PLASMA CYTOKINE LEVELS AND ACTIVATED T CELL MARKERS IN HUMAN IMMUNODEFICIENCY VIRUS POSITIVE INDIVIDUALS IN RWANDA

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RESEARCH THESIS SUBMITTED TO MOI UNIVERSITY, SCHOOL OF MEDICINE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR AN AWARD OF THE DEGREE OF MASTERS OF SCIENCE IN IMMUNOLOGY OF MOI UNIVERSITY.

DECLARATION

Declaration by the candidate:

This thesis report is my original work, and has not been presented to any other university. No part of this research work may be reproduced without principal investigators prior permission.

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DEDICATION

This study work report is dedicated to all individuals who know their HIV status.

ABSTRACT

Background: Cytokines network impact on Human Immunodeficiency Virus (HIV) pathogenesis, viral latency; infection reservoirs; persistent immune activation and chronic inflammation. Highly Active Antiretroviral Therapy (HAART) maintains low HIV Viral Load (VL) but immune activation level and chronic inflammation remain high in HIV infected /AIDS individuals compared to HIV naïve population. Immunological markers to assess immune status, chronic inflammation in HIV infected /AIDS individuals have not been resolved.

Objective: To determine plasma cytokine levels, peripheral T cell activation markers and their relationship with HIV VL in HIV infected /AIDS individuals initiating and those who had completed six months on HAART in Rwanda.

Methods: This was a comparative cross sectional study in HAART naïve and with HAART for six months for population above 18 years in five provinces of Rwanda. The matching groups approach based on sex and systematic sampling within the groups were used to enroll fifty patients in each group. Fifteen HIV naive individuals were included in the study as controls. Flow cytometry was used to determine cytokine levels and T cell activation markers expression while a revised WHO questionnaire was used to collect sociodemographic and clinical data for study subjects. R Studio statistic package and graph Pad Prism 7 package were used for descriptive and analytical statistic of the data at initiation and after six months of HAART treatment to address specific objectives. Statistical significance differences and the correlation between assessed parameters were determined at p-value ≤ 0.05.

Results: At the initiation of HAART, Interleukin (IL)-2, IL-10, IL-6 plasma levels, Cluster of Differentiation (CD)69, CD154 expression were high while IFN-γ and TNF-α were low compared to its levels after six months HAART. There was no statistically significant difference between CD69 and CD154 activation markers co-expression on $CD8^+$T cell at initiation and after six months of HAART (p-value = 0.167). IL-10, IL- 6 and CD69 expression on $CD4^+$T cell correlated more with HIV VL (p value = 0.001).

Conclusions: Th-2 cytokines milieu mediate HIV/AIDS immunity before HAART and tended to shift to Th-1 cytokines milieu following successful HAART treatment after six months. CD69 and CD154 express differently on T cells at initiation and after six months of HAART. IL- 6, IL-10 plasma level and CD69 T cell activation markers are good correlate for HIV VL.

Recommendations: This study recommended IL-10, IL-6 plasma levels and CD69 expression on $CD4^+$T cell as alternative immunological biomarkers and a longitudinal study design that to address cytokine levels, T cell activation markers expression and T cell exhaustion in HIV/AIDS.

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DEFINITION OF TERMS AND ACRONYM

cART Recombinant Antiretroviral Therapy

CBA Cytometric Bead Array

CCR5 Chemokine Receptor-5

CD Cluster of Differentiation

CHUK Centre Hospitalier Universitaire de Kigali

CXCR4 C-X-C Chemokine Receptor Type 4

Cytokines Cytokines are polypeptides produced by different cell types after their activation. The produced cytokines affect cell that bear the matching receptors (Sabat, 2010) to mediate inflammatory and immune reactions. These cytokines are principal mediators of communication between cells of the immune system (Abul et al., 2007).

HAART Highly Active Anti Retroviral Therapy

HIV Human Immunodeficiency Virus

VL Viral Load

IFN-γ Interferon Gamma

IL Interleukin

IREC Institutional research and ethics committee for Moi University College of Health Sciences / Moi Teaching and Referral Hospital

MHC Major Histocompatibility Complex

NRL National Reference Laboratory

RBC Rwanda Biomedical Center

RNEC Rwanda National Ethics Committee

T- Cell activation and activation markers: T cell activation is a crucial checkpoint in adaptive immunity mostly through cytokines network, and this activation depends

on the binding parameters that govern the interactions between T cell receptors (TCRs) and peptide–major histocompatibility (MHC) complexes (Lever et al., 2014). There are several T cell activation markers. In this study, CD69 and CD154 expression were used to evaluate T cell activation markers.

TCR T cell receptor

TNF-α Tumor Necrosis Factor alpha

VL Viral load

CHAPTER ONE

1.1 Background Information

The burden of Human Immunodeficiency Virus (HIV) disease remains a global challenge despite effort made in HIV diagnosis and treatment due to persistent immune activation and chronic inflammation in HIV patient despite HIV virological success (Nasi et al., 2017; Slim & Saling, 2016). The magnitude seems to be high in Africa compared to the developed countries due to delay in diagnosis and HAART initiation. HIV Viral Load (VL) is the right arm of HIV biological follow up. Although progress has been made to improve HIV VL access, distance between facilities and complexity VL procedure remains a challenge for accessibility mostly in Africa. To date, there is no biomarker that gives the hallmark picture of immune activation, chronic inflammation and HIV progression outcomes while it is clear that higher morbidity and mortality rates are correlated with inflammatory markers and are attributed to chronic inflammation and immune system dysfunction in HIV/AIDS patients even with controlled HIV VL (Slim & Saling, 2016). Studies evaluated the value of plasma cytokines levels as predictors of immune activation, persistence inflammatory status and HIV disease prognosis (de Medeiros et al., 2016; Huang et al., 2016). In HIV infection, T- Helper (Th)-1 and Th-2 cytokines are some of the cytokines that had been evaluated (Gorenec et al., 2016). Some studies demonstrated a Th1/Th2 cytokines switch as HIV infection progress to end-stage HIV/AIDS while others were not able to support this cytokines imbalance (Osakwe et al., 2010). Plasma cytokines demonstrated the ability to interfere with the molecular mechanisms responsible for HIV latency (Vandergeeten et al., 2012; Williams et al., 2013) and this makes particular pro-inflammatory cytokines, TNF-α, IFN-γ, IL-2, IL-6 and anti-inflammatory cytokines IL-4 and IL-10 to be more attractive candidate

immunological marker for HIV prognostic follow up due to its reproducibility in many studies (Williams et al., 2013). The immune disturbance had been argued in different studies to be responsible of persistence immune activation, chronic inflammation as well as HIV replication and infection reservoir establishment (Wang et al., 2011) and Cluster of Differentiation (CD)-69 expression was considered as an early leukocyte activation marker. Current studies suggest other role of CD69 that include involvement in complete immune activation including CD154 expression, cytokines secretion and latter in T cell differentiation, regulatory $CD4^+$T cells (T-reg) and Th17 (Cibrián & Sánchez-Madrid, 2017; K. Radulovic et al., 2012; Katarina Radulovic & Niess, 2015) . In HIV infection, it has urged that CD154 seems to be down regulated as an escape immune mechanism (impaired innate and adaptive response) and study findings show far less expression of CD154 on $CD4^+$ T cell considering CD69 expression (Ballesteros et.al., 2013; Olson et al., 2014; Subauste, Subauste, & Wessendarp, 2007) . In this perspective, this study aimed to examine the relationship between cytokines, T cell activation markers and HIV VL with a view of understanding cytokine networks during HIV infection, T cell activation markers and determining whether they can be used as alternative biomarker for persistent immune activation and chronic inflammation

1.2 Statement of the Problem

$CD4^+$T cell count test, the most common prognostic immunological marker, is no longer a routine test in the test and treat HIV protocol (World Health Organization, 2015) while chronic inflammation and immune system dysfunction are key drivers of higher non HIV infection/AIDS morbidity and mortality even with controlled HIV Viral Load (HIV VL) (Slim & Saling, 2016). Limited data is available for other routine immunological markers to monitor HIV infection and HIV disease

progression in the advent of high morbidity and mortality rate attributed to chronic inflammation and immune system dysfunction in HIV infected /AIDS patients. Cytokines levels had been evaluated but limited data is available in relation to cytokine levels, HIV VL and T cell activation markers. This study will evaluate cytokines plasma and T cells expression level in HIV population, correlate their level with HIVVL to explore their potentials as immune biomarker for persistent immune activation and chronic inflammation in HIV.

1.3 Justification of Study

HIV VL access is challenging in Low and Mid Income (L&MI) countries, including Rwanda, due to cost, the stringent requirements of the technique itself, as well as the distance between community and VL testing sites. This brings in the need to assess alternative markers to monitor HIV infection and HIV treatment. Several studies have been done to detail cytokine interactions in HIV infection and recommended the use of groups of soluble biomarkers Keating et al., 2016; Mora et al., 2015; Williams et al., 2013)**.** Although studies attempt to detail cytokines, T cells activation markers expression data is limited and very little is known in relation with cytokines level, T cell activation and HIV VL relationship (Slim & Saling, 2016). This study will and activated T cell ($CD3^+CD4^+CD69^+CD154^+$/ $CD3^+CD8^+$ $CD69^+CD154^+$) markers were determined, described and compared with HIV viral load at the initiation of cART and six months after cART initiation among HIV positive patients in Rwanda using matching groups for association. This study will evaluate cytokines (TNF-α, IFN-γ, IFN-α, IL-2, IL-4, IL-6, IL-10) plasma and T cells activation markers ($CD69^+CD154^+$) expression level on T cells in HIV^+ population, correlate their level with HIVVL to explore their potentials as immune biomarker for persistent immune activation and chronic inflammation in HIV.

1.4 Hypothesis

The current study hypothesized that Cytokines plasma levels and T cell activation markers are highly expressed in untreated HIV cases compared to treated HIV cases. They are good correlate of HIVVL at initiation and after 6 months of HAART.

1.5 Objectives of the study

1.5.1 General Objective

To determine cytokines levels and T cell activation markers in patients initiating cART and patients who have completed six months cART regimens in Rwanda.

1.5.2 Specific Objective

1. To determine plasma cytokines levels (IL-2, IL- 4, IL-6, IL10, TNF-α, and IFN-γ) in patients initiating cART and in patients who have been on cART for six months in Rwanda.
2. To determine the expression levels of $CD69^+$, $D154^+$ and $CD69^+CD154^+$ co-expression on $CD4^+$ and $CD8^+$ T cell in patients initiating cART and in patients who have been on cART for six months in Rwanda.
3. To determine the relationship between cytokines and activated T cell markers in patients initiating cART and those who have been on cART for six months in Rwanda.

CHAPTER TWO: LITERATURE REVIEW

2.1 Immune activation in HIV infection

Increased evidences that the cytokines milieu influence immune response and persistence immune activation in HIV infection continued to rise (Stiksrud et al.,2016; Gori et al., 2016; Maharaj et al., 2017; Slim & Saling, 2016; Lucie et. al, 2013; Z. Wang, Shang, & Jiang, 2017)). HIV infected /AIDS individuals had inadequate CD4 responses, more activated , differentiated T-cell markers and regulatory T cell, compared to patients with normal CD4 (Lubanga et al., 2016; Stiksrud et al., 2016). It was known that cytotoxic T cells play a central role in controlling HIV infection while cytotoxic T cell were normal and functional despite the HIV suppressive effect in subjects who were controlling HIV in time as results of balanced cytokines, immune activation and regulation (Stiksrud et al., 2016).

The role of regulatory T cells in HIV infection remained controversial. Some findings support the positive role of regulatory T cell whereas others concluded to the contrary that regulatory T cell inhibit HIV-specific immune responses thus causing HIV disease progression (Lubanga et al., 2016; Miles et al., 2015). Host cell mediated immunity played a role in the mechanisms of chronic HIV infection, and data from studies supported the role of regulatory T cells at HIV replication sites (Chevalier et al., 2015). It had been demonstrated that HIV infect regulatory T cells and this impact a lot on the functional capacity of the overall immune response to HIV and results in continuous immune activation, chronic inflammation and HIV infection progression (Stiksrud et al., 2016).

