL-10 had a negative correlation with CD4 count at baseline and

after 6 months of therapy.

Schulbin and colleagues assessed the cytokine expression in the colonic mucosa of ten HIV infected patients and controls in a nine month longitudinal study after HAART initiation (Schulbin *et al.*, 2008). In this study mRNA of a number of cytokines including IFN- γ , IL-2, IL-4, IL-6, and IL-10 among others was quantified. The levels of mRNA of these cytokines as well as viral load and T-cell dynamics were determined in the colonic mucosa and the bloodstream, at both the baseline and after nine months of HAART. Though there was a decline in blood and mucosal HIV RNA levels, an increase in the level of CD4⁺ T lymphocytes and a decrease in the expression of these cytokines, HAART failed to restore the normal colonic immunologic environment. The mucosal mRNA expression of TNF-alpha, IFN-gamma, IL-4, IL-6, and IL-10 was significantly higher in HIV-infected patients than in control patients and remained elevated in the course of the nine months of HAART.

To provide evidence of changes in relation to type 1 and type 2 cytokines during HIV-1 infection, cytokine-specific mRNA expression before and after initiation of HAART were studied by Imami and colleagues in unstimulated peripheral blood mononuclear cells from nine HIV-1-infected individuals obtained at different time intervals before and after the initiation of HAART. In this study, mRNAs for IFN-y, IL-2, IL-4, and IL-10 were assessed and their levels correlated with CD4⁺ T cell counts and viral load. At the baseline, there was a little expression of specific IFN- γ and IL-2 (type 1 cytokine) mRNA in all patients compared to the readily detectable expression of specific IL-4 and/or IL-10 (type 2) mRNA in the majority of patients. After initiation of HAART, there was a continuous increase in IFN-γ and IL-2 mRNA expression in parallel to a dramatic reduction in viral load and an increase in CD4⁺ T cell counts and a drop of expression of Type 2 cytokine-specific mRNA to undetectable levels. The predominant expression of type 2 cytokine mRNA, before initiation of HAART, concurs with previous findings of a dominant antiproliferative; type 2 cytokine profile during HIV-1 infection. Reversion of the cytokine profile to a strong type 1 profile, after HAART, suggests that in addition to suppressing viral replication directly, treatment may offer the immune system a chance to recover (Imami et al., 1999).

In a cross-sectional study at St. Justine Hospital Research Center, the relationship between

Th1, Th2 cytokines, CD4 count and viremia in patients on HAART was assessed by Sindhu *et al.* (2006). The aim of the study was to understand the critical role played by the imbalance between Th1 and Th2 cytokines in the progression and pathogenesis of HIV-1. Serum samples from 17 patients on HAART with opportunistic infections, 43 without opportunistic infections, and 20 HIV-seronegative controls were assessed for the levels of IL-2, IFN-gamma, IL-4, and IL-10 and their mRNAs. Results showed lower serum IL-2 and IFN-gamma levels in patients compared to controls and a clear reduction of IFN-gamma in patients without opportunistic infections. The levels of IL-4 and IL-10 were higher in patients than in controls and this elevation was more remarkable in patients with opportunistic infections. In *in vitro* cytokine production assays, CD4⁺ T cells from low viremia patients produced mainly IL-2 and IFN-gamma. Positive correlations between sera and supernatant proteins and cellular mRNAs were noted.

In a study in which 18 HIV positive patients were given a tritherapy consisting of Efavirenz Nelfinavir, and Stavudine; the relationship between cytokines levels was also investigated. Patients received treatment for 10 months and levels of IL-2, IFN- γ , IL-4, and IL-10 as well as quantitation of mRNA for the same cytokines were assessed at baseline and 2 weeks, 2 months, and 10 months into therapy. It was noticed that IL-2 and IFN- γ production was augmented and IL-10 production was reduced after treatment. Treatment was also associated with a reduction in HIV RNA in plasma and an increase in CD4⁺ cell count (Trabattoni *et al.*, 2002).

To investigate the short-term effects of HAART on programmed death 1 receptor (PD-1) expression and lymphocyte function, Spitsin and co-workers used mitogens including phytohemagglutinin (PHA), HIV antigen p24 and Candida recall antigen to test the function of lymphocytes from HIV infected adults before and after HAART initiation. PHA-stimulated peripheral blood mononuclear cells from samples obtained 2 months after HAART produced higher levels of IFN- γ and TNF- α than the levels observed for samples taken before HAART. However, there were no significant changes in IL-2 or Th-2 cytokines (IL-4, IL-5, and IL-10) in the corresponding samples. A significant reduction in viral load and recovery of CD4⁺T cells was also noted (Spitsin *et al.*, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Study sites

Patients who participated in this study were recruited from Biryogo, Muhima, Kabusunzu, Gitega Health Centers as well as Centre Medico-Social Cornum (located in Nyarugenge district of Kigali City); Kagugu and Kimironko (located in Gasabo district of Kigali city). The sites were selected because of being among sites in Rwanda where HIV positive patients can get ART services and could help in achieving the desired number of study participants within the study duration. In addition, these sites are near the Rwanda National Reference Laboratory where blood samples were analyzed. This presents opportunity that guarantees quick analysis of samples.



(in capital letters)

3.2. Study Design

This was a longitudinal study involving HIV positive patients within selected health facilities in Kigali/Rwanda, from August 2013 to January 2014.

3.3. Study Population

This study was carried out using HIV positive patients of all age groups who presented themselves for HAART initiation at the above-mentioned sites. Data were collected at the start of HAART and then at six months of the treatment.

3.3.1 Inclusion Criteria

To be included in the study population sample, a patient had to be:

- HIV positive
- Coming for HAART initiation.
- Willing to provide informed consent

3.3.2 Exclusion criteria

- HIV positive patients who had started HAART before the beginning of this study
- Newborns with unconfirmed HIV status (by early infant diagnostic procedures using DBS and nucleic acid detection tools)

3.4. Sample size and sampling strategy

A correlation of -0.5 (r = - 0.5) between IL-10 and CD4⁺ T cells count was anticipated (Miguel et al., 2006). The minimum sample size to estimate the same correlation with a Probability of Type I Error (α) of 0.05, a Power (1 - β) of 0.8 was 20 patients (sample size estimated using statstodo software available at (Chang, 2013).

Due to loss to follow up and death cases that are normally recorded at the selected health facilities (about 30% of total patients coming for HAART initiation were dying or being lost to follow up based on records at these facilities); the sample size was raised to 40 HIV positive patients who came for HAART initiation. Any patient who came for HAART initiation was included in the sample size until the desired number of 40 patients was reached.

3.5. Data Collection

Variables considered during this study included CD4 count, HIV plasma RNA, IL-10, IL-2

and IFN-*γ*. Measurements were done on whole blood (CD4 count) and on its plasma (cytokines and the viral load). Blood was collected in 4 ml tubes containing EDTA at two time points namely before HAART (August, 2013) and six months following HAART initiation (February, 2014). After collection, samples were transported immediately to the NRL for analysis was being done immediately for CD4 count. Whole blood was centrifuged to get plasma that was kept according to the instructions on test kits, for subsequent tests including cytokines and the viral load. Analyses were done at the Rwanda National Reference Laboratory according to the kits manufacturer's instructions. CD4 count was done using flow cytometry while the viral load and cytokines were measured using the COBAS[®] AmpliPrep/COBAS[®] TaqMan[®] HIV-1 Test and ELISA techniques, respectively.

3.5.1. Determination of CD4 count by flow cytometry

Cluster of Differentiation 4 marker positive cells count was performed using flow cytometry with Becton Dickinson FACSCalibur (Software: MultiSET V3.0.2). When cells labeled with a fluorescent antibody pass a beam of laser light, the labeled cells to fluoresce. This fluorescent light provides the information necessary for the instrument to identify and count the lymphocytes and CD4 T lymphocytes. In addition, the reagent tubes contain a known number of fluorescent reference beads to which a precise volume of whole blood is added. The software automatically identifies the lymphocyte populations of interest and calculates the CD4 counts (cells/µL) by comparing cellular events to bead events.

To 20µl of tritest (CD3/CD45/CD4 monoclonal antibodies) reagent in TruCOUNT tube, 50µl EDTA whole blood was added. Vortexing was done followed by a 15-minute incubation in the dark. This was followed by fixation and lysing for a further 15 minutes in dark. The sample analysis was then done using multiset software (Appendix 3).

3.5.2. Determination of the Viral Load

The viral load was measured using The COBAS[®] AmpliPrep/COBAS[®] TaqMan[®] HIV-1 Test which is a nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma (Appendix 4). In this procedure, specimen preparation is automated using the COBAS[®] AmpliPrep Instrument with amplification and detection automated using the COBAS[®] TaqMan[®] Analyzer or the COBAS[®] TaqMan[®] 48 Analyzer.

The test is based on three major processes according to Roche molecular systems, 2007:

3.5.2.1 Specimen preparation:

This was an automated specimen preparation on the COBAS® AmpliPrep Instrument by a generic silica-based capture technique. The procedure processes 850µL of plasma. The HIV-1 virus particles were lysed by incubation at elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HIV-1 RNA from Rnases in plasma. Protease and a known number of HIV-1 QS Armored RNA molecules are introduced into each specimen along with the lysis reagent and magnetic glass particles. Subsequently, the mixture is incubated and the HIV-1 RNA and HIV-1 QS RNA are bound to the surface of the magnetic glass particles. Unbound substances, such as salts, proteins and other cellular impurities, are removed by washing the magnetic glass particles. After separating the magnetic glass particles and completing the washing steps, the adsorbed nucleic acids are eluted at elevated temperature with an aqueous solution. The processed specimen, containing the magnetic glass particles as well as released HIV-1 RNA and HIV-1 QS RNA, is added to the amplification mixture and transferred to the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer;

3.5.2.2 Reverse transcription of the target RNA

To generate complementary DNA: The reverse transcription and PCR amplification reaction was performed with the thermostable recombinant enzyme Thermus species DNA Polymerase (Roche, 2007). In the presence of manganese (Mn^{2+}) and under the appropriate buffer conditions, Z05 has both reverse transcriptase and DNA polymerase activity. This allowed both reverse transcription and PCR amplification to occur together with real-time detection of the amplicon;

3.5.2.3 Simultaneous PCR amplification of target cDNA and detection of cleaved duallabeled oligonucleotide probe specific to the target

Following reverse transcription of the HIV-1 target RNA and the HIV-1 QS RNA, the

Thermal Cycler in the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer heated the reaction mixture to denature the RNA:cDNA hybrid and expose the specific primer target sequences. As the mixture cooled, the primers annealed to the target DNA. The thermostable Thermus species Z05 DNA Polymerase extended the annealed primers along the target template to produce a double-stranded DNA molecule termed an amplicon. The required number of cycles was preprogrammed into the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer by the manufacturer.

The detection of amplified DNA was performed using a target-specific and a QS-specific dual-labeled oligonucleotide probe that permitted independent identification of HIV-1 amplicon and HIV-1 QS amplicon. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 test utilizes real-time PCR technology. The detection of PCR product accumulation was done by monitoring the emission intensity of fluorescent reporter dyes released during the amplification process.

3.5.3. Determination of plasma level of IL-10 using *InvitrogenTM* ELISA/Human IL-10 kit

The Invitrogen Hu IL-10 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). This assay operates on the following principle. A monoclonal antibody specific for Hu IL-10 comes pre-coated onto the wells of the microtiter strips. Samples, including standards of known Hu IL-10 content, control specimens, and unknowns, are pipetted into these wells. Human IL-10 binds to the immobilized (capture) antibody. A biotinylated monoclonal antibody specific for human IL-10 is used to trace the captured IL-10.

Fifty (50) μ L of standards, samples or controls were added to the appropriate microtiter wells and 50 μ L of incubation buffer were added to the wells containing standards and samples. The plate was then covered with plate cover and incubated for 2 hours at room temperature. This was followed by washes after which 100 μ L of biotinylated anti-IL-10 (Biotin Conjugate) solution were added into each well. The test palate was covered and incubated for 2 hours at room temperature followed by another four washes was done. Streptavidin-HRP Working Solution (100 μ L) was then added to the wells and the test plate

covered and incubated for 30 minutes at room temperature. Following another 4 washes; 100 μ L of Stabilized Chromogen was added to each well and plate incubated in the dark for 30 minute at room temperature and in the dark. 100 μ L of stop solution was added to each well and optical densities measured at 450 nm. IL-10 concentrations were determined using a standard curve (Appendix 5).

3.5.4. Determination of IL-2 levels in plasma using *BIOSOURCE IL-2 EASIA/Human IL-2 Immunoassay*

This was a solid phase Enzyme Amplified Sensitivity Immunoassay performed on microtiter plate. The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of IL-2 is used. Standards or samples containing IL-2 react with capture monoclonal antibodies (MAbs 1) coated on the microtiter well and with a monoclonal antibody (MAb-2) labeled with horseradish peroxidase (HRP).

100 μ L of solution B (buffer) was pipetted into the wells for the standards, samples and controls to which 100 μ L of standard, control, or sample into the appropriate was added specifically. 50 μ L of anti-IL-2 Conjugate were transferred into all the wells followed by a 2 hour incubation at room temperature on a horizontal shaker set at 700 rpm. The plate was then washed three times and100 μ L of Chromogen were added into each well. Wells were incubated for 15 minutes at room temperature on a horizontal shaker at 700 rpm, avoiding direct sunlight. 100 μ L of stop solution were put into each well and absorbances were read at 450 nm within 3 hours and IL-2 concentrations calculated using standard curve (Appendix 6).

3.5.5. Determination of IFN- γ levels in plasma using *Quantikine^R ELISA/Human IFN-\gamma Immunoassay*

The Invitrogen IFN- γ kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs 1) directed against distinct epitopes of IFN- γ are used. All reagents were allowed to reach room temperature before use. Fifty (50) µL of each Standard, Control, or Sample was pipetted into specific wells. 50 µL of anti-IFN- γ HRP Conjugate was added

into all wells. After a 2 hour incubation at room temperature on a horizontal shaker set at 700 rpm, there was aspiration of the solution and a 4 times wash of wells was done. 200 μ L of freshly prepared chromogen-substrate (H2O2-TMB mixture) solution was added into each well (within 15 minutes after washing) followed by a 15 minutes incubation of the plate at room temperature on a horizontal shaker set at 700 rpm, avoiding direct sunlight. 50 μ L of Stop Solution (1.8 Normal sulfuric acid) was added into each well and absorbance was read at 450nm. IFN- γ concentrations were calculated using a curve done with standards (Appendix 7).

3.6. Data Analysis

Laboratory data for the five parameters were analyzed using R programming language as described by (John and Duncan, 2007). Mean for CD4 count, viral load, IL-10, IL-2 and INF- γ were computed. Significances of changes in these parameters following HAART were assessed using Wilcoxon test. Association between levels of IL-10, IL-2, INF- γ , CD4 count, viral load and age or gender were assessed using chi-square, t-test and ANOVA. Finally, Pearson's correlations between these parameters were determined. The level of significance was set at $p \le 0.05$.

3.7. Ethical Consideration

The National Health Research Committee of the Rwanda Biomedical Center/Medical Research Center did a scientific review of the research proposal (Appendix 10). Ethical clearance was obtained from the Rwanda National Ethics Committee (Appendix 8) after submission of research permit from the Ministry of Education-Rwanda (Appendix 9). These approvals were presented to research sites administration before starting data collection. Eligible HIV positive patients received details about the study including anonymous sample collection, analysis and confidentiality of results as only codes representing study participants were to be used. It is only when informed consent was provided that blood collection proceeded (Appendix 1 and 2 for English and Kinyarwanda versions respectively).

CHAPTER FOUR

4.0 RESULTS

Specific objectives of this study were to determine levels of CD4 positive cells (CD4 count), viral load, Interleukin-10, Interleukin-2 and gamma-Interferon before HAART and at six months of treatment; to determine changes in levels of these variables following HAART initiation and finally, to correlate these variable between themselves both before HAART and at six months of treatment among HIV positive patients in Kigali-Rwanda. At six months of HAART, thirty three (33) patients were still under the follow up (four and then three patients were dead and lost to follow up respectively).

4.1. Characteristics of study participants

Thirty three (33) HIV positive patients participated in this study. These comprised of 20 (60.6%) men and 13 (39.4%) women distributed into three age groups for data analysis. The minimum age was 20 years and the maximum age was 51 years. The mean age of study participants was 33 years. Study participants were grouped into three age groups. Group one was composed of patients aged between 20 and 29 years, group two comprised patients aged between 30 and 39 years and group three consisted of study participants aged between 40 and 51 years. Thirteen (39.4%) study participants were aged between 20 and 29 years; 13 (39.4%) were aged between 30 to 39 years whereas 7(21.2%) were between 40 and 51 years (Figure 4.1).



Figure 4.1: Distribution of study participants with regard to age and sex. Thirteen (13) females and 20 males participated to this study and they were stratified into four age groups (20-29 years, 30-39 years and 40 to 51 years). Most of study participants were young as they had below 40 years

4.2. Levels of measured variables before HAART

4.2.1. CD4 count Before HAART initiation

At HAART initiation, 12 (36.4%) patients had CD4 counts below 200 cells/ μ of blood. Twenty (63.6%) study participants had CD4 counts of 200 cells/ μ l of blood and above and only 1(3%) patient had a CD4 count of more than 350 cells/ μ l. The mean CD4 count at HAART initiation was 213 cells/ μ l of blood. The minimum CD4 count was 4 cells/ μ l whereas the maximum was 509 cells/ μ l (Table 4.1).

Association between CD4 count level and gender at HAART initiation was assessed using chi-square statistics. Results indicated that there was no association between CD4 count and gender (p value: 0.34: Table 4.1).

Table 4.1 presents CD4 Count and Gender before HAART. The mean CD4 count was 213 cells/ μ l. Seven (7) men out the 20 men who participated to this study, were having CD4 count below 200 while 13 had CD4 count \geq 200 cells/ μ l. Five (5) and 8 women started HAART with CD4 count below 200 and \geq 200 cells/ μ l respectively. CD4 count was not influenced by gender.

Gender	<200 cells/µl	≥200 cells/µl	Total	Overall Mean CD4 count	Association CD4 count-Gender
Female: n(%)	5(15.2)	8(24.2)	13(39.4)	213 cells/µl of blood	None (p value >0.05)
Male: n(%)	7(21.2)	13(39.4)	20(60.6)		
Total: n(%)	12(36.4)	21(63.6)	33(100)		

The CD4 counts were also analyzed based on age. Five (15.2%) patients aged between 20 and 29 had CD4 counts below 200 cells/µl and 8 (24.2%) from same age group had CD4 counts of 200 cells/µl and above at HAART initiation. Five (15.2%) and 8(24.2%) in the age group of 30 to 39 years had CD4 counts below 200 cells/µl and 200 cells/µl and above respectively. The age group of 40 to 51 years had 2 (6.1%) study participants with CD4 counts below 200 cells/µl and 5(15.2%) with 200 cells/µl and above at HAART initiation. Chi-square statistics showed that there was no statistically significant difference in CD4 counts between age groups (*p* value: 0.55: Table 4.2).
Table 4.2 is about distribution of CD4 count at HAART initiation based on age of study participants. For example: Five (5) of the 12 patients who started HAART with CD4 count <200 cells/µl had between 20 and 29 years. CD4 count was not influenced by age as indicated by p value >0.005.

Age group	<200 cells/µl	≥200 cells/µl	Total	Association CD4 count- Age
20-29: n (%)	5(15.2)	8(24.2)	13(39.4)	
30-39: n (%)	5(15.2)	8(24.2)	13(39.4)	None (p value
40-51: n (%)	2(6.1)	5(15.2)	7(21.2)	>0.05
Total: n(%)	12(36.4)	21(63.6)	33(100)	

4.2.2. Viral load

At the time of HAART initiation, all study participants had high viremia. The median viral load was 23400 HIV RNA copies/ml of plasma with a mean log of 4.5. Men had a median viral load of 22950 RNA copies/ml and a mean log of 4.5 compared to 39500 RNA copies/ml and a mean log of 4.7 among women (Table 4.3).

One (3%) patient had a viral load equivalent to 2 logs. Six (18.2%) study participants had a 3 log viral load whereas 15(45.5%) participants had a 4 log viral load. Ten (30.3%) and 1 (3%) study participants had a viral load of 5 and 6 logs respectively. Association between viral load level, sex and age was assessed using t-test and transformed viral load results (log transformation). There was no difference in levels of viral load between men and women (*p values: 0.54:* **Table 4.3**).

Table 4.3 distributes viral load on gender basis before HAART. Viral load level is presented as number of logs. The lowes viral load was equivalent to 2 logs and the highest corresponded to 6 logs. The table also shows that at HAART, the median viral load was 23400 RNA copies/ml and this corresponds to a log_{10} of 4.5. Data analysis has indicated that viral load was not influenced by gender as the p value was >0.05.

Gender	2 Log VL	3 log VL	4 log VL	5 log VL	6 log VL	Median VL (log)	Assoc. VL-Sex		
Female:n(%)	0(0.0)	3(9.1)	5(15.2)	5(15.2)	0(0.0)	-	-		
Male: n(%)	1(3)	3(9.1)	10(30.3)	5(15.2)	1(3)	23400 copies/ml (4.5)	None (p value >0.05)		
Total: n(%)	1(3)	6(18.2)	5(45.5)	10(30.3)	1(3)				
VL: Viral Load; Assoc: Association									

Analysis of levels of viral load was also done with regard to age groups. The minimum viral load in the age group of 20 and 29 years that was composed of 13(39%) patients was equivalent to three logs and the maximum viral load was equivalent to 6 logs. The minimum viral load in the second age group (patients aged between 30 and 39 years) was equivalent to three logs whereas the maximum viral load in this age group was equivalent to 5 logs. The remaining age group (study participants aged between 40 and 51 years) had a minimum viral load of 1 log and a maximum viral load of 5 log (Table 4.4). In general, 1(3%) patients had viral loads in the range of 1 log, 6(18.2) had viral loads in the range of 2 logs, 15(45.5%) had viral loads in the range of 4 logs, 10(30.3%) had viral loads of 5 logs whereas 1 (3%) patient was having a viral load equivalent to 6 logs.