2.2. T cell activation markers and immune activation

There was numerous T cell activation markers, CD25, CD69, CD154 (CD40 Ligand), and CD45RO and now FoxP3 expression was known to be induced upon T cells activation (Keating et al., 2016; Ziegler, 2007). Findings from recent studies had shown the persistence of immune activation and strongly suggested that it drives HIV/AIDS disease progression (Keating et al., 2016). The mechanisms leading to immune activation were not well understood. Recent reports showed correlation between $CD4^{+}CD25^{+}$T regulatory cells with immune activation and HIV viral load (Lubanga et al., 2016)

The role and mechanism of CD154 in regulation of numerous aspects of the immune response prompted different studies that explored whether induction of CD154 is defective in HIV-1 infection. In cell culture and in animal model research findings demonstrated that $CD4^{+}$ T cells from HIV-1 patients had defective CD154 induction compared with cells from HIV naïve (Carlos S Subauste et al., 2007) and it was suggested to be secondary to CD40 induced down-modulation of CD154 (Ballesteros-Tato et al., 2013). On the other hand, it had demonstrated that HIV gp120 impaired induction of CD154 in HIV-1 infection when T cells were exposed to high concentrations of virions and impaired CD154 protein expression as well as CD154 mRNA levels (Zhang et al., 2004). Induction of CD154 and its regulation of expression were two mechanisms of regulation of CD154 expression and were all dependent on the binding of CD40 to CD 154 (Subauste et al., 2017).

CD69 protein expression had been studied in immune reconstitution in the sigmoid colon after long term HIV therapy and demonstrated to be highly expressed in colon rather than in T cells peripheral blood for both $CD4^{+}$ and $CD8^{+}$ T cells (X. Wang et

al., 2011). Results for CD69 and HLA DR expression did not differ in colon sigmoid. A Positive correlation was only observed between % CD8 CD69 expression and HIV proviral DNA in sigmoid colon (Sheth et al., 2015)

2.3. Cytokine production and immune function in HIV infection

In the HIV infection process, cytokines controlled the proliferation, differentiation, functioning and maintenance of T cells as central elements of human immunity against HIV(Speiser et al.,2014). HIV infection was associated with cytokines secretion imbalances that lead to cytokines network disparity and lymphocyte dysfunction (Keating et al.,2016). Data showed that cytokines imbalance is key in HIV pathogenesis by harmonizing viral replication and latency formation, innate immune response and adaptive immune responses (Huang et al., 2016, Keating et al., 2016). It had been reported that HIV infection is characterized by modified cytokines production and responsiveness particularly for T cells response that lead to an upsurge in virus replication and delay in the immune response (de Medeiros et al., 2016; Keating et al., 2016). It had further been reported that levels of IL-2 (Th-1 cytokine) decreased while IL-4 and IL-10 (both Th-2 cytokines) increase with the $CD4^+$ T cells decreasing gradually (de Medeiros et al., 2016; Reuter et al., 2012). Even though the idea of Th-1 and Th-2 cytokines shift rest inconclusive, Th-1 cytokines activities are linked directly to HIV/AIDS development (Reuter et al., 2012).

On the other hand, cytokines were reported to have a crucial role in the failure of the immune system to eradicate HIV by promoting mechanisms of HIV persistence in HIV-infected subjects receiving suppressive cART by exacerbating low levels of ongoing viral replication in lymphoid tissues (Bortolin et al., 2012; Huang et al., 2016; Vandergeeten et al., 2012). Studies reported cytokines to be actively

participating in the maintenance process of memory $CD4^+$ T cells that enhance the persistence of these cells during cART while pro- inflammatory cytokines may favor HIV persistence even after prolonged therapy (de Medeiros et al., 2016; Vandergeeten et al., 2012; Williams et al., 2013). Furthermore, studies had shown that IL-2 and IL-12 act synergistically to improve NK cytotoxic effects (Nielsen et al., 2016) while IFN-γ stimulates the expansion and livelihood of immune effectors cells (Zhao et al., 2006). It was demonstrated that IFN-γ triggers transcription of a large number of genes that played a critical role in antiviral activity, apoptosis, antigen processing, MHC protein expression, and Th-1 cells clonally expansion.

Th-2 cytokines, mostly IL-10 had shown inhibitory effects on expression of co-stimulators and class II MHC molecules on antigen presenting cells (APC) thus inhibiting T cells activation and terminate cell-mediated immune reactions (Sabat et al., 2017; Williams et al., 2013).

Correlation between $CD4^+$ T cells count and cytokines remained inconclusive. Some studies had supported a positive correlation between CD4 count, IL-2 and IFN-γ, others had observed a negative correlation between IFN-γ, $CD4^+$ T cells count and IL-2 (Watanabe et al., 2010; Spitsin et al., 2012).

2.4 Cytokine production and viral load in HIV Infection.

Different cytokines correlated differently with HIV VL. Findings from studies demonstrated a decrease of Interleukin (IL-)-4, IL-10, and IL-6 together with HIV VL while IL-2, TNF-α, IFN-γ levels improved significantly in peripheral blood and genital secretions from HIV infected /AIDS patients person on HAART therapy (Sachdeva et al., 2010). In mucosal lymphoid tissue, HIV infection was associated with significant increases of TNF-α, IFN-γ, IL-4, IL-5, and IL-10 compared to control patients. (Schulbin et al., 2014; Bortolin et al., 2012; Vandergeeten et al., 2012)

Establishment of type 1 cytokines profile and suppression of viral replication in HIV patients on cART suggested that cART treatment may give opportunity to the immune system to reconstitute (Williams et al., 2013). These findings were supported by another studies aimed at recognizing the critical role played by the imbalance between Th1 and Th2 cytokines in the progression and pathogenesis of HIV-1 on patients on cART with opportunistic infections, without opportunistic infections, and HIV-seronegative controls (Sindhu et al., 2016). Results of this study demonstrated lower levels of serum IL-2 and IFN-γ in patients compared to controls and a significant decreased levels of IFN-γ in HIV population without opportunistic infections (Sindhu et al,. 2016). IL-4 and IL-10 were more increased in patient's population with opportunistic infections compared to other groups (Sindhu et al. 2016). To highlight on the above reports, the In Vitro studies, reported that $CD4^+$ T cells from low viremia patients produce mostly IL-2 and IFN-γ (Trabattoni et al., 2012, Spitsin et al., 2016; Trabattoni et al., 2015). Cytokines dynamism showed to be affected by cART dosage (Spitsin et al., 2016). Using PHA-stimulated peripheral blood mononuclear cells from samples obtained 2 months after cART it had shown that only levels of IFN-γ and TNF-α increased compared to the levels observed for samples taken before initiation of cART (Spitsin et al., 2016). There were no significant variations in IL-2 or Th-2 cytokines (IL-4, IL-5, and IL-10) in the samples taken before and after 2 months of treatment (Spitsin et al., 2016).

2.5 Cytokine production as Soluble Plasma Biomarker for HIV Prognosis

Facts supported the idea that a chronic disease like HIV was associated with changes in the expression of numerous soluble proteins, and most of them were cytokines (Neaton et al., 2014). These changes were directly or indirectly the cause of onset of disease development (Neaton et al., 2014, Edwin et al., 2013). Regardless of the

causative relationship those proteins were considered as plasma biomarkers to enable the researchers understand HIV pathogenic processes (Neaton et al., 2014, Edwin et al., 2013). Plasma biomarkers including cytokines assisted researchers to comprehend HIV immunopathogenesis (Neaton et al., 2014). There were suggestions of using groups of soluble biomarkers rather than one individual biomarker to relate innate and adaptive immune activation, cell death and tissue destruction to predict a patient's clinical course and or response to therapy (Neaton et al., 2014).

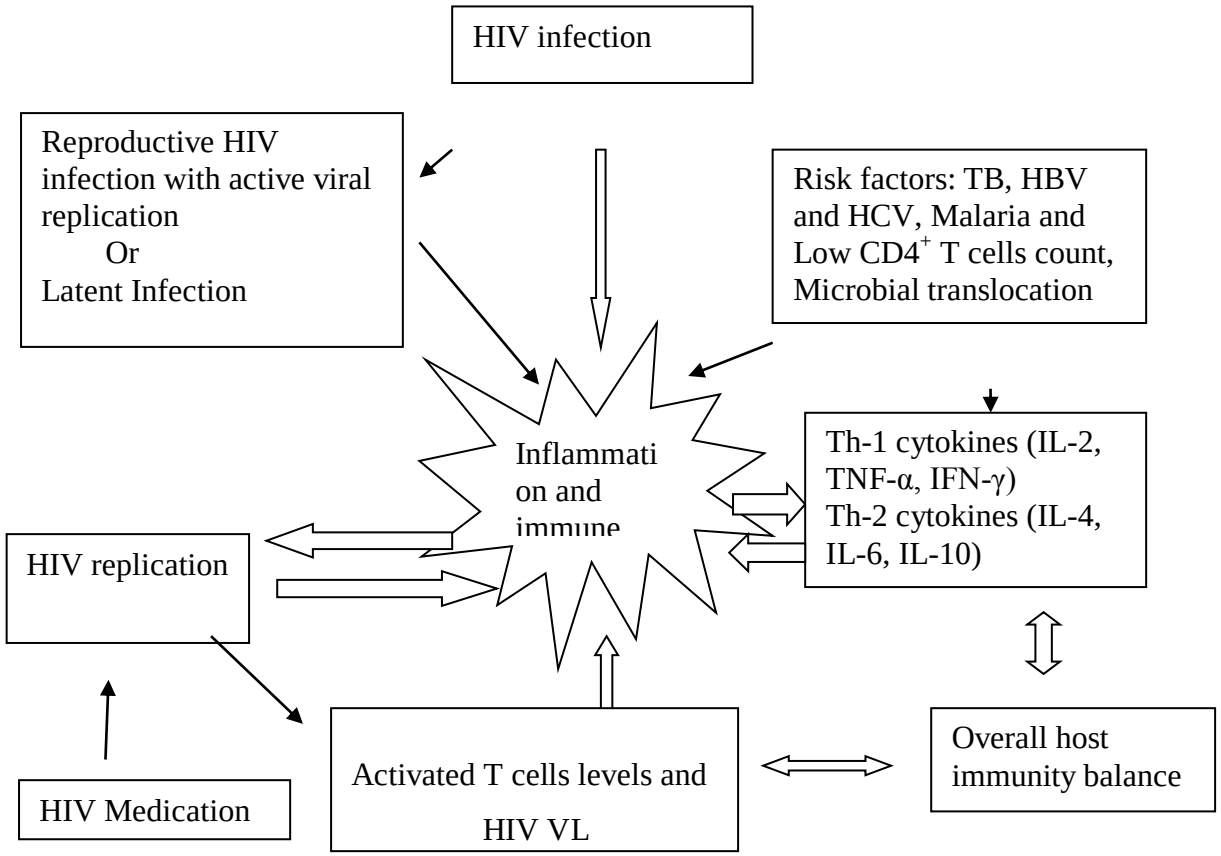
2.5.1. Cytokine production as soluble plasma biomarker for HIV acute infection.

Increased level of TNF-α, IP-10 and IL-10 six weeks after initial HIV infection had been demonstrated. A model for forecasting viral load set-point 12 months after infection had also been established using five cytokines concentration levels (IFN-γ, IL-2, IL-7, IL-12, and IL-15) measured during acute infection at 66%, p- value less than 0.001 (Katsikis et al., 2011, Roberts et al., 2010). Building on these results and the demonstration that higher levels of IFN-γ induced protein 10 (IP-10) during stages III, IV and V of HIV was strongly associated with low CD4 cell counts 2 years after the initial infection. IP-10 might therefore be a useful acute stage biomarker of HIV disease progression (Mahle et al, 2014). Further studies had shown that IFN-α, IP-10, IL-10, IFN-γ rise respectively with serum amyloid A in its second phase during HIV viral expansion period ,high replication rate and HIV viral load first pick while IL-12, IL-6 and IL-7 rise soon after in viral set point period (Keating et al., 2016). From these findings cytokines could be considered as candidate biomarkers for acute HIV infection and may indicate viral set-point variation and disease progression in HIV infected /AIDS patients both on and off antiretroviral therapy (Stacey et al., 2015; Keating et al., 2016).