ANOVA was used to compare levels of viral load based on age groups. With a *p* value of 0.26, there was no statistically significant difference in levels of viral load across the age groups.

Table 4.4: Distribution of levels of viral load based on age groups before treatment. The age group. Fifteen patients had a viral load of 4 logs and these included 6 patients in the age group of 20-29, 5 participants in the age group of 30-39 and 4 patients in the group of 40 to 51 years. However, viral load level was not age dependent (*p* value >0.05).

Age Group	2 Log VL	3 log VL	4 log VL	5 log VL	6 log VL	Total (%)	Assoc. VL-Age
20-29:n(%)	0(0.0)	2(6.1)	6(18.2)	4(12.1)	1(3)	13(39.4)	
30-39:n(%)	0(0.0)	3(9.1)	5(15.2)	5(15.2)	0(0.0)	13(39.4)	None (p
40-51:n(%)	1(3)	1(3)	4(12.1)	1(3)	0(0.0)	7(21.2)	>0.05)
Total: n(%)	1(3)	6(18.2)	15(45.5)	10(30.3)	1(3)	33(100)	

4.2.3. Cytokines

4.2.3.1. Interleukin-10

Three cytokines were measured during this study. These were IL-10, IL-2 and IFN- γ . The mean IL-10 concentration was 120.2pg/ml of plasma (**Table 4.5**). The mean of this Th2 cytokine was 120.4pg/ml among men while it was 119.8pg/ml among women. A comparison of the two means (mean IL-10 among men and mean among women) using t-test has shown a statistically non-significant difference (*p* value: 0.99).

ANOVA was used to test differences in levels of IL-10 between age groups and no statistically significance of these differences was found (*p* value: 0.24).

4.2.3.2. Interleukin-2

Before HAART, mean IL-2 among the 33 study participants was 1.29pg/ml (**Table 4.5**). Men had a mean of 0.88pg/ml and women had a mean IL-2 of 1.93 pg/ml. Using t-test, mean IL-2 was compared between men and women and results indicated no significant difference of IL-2 concentration between men and women before HAART (*p* value: 0.34).

To test differences in levels of IL-2 between age groups, ANOVA was used and no statistically significance of these differences was found (*p* value: 0.29).

4.2.3.3. Gamma-interferon

Results of Th1 cytokine before HAART showed a mean IFN- γ of 0.84pg/ml (**Table 4.5**). Mean IFN- γ among study participants was 0.92pg/ml among men and 0.73 pg/ml among women. No statistically significant difference between levels of this cytokines between men and women was found (*p* value: 0.52).

Significance of difference in levels of IL-2 between age groups was assessed using ANOVA and no statistically significance of these differences was found (*p* value: 0.9).

Table 4.5: Levels of IL-10, IL-2, IFN- γ with regard to sex and age groups. Mean levels of the three cytokines are compared between men and women and then across age groups using t test and ANOVA respectively. No difference found as p values are >0.05.

Cytoki ne	Overall mean	Mean (Men)	Mean (Women)	T-test: cytokine and Sex (p value)	ANOVA(F): Cytokine and age groups (20-29yrs, 30- 39 yrs and 40-51yrs): (p value)				
IL-10	120.2	120.4	119.8	-0.02(0.99)	1.49(0.24)				
IL-2	1.29	0.88	1.93	0.98(0.34)	1.29(0.29)				
IFN-γ	0.84	0.92	0.73	-0.65(0.52)	0.11(0.9)				
Results for mean are in pg/ml									

Means of levels of cytokines were also compared based on whether a study participant started HAART with a CD4 count of less than 200 cells/µl or 200 cells and above/µl. The Mean of IL-10 among patients who started HAART with a CD4 count below 200 cells/µl was 151pg/ml compared to 102.6pg/ml for patients who were enrolled for HAART with a CD4 count of 200 cells/µl and above. As for IL-2 and IFN- γ ; their means were 0.4 and 1.7pg/ml for IL-2, and 0.39 then 1.1 pg/ml for gamma-interferon. When these means were compared using t-test, statistically significant low level of IFN- γ was found in the group of patients who started HAART with CD4 count below 200 cells/µl (*p* value: 0.02). As for IL-10 (*p* value: 0.26) and IL-2 (*p* value: 0.21), there was no statistically significant difference was found between the two groups (**Table 4.6**).

Table 4.6: Levels of cytokines with regard to CD4 count before HAART. This table compares levels of cytokines based on whether participants had CD4 count <200 cells/µl or \geq 200 cells/µl. While there was no difference in levels of IL-10 and IL-2 among the two groups (p value >0.05), IFN- γ levels were lower among patients with CD4 count <200 cells/µl.

Cytokine	<200 cells/µl	≥200 cells/µl	t-test for difference (p value)
Mean IL-10	151	102.6	-0.03(0.26)
Mean IL-2	0.4	1.7	-1.27(0.21)
Mean IFN-γ	0.39	1.1	-2.7(0.02)
	ŀ	Results for mean	are in pg/ml

4.3. Levels of measured variables following HAART

4.3.1. CD4 count at six months of HAART

At six months of HAART, the mean CD4 count was 369 cells/ μ l. Five (15.2%) patients including one woman and four men were still having CD4 counts below 200 cells/ μ of blood. Twenty eight (84.8%) study participants namely 12 women and 16 men had CD4 counts of 200 cells/ μ of blood and above. Association between CD4 count level and gender was assessed using chi-square. No association between CD4 count and gender was found (*p* value: 0.33: Table 4.7).

Table 4.7 highlights levels of $CD4^+$ cells with based on age groups and shows that after six months of HAART, 5 patients (4 men and 1 woman) were still having less 200 cells/µl. The table also shows that number of CD4 positive cells is not influenced by gender as the p value is >0.05.

Gender	<200 cells/µl	≥200 cells/µl	Total	Overall Mean CD4 count	Difference in CD4 count
Female: n(%)	1(3)	12(36.4)	13(39.4)		
Male: n(%)	4(12.1)	16(48.5)	20(60.6)	369 cells/μl of blood	None. <i>p</i> value >0.05
Total: n(%)	5(15.2)	28(84.8)	33(100)		

Results of analysis of CD4 count based on age have shown that 1(3%) patient in the group of 20 to 29 years had a CD4 count below 200 cells/µl and 12 (36.4%) had CD4 counts of 200 cells/µl and above. The group of 30 to 39 years comprised of 3 (9.1%) patients with CD4 counts below 200 cells/µl and 10 (30.3%) patients with CD4 counts of 200 cells/µl and above. There were 1(3%) and 6(18.2%) patients with a CD4 count below 200 cells/µl and a CD4 count of 200 cells/µl and above respectively in the age group of 40 to 51 years. A total of 5(15.2%) patients had CD4 counts of less than 200 cells/µl whereas 21(84.8%)had CD4 counts of 200 cells/µl and above. Association between CD4 count level and age was assessed using chi-square. No association between CD4 count and age was found (*p* value: 0.55: Table 4.8).

Age group	<200 cells/µl	≥200 cells/µl	Total	Difference in CD4 count between Age groups
20-29: n(%)	1(3)	12(36.4)	13(39.4)	
30-39: n(%)	3(9.1)	10(30.3)	13(39.4)	None
40-51: n(%)	1(3)	6(18.2)	7(21.2)	(<i>p</i> value >0.05)
Total: n(%)	5(15.2)	28(84.8)	33(100)	

Table 4.8 highlights levels of $CD4^+$ cells with based on age groups and shows that 5 patients were still having less 200 cells/µl. The table also shows that number of CD4 positive cells is not influenced by age as the *p* value is >0.05.

At six months of HAART, CD4 counts were also assessed based on whether a patient had a CD4 count of 350 cells/ μ l, the normal low CD4 count below which HIV positive patients start HAART. Five (15.2%) and 8(24.2%) women had CD4 counts below 350 cells/ μ l and CD4 counts of 350 cells/ μ l and above respectively whereas 10 (30.3%) and 10 (30.3%) men had CD4 counts below 350 cells/ μ l and CD4 counts of more than 350 cells/ μ l respectively on the other side. CD4 counts were compared between sex and chi-square showed the difference in number of these cells was significant (*p* value: 0.52) (Table 4.9).

Table 4.9 shows CD4 counts and sex with regard to 350 cells/ μ l limit. Eighteen (18) patients were having a CD4 count of 350 cells/ μ l and above (the limit below which it is assumed that the immune system is weakened enough). No association was found between CD4 count and gender.

Gender	ender CD4 count Cl <350 cells/µl 3		Total	Difference in CD4 count between sex
Female:n(%)	5(15.2)	8(24.2)	13(39.4)	-
Male:n(%)	10(30.3)	10(30.3)	20(60.6)	None (p value >0.05)
Total:n(%)	15(45.5)	18(54.5)	33(100)	

Seven (21.2%) study participants in the age group of 20 to 29 years had CD4 counts below 350 cells/ μ l while 6(18.2%) patients in the same age group had CD4 counts of 350 cells/ μ l and above. Six (18.2%) and 7(21.2%) aged between 30 and 39 years had CD4 counts below 350 cells/ μ l and CD4 counts of 350 cells/ μ l and more sequentially. As for the age group of 40 to 51 years, 2(6.1%) had CD4 counts of less than 350 cells/ μ l while 5(15.5%) in this group had CD4 counts of 350 cells/ μ l and above (Table 4.10).

Association between CD4 count levels with regard to the limit of 350 cells/ μ l and age was assessed using chi-square. No association between CD4 count and age was found (*p* value: 0.56).

Age group	oup CD4 count CD4 count ≥ T <350 cells/µl 350 cells/µl T		Total	Difference in CD4 count between Age groups
20-29: n(%)	7(21.2)	6(18.2)	13(39.4)	
30-39: n(%)	6(18.2)	7(21.2)	13(39.4)	
40-51: n(%)	2(6.1)	5(15.2)	7(21.2)	None (p value >0.05)
Total: n(%)	15(45.5)	18(54.5)	33(100)	

Table 4.10 shows CD4 counts and age with regard to 350 cells/ μ l limit. Fifteen (15) patients were still having a CD4 count of less than 350 cells/ μ l (the limit below which it is assumed that the immune system is weakened enough).

4.3.2. Viral load following HAART

After six months of HAART, the median viral load dropped from 23400 HIV RNA copies/ml of plasma to 661 RNA copies/ml equivalent to a 2.7 log. Three (9.1%) patients namely 1 woman and 2 men achieved undetectable viral load. Two (6.1%) women and 4(12.1%) men had viral loads in the range of 1 log. Five (15.2%) women and 7(21.2%) men had viral loads equivalent to 2 logs. Three (9.1%) and 3(9.1%) men had viral loads of 3 logs while 2(6.1%) women and 4(12.1%) men had viral load equivalent to one log; 12 (36.4%) patients had viral load equivalent to two logs; 6 (18.2%) patients had viral load equivalent to four logs. When looked at individually, 5 (15.2%) patients had their viral load unchanged despite the six month HAART. Differences in viral load decrease were assessed between men and women and between age groups. There was no difference in decrease of viral load between men and women following HAART (p value: 0.3) (**Table 4.11**).

Table 4.11 shows levels of viral load following HAART based on gender. While some patients achieved undetectable viral load (3 patients), others saw their viral load undiminished (5 patients). Median for viral load dropped from 23400 RNA copies/ml before HAART (not shown here) to 661 RNA copies/ml Viral load was not influenced by sex as the *p* value for difference in viral load between men and women was >0.05.

Gender	Udt.	1 Log VL	2 Log VL	3 Log VL	4 Log VL	UD VL	Med. VL (log)	Assoc. VL-Sex
Female: n(%)	1(3)	2(6.1)	5(15.2)	3(9.1)	2(6.1)	_		_
Male: n(%)	2(6)	4(12.1)	7(21.2)	3(9.1)	4(12.1)	5 (15.2)	661 copies/ml (2.7)	None (<i>p</i> value >0.05)
Total: n(%)	3(9)	6(18.2)	12(36.4)	6(18.2)	6(18.2)			

Udt: Undetectable viral load; *VL:* Viral load; *UD:* Undiminished; *Assoc:* Association; Viral load median is in RNA copies/ml of plasma and Viral load logs are in *log*₁₀

When viral loads were analyzed based on age groups, results showed that 1(3%) in the age group of 20 to 29 years and 2(6.1%) aged between 40 and 51 years achieved undetectable viral load after six months of HAART. Two (6.1%) patients in the age group of 20 to 29; 4(12.1%) patients in the age group of 30 to 39 years had log viral loads in the range of 4 logs **(table 12)**. ANOVA for difference in viral loads among age groups did not show a significant deference in decrease in viral load between age groups following HAART (*p* value: 0.3: Table 4.12).

Table 4.12 shows changes in viral load levels following HAART based on age groups. While some patients achieved undetectable viral load, others were still having a viral load equivalent to 4 logs. Following HAART, viral load was not influenced by age as the p value for difference between age groups was >0.05

Age Group	Undetec-	1 Log	2 Log	3 Log	4 Log	Association
	table vL	٧L	٧L	٧L	٧L	VL-Age
20-29: n(%)	1(3)	4(12.1)	4(12.1)	2(6.1)	2(6.1)	
30-39: n(%)	0(0.00)	1(3)	5(15.2)	3(9.1)	4(12.1)	None (p value
40-51: n(%)	2(6.1)	1(3)	3(9.1)	1(3)	0(0.00)	>0.05)
Total: n(%)	3(9.1)	6(18.2)	12(36.4)	6(18.2)	6(18.2)	
	VL: Viral l	load;	Viral loa	d logs are	in log ₁₀	

4.3.3. Cytokines levels following HAART

4.3.3.1. Interleukin-10

Following HAART, the mean IL-10 concentration dropped to 48.06 pg/ml of plasma. The mean IL-10 was 42.4pg/ml among men whereas the mean of this Th2 cytokine was 51.5 pg/ml among women (Table 4.13). A comparison of the two means (mean IL-10 among men and mean among women) using t-test has shown no difference (*p* value: 0.76).

After six months of HAART, ANOVA was used to test differences in levels of IL-10 between age groups and no difference was found (p value: 0.27).

4.3.3.2. Interleukin-2

After six months of highly active antiretroviral treatment, mean IL-2 for all study participants was 10.08 pg/ml (**Table 4.13**). Men had a mean of 8.7pg/ml and women had a mean IL-2 of 12.16 pg/ml. Using t-test, mean IL-2 was compared between men and women and results indicated no difference of IL-2 concentration between men and women (*p* value: 0.41).

To test differences in levels of IL-2 between age groups, ANOVA was used and no significant difference in IL-2 levels was found (*p* value: 0.79).

4.3.3.3. Gamma-interferon

Results of IFN- γ concentration after six months of HAART showed a mean IFN- γ of 5.24pg/ml. Mean I IFN- γ was 5.27pg/ml among men and 5.19pg/ml among women (**Table 4.13**). No difference between levels of this cytokine was found between men and women (*p* value: 0.97).

Significance of difference in levels of IFN- γ between age groups was assessed using ANOVA and no difference was found (*p* value: 0.44).

Table 4.13: Levels of cytokines following HAART. This table highlights changes in levels of cytokines following HAART. Results are with regard to sex and age groups. Overall means for the three cytokines changed. Example: Mean IL-10 dropped from 120.2 before HAART (not shown here) to 48.06pg/ml following HAART. There was no association levels of cytokines-sex or age as depicted for under t test and ANOVA areas respectively.

Cytokine	Overall	Men	Women	t-test (p value)	ANOVA: F(p value)
Mean IL-10	48.06	42.4	51.5	-0.31(>0.05)	1.36(>0.05)
Mean IL-2	10.08	8.7	12.16	0.83(>0.05)	0.23(>0.05)
Mean IFN-γ	5.24	5.27	5.19	-0.03(>0.05)	0.85(>0.05)

Results for mean are in pg/ml

4.4. Significance of change in levels of measured variables following HAART

Following HAART, the mean CD4 count increased from 213 to 369 cells/ μ l. Viral load median dropped from 23400 HIV RNA copies/ml to 661 RNA copies/ml. Mean IL-10 decreased from 120.2pg/ml to 48.06pg/ml whereas the means for IL-2 and IFN- γ increased from 1.29 and 0.84 pg/ml to 10.08 and 5.24pg/ml sequentially (**Table 4.14**).

Significance of changes in levels of measured variables was assessed using Wilcoxon

signed rank test and these changes were all statistically significant after six months of HAART (all *p* values <0.05).

Table 4.14: This table shows significance of changes in measured variables following HAART. Following HAART, all parameters changed significantly as indicated by p values below 0.05. Viral load and IL-10 dropped while CD4 count and the two Th1 cytokines increased.

Variable	Mean/Median before HAART	Mean/Median following HAART	Wilcoxon	<i>p</i> value
IL-10	120.2	48.06	12	0.00001
IL-2	1.29	10.08	554	0.00001
IFN-γ	0.84	5.24	525	0.00003
Viral load	23400	661	15	0.00001
CD4 Count	213	369	31	0.00008

Viral load median is in RNA copies/ml; IL-10,IL-2, and IFN- γ means are in pg/ml; CD4 count mean is in Cells/ μ l

4.5. Correlation between variables

Variables were finally correlated between themselves before HAART initiation and then after six months of HAART (Table 4.15).

Before HAART, viral load correlated negatively with CD4 count (r: -0.42, *p* value: 0.01). A negative correlation (r = -0.46; p values: 0.01) was also observed between CD4 count and IL-10. However, CD4 count did not correlate with IL-2 (r: 0.15 and *p* value: 0.37) but correlated positively with IFN- γ (r: 0.29 and *p* value: 0.003). As for viral load, there was a strong correlation with IL-10 (r: 0.7 and *p* value: 0.00001) at HAART initiation. Viral load did not correlate with the two Th1 cytokines (*p* values >0.05).

Following HAART, there was a negative correlation between CD4 count and HIV plasma RNA (r: -0.46 and *p* value: 0.006). CD4 count did not correlate with IL-10, IL-2 and IFN- γ (all *p* values >0.05). A strong positive correlation (r: 0.7 and *p* value: 0.000002) was observed between viral load and IL-10. Viral load did not correlate with IL-2 and IFN- γ at six months of HAART.

Table 4.15: Correlation between measured variables. Higlighted areas in the table showsignificant correlation between parameters while unhiglighted show non-significantcorrelations as p values are >0.05

	Before HAART		At Six months of HAART	
Variable	CD4 Count (p value)	Viral load (p value)	CD4 count (p value)	Viral load (p value)
IL-10	-0.4(<0.05)	0.7(<0.05)	-0.2(>0.05)	0.7(<0.05)
IL-2	0.15(>0.05)	-0.1(>0.05)	0.27(>0.05)	-0.19(>0.05)
IFN-γ	0.4(<0.05)	-0.28(>0.05)	0.2(>0.05)	-0.2(>0.05)
CD4 Count		- 0.42(<0.05)		- 0.46(<0.05)

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Human Immunodeficiency virus disease is still a global challenge. While efforts have been made in providing ARV drugs to infected people particularly in Africa; monitoring of HIV positive patients to assess the response to ARV as well as infection progression remains a challenge especially due to scarcity of adequate facilities for the biological follow up. CD4 count facilities have been scaled up but the viral load measurement (the current most reliable test to assess the progression of the infection and response to treatment) is still limited to a small number of health facilities and this is particularly critical in Africa. Furthermore, cytokines networks in the course of HIV infection is yet to be fully understood. This study was done to examine the relationship between cytokines, viral load and CD4 count levels with a view to understand cytokine networks during HIV infection as well whether they can be used as alternative markers of the disease progression which are easily implementable and affordable.

Cytokines regulate the intensity and duration of immune response by stimulating or inhibiting activation, proliferation, and/or differentiation of various cells and by regulating secretion of antibodies and other cytokines (Vandergeeten *et al.*, 2012). In the course of HIV disease, cytokines are produced by activated immune system engaged in trying to control this viral infection through supporting the expansion of antiviral T cells and antibody responses. While some cytokines like IL-2 and IFN- γ (TH1 cytokines) have been found helping in the control of HIV disease, others like IL-10 (Th2 cytokine) have been found impairing mechanisms that aim at producing Th1 cytokines and playing a role in HIV/AIDS-associated pathologies (Keating *et al.*, 2012).

In this study thirty three (33) HIV^+ patients were recruited and CD4 count, viral load, IL-10, IL-2, and IFN- γ were measured; changes and correlations were assessed both before HAART and at six months after initiation of treatment.

Before HAART and at six months of HAART, CD4 count, viral load and cytokines levels

were not influenced by age or sex in this study. This is consistent with previous studies (Fricke *et al.*, 2012; Song *et al.*, 2011) and it is a good property for any marker if it does not change with sex, age or other normal conditions.

Findings from the present study have shown that higher levels of IL-10 were associated with high plasma HIV RNA, low CD4 counts as well as low levels of two of the Th1 cytokines (IL-2, IFN- γ) at the time of HAART initiation. These findings are in agreement with other studies which have reported a shift from Th1 to Th2 cytokine profiles (Osakwe *et al.*, 2010; Stylianou *et al.*, 1998). However, a study carried out by Sachdeva *et al.* (2010) has shown discrepant results on cytokines like IL-10 levels with regard to the status of immune response in the course of HIV disease. Low CD4 count, IL-2 and IFN- γ with high viral load and high IL-10 indicated a depressed immune system that lead to HAART initiation among these study participants. This highlights the anti-inflammatory role of IL-10 as well as viral replication negative effect on CD4 positive cells and this can also be seen through correlation between measured parameters at HAART initiation (Sindhu *et al.*, 2006; Agarwal *et al.*, 2001).