2.5.2 Cytokine production as soluble plasma biomarker of chronic HIV disease

Chronic untreated HIV-1 infection was reported to be characterized by a state of persistent immune activation, and this broad activation was a strong contributor to disease progression (Douek et al., 2009). It was demonstrated that cytokines in HIV acute infection stage may be plasma candidate biomarkers to foresee the viral load set-point in immune activation set-point (T-cell activation level) and forecast disease progression independently of HIV viral load (Williams et al., 2015)However, the relationship between the acute cytokine storm and the T-cell activation set-point and chronic stage activation levels remained to be defined (Turk et al., 2018; Wu et al., 2017)

2.6 Conceptual Framework



The stimuli and mechanism of persistent inflammation and immune activation in HIV patients remained unclear. This study was evaluating IL-2, IL-4,IL-6 and IL-10 cytokines together with T cells activation level (the expression level of CD69 and CD154) to predict the outcome of HIV disease (Inflammation and activation level). The risk factors for viral replication and cytokines production was excluded in patient's population. Cytokines production, and T cell activation levels was compared to predict associations between immune activation and cytokines in HIV patients.

CHAPTER THREE: RESEARCH DESIGN AND METHODOLOGY

3.1 Study Area

The data were collected in five health facilities, representing five provinces of Rwanda: Kigali city, East; North; South and West from April 2018 to June 2018. The sites were selected because of being among established HIV care sites in Rwanda and they are coordinating other satellites sites in catchment area. For that, they helped to achieve the desired sample population of the study in allocated time.

Rwanda is a country located in East Africa. It shares its border with Uganda in the north, Burundi in south; Tanzania in east and Democratic Republic of Congo in west. In this country, HIV prevalence have been stable for the last 5 years and was estimated at 3% although it varies widely in the 5 provinces of the country with the North: 2.3%, South: 3.1%; East: 3.6%; West: 2.6% and Kigali city 7.4%. Incidences of 0.22% in rural and 0.65% in urban have been documented (Rwanda AIDs indicator and HIV incidence survey 2013).

A map of Rwanda, showing provinces in different colors.

3.2 Study Design

It was a comparative cross sectional study, to determine cytokines level and T cell activation markers in HIV positive patients using matching groups based on sex before initiation and after six months of HAART

3.3 Target and study population

3.3.1 Target Population

The target population of the study was HIV infected/AIDS individuals above 18 years old.

3.3.2 Study Population

The study population was obtained from target population based on eligibility criteria.

3.4 Eligibility Criteria

New patients for this current study was all patients eligible to start HAART from 1st April 2018 to 30th May 2018 and willing to consent. Existing patients are patient who had completed at least six months on treatment from 31st may to 30 June 2018, matching new patients and willing to consent. Patients who had malaria, Hepatitis B and or C , Tuberculosis (TB) at the time of enrollment and those who had been lost to follow- up were excluded in this study.

3.5 Sample size calculation and Sampling Procedures

3.5. 1 Sample Size Calculation

The minimum sample size was calculated using the formula named: Estimated sample size for two groups comparison design using means (Lemeshow & Lwanga, 1990; Lwanga & Lemeshow, 1991)

$$N = \frac{(2\,SD)^2 \left(Z\,1 - \frac{\alpha}{2}\right)^2}{d^2}$$

Where:

SD = Standard deviation for the means of the groups populations. The two SD was assumed the same. In this study, cytokines was considered as major variable. SD and precision used in this formula was for cytokines results in HIV positive patients ART naïve and HIV patients on ART. Considering the highly varied cytokine, (IL-4), SD is as follow, 38 pg/ ml and 41 pg/ ml for ART naïve and patients on ART respectively (Gori et al., 2016)

d = Expected precision. Mean difference of variables between the groups that is associated with clinical difference. The mean IL-4 for ART naïve patients (µ1) was estimated at 138 pg/ml for ART naïve patients and 116 pg/ml for HIV patients on treatment (µ2). Precession (d) = **µ1 - µ2 = 22pg/ml** (Gori et al., 2016)

Z1-α/2= Standard normal variate for level of significance. At 5% type1 error p value ≤ 0.05, is 1.96 as from normal distribution curve. The minimum sample size (**N)** for each group equal = $\frac{(38+41)^2\,(1.96)^2}{22^2}$ **= 49 patients.** The sample size was rounded up to fifty patients for new patients and existing group based on cytokines kit size.

3.5.2 Sampling Procedures

Considering the five sites distributed countrywide, incidence in Rural (0.22) and urban (0.65), a following assumption was made: N= 3X+4X= 50 HIV $^{+}$ People where N= sample size and X = equal distribution of patients per site was used to estimate 28 patients for rural hospitals and 22 HIV $^{+}$ People for Kigali city. The K^{th} = four (4) was calculated Based on ground registers and HMIS data and it was used to recruit 7 patients per site in rural and 22 HIV $^{+}$ People in Kigali city. A matching group by sex was recruited between 31^{st} may to 15^{th} June 2018, as they are coming for biological follow up after six months on cART. Each selected client was screened

for Malaria, TB, HBV and HCV using appropriate rapid tests. If he or she is positive for one of the screened conditions, he or she was excluded and looked for the next client.

3.6 Data Sources and Instruments

This study collected primary data for subject social demographic data and laboratory data. Subject's data was gathered at enrolment using patient's file and patient's conversation. Laboratory data were generated by laboratory measurement of variables named cytokines (IL-2, TNF-α, IFN-γ, IL-4, IL-6, and IL-10) in plasma and activated T cell (CD3+CD4+CD69+CD154+/ CD3+CD8+ CD69+CD154+) markers in peripheral circulation at enrollment and after six months of follow up using EDTA whole blood. Two tubes whole blood were collected in 4 ml EDTA tubes before starting cART and for matched group that had been on cART for at least six months. Samples were transported at Rwanda National Reference Laboratory (NRL) for processing and analysis. One tube was used for T cell immunophenotyping the same day. While the second tube was centrifuged to get plasma that was kept according to the kit insert, The BD™ CBA Human Th1/Th2 Cytokine Kit II (Catalog No. 551809) before analysis. Analyses for all parameters were performed at NRL according to the kits manufacturer's instructions and standard operating procures in place. Cytokine plasma levels and T cell activation markers immunophenotyping was performed using the BD FACSCalibur flow cytometer (https://www.bdbioscences .com > research>cba-kits)

3.6.1 Subject Data Collection Procedure

On enrollment, a revised WHO questionnaire (Appendix 3 in this study) was used to collect the relevant demographic characteristics data for this study by interview. Using this questionnaire, we collected also, baseline $CD4^+$ T cell count levels, the type of ART in use and HIV VL data in patient file.

3.6.2 Laboratory based data collection

3.6.2.1 Procedure for Cytokines Level Determination

Cytokines were measured using Flow cytometry technology powered with Cytometric Bead Array (CBA). CBA was improved analysis tool compare to conventional ELISA using BD™ Cytometric Bead Array (CBA) and allowed analyst to discriminate different particles based on size and color in a single sample. The BD CBA system used the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a CBA provided a capture surface for a specific protein.

The cytokine capture beads were mixed with the PE-conjugated detection antibodies and then incubated with recombinant standards or test samples to form sandwich complexes. Following acquisition of sample data using the flow cytometry, the sample results were generated in graphical and tabular format using the BD™ CBA Analysis Software. The detailed procedures for all steps needed for cytokines assay (Instrument Setup with BD FACSComp™ Software and BD CaliBRITE™ Beads Procedure, Cytometer Setup Bead and Cytokine Standards Preparation Procedure; Cytokines Plasma assay procedure; Cytometer Setup; Data Acquisition and Analysis procedure) was detailed in the annex 1 for this study, cytokines procedure

3.2.2. T cell Immunophenotyping Procedure

Immunophenotyping by flow cytometry was applied to study the expression of antigens on surface or in cytoplasm of leukocytes and other cells including T cell, monocytes /macrophages markers.

Furthermore, intracellular antigens were detected after permeabilization of the cells.

A defined set of fluorochrome-conjugated monoclonal antibodies allowed analysis of several human specimens such as peripheral blood, bone marrow and lymph nodes for the presence of markers of interest. In this case, T cell activation markers to measure the phenotypes ($CD3^{+}CD4^{+}$ $CD69^{+}CD154^{+}$/ $CD3^{+}CD8^{+}CD69^{+}CD154^{+}$) in peripheral blood.

Flow cytometer used one or more LASER (s) to evaluate the size (forward light scatter), granulation (sideward light scatter) and emitted fluorescence on a single cell basis. The fluorochromes were coupled to antibodies specific CD3, CD4; CD69 and CD154 on the surface of a cell. Immunophenotyping using four-color flow cytometry served as an adequate platform to combine markers for maturation, activation and lineage identification markers (annex 2-immunophenotying procedure). The following antibodies were used for T cell activation markers determination:

CD3- FITC BD Biosciences,

CD8/4- PE BD Biosciences;

CD69- PerCp BD Biosciences;

CD154- APC BD Biosciences.

3.7 Framework for Data Analysis

Data analysis was based on assessing significant changes between dependent variables, Plasma cytokines levels and T cell activation markers expression before and after six months of HIV treatment and their relationship with independent HIV

VL. Only age in categorical variables expressed in age group (18-24 years, 25-44 years and 45-65years) was considered in analysis

3.8 Data processing, Presentation and Analysis

3.8.1 Data Processing

After laboratory samples analysis, the results for each variable (cytokines, activated T cell) were cleaned and computed using R studio and graph pad prism 7. Hard copies were kept in locked cupboard in NRL, Flow cytometry laboratory. While Soft data was kept in folder that was secured with password and external hard disk is used to store backup data. Based on record keeping guideline in Rwanda, the data for this study will be stored for 10 year for hard copies and fifty years for soft data files.

3.8.2 Data Presentation

3.8.2.1 Subjects data presentation

R studio, descriptive statistics package was used to compute the mean, standard deviation, and 95% confidence interval for continues variables. The data were presented in figures from R statistic and graph pad prism 7.

3.8.2.2 Laboratory data presentation

Cytokine levels and T cell activation markers expression results before treatment and after six months were described and summarized using the mean, standard deviation and p-value. Data were presented using R Studio and graph pad figures in form of box whisker plot and correlation tables.

3.8.3 Data Analysis

The data from this study were analyzed based on objectives and hypothesis. Hypotheses were tested using independent t test for normally distributed data and similar variance for two group's data analysis. Otherwise, Wilcoxon ranks test was used to test hypothesis. For more than two groups ANOVA was used accordingly. Correlation was determined using Pearson r correlation test with statistical significance at 95% Confidence interval, p-value ≤ 0.05

Normal distribution was assessed using Shapiro-Wilk normality test (W) and variances similarities were computed using F test where disparity in data were significant at 95% confidence interval p-value ≤ 0.05

3.9 Study limitation

Since this study was cross sectional, temporal relationship could not established and it may however generate hypothesis for further stronger study designs for relationship establishment.

The study was not able to distinguish T cell activation due to HIV antigens from other possible causes of activation not controlled during this study. There were no T cells culture and T cells challenge.