It has been indicated that interleukin-10 enhances viral replication by supressing IL-2 (a Th1 cytokine) secretion as well as blocking cytolysis of infected cells; giving time to the virus to replicate, resulting in an increase in viraemia as well as loss of CD4 positive cells. Similarly, studies in animal models have revealed that IL-10 gene-knockout or signaling blockade can enhance resistance to pathogens, and substantially facilitate viral clearance (Kwon *et al.*, 2010; Elrefaei *et al.*, 2007). Moreover, interleukin-2 and IFN- γ stimulate pathways of immune responses production to viruses through promoting differentiation and proliferation of T cell, Natural Killer cells as well as stimulating antigen presentation by Macrophages (Vandergeeten *et al.*, 2013). This can explain the low levels of these two Th1 cytokines among study participants and high levels of IL-10 at the start of HAART; in addition to low CD4 counts and high viraemia.

Even though, other studies have shown low levels of IL-2 and high IL-10 serum concentrations in HIV-1-infected patients before HAART; particularly when CD4 count was less than 200 cells/ μ l (Orsilles *et al.*, 2006), there was no significant differences in this

stduy. This difference might be due to dispersion of CD4 counts as well as clinical characteristics of the study particcipants which were not considered in the present study.

Human Immuno-deficiency virus targets CD4 positive cells and replicates inside, causing several cytopathic effects that lead to depletion in number of these cells. In an *in vitro* experiment, removal of IL-10 producing cells from a population of lymphocytes from HIV positive patients has been associated with anti-HIV replication activities that include an increase in IL-2 level as well as increase in cytotoxicity of CD8 positive infected cells (Prescott *et al.*, 2002; Elrefaei *et al.*, 2007). In addition, HIV-1 may directly subvert specific immune responses by IL-10 induction as it was found that circulating frequencies of CD4⁺ T cells constitutively producing IL-10 are significantly higher in individuals with infection progression or active viral replication (Ostrowski *et al.*, 2001).

Highly active antiretroviral treatment has been shown to have a profound down-regulatory effect on HIV replication as well as effect on levels of cytokines and promotes CD4 positive cells recovery (Watanabe *et.,al*, 2010; Stylianou *et al.*, 1998). In agreement with findings from the present study, other studies have shown that following HAART, there is a significant drop in the viral load accompanied by a significant drop in IL-10 levels (Spitsin *et al.*, 2012; Ostrowski *et al.*, 2001), CD4⁺ cells recovery and a significant increase in IL-2 and IFN- γ (Trabattoni *et al.*, 2002). Furthermore, following HAART; there is evidence that IL-10 is anti-inflammatory while IL-2 and IFN- γ have pro-inflammatory effects (Osakwe *et al.*, 2010; Paris *et al.*, 2012; Song *et al.*, 2011)

The contribution of IL-10 to HIV disease progression was also highlighted by its strong positive correlation (r > 0.5 at *p* values <0.05 at both time points) with viraemia. On the other hand, this anti-inflammatory cytokine negatively correlated with CD4 count which concurs with other studies (Osakwe *et al.*, 2010; Song *et al.*, 2011).

The significant drop in the viral load as well as a significant increase in secretion of IFN- γ following HAART as found in the present study, are in agreement with what has been reported by Sadeghi *et al.* (2007). Contrary, Sachdeva *et al.* (2010) reported a decrease in this Th1 cytokine following antiretroviral treatment.

Findings from this study have demonstrated suppressive property of IL-10 on the synthesis of Th1 cytokines (IL-2 and IFN- γ). This down-regulatory effect of IL-10 was also associated with high viremia and a drop in CD4 count at HAART initiation. Following HAART, decrease in viremia was associated with a significant down-regulation of the synthesis of IL-10. This led to a significant recovery of CD4 positive cells as well as the duo of two Th1 cytokines (Osakwe *et al.*, 2010)

5.2 Conclusion

Cytokines have a complex effect on the replication of HIV and conversely in infected individuals; HIV directly affects cytokine production. Different studies postulate that the progression of disease in HIV infected individuals may be controlled by the balance between the levels of type 1 (Th1) and type 2 cytokines (Th2). In this study, Highly active antiretroviral treatment has been associated with significant drop in viral load and IL-10 levels as well as significant recovery of CD4⁺ cells and an increase in the levels of the two Th1 cytokines (IL-2 and IFN- γ) measured during this study. Interleukin-10 strongly and positively correlated with viral load before HAART and after six months of treatment while it weakly correlated (negative correlation) with CD4 count at both time points. The down-regulatory effect of IL-10 on Th1 cytokines as well as a shift from Th1 to Th2 cytokines with HIV disease progression has been demonstrated. Cytokines levels were not influenced by sex or age and this is a characteristic of stable and good markers, particularly IL-10.

5.3 Recommendations

The present study has shown that there is a shift from Th1 to Th2 cytokines in the course of HIV infection progression, and has confirmed the suppressive effect of IL-10 on other cytokines.

• If additional studies support findings in the present study, IL-10 should be considered as an alternative marker for assessment of the HIV infection prognosis but appropriate reference values should be established for Rwandan population and elsewhere.

• Laboratory personnel should receive training on ELISA and flow cytometric measurements of this cytokine as one of these techniques can be used to quantify cytokines. Multiplex measurements are preferred because many monoclonal antibodies can be used in a single reaction thereby reducing the cost and minimizing volumes of hazardous wastes. Based on the cost of ELISA kits for cytokines during this study, cytokine measurement would cost 30% of the current cost of viral load assessment in Rwanda.

• During this study, some patients had unchanged viral load after six months of HAART which suggested a treatment failure. While the viral load is the only current marker of a successful or failed HAART and this being done once in 12 months for HIV

patients in Rwanda, an improved biological follow up that will be able to detect treatment failure at an early stage of the treatment is recommended. If considered, cytokines measurement; particularly IL-10 would reduce the cost of follow up.

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APPENDICES

Appendix 1: Informed Consent Form

Title: IMMUNOLOGICAL PROFILES IN HIV POSITIVE PATIENTS HAART FOLLOWING INITIATION IN KIGALI-RWANDA

I am Augustin Penda Twizerimana, a student at Jomo Kenyatta University of Agriculture and Technology/Institute of Tropical Medicine and Infectious Diseases at Kenya Medical Research Institute (ITROMID-KEMRI). I am doing a research on HIV to assess the level of some parameters in the blood (IL-10, IL-2, IFN- γ , CD4 count and viral load) from HIV positive patients in Rwanda. The research consists in measuring above mentioned parameters both at the start of the ARV treatment and six months later on the same patients.

Results from this study will help to know changes in and interactions between these parameters; that occur after starting ARV treatment, to improve care offered to HIV positive patients. Results will be kept confidential and no one else will be able to have access to them.

If you accept to participate to this research, venous blood sample (8 ml) will be taken at the start of ARV treatment and then six months later while on treatment. Taking blood will take a little time and during the collection of blood from the vein, you might feel a little pain but this will only be for a very short time. Participation to the study is voluntary. At any time, you can end your participation to the study. There will be no negative consequences to you because of that. If you have any question even after starting participating to the study, feel free to ask me.

Thank you

Signature/thumb of the participant or representative For any concern, feel free to Contact me at: 0789391413 or at E-Mail: <u>p.amylase5@gmail.com</u> Rwanda National Ethics Committee: Dr Jean Baptiste MAZARATI at 0788309807 Dr Laetitia NYIRAZINYOYE at 0738683209

Appendix 2: Consent Form (Kinyarwanda version)

Urupapuro Rugaragaza Kwemera Kugira Uruhare Mu Bushakashatsi Ku Bushake Inyito y'ubushakashatsi: Immunological Profiles In HIV positive Patients Following HAART Initiation In Kigali/Rwanda

Nitwa TWIZERIMANA Augustin Penda; umunyeshuri muri kaminuza ya Jomo Kenyatta University of Agriculture and Technology/Institute of Tropical Medicine and Infectious Diseases at Kenya Medical Research Institute (ITROMID-KEMRI) mu gihugu cya Kenya. Ndimo gukora ubushakashatsi bugamije gupima uko bimwe mu bintu biboneka mu maraso bingana mu bantu babana n'ubwandu bw'agakoko gatera SIDA. Ibyo bipimwa ni IL-10, IL-2, IFN-γ, CD4 count and viral load. Ibi bizapimwa mu maraso y'umurwayi mbere yo gutangira imiti ndetse na nyuma y'amezi atandatu uyu murwayi atangiye iyi miti igabanya ubukana bw'agakoko gatera SIDA.

Ibisubizo by'ibyabonetse mu maraso yanyu bizagirwa ibanga hagati yanjye namwe. Ibizava muri ubu bushakashatsi bizatuma hamenyekana uko ibintu bimwe na bimwe biboneka mu maraso biba bingana ndetse n'uko bikorana hagati yabyo mbere y'uko umuntu ubana n'ubwandu bw'agakoko gatera SIDA atangira imiti igabanya ubukana bw'ako gakoko ndetse na nyuma y'amezi atandatu umurwayi atangiye iyi imiti.

Ibi kandi bizafasha mu kurushaho kwita ku bantu babana n'ubwandu bw'aka gakoko gatera SIDA.

Uramutse wemeye kugira uruhare muri ubu bushakashatsi, turagufata amaraso yo mu mutsi (ml 8) mbere yo gutangira imiti igabanya ubukana bw'agakoko gatera SIDA ndetse na nyuma y'amezi atandatu (6) umuntu afata iyo miti. Hashobora kubaho ububabare buke cyane mu gihe amaraso aza kuba afatwa ariko ubwo bubabare buramara agahe gato cyane.

Kugira uruhare muri ubu bushakashatsi ni ubushake kandi ushobora guhagarika uruhare rwawe mu bushakashatsi n'igihe waba waramaze gutangira. Nta ngaruka mbi zabaho ziturutse ku kwanga cyangwa guhagarika kugira uruhare muri ubu bushakashatsi. Wemererwa kumbaza ikibazo icyo ari cyo cyose wagira n'iyo byaba nyuma yo gutangira kugira uruhare mu bushakashatsi.