3.10 Variables and Measurements

During this study cytokines were considered as Dependent variables while T cell activation markers were considered as Independent variables

Table 3.1 Study Variables and measurements

Variables	**Outcome variable**	**Measurement**
Dependent variables		
Cytokines IL-2, IL- 6, TNF-α, IFN-γ, IL-4, IL-10	Plasma level of each cytokine before and after six months of cART treatment	pg. per ml as measured by CBA
Independent variable		
Activated T cell	% of activated T cell in peripheral blood	% of activated T cell as measured by Cell QUEST software

3.11 Ethical consideration.

Ethical clearance was obtained from Moi University (IREC) and endorsed by Rwanda National Medical Ethics Committee and Ministry of health .These approvals was presented to research sites administration before starting data collection. Eligible HIV positive patients received details of study including anonymous sample collection, analysis, and confidentiality of results and all right to withdrawn them self from study any time as they want. Only codes were used as unique identify for participants during all phases of study. Blood collection was performed for those who consented and fulfilling inclusion criteria.

CHAPTER FOUR: RESULTS

The aim of this study was to determine the changes in plasma cytokines levels and activated T cell markers expression levels following HAART and their relationship with HIV VL before and after six months of HAART treatment. In the above context, the study performed and determine relationship between IL -2, IL-4; IL-6; IL-10;IFN-γ;TNF-α; plasma levels; T cell activation markers ($CD4^+CD69^+$%, $CD8^+CD69^+$%, $CD4^+CD154^+$%, $CD8^+CD154^+$%, $CD4^+/CD69^+/CD154^+$% and $CD8^+/CD69^+/CD154^+$%) and HIV VL at initiation and after 6 months of HAART for one hundred (100) study participants and Fifteen (15) participants HIV naïve as controls. The study found statistically significant differences in plasma cytokines levels and peripheral T cell activation markers expression at initiation and after six months of HAART. A positive relationship was found between IL-10, IL-6; CD69 expression on $CD4^+$ and $CD8^+$ T cell with HIV VL and a negative relationship was found between IFN-γ, TNF-γ and HIV VL

4.1. Characteristics of study participants

They were One hundred (100) HIV positive clients and fifteen (15) HIV naïve clients participated in this study. Among HIV positive clients, 50 clients were HAART naïve and 50 clients were on HAART for at least 6 months. Based on sex, there were no statistically significance difference in male 24(48%) / 23(46%) and female 26(52%) /27(54%) who participated in this study at initiation and after 6months of HAART respectively, Chi-square p value 0.8411 (Table 4.1). The dominant groups of study participants (56% and 62%) were between 25 and 44 years old at initiation and after 6 months respectively. The smokers were five (10%) in HAART naïve clients group while 1 (2%) client was smoker in clients group on HAART. The alcohol was consumed by 4 (8%) and 2(4 %) clients before HAART and after 6 months HAART

respectively. Before and after 6 months of HAART, 43 (86%) and 46 (92%) clients were able to have at least two meals per day respectively while 35 (70%), 28 (56%) of patients were able to have fruits and vegetables at least At least once week before HAART and after 6 months HAART respectively (Table 4.1).

Table 4.1: Social demographic distribution of study participants (N=100)

Parameters	HIV+HAART Naïve N=50, (%)	HIV+ HAART after 6 months N=50, (%)	Chi-square probability(p-value)
Sex			
Males	24(48)	23(46)	0.841
Females	26(52)	27(54)	
Age			
18- 24	6(12)	7(14)	
25-44	28(56)	31(62)	0.003
45-64	16(32)	12(24)	
Smoking			
Yes	5(10)	1(2)	0.212
No	45(90)	49(98)	
Alcohol consumption			
Yes	4(8)	2(4)	0.399
No	46(92)	48(96)	

Note that male and female were almost equal and more than half of study participants were between 25 and 44 years old. NA: Not applicable

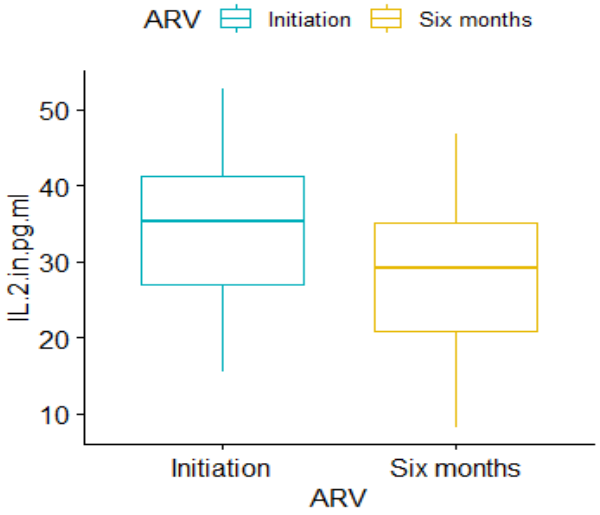
4.2. Cytokine levels before and after HAART treatment

The objective one (1) of this study was to determine the plasma levels of IL-2, IL- 4, IL-6, IL10, TNF-α and IFN-γ in HIV^+ people before they initiate HAART and after six months of HAART. We hypothesized that there was no statistically significant difference between mean cytokines at the initiation and after 6 months of HAART under null hypothesis. Before hypothesis testing, we described the cytokines data frame for each cytokine and we verified assumptions required for hypothesis testing

using independent T test as follow : 1) The two groups were independent given that they include different individual patients data matched by sex, 2) the two groups data set were normally distributed and it was assessed using Shapiro-Wilk normality test (W) with significance level estimated at 95% confidence interval and p-value ≤ 0.05 suggesting a normal distribution among the two groups if failed to reject null hypothesis, and 3) Two variances similarity was assessed by computing F test with significance estimated at 95% confidence interval and p-value ≤ 0.05 suggesting variance similarity among the two groups if failed to reject null hypothesis. If not verified, Wilcoxon rank sum test (W) was used to test hypothesis.

4.2.1. IL-2 plasma levels before and after HAART treatment.

IL-2 was measured for 100 clients. 50 clients at initiation and 50 clients at month 6 of HAART treatment. The mean (SD) concentration in pg/ml was 34.7 (9.43) and 28(9.98) at initiation and after 6 months of HAART treatment respectively (Figure 4.2.1)



Statistic	Value (p-value)
Mean (SD) at initiation	*34.7(9.43)*
Mean (SD) after 6 months	*28.3(9.98)*
W at initiation	*0.975(0.381)*
W after 6 months	*0.974(0.364)*
F test	*0.892(0.690)*

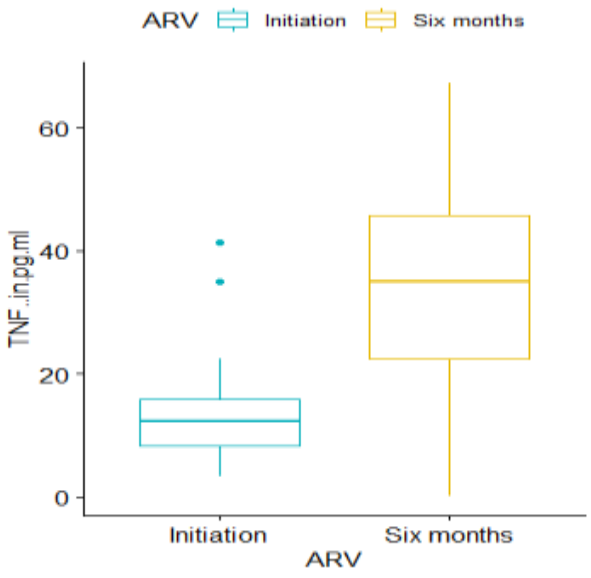
Figure 4.2.1: Summary data of IL-2 plasma level at initiation and after 6 months: Extract from R statistic. N=50 at Initiation and after 6months, Mean (SD) 34.7(9.43) versus 28.3(9.98), W (p-value)=0.975(0.381) versus 0.974(0.364) and F (p-value) = 0.892(0.69)

To test whether the mean concentration of IL-2 at initiation differ from mean concentr ation after 6 months of HAART treatment, The independent t-test was performed whe reby T test at 95% confidence interval , T (98) = 3.309 with a p-value = 0.001. The n ull hypothesis was as rejected with p-value = 0.001 and the alternative hypothesis ado pted.

The data from this study indicated showed IL-2 plasma concentration high at initiati on compared to it level after 6 months of HAART.

4.2.2 TNF-α plasma levels before and after HAART treatment.

TNF-α was measured for 100 clients. 50 clients at initiation and 50 clients at month 6 of HAART treatment. The mean (SD) concentration in pg/ml was 11.9 (5.21) and 35. 3(16.2) at initiation and after 6 months of HAART treatment respectively (Figure 4.2. 2)



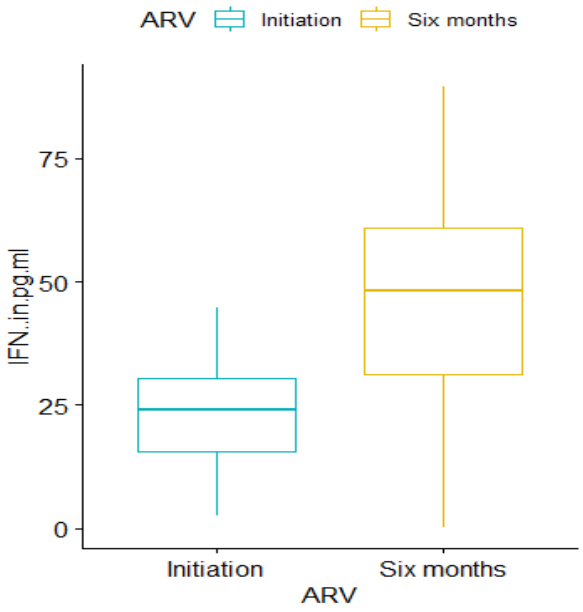
Statistic	Value (p-value)
Mean (SD) at initiation	*11.9(5.21)*
Mean (SD) after 6 months	*35.3(16.2),*
W at initiation	*0.9804(0.5712)*
W after 6 months	*0.9860 (0.8157)*
F test	0.2337 *(0.0001)*

Figure 4.2.2: Summary data of TNF-α plasma level at initiation and after 6 months: Extract from R statistic. N=50 at Initiation and after 6months, Mean (SD) 11.9(5.21) versus 35.3(16.2), W (p-value) = 0.980(0.571) versus 0.986 (0.815) and F (p-value) = 0.233 (0.0001)

To test whether the mean concentration in pg/ml of TNF-α at initiation differ its mean concentration after 6 months of HAART treatment, the Wilcoxon rank sum test (W) w as performed for the means whereby W = -9.770 with a p-value = 0.0001. The null h ypothesis was rejected with p-value = 0.0001 and the alternative hypothesis was endor sed. Before HAART treatment, TNF-α plasma level was high in group after 6 months of HAART treatment compared to its level in HAART naïve group (p-value = 0.0001) . More than three time increases in TNF-α plasma concentration was observed after 6 months of HAART (Figure 4.2.2 and 4.2.4).

4.2.3 IFN-γ plasma levels before and after HAART

IFN-γ was measured for 100 clients. 50 clients at initiation and 50 clients at month 6 o f HAART treatment. The mean (SD) concentration in pg/ml was 23.8 (10.4) and 47.1(21.6) in pg/ml at initiation and after 6 months respectively *(Figure 4.2.3)*



Statistic	Value (p-value)
Mean (SD) at initiation	23.8 (10.4)
Mean (SD) after 6 months	47.1(21.6)
W at initiation	*0.97552(0.3818)*
W after 6 months	*0.97498 (0.3642)*
F test	0.89206 *(0.6909)*

Figure 4.2.3: Data summary of IFN-γ plasma level at initiation and after 6 months: ***Extract fro m R statistic. N=50 at Initiation and after 6months, Mean (SD)*** 23.8 (10.4) ***versus*** 47.1(21.6) ***W (p-va lue) = 0.9755(0.3818) versus 0.97498 (0.3642) and F (p-value)*** **= 0.8920** ***(0.6909)***

To test if the difference between IFN-γ plasma concentration was statistically differen t, the study tested hypothesis using independent t-test whereby t (98) = -6.9009 with a p-value = 0.0001. Null hypothesis was rejected with a p-value = 0.0001 and adopted a lternative hypothesis. IFN-γ was found to increase after 6 months of HAART (Figure 4.2.4)

The IL-2, IFN-γ and TNF-α plasma levels at initiation and after six months of HAAR T were also compared to HIV naïve healthy controls and observed difference in IL-2, IFN-γ and TNF-α plasma level at initiation and after six months of HAART compare d to control (p value 0.001 and 0.0001) (Figure 4.2.4)

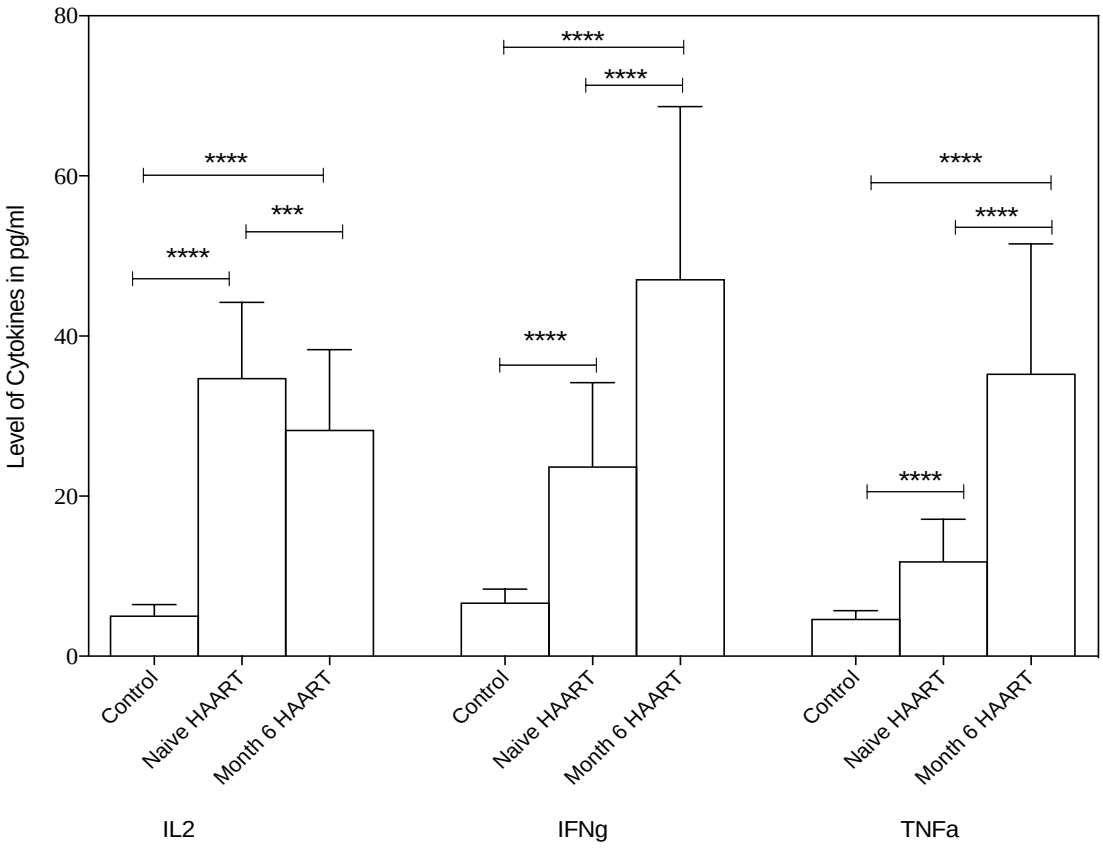
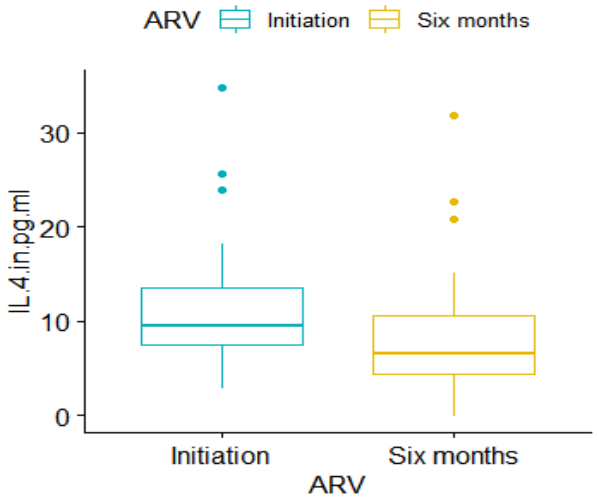


Figure 4.2.4: IL-2,IFN- γ and TNF-α cytokines variation at initiation and after 6 months. ***Unpaire d t test result from graph Pad prism 7: N=50 at Initiation and after 6months and 15 health control. ***: p-value ≤ 0.001, ****: P-value ≤ 0.0001***

4.2.5. IL-4 plasma levels before and after HAART treatment

IL-4 was measured for 100 clients. 50 clients at initiation and 50 clients at month 6 of HAART treatment. The mean (SD) concentration in pg/ml was 10.7 (15.1) versus 7.7 2(6.19) in pg/l at initiation and after 6 months of HAART respectively (Figure 4.2.5)



Statistic	Value (p-value)
Mean (SD) at initiation	*10.7(15.1)*
Mean (SD) after 6 months	*7.72(6.19)*
W at initiation	*0.8467(0.001)*
W after 6 months	*0.80776 (0.001)*
F test	*0.9462(0.847)*

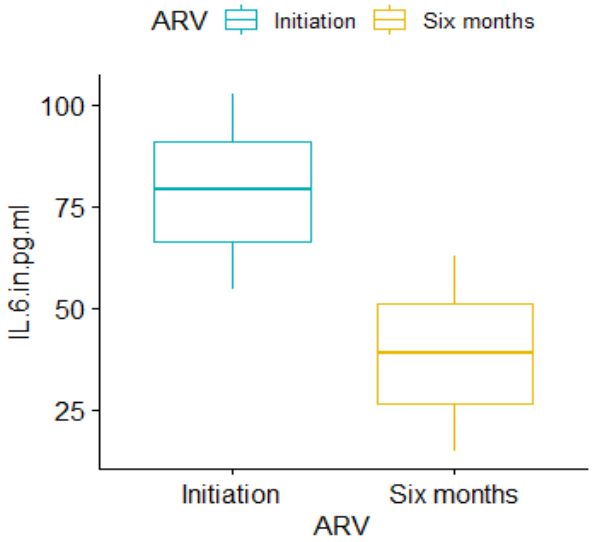
Figure 4.2.5: IL-4 plasma level at initiation and after 6 months. N=50 at Initiation and after 6month s, Mean (SD) 10.7(15.1) versus 7.72(6.19); W (p-value)= 0.846(0.001), versus 0.8077(0.0013);F = 0. 946, p-value = 0.847

To test whether the mean concentration IL-4 in pg/ml at initiation differ its mean conc entration after 6 months of HAART, wilcoxon rank sum test (W) was performed beca use IL-4 results at initiation and after 6 months of HAART were not normally distrib uted (p-value = 0.001 versus p-value = 0.001) whereby W = 1721, p-value = 0.011. N ull hypothesis was rejected with p-value = 0.001 and alternative hypothesis was adopt ed. Before HAART treatment, IL-4 plasma level was high in HAART naive group su bjects compared to its level in group that was on HAART for 6 months (p-value = 0.0 11) (figure 4.2.8)

4.2.6. IL-6 plasma level before and after HAART

IL-6 was measured for 100 clients. 50 clients at initiation and 50 clients at month 6 of HAART treatment.

The mean IL- 4 was Mean (SD) 78.6*(*13.8*) versus* 54.8*(*17.0*)* in pg/ml at initiation and after 6 months respectively (*Figure 4.2.6*)



Statistic	Value (p-value)
Mean (SD) at initiation	*78.6(13.8*
Mean (SD) after 6 months	*54.8(17.0)*
W at initiation	*0.9758(0.3927)*
W after 6 months	*0.9757(0.393)*
F test	*0.945 (0.84)*

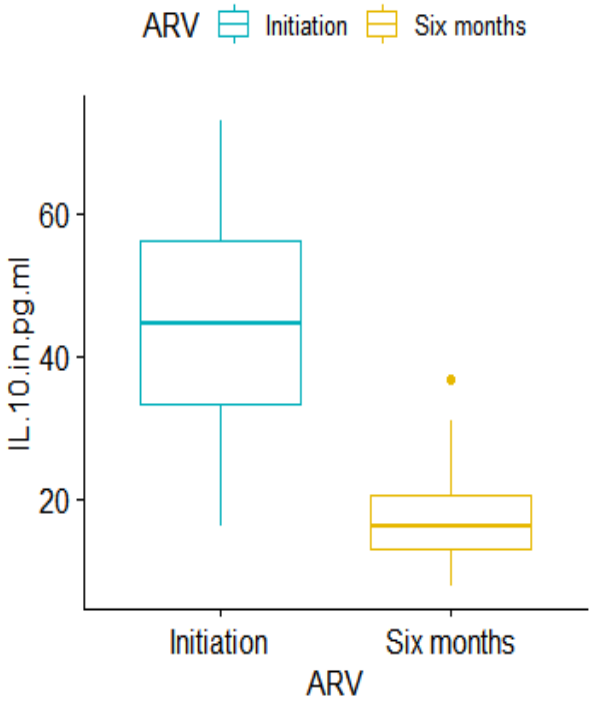
Figure 4.2.6: IL-6 plasma level at initiation and after 6 months. ***N=50 at Initiation and after 6months, Mean (SD) 78.6(13.8) versus 54.8(17.0); W (p-value) = 0.975(0.392) versus 0.975(0.393); F = 0.945, p-value = 0.84***

To test whether the mean concentration of IL-6 at initiation differ its mean after 6 months of HAART treatment, independent t-test was performed whereby t (98) = 5.78 with a p-v alue 0.0001. Null hypothesis was rejected with p- value 0.0001 and alternative hypothesi s was adopted. The data from this study indicated high plasma level of IL-6 at initiation c ompared to it level after 6

months of HAART (Figure 4.2.8).

4.2.7. IL-10 plasma levels before and after HAART treatment

During this study, IL-10 was performed for 100 subjects enrolled in this study. 50 pat ients were HAART naïve and 50 patients were on HAART treatment for 6 months. The mean (SD) IL- 10 was *45.7(15.1) versus 17.4(6.79)* in pg/ml at initiation and after 6 months respectively (Figure 4.2.7).



Statistic	Value (p-value)
Mean (SD) at initiation	45.7*(15.1)*
Mean (SD) after 6 months	*17.4(6.79)*
W at initiation	*0.966(0.159)*
W after 6 months	*0.941(0.016)*
F test	*4.97 (0.001)*

Figure 4.2.7: Data summary of IL-10 pla sma level at initiation and after 6 months. ***N=50 at Initiation and after 6months. Mea n (SD) 45.7(15.1) versus 17.4(6.79); W (p-v alue)= 0.966(0.159) versus 0.941(0.016) ; F = 4.97, p-value = 0.001***

IL-10 results were normally distributed before initiating HAART and after at month 6, Variance test (F) showed none similar variance (p-value = *0.001*). To test whether t he mean IL-10 at initiation differ the mean IL-10 after 6months of HAART treatment (hypothesis one of this study), Wilcoxon rank sum test (W) was performed due to diff erence in variance whereby W = 2413 with a p-value <0.0001. Null hypothesis was re jected with p-value = 0.0001 and alternative hypothesis was adopted.