Murakoze cyane

Umukono cyangwa igikumwe w/cy'ugira uruhare mu bushakashatsi cyangwa umuhagarariye

Mugize ikibazo mwambaza kuri telefoni ifite nomero: 0789391413 no kuri E-Mail: p.amylase5@gmail.com

Mushobora no kubaza Urwego rw'igihugu rushinzwe iyubahirizwa ry'amategeko mu bushakashatsi, mukabaza uwitwa:

Dr Jean Baptiste MAZARATI at 0788309807, Umuyobozi mukuru

Dr Laetitia NYIRAZINYOYE at 0738683209, Umunyamabanga

representative of Standard Operating refocutive for OD recount
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	Title:CD4 count FACSCalibur manually			
RWANDA BIOMEDICAL CENTER	SOP Number: NRL-IMM-SOP 014- VERS 003	Effective date: 16/01/2013	Revision date: 16/01/2014	
A Healthy People. A Wealthy Nation National Reference Laboratory (NRL) Division	Prepared and signed by: TUYIZERE Samuel (Team leader)	Authorized and signed by: Dr Odette MUKABAYIRE Head of NRL Division		

Background and Purpose

The purpose of this SOP is to explain the analysis of a CD4 sample prepared using Tri test antibody (Anti-CD3/CD4/CD45) +Tru count tubes and the run of CaliBRITE beads on FACSCalibur without a loader. The CD4 sample will be prepared according to the CD4 sample preparation SOP and the CaliBRITE beads prepared as stated in the SOP of Calibration of FACSCalibur. The SOP will be used when the loader is out of work. This SOP is aimed to provide guideline in order to ensure the quality of the CD4 enumeration and running CaliBRITE beads using FACS Calibur machine.

Responsibilities have been assigned as follows:

Task	Person responsible
Ensure that all relevant people have read this SOP, are trained accordingly and that this is documented appropriately	Director of Immuno-virology unit Immunology Laboratory In-charge
Conduct the preparation of samples	Laboratory biotechnologists
Conduct the preparation of CaliBRITE beads	Laboratory biotechnologists
Ensure adequate stock of test kits and supplies needed	Immunology Laboratory In-charge
Ensure that all procedures are performed according to the SOP	Immunology Laboratory In-charge, Laboratory biotechnologists

Ensure validation, recording and reporting of results	Director of Immuno-virology unit
	Immunology Laboratory In-charge

Equipment and Consumables

- -Vortex mixer
- -BD FACSCalibur machine
- -FACS Flow (sheath fluid)
- -Antibody (Tri-Test 3 color reagents)
- -FACS Lyse solution
- -FACS Clean solution
- -FACS Rinse solution
- -TruCOUNT Tubes
- -Latex gloves

Procedures

Running CaliBRITE beads

- CaliBRITE beads are prepared according to the SOP NRL-LAB-CD4 03 vers 02
- Starting up the FACS Calibur using the SOP NRL-LAB-CD4 01 vers 01
- On the main menu, click on FACSComp, then enter operator name
- Enter Lot IDs using card provided in CaliBRITE beads kit:
- Type FITC bead lot ID
- Type PE bead lot ID
- Type Unlabeled lot ID
- Type PerCP bead lot ID
- On the top of the sreen, choose FACSComp, the prefernces.
- On the left side of the screen where there are: Optimization, Export, Printing, LJ Data, Loader, LeucoCOUNT, Deactivite Run with the Loader.
- Place Click on SAVE.
- Remove the tube of distilled water on FACSCalibur.
- Vortex gently the tube A
- Put FACSCalibur on RUN and HI mode

- Put the tube A on support arm of the FACSCalibur
- Click on Run, then the FACSCalibur start to run tube A
- After running the tube A, it asks the tube B
- Put the tube B on support arm of the FACSCalibur
- Click on RUN, the FACSCalibur run tube B and print the calibration report on printer
- Check the calibration printout and verify if all parameters passed
- If all parameters passed click on Quit to exit the program
- Fill out the daily maintenance log sheet
- Put the signature of operator and date on the report
- File the calibration report in calibration file
- If the calibration failed, repeat the point 6.2.15 to 6.2.20
- Run sample using the protocol below

Running sample

- Launch Multiset
- Type operator name then click on Accept
- In data source, select From Cytometer: Acquisition with Analysis
- Click on Accept
- Click on OK
- Skip FACSComp
- Click Accept and you reach to Worklist
- Type Sample name, Sample ID, sex, age, site and treatment according to the number of sample
- Select the panel name CD3/CD4/CD45 at each sample
- stained samples must be kept at room temperature in dark
- Vortex the Trucount tube
- Remove the tube of distilled water on FACSCalibur
- Activate RUN and HI mode on FACSCalibur
- Put the first tube on FACSCalibur
- Click on RUN
- Wait until the cell's histograms appear on the screen
- Click on Acquire
- The machine run the sample and print the result on printer

- Remove the Trucount tube on FASCalibur immediately
- Put the second tube and repeat 6.3.22 to 6.3.26 until you finish all samples
- After running all samples, click on Quit to quit Multiset
- Remove the last Trucount on FACSC alibur
- Shut down the FACSCalibur according to the SOP NRL-LAB-CD4 01 vers 01
- Discard the Trucount tube into the infectious waste bin

Appendix 4: Viral Load Assay

	Title:The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test			
BOMEDICAL	SOP Number:	Effective date:	Revision	
A Health People, A Wealthy Nation	NRL-IMM-SOP 014-VERS 003	16/01/2013	date: 16/03/2014	
National			10/00/2011	
Reference	Prepared and signed by:	Authorized and signed by:		
Laboratory (NRL) Division	Musabyimana Jean Pierre	Dr Odette MUKABAYIRE		
		Head of NRL Division		

1. Intended Use

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is an in vitro nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma using the COBAS® AmpliPrep Instrument for automated specimen processing and COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer for automated amplification and detection. This test is intended for use in conjunction with clinical presentation and other laboratory markers of disease progress for the clinical management of HIV-1 infected patients. The Test can be used to assess patient prognosis by measuring the baseline HIV-1 RNA level or to monitor the effects of antiretroviral therapy by measuring changes in EDTA plasma HIV-1 RNA levels during the course of antiretroviral treatment. The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is not intended for use as a screening test for the presence of HIV-1 in blood or blood products or as a diagnostic test to confirm the presence of HIV-1 infection. The test can quantitate HIV-1 RNA over the range of 48 – 10,000,000 copies/mL. One copy of HIV-1 RNA is equivalent to 1.7 ± 0.1 International Units (IU) based on the WHO 1st International Standard for HIV-1 RNA for Nucleic Acid-Based Techniques (NAT) (NIBSC 97/656).

2. Principle

The COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test is a nucleic acid amplification test for the quantitation of Human Immunodeficiency Virus Type 1 (HIV-1) RNA in human plasma. Specimen preparation is automated using the COBAS® AmpliPrep Instrument with amplification and detection automated using the COBAS® TaqMan® Analyzer. Test is based

on three major processes: (1) specimen preparation to isolate HIV-1 RNA; (2) reverse transcription of the target RNA to generate complementary DNA (cDNA), and (3) simultaneous PCR amplification of target cDNA and detection of cleaved dual-labeled oligonucleotide probe specific to the target.

The quantitation of HIV-1 viral RNA is performed using the HIV-1 QS. The HIV-1 QS is added to each specimen at a known copy number.

The COBAS® TaqMan® Analyzer calculates the HIV-1 RNA concentration in the test specimens by comparing the HIV-1 signal to the HIV-1 QS signal for each specimen and control. Generic silica based specimen preparation is used to capture the HIV-1 RNA and HIV-1 QS RNA and defined oligonucleotides are used as primers in amplification of the HIV-1 RNA and HIV-1 QS RNA. A target-specific and a QS-specific dual-labeled oligonucleotide probe permit independent identification of HIV-1 amplicon and HIV-1 QS amplicon. Titer results are reported in copies/mL (cp/mL). The test can quantitate HIV-1 RNA over the range of 48 - 10,000,000 copies/mL.

3. Reagents

COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test.

COBAS® AmpliPrep/COBAS® TaqMan® Wash Reagent

Disposables

- Sample processing units: SPUs (P/N: 03755525001)
- Sample input tubes (S-tubes) with barcode clips (P/N: 03137040001)
- Racks of K-tips (P/N: 03287343001)
- K-tube Box of 12 x 96 (P/N: 03137082001)

4. Equipment

COBAS® AmpliPrep

COBAS® TaqMan
5. Procedure

Preparing specimens for testing

- Receive double labeled (TRACNET number and lab code) specimen together with its corresponding laboratory request form.
- Inspect the specimen for acceptance criteria such as volume (minimum 4 ml), nonhemolysis, and cross-check to ensure good labeling. Non-compliant specimens should be rejected and documented then communicated to the sender with a suggested corrective action.
- Centrifuge the specimen at 3000 rpm for 20 minutes.
- Aliquot the total plasma to sterile micro vial tube and store between 2 and 8° Celsius if testing is going to occur not later than 6 days. If not, store at -20 for no later than 6 weeks.
- Start of COBAS® AmpliPrep/ COBAS® TaqMan system Run
- Turn the Data Station for the AMPLILINK software ON. Prepare the Data Station as follows:
- Log into Windows® XP.
- Double click the AMPLILINK software icon.
- Log into AMPLILINK software by entering the assigned User ID and password.
- Check the supply of PG WR using the Status Screen and replace if necessary.

6. Maintenance

- Perform all Maintenance that is listed in the Due Tab, as outlined in the COBAS® AmpliPrep
- Instrument Manual for use with the COBAS® TaqMan® Analyzer and the AMPLILINK Software, Version 3.3.x Series. The COBAS® AmpliPrep Instrument will automatically prime the system.

Ordering and Loading of Specimens

Prepare sample racks as follows:

- Prepare a laboratory work sheet by making an order of controls and specimens as designated on the work sheet
- Attach a barcode label clip to each sample rack position where a specimen (S-tube) is to be placed.

- Attach one of the specific barcode label clips for the controls [CTM (–) C, HIV-1 L(+)C and HIV-1 H(+)C] to each sample rack position where the controls (S-tube) are to be placed.
- The barcode label clips for controls must have the same control lot number as the lot number on the control vials in the kit.
- Take care in assigning the correct control to the position with the appropriate control barcode clip.
- Place one Input S-tube into each position containing a barcode label clip.
- Using the AMPLILINK software, create specimen orders for each specimen and control in the Orders window Sample folder referring to the laboratory work sheet. The Test file name in the Orders window will be HI2CAP96 Select the appropriate test file and complete by saving.
- Assign specimen and control orders to sample rack positions in the Orders window Sample Rack folder.
- Prepare specimen and control racks in the designated area for specimen and control addition as follows: Vortex each specimen and control [CTM (–) C, HIV-1 L(+)C and HIV-1 H(+)C] for 3 to 5 seconds.
- Avoid contaminating gloves when manipulating the specimens and controls.
- Transfer 1000 to 1050 µL of each specimen and control [CTM (–) C, HIV-1 L(+)C and HIV-1 H(+)C] to the appropriate barcode labeled Input S-tube using a micropipette with an aerosol barrier or positive displacement RNase-free tip. Avoid transferring particulates and/or fibrin clots from the original specimen to the Input S-tube.
- Specimens and controls should be transferred to tube positions as assigned and recorded on the worksheet. The barcode label clips for controls must have the same control lot number as the lot number on the control vials in the kit. Assign the correct control to the position with the appropriate control barcode clip. Avoid contaminating the upper part of the S-tubes with specimens or controls.
- Load the prepared sample rack to the sample loading panel of COBAS® AmpliPrep instrument.
- Start the COBAS® AmpliPrep Instrument using the AMPLILINK software as described in the AMPLILINK Software Version 3.3.x Series Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer, The

COBAS® AmpliPrep Instrument is ready for operation in stand-by mode.