The IL-4, IL-6, IL-10 A for HIV naïve healthy controls (15), HAART Naïve HIV gro up (50) and a group on HAART for 6 months (50) were compared. The mean concent

ration for control, a group before and after HAART treatment showed statistically diff erent p value 0.001/0.0001 for IL-6 and IL-10 and p value 0.01 for both before and aft er HAART for IL-4 (Figure 4.2.8)

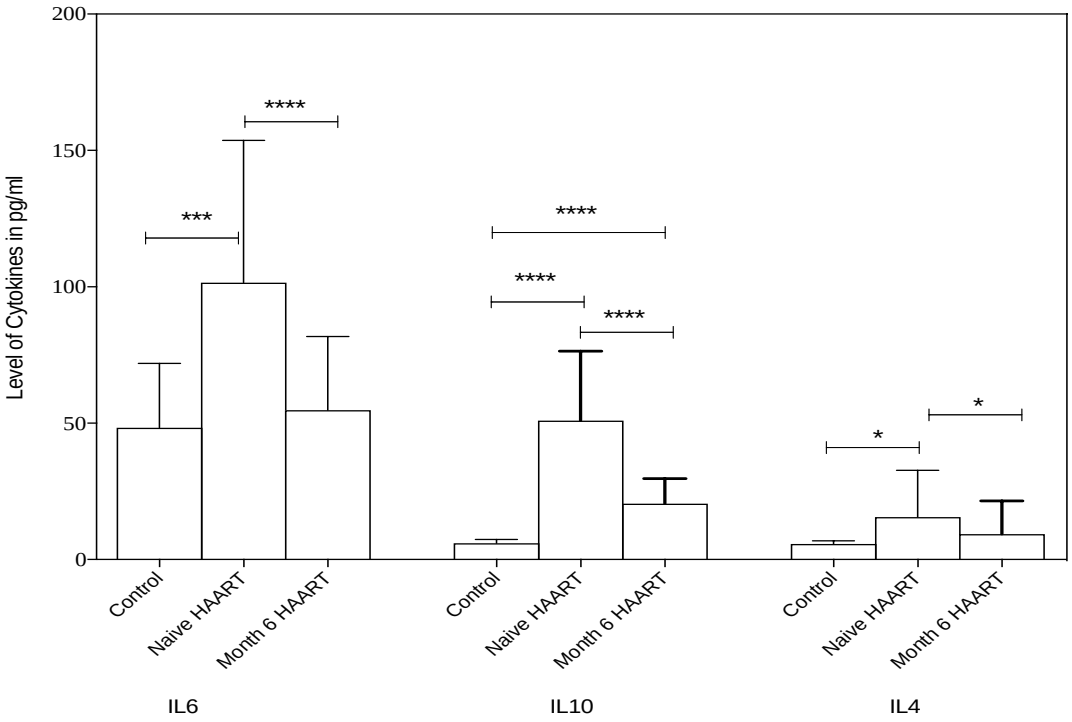
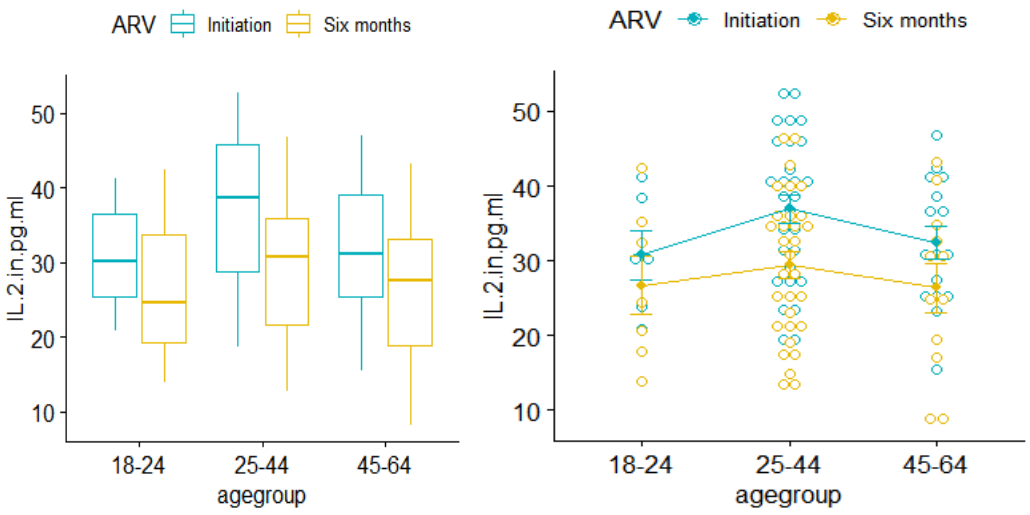


Figure4.2.8: IL-4, IL-6 and IL-10 cytokines variation at initiation and after 6 months. ***n=50 at initiation and after 6months and HIV naïve health control. *: 0.01,***:0.001;****:0.0001***

4.3 Cytokine variation based on age and sex

The study examined if the age and sex do not have effect on cytokines plasma level at initiation and at least at month 6. Although age and sex did not show statisticaly difference affect in cytokines plasma level across all group of age with ANOVA p-value 0.1271, the study observed a high concentration and data overlap mostly for IL-2 and IL-6 in 25-44 years old subpopulation (Figure 4.3)

a) Il-2 box plot ***b)Il-2 dots presentation***



c) ***Il-6 box plot*** d) ***Il-6 dots presentation***

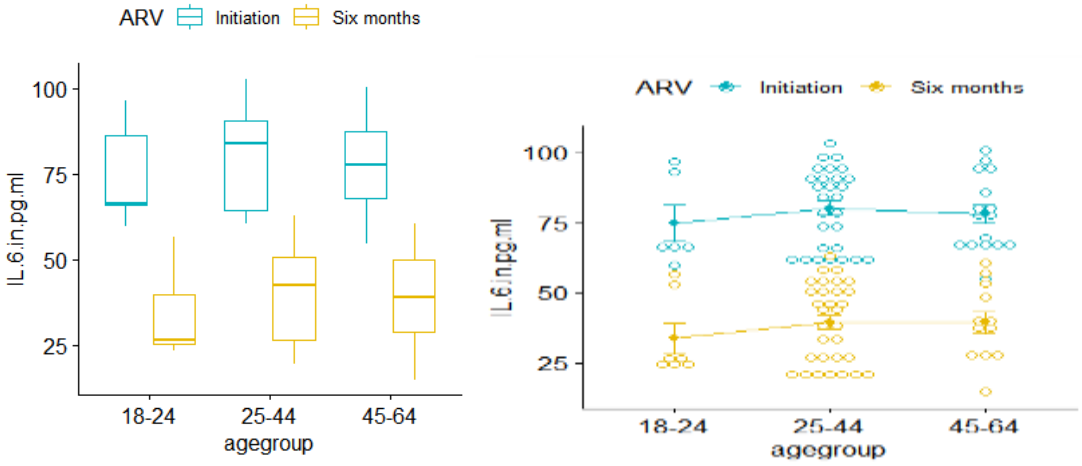


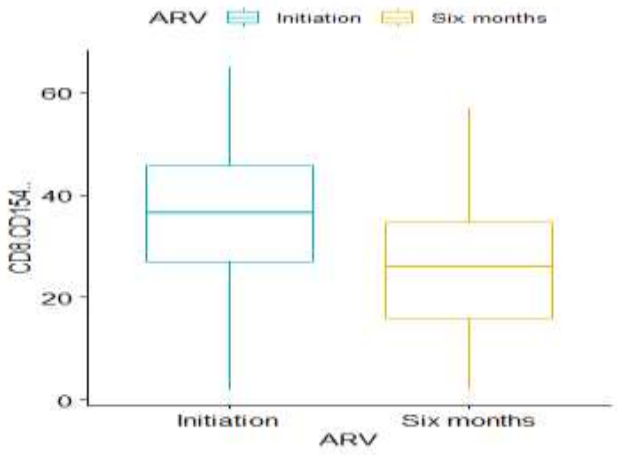
Figure 4. 3. Summary data of Cytokines variation based on Age group at initiation and after 6 months of HAART*: a&b IL-2 , c&d): IL-6 variation. Note high level of Il-2 and data overlap in age group 25-44 years old. Dot plot represent individual data and box represent 25-75 interquatile range. Line between box is mean.*

4.4 Levels of activated T cell markers before and after HAART

The objective two (2) of this study was to determine the peripheral levels (percentage) of activated T cell markers, $CD4^{+}CD69^{+}$ T cell, $CD8^{+}CD69^{+}$ T cell, $CD4^{+}CD154^{+}$ T cell $CD8^{+}CD154^{+}$ T cell $CD4^{+}CD69^{+}CD154^{+}$ T cell and $CD8^{+}CD69^{+}CD154^{+}$ T cell in patients initiating HAART and in patients who have been on HAART treatment for six months.

Under null hypothesis, it was hypothesized that there was not statistically significant difference between mean activated T cell markers at the initiation and after HAART under null hypothesis. Activated T cell markers data description demonstrated normal distribution of activated T cell markers data frame (Shapiro-Wilk normality test with a p-value > 0.05) and variance similarity (F test with a p-value > 0.05) for $CD4^{+}/CD154^{+}$ T cell marker, $CD8^{+}CD154^{+}$ T cell marker, $CD8^{+}/CD69^{+}/CD154^{+}$ T cell markers, $CD4^{+}/CD69^{+}/CD154^{+}$T cell markers data frame and for that, the second hypothesis was tested using independent T test for those mentioned above activated markers and for $CD4^{+}/CD69^{+}$, $CD8^{+}/CD69^{+}$ T cell markers, Wilcoxon rank sum test was used to test hypothesis two.

4.4.1 $CD8^{+}CD154^{+}$ T cell markers before and after HAART



The activated $CD8^{+}CD154^{+}$ T cell markers were measured (in Percentage) for 50 HIV+ before HAART initiation and 50 HIV+ clients after 6 months of HAART treatment in peripheral blood. The mean (SD) of percentage of activated $CD8^{+}CD154^{+}$ T cell markers was 37.4 (13.2) and 26.5(14.6) at initiation and after 6 months HAART respectively.

Statistic	Value (p-value)
Mean (SD) at initiation	*34.4(13.2)*
Mean (SD) after 6 months	*26.51(14.6)*
W at initiation	*0.9733(0.316)*
W after 6 months	*0.9739(0.331)*
F test	0.8076 (0.457

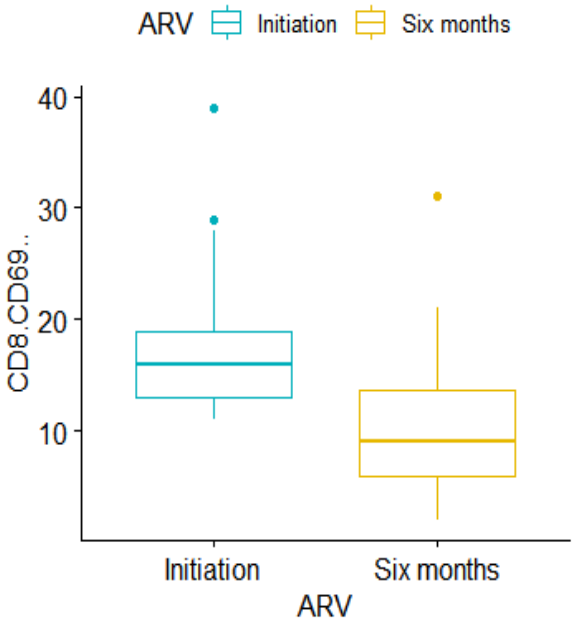
Figure 4.4.1 activated CD8+CD154+ T cell markers at initiation and after 6 months: ***N=50 at Initiation and after 6months, Mean % (SD) 34.4(13.2) versus 26.51(14.6) and W(p-value) = 0.9733 (0.316) versus 0.9739 (0.331) ; F = 0.8076, p-value = 0.4574***

The hypothesis two for activated $CD8^{+}CD154^{+}$ T cell markers was tested using independent t-test, whereby t (98) = 3.9063 with a p-value = 0.0001. Null hypothesis was rejected with a p-value = 0.0001 and adopted alternative hypothesis (Figure 4.4.4). The data from this study indicated the CD154 on $CD8^{+}$ T cell is more expressed at initiation compared to its level after 6 months of HAART with a p-value = 0.0001 (Figure 4.4.4).

4.4.2 $CD8^+CD69^+$ T cell markers before and after HAART treatment

The activated $CD8^+/CD69^+$ T cell markers was measured (in percentage) for 50 HIV + clients before initiation of HAART and after 6 months of HAART.

The mean (SD) percentage of activated $CD8^+/CD69^+$ T cell markers was 17.4 (5.91) and 9.38(6.11) at initiation and after 6 months respectively (Figure 4.5.2)



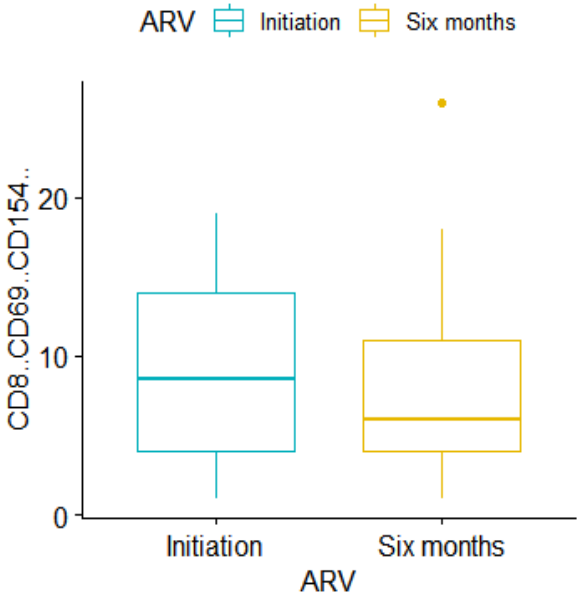
Stat.	Value (p-value)
Mean (SD) at initiation	*17.4(5.91)*
Mean (SD) after 6 months	*9.38(6.11)*
W at initiation	*0.83929(0.0008)*
W after 6 months	*0.91425(0.00147)*
F test	*1.1355(0.619)*

Figure 4.4.2 Activated CD8+/CD69+ T cell markers at initiation and after 6 months*: N=50 at Initiation and after 6months, Mean % (SD) 17.4(5.91) versus 9.38(6.11) and W(p-value) = 0.83929(0.0008)versus W = 0.91425(0.0014) and F(p-value) = 1.1355(0.619)*

Under null hypothesis, it was hypothesized that activated $CD8^+/CD69^+$ T cell markers levels are equal before and after HAART. To test this hypothesis, wilcoxon sum rank test (W) was used whereby W = 2091.5 with a p-value 0.0006 thus rejected null hypothesis with a p-value = 0.0006 and adopted alternative hypothesis. The data from this study indicated the CD69 on $CD8^+$ T cell is more expressed at initiation compared to its level after 6 months of HAART with a p-value 0.0006 (Figure 4.4.4)

4.4.3 $CD8^+/CD69^+/CD154^+$ T markers before and after HAART

The activated $CD8^+/CD69^+/CD154^+$T cell markers was measured (in percentage) for 50 patients at initiation and 50 patients after 6 months of HAART. The mean (SD) wa s 8.84 (5.43) and 7.38(5.05) at initiation and after 6 months respectively.



Statistic	Value (p-value)
Mean (SD) at initiation	*8.84 (5.43)*
Mean (SD) after 6 months	*7.38(5.05)*
W at initiation	*0.925(0.0038)*
W after 6 months	*0.89(0.0003)*
F test	*1.156(0.613)*

Figure 4.4.3 Activated CD8+/CD69+/CD154+T cell markers at initiation and after 6 months*: N=5 0 at Initiation and after 6months, Mean % (SD) 8.84 (5.43) versus 7.38(5.05) W(p-value) = 0.925(0.0 038)versus 0.89(0.0003); F (p-value) = 1.156(0.613)*

To verify hypothesis two for activated $CD8^+/CD69^+/CD154^+$ T cell markers we perf ormed independent t-test for the mean whereby t(98) = 1.3922 with a p-value = 0.167 and failed to reject null hypothesis with independent t-test p-value = 0.167. The data f rom this study strongly suggested no statistically significant difference between mean activated $CD8^+/CD69^+/CD154^+$ T cell markers at initiation and after 6 months of HA ART (p-value = 0.167) (Figure 4.4.4)

The study compared CD69 and CD154 coexpression on $CD8^+$T cell in HAART naïve (50) and after 6 months group (50) with health HIV naïve control (15). The study obse rved statistically different mean expression in percentage CD8 expressing CD69 and

CD154 for control, group before and after HAART treatment with a p value 0.0001 (F igure 4.4.4)

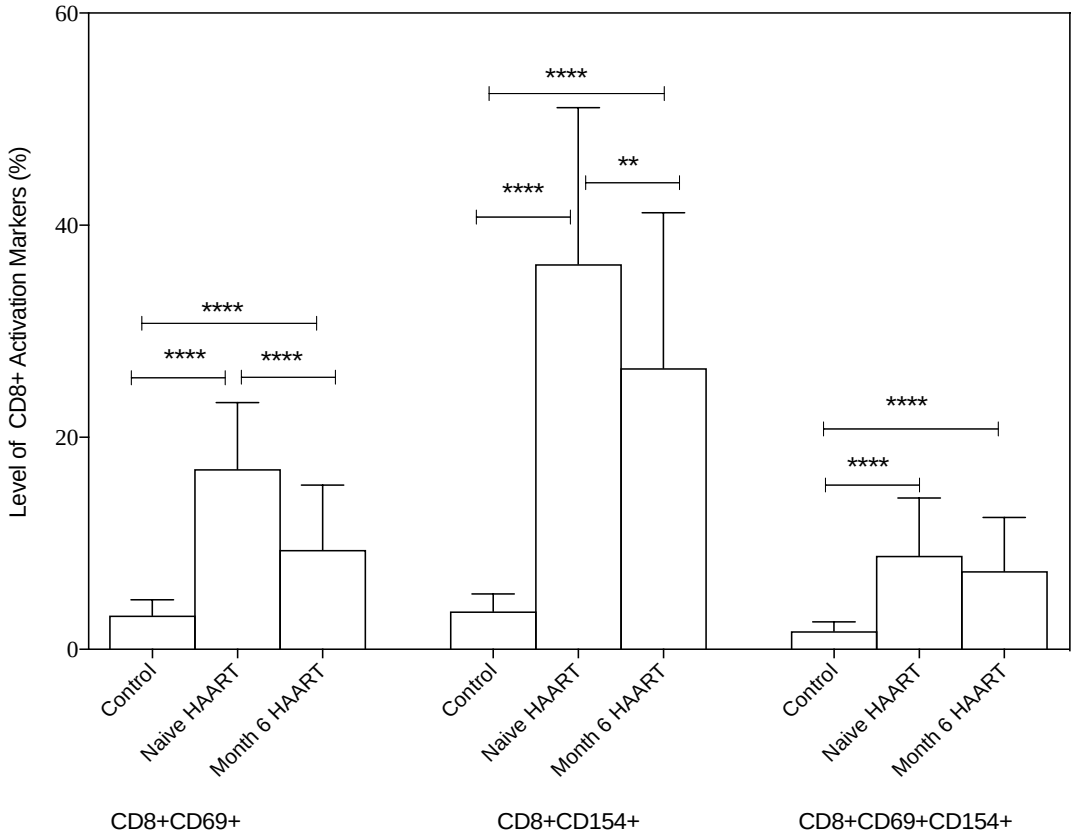
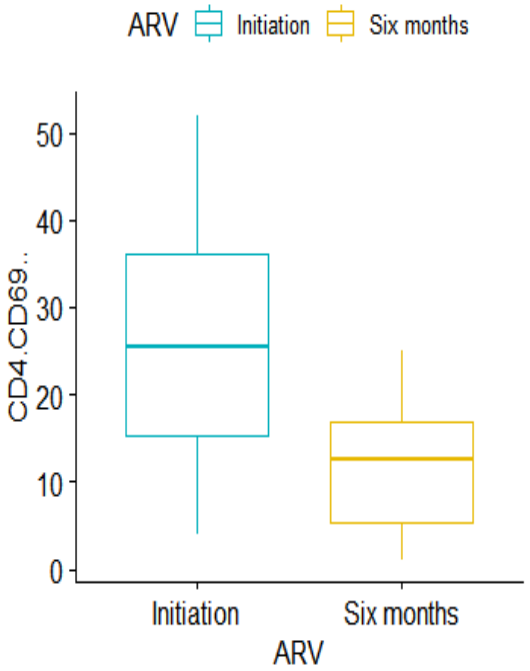


Figure 4.4.4: CD8+T cell activation markers: CD69, CD154 expression and CD69, CD154 co-exp ression at initiation and at least at month6 following HAART. ***Unpaired t test from graph pad pris m 7*: 0.1, ***:0.001; ****:0.0001***

4.4.5 $CD4^+CD69^+$ T cell markers before and after HAART treatment

The activated $CD4^+/CD69^+$ T cell markers was measured (in percentage) for 50 patien ts at initiation and after 6 months of HAART treatment. The mean of percentage of ac tivated $CD4^+/CD69^+$ T cell markers were 26.8 (13.1) and 11.7 (6.94) at initiation and after 6 months respectively.



Statistic	Value (p-value)
Mean (SD) at initiation	*26.8(13.1)*
Mean (SD) after 6 months	*11.7(6.94)*
W at initiation	*0.95196, (0.0411)*
W after 6 months	*0.95312 (0.04588)*
F test	*3.*4852(0.0002)

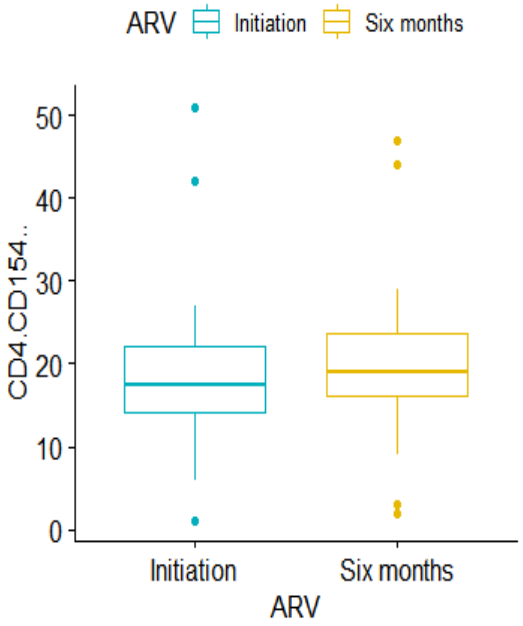
Figure 4.4.5 activated CD4+/CD69 + T cell markers at initiation and after 6 months: ***N=50 at Initiation and after 6months, Mean % (SD) 26.8(13.1) versus 11.7(6.94) W(p-value)= 0.95196, (0.0411) versus 0.95312(0.04588); F = 3.4852, p-value =0.0002***

The hypothesis two for activated $CD4^+/CD69^+$ T cell markers was tested using wilcoxon rank sum test (w) whereby W = 2059.5 with a p-value = 0.0002. rejected null hypothesis with a p-value = 0.0002 and adopted alternative hypothesis. The data from this study indicated statistically significant difference between mean activated $CD4^+/CD69^+$ T cell markers at initiation and after 6 months of HAART (Figure 4.4.8.)

4.4.6 $CD4^+$ $CD154^+$ T markers before and after HAART

The percent expression of activated $CD4^+CD154^+$ T cell markers was measured for 50 patients at initiation and after 6 months of HAART.