- End of COBAS® AmpliPrep Instrument Run and Transfer to COBAS® TaqMan® Analyzer
- Check for flags or error messages in the system screen as described in (a) the COBAS® AmpliPrep Instrument Manual for use with the COBAS® TaqMan® Analyzer and the AMPLILINK Software, Version 3.3.x Series and (b) the AMPLILINK Software Version 3.3 Series Application Manual for use with the COBAS® AmpliPrep Instrument, COBAS® TaqMan® Analyzer
- Remove processed specimens and controls from the COBAS® AmpliPrep Instrument on either sample racks
- Remove waste from COBAS® AmpliPrep Instrument.
- Results review and acceptance
- When the run is complete, check the results icon of the AMPLILINK Software or click F8. Review, accept and then print results.
- Transcribe the results to the laboratory register then to the request form and send them to the laboratory manager for validation.

Appendix 5: Assay Method for IL-10

1. REAGENT PREPARATION AND STORAGE

REAGENTS PROVIDED

Note: Store all reagents at 2 to 8°C.

Reagent	96 Test Kit	192 Test Kit	480 Test Kit
<i>Hu IL-10 Standard</i> , recombinant Hu <i>IL-10</i> . Refer to vial label for quantity and reconstitution volume.	2 vials	4 vials	10 vials
Standard Diluent Buffer. Contains 8 mM sodium azide; 25 mL per bottle.	1 bottle	2 bottles	5 bottles
Incubation Buffer. Contains 8 mM sodium azide; 25 mL per bottle.	1 bottle	1 bottle	5 bottles
Hu IL-10 Antibody-Coated Wells. 96 wells per plate.	1 plate	2 plates	5 plates
<i>Hu IL-10 Biotin Conjugate</i> , (Biotin-labeled anti- <i>IL-10</i>). Contains 8 mM sodium azide; 11 mL per bottle.	1 bottle	2 bottles	5 bottles
Streptavidin-Peroxidase (HRP), (100x concentrate). Contains 1.3 mM thymol; 0.125 mL per vial.	1 vial	2 vials	5 vials
Streptavidin-Peroxidase (HRP) Diluent. Contains 1.3 mM thymol and 0.05% Proclin® 300; 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Wash Buffer Concentrate (25x); 100 mL per bottle.	1 bottle	1 bottle	2 bottles
<i>Stabilized Chromogen</i> , Tetramethylben- zidine (TMB); 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Stop Solution; 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Plate Covers. Adhesive strips.	4	6	15

Reconstitution and Dilution of Hu IL-10 Standard

- Reconstitute standard to 5000 pg/mL with Standard Diluent Buffer. Refer to standard vial label for instructions.
- Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use standard within 1 hour of reconstitution.
- Add 0.050 mL of the reconstituted standard to a tube containing 0.450 mL Standard Diluent Buffer. Label as 500 pg/mL Hu IL-10.
- Mix. Add 0.200 mL of Standard Diluent Buffer to each of 6 tubes labeled 250, 125,

62.5, 31.2 ,15.6 and 7.8 pg/mL Hu IL-10.

• Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

Standard:	Add:	Into:
500 pg/mL	Prepare as described in Step 2.	
250 pg/mL	0.200 mL of the 500 pg/mL std.	0.200 mL of the Diluent Buffer
125 pg/mL	0.200 mL of the 250 pg/mL std.	0.200 mL of the Diluent Buffer
62.5 pg/mL	0.200 mL of the 125 pg/mL std.	0.200 mL of the Diluent Buffer
31.2 pg/mL	0.200 mL of the 62.5 pg/mL std.	0.200 mL of the Diluent Buffer
15.6 pg/mL	0.200 mL of the 31.2 pg/mL std.	0.200 mL of the Diluent Buffer
7.8 pg/mL	0.200 mL of the 15.6 pg/mL std.	0.200 mL of the Diluent Buffer
0 pg/mL	0.200 mL of the Diluent Buffer	An empty tube

• Discard all remaining reconstituted and diluted standards after completing assay. Return the Standard Diluent Buffer to the refrigerator.

Storage and Final Dilution of Streptavidin-HRP

 Dilute 10 µL of this 100x concentrated solution with 1 mL of Streptavidin-HRP Diluent for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.

For Example:

# of 8-Well Strips	Volume of Streptavidin-HRP Concentrate	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	$120 \ \mu L$ solution	12 mL

2. Return the unused *Streptavidin-HRP* concentrate to the refrigerator.

Dilution of Wash Buffer

• Allow the 25x concentrate to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the 25x wash buffer

concentrate with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer. Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

2. Directions for Washing

Incomplete washing will adversely affect the test outcome. All washing must be performed with Wash Buffer provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under ASSAY METHOD. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue. Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, first tap out the contents of wells. Flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue. If using an automated washer, the operating instructions for washing equipment should be carefully followed. If your washer allows, 30 second soak cycles should be programmed into the wash cycle.

3. Procedure and Storage

Be sure to read the Procedural Notes/Lab Quality Control section before carrying out the assay. Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

- Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
- Add 50 µL of the Standard Diluent Buffer to zero wells. Well(s) reserved for chromogen blank should be left empty.
- Add 50 µL of standards, samples or controls to the appropriate microtiter wells. (See

REAGENT PREPARATION AND STORAGE, Section B.)

- Add 50 μ L of Incubation Buffer to the wells containing standards and serum/plasma samples or 50 μ L of Standard Diluent Buffer to the wells containing cell culture samples.
- Cover plate with plate cover and incubate for 2 hours at room temperature.
- Thoroughly aspirate or decant solution from wells and discard the liquid.
- Wash wells 4 times. See DIRECTIONS FOR WASHING.
- Pipette 100 μL of biotinylated anti-IL-10 (Biotin Conjugate) solution into each well except the chromogen blank(s).
- Tap gently on the side of the plate to mix. Cover plate with plate cover and incubate for 2 hours at room temperature.
- Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See DIRECTIONS FOR WASHING.
- Add 100 µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in REAGENT PREPARATION AND STORAGE, Section C.)
- Cover plate with the plate cover and incubate for 30 minutes at room temperature.
- Thoroughly aspirate or decant solution from wells and discard the liquid.
- Wash wells 4 times. See DIRECTIONS FOR WASHING.
- Add 100 μ L of Stabilized Chromogen to each well. The liquid in the wells will begin to turn blue.
- Incubate for 30 minutes at room temperature and in the dark. Please Note: Do not cover the plate with aluminum foil or metalized mylar.
- The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the Stop Solution has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested Add 100 μ L of Stop Solution to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 μ L each of Stabilized Chromogen and Stop

Solution. Read the plate within 2 hours after adding the Stop Solution.

- Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.)
- Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit. Read the Hu IL-10 concentrations for unknown samples and controls from the standard curve plotted in Step 17. Samples producing signals greater than that of the highest standard (500 pg/mL) should be diluted in Standard Diluent Buffer for serum/plasma samples or corresponding medium for cell culture samples and reanalyzed, multiplying the concentration found by the appropriate dilution factor.

Appendix 6: The Biosource IL-2 EASIA Assay

REAGENTS PROVIDED

Reagents	96 tests Kit	192 tests Kit	Reconstitution
Microtiter plate with 96 anti- IL-2 coated wells	1 x 96 wells	2 x 96 wells	Ready for use
Standards 0 to 5 in human plasma with preservatives : see vial label for exact concentrations	6 vials lyophil.	6 vials lyophil.	Add 1 ml distilled water
Solution A (human plasma with preservatives) for cell culture	2 vials Iyophil.	4 vials lyophil.	Add distilled water (see vial label for exact volume)
Solution B (buffer with preservatives) : for serum/plasma	1 vial 11 ml	2 vials 11 ml	Ready for use
Anti-IL-2-HRP Conjugate in a buffered solution with proteins and preservatives	1 vial 6 ml	2 vials 6 ml	Ready for use
Controls 1 and 2 in human plasma with preservatives	2 vials lyophil.	2 vials lyophil.	Add 1 ml distilled water
Washing Solution Concentrate (buffer with preservatives)	1 vial 10 ml	1 vial 10 ml	Dilute 2 ml in 400 ml distilled water or the vial contents in 2000 ml distilled water
Chromogen : TMB	1 vial 25 ml	1 vial 25 ml	Ready for use
Stop Solution	1 vial 25 ml	2 vials 25 ml	Ready for use

Reagent Preparation

Standards, Controls and Solution A : Reconstitute the lyophilized Standards, Controls and Solution A to the volume specified on the vial label with distilled water (1 ml for Standards and Controls). Allow them to remain undisturbed until completely dissolved, then mix well by gentle inversion.

Wash Solution: Dilute 2 ml of Washing Solution Concentrate in 400 ml distilled water or all the contents of the Washing Solution Concentrate vial in 2000 ml distilled water (use a magnetic stirrer).

Storage and Shelf Life of Reagents

A. UNOPENED vials Store the unopened vials at 2/°C to 8/ °C. All kit components are stable

until the expiry date printed on the labels.

B. OPENED vials: The Conjugate vial must be stored at 2/ to 8/ $^{\circ}$ C. 2. The reconstituted Standards, Controls and Solution A are stable for 4 days at 2/ $^{\circ}$ C to 8/ $^{\circ}$ C. Aliquots held for longer periods of time should be frozen, a maximum of two times, at -20/ $^{\circ}$ C (maximum 2 months) or at -70/ $^{\circ}$ C for longer storage (until expiration date).

Store the unused strips at $2/{^{\circ}C}$ to $8/{^{\circ}C}$ in the sealed bag containing the dessicant until expiration date.

The Wash Solution Concentrate is stable at room temperature until expiration date. In order to avoid washerhead obstructions, it is recommended to prepare a fresh diluted Wash Solution each day.

Equipment and Supplies Required But Not Provided

High quality distilled water. 2. Precision pipettes 3. Vortex mixer and magnetic stirrer. 4. Horizontal microtiter plate shaker capable of 700 rpm \pm 100 rpm, 5. microtiter plate reader capable of reading at 450 nm and 490 nm and 6. microtiter plate washer.

Procedure

The instructions of the assay procedure must be followed to obtain reliable results. A. Procedural notes

1. Allow the samples and reagents to equilibrate to room temperature (18/C to 25/C) before commencing the assay. Thoroughly mix the reagents and samples before use by gentle agitation or swirling.

2. Do not use kit components beyond the expiration date.

3. Do not mix materials from different kit lots.

4. Do not mix strips from different plates.

5. Perform Standards, Controls and Unknowns in duplicate. Vertical alignment is recommended.

6. A standard curve should be run with each assay run or each plate run.

7. To avoid drift, the time between pipetting of the first standard and the last sample must be

no longer than 30 minutes. Otherwise, results will be affected.

8. Use a clean disposable plastic pipette for each reagent, standard, control or specimen addition in order to avoid cross contamination .

9. For the dispensing of the Chromogenic Solution and Stop Solution avoid pipettes with metal parts. 10. Use a clean plastic container to prepare the Wash Solution.

11. The Chromogenic Solution should be colourless. If a blue colour develops within a few minutes after preparation, this indicates that the reagent is unusable, and must be discarded. Dispense the Chromogenic Solution within 15 min. following the washing of the microtiter plate.

12. During incubation with Chromogenic Solution, avoid direct sunlight on the microtiter plate.

13. Respect the incubation times described in the assay

B. Assay Procedure

1. Select the required number of stripsfor the run. The unused stripsshould be resealed in the bag with desiccant and stored at 2-8/°C. 2. Secure the strips into the holding frame.

3. Pipette 100 μ l of Solution B into the appropriate wells foreseen for the Standards and Controls.

4. Pipette 100 μ l of Solution B into the appropriate wells for serum/plasma samples, or , Pipette 100 μ l of Solution A into the appropriate wells for cell culture supernatant/urine samples.

5. Pipette 100 µl of each Standard, Control, or Sample into the appropriate wells.

6. Pipette 50 µl of anti-IL-2 Conjugate into all the wells.

7. Incubate for 2 hours at room temperature on a horizontal shaker set at 700 rpm \pm 100 rpm.

8. Aspirate the liquid from each well;

9. Wash the plate three times by : a) dispensing of 0.4 ml of Wash Solution into each well ; b) aspirating the content of each well.

10. Pipette 100 µl of Chromogen into each well within 15 min. following the washing step.

11. Incubate the plate for 15 min. at room temperature on an horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight.

12. Pipette 200 µl of Stop Solution into each well.

13. Read absorbances at 450 nm and 490 nm (reference filter : 630 or 650 nm) within 3 hours and calculate the results using standard curve.

Appendix 7: Human IFN-γ ELISA

Reagents Provided	96 Test Kit	Color
Standard 0 in human serum, with benzamidin and thymol. Lyophilized. Refer to vial label for quantity and reconstitution volume.	3 vials	Black
Standards 1 - 5 in human serum, with benzamidin and thymol. Lyophilized. Refer to vial label for concentration and reconstitution volume. 1 U of the standard preparation is equivalent to 1 IU NIBSC 87/586.	5 vials	Yellow
Controls 1 and 2 in human serum, with benzamidin and thymol. Lyophilized. Refer to vial label for reconstitution volume and range.	2 vials	Silver
IFN-y Antibody-Coated Wells, 96 wells per plate.	1 plate	Blue
Anti-IFN- ₇ -HRP Conjugate in Tris-Maleate Buffer with BSA and thymol; 6 mL per bottle.	1 bottle	Red
Wash Buffer Concentrate (200x); 10 mL per bottle.	1 bottle	Brown
Concentrated Chromogen, Tetramethylbenzidine (TMB) in DMF, 1 mL per vial	1 vial	Green
Substrate Buffer: H ₂ 0 ₂ in acetate/citrate buffer; 21 mL per botttle	3 bottles	White
Stop Solution, 1.8 N H ₂ SO ₄ ; 6 mL per bottle	1 bottle	Black
Note: Standard 0 is recommended for sample dilutions	22	

Materials Needed But Not Provided

Microtiter plate reader (at or near 450 nm) with software \cdot Horizontal microtiter plate shaker capable of 700 rpm \pm 100 rpm \cdot Calibrated adjustable precision pipettes \cdot Distilled or deionized water \cdot Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.) \cdot Glass or plastic tubes for diluting solutions \cdot Absorbent paper towels \cdot Calibrated beakers and graduated cylinders

Preparation of Reagents

Standards	Reconstitute lyophilized Standard 0 according to the instructions on the vial.
and Controls	Reconstitute the lyophilized Standards and Controls to the volume specified on the vial label with distilled water (0.5 mL). Allow them to remain undisturbed until completely dissolved, then mix well by gentle inversion. See vial label for exact concentration.
	The reconstituted Standards and Controls are stable for 4 days at 2°C to 8°C. Aliquots held for longer periods of time should be frozen at -20°C (maximum 2 months) or at -70°C for longer storage (until expiration date). Avoid freeze-thaw cycles.
Wash Buffer	Dilute 2 mL of Washing Solution Concentrate in 400 mL distilled water or all the contents of the Wash Solution Concentrate vial in 2000 mL distilled water (use a magnetic stirrer).
	The Wash Solution Concentrate is stable at room temperature until expiration date. In order to avoid washerhead obstructions, it is recommended to prepare a fresh diluted Wash Solution each day.

Chromogen Solution

Pipette 0.2 mL of the Concentrated Chromogen (TMB) into one of the vials of Substrate Buffer (H2O2 in acetate/citrate buffer). Extemporaneous preparation is necessary. Use only at room temperature. Avoid direct exposure to sunlight. The freshly prepared Chromogen Solution is stable for a maximum of 15 min. at room temperature and must be discarded afterwards.

Assay Procedure

Be sure to read the Procedural Notes section before carrying out the assay. Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use. Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame for current use. (Re-bag extra strips. Store these in the refrigerator for future use.)

2. Pipette 50 µL of each Standard, Control, or Sample into the appropriate wells.

3. Pipette 50 μ L of anti-IFN- γ HRP Conjugate into all the wells.

4. Incubate for 2 hours at room temperature on a horizontal shaker set at 700 rpm \pm 100 rpm.

5. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See Direction for Washing.

6. Pipette 200 µL of freshly prepared Chromogen Solution into each well within 15 min.

7. Incubate the plate for 15 min. at room temperature on an horizontal shaker set at 700 ± 100 rpm, avoiding direct sunlight. Note: Do not cover the plate with aluminum foil or metalized mylar. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument.

8. Pipette 50 μ L of Stop Solution into each well. The solution in the wells should change from blue to yellow.

9. Read absorbance at 450 nm and 490 nm (reference filter: 630 or 650 nm) within 3 hours. Read the plate within 30 minutes after adding the Stop Solution.

10. Use a curve fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit.

11. Read the concentrations for unknown samples and controls from the standard curve. (Samples producing signals greater than that of the highest standard (500 pg/mL) should be further diluted with Standard 0 and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

12. If any Control or Sample has an absorbance greater than the absorbance of the last standard read at 450 nm, a second reading at 490 nm (reference filter: 630 or 650 nm) is needed.

Construct a second standard curve at 490 nm using all the standard points. The segment of the curve drawn between the last standard read at 450 nm and the most concentrate standard will be considered at 490 nm.

The concentration of Samples and Controls for which absorbance is included in this segment, is read at 490 nm. So, the first reading gives the high sensitivity of the assay and the second reading allows an extended standard range. Note: The readings at 490 nm are only for off scale values at 450 nm (above the limit of reader linearity) and should not replace the reading at 450 nm for values below the limit of reader linearity.

Appendix 8: Ethical Clearance from Rwanda National Ethics Committee

REPUBLIC OF RWANDA/REPUBLIQUE DU RWANDA NATIONAL ETHICS COMMITTEE / COMITE NATIONAL D'ETHIQUE Telephone: (250) 2 55 10 78 84 **Ministry of Health** E-mail: info@rnecrwanda.org P.O. Box. 84 Web site: www.rnecrwanda.org Kigali, Rwanda. FWA Assurance No. 00001973 IRB 00001497 of IORG0001100 August 01, 2013 No.583/RNEC/ 2013 Augustin PENDA TWIZERIMANA Principal Investigator (A student) Your Project title: "IMMUNOLOGICAL PROFILES IN HIV POSITIVE PATIENTS FOLLOWING HAART INITIATION IN KIGALI/ RWANDA" has been evaluated by the **Rwanda National Ethics committee.** Involved in the decision No (Reason) Name Institute Yes Absent Withdrawn from the proceeding Dr.Jean-Baptiste MAZARATI National Referral Х Laboratory Prof. Eugène RUTEMBESA National University of X Rwanda Dr.Laetitia NYIRAZINYOYE National University of X Rwanda(school of public Health) National Prof.Alexandre University of X Rwanda LYAMBABAJE Ms.Françoise UWINGABIYE Lawyer at Musanze X National University X Dr. Egide KAYITARE of Rwanda

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After reviewing your protocol during the RNEC meeting of 13 July 2013 where quorum was met, and revisions made on the advice of the RNEC submitted on 01 August 2013, **Approval letter has been granted to your study**.

Please note that approval of the protocol and consent form is valid for **12 months**. You are responsible for fulfilling the following requirements:

- Changes, amendments, and addenda to the protocol or consent form must be submitted to the committee for review and approval, prior to activation of the changes.
- 2. Only approved consent forms are to be used in the enrollment of participants
- All consent forms signed by subjects should be retained on file. The RNEC may
 conduct audits of all study records, and consent documentation may be part of such
 audits.
- 4. A continuing review application must be submitted to the RNEC in a timely fashion and before expiry of this approval.
- Failure to submit a continuing review application will result in termination of the study.
- 6. Notify the Rwanda National Ethics committee once the study is finished.

NATIONA

Sincerely,

BATE.O

Dr. Jean- Baptiste MAZARATI Chairperson, Rwanda National Ethics Committee.

C.C.

Minister of Health

Expiration date: July 31, 2014

Date of Approval: August 01, 2013

- Hon. Minister of Health.

- The Permanent Secretary, Ministry of Health.

Appendix 9: Research Permit from the Ministry of Education/Rwanda

REPUBLIC OF RWANDA



MINISTRY OF EDUCATION P.O BOX 622 KIGALI Kigali, 2510.812043.... Ref: 20.5.4..../12.00/2013



Re: Permission to carry out research in Rwanda - No: MINEDUC/S&T/0157/2013

Permission is hereby granted to Mr. PENDA TWIZERIMANA Augustin, a Master's student in Molecular Medicine at the Institute of Tropical Medicine and Infectious Diseases (ITROMID) a joint programme between Kenya Medical Research Institute (KEMRI) and Jomo Kinyatta University of Agriculture and Technology (JKUAT) to carry out research on: "Immunological Profiles in HIV Positive Patients following HAART Initiation in Kigali/Rwanda ".

The research will be conducted in Nyarugenge, Nyakabanda, Muhima and Kimironko sectors in Gasabo and Nyarugenge Districts. He will interview 33 HIV Positive Patients coming from HAART Initiation at selected Health Centers.

The period of research is from 22nd, August, 2013 to 31st, July, 2014. This period may be renewed if necessary, in which case a new permission will be sought by the researcher.

Please provide Mr. PENDA TWIZERIMANA Augustin any support he may require in the course of conducting this research.

Yours sincerely,

Mr.TWIRINGIYIMANA Remy Acting Director General, Science, Technology and Research Ministry of Education

Appendix 10: Scientific review approval



Dr. Jean de Dieu Norrab Chairpenson of NHRC Signature Date

Dr. Leon Mutesa Vice-Chairperson of NHRC Signature -Date: 16-04-2013