The mean (SD) of percentage of activated $CD4^+CD154^+$ T cell markers was 18.5 (7.87) and 20.0(8.43) at initiation and after 6 months respectively (Figure 4.4.6)



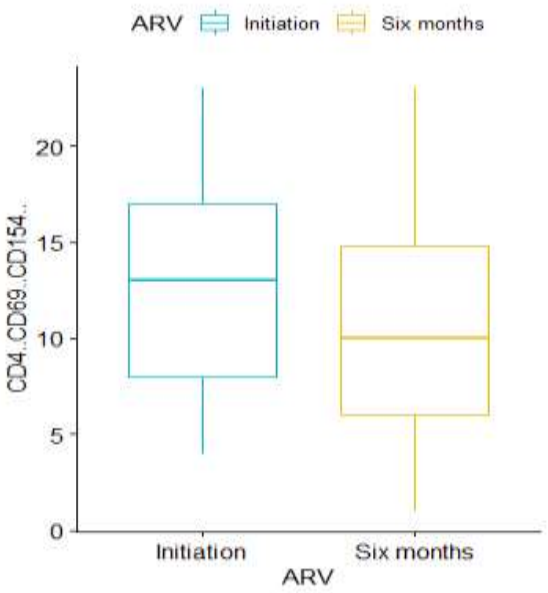
Statistic	Value (p-value)
Mean (SD) at initiation	*18.5 (7.87)*
Mean (SD) after 6 months	*20.0 (8.43)*
W at initiation	*0.8568 (0.0023)*
W after 6 months	*0.9058 (0.0075)*
F test	*0.871(0.6307)*

Figure 4.4.6 Activated CD4+CD154+T cell markers at initiation and after 6 months: ***N=50 at Initiation and after 6months, Mean % (SD) 18.5 (7.87) versus 20.0 (8.43) W(p-value)=0.85683 (0. 0023) versus 0.9058 (0.0075); F (p-value) = 0.871(0.6307)***

The hypothesis two for activated $CD4^{+}CD154^{+}$ T cell markers was tested using independent t-test whereby T (98) = 2.3848 with a p-value = 0.0190. Rejected null hypothesis with a p-value = 0.01901 and adopted alternative hypothesis. The data from this study indicated statistically significant difference between mean activated $CD4^{+}$ $CD154^{+}$ T cell markers at initiation and after 6 months of HAART (Figure 4.4.8)

4.4.7 $CD4^{+}/CD69^{+}/CD154^{+}$T markers before and after HAART

Percentage of activated $CD4^{+}/CD69^{+}/CD154^{+}$ T cell markers was measured for 50 patients at initiation and after 6 months of HAART. The mean of percentage of activated $CD4^{+}/CD69^{+}/CD154^{+}$ T cell markers was 12.8 (5.15) and 10.2(5.97) at initiation and after 6 months respectively (*Figure 4.4.7*)



Statistic	Value (p-value)
Mean (SD) at initiation	12.8(5.15)
Mean (SD) after 6 months	10.2(5.97)
W at initiation	0.9563(0.6221)
W after 6 months	0.9604 (0.0927)
F test	0.7430 (0.3019)

Figure 4.4.7 activated CD4+/CD69+/CD154+T cell markers at initiation and after 6 months:. ***N=50 at Initiation and after 6months, Mean % (SD) 12.8(5.15) versus 10.2(5.97) W = 0.9563(0.6221) versus W = 0.9604 (0.0927) and F(p-value) = 0.7430 (0.3019)***

The hypothesis two for activated $CD4^+/CD69^+/CD154^+$ T cell markers was tested using independent t-test whereby t (98) = 2.3848 with a p-value = 0.0190 rejected null hypothesis with a p-value = 0.0190 and adopted alternative hypothesis. The data from this study indicated statistically significant difference between mean activated $CD4^+/CD69^+/CD154^+$ T cell markers at initiation and after 6 months of HAART (Figure 4.4.8)

The study compared CD69 and CD154 expression on $CD4^+$ T cell in HAART naïve (50), after 6 months group (50) and healthy HIV naïve control (15).

The findings showed statistically different difference in mean expression percentage of $CD4^+$ T cell expressing CD69 and CD154 considering controls, a group before and after HAART treatment with a p value 0.0001 (Figure 4.4.8)

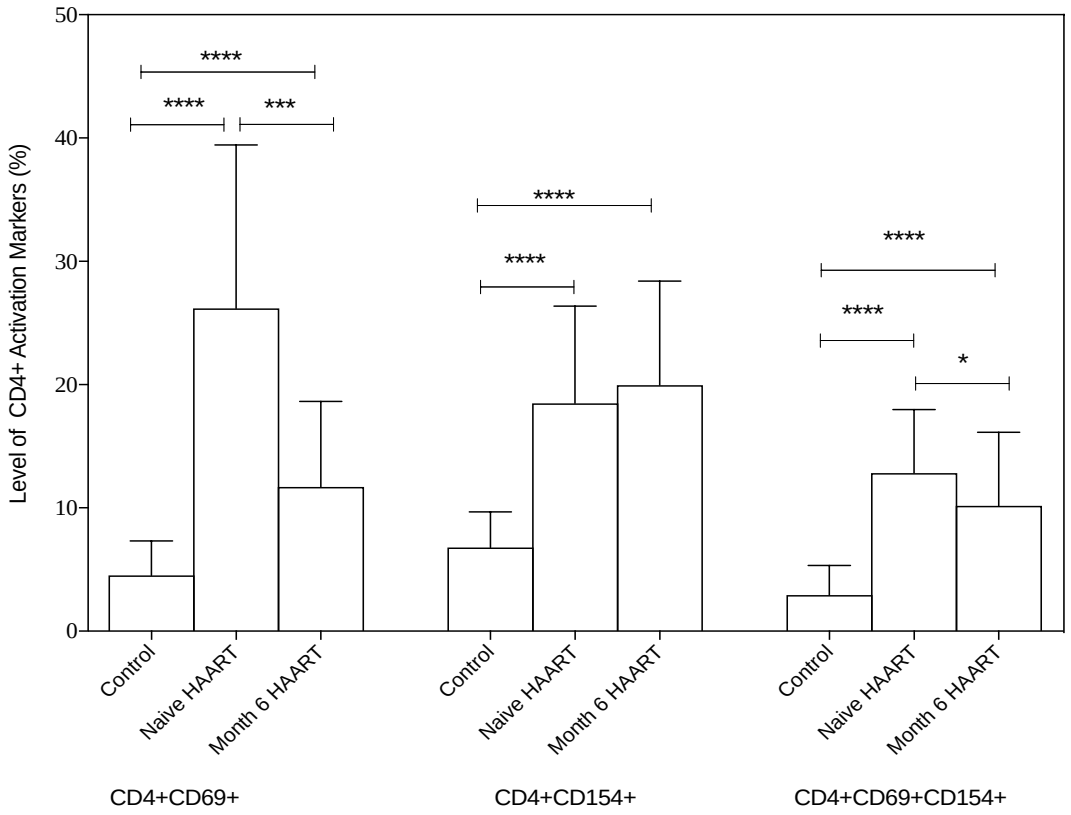


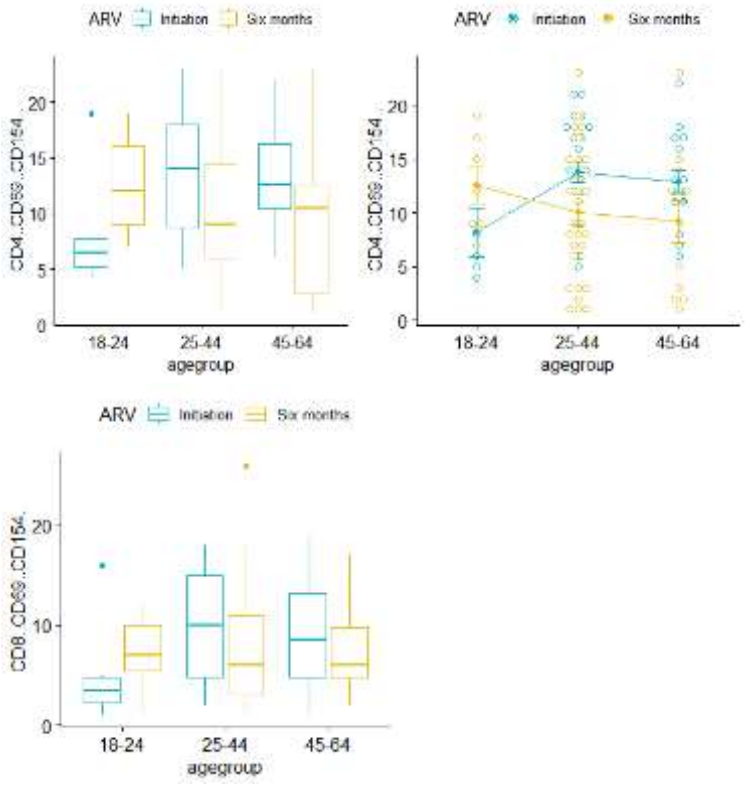
Figure 4.4.8: CD4+T cell activation markers: CD69, CD154 expression and CD69, CD154 co-exp ression at initiation and at least at month6 following HAART: ***Unpaired t test from graph pad pris m 7*: 0.1, ***:0.001; ****:0.0001***

4.5 T cell activation markers based on age and sex.

The study examined if the age and sex do not have effect on perpheral T cell activation markers at initiation and at least at month 6 of HAART treatment.

Although age and sex did not show statisticaly difference affect on CD69 and CD154 expression level across all group of age with the lowest ANOVA p-value 0.737 considering p-value for each marker in all subgroups of age, study noted the highest T cell activation marker and data overlap mostly for double expression, $CD69^{+}CD154^{+}$ on both CD4 and CD8 T cell in 25-44 years old subgroup (Figure 4.5)

a) ***b)*** ***c)***



d) e) f)

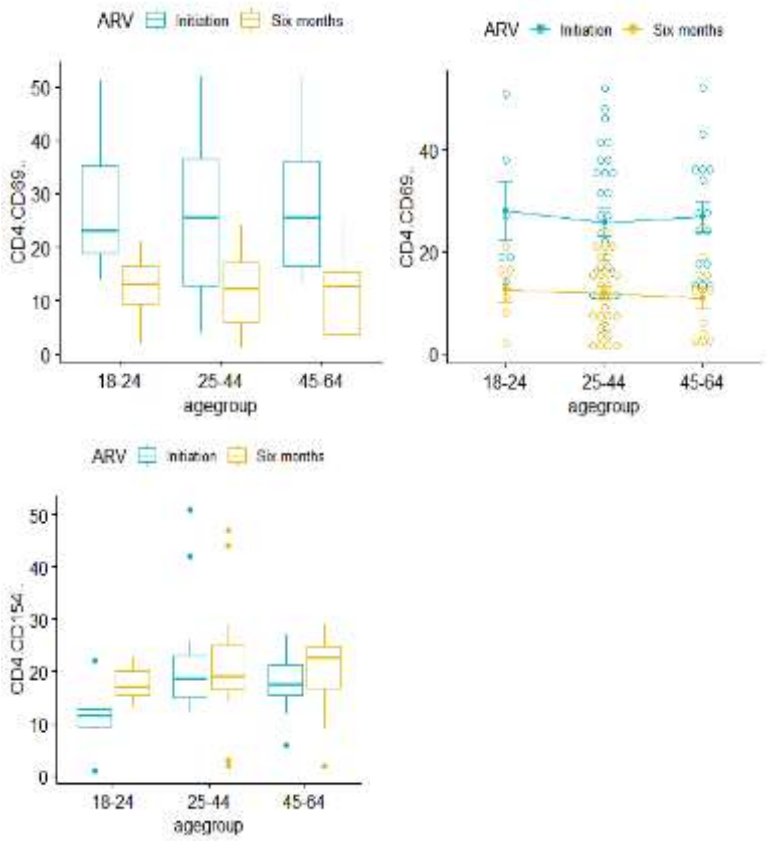


Figure 4.5: Cytokines variation based on Age group folowing HAART: ***Blue box plot represent initiation data summary while orange represent data summary after 6 months. Dot plot represent individal data Bue initiation and yellow after 6 months of HAART***

4.6. Relationship between cytokine production, T cell activation markers expression and HIV VL at initiation and after six months of HAART

The objective three of this study was to determine the relationship between cytokines and activated T cell markers in patients initiating HAART and those who have been on HAART for six months. Study hypothesized that there is relationship between cytokines plasma level and activated T cell markers under null hypothesis.

4.6.1 Relationship between T cell activation markers and HIV VL.

The linear dependence of $CD4^{+}CD69^{+}CD154^{+}$ T cell markers, $CD8^{+}CD69^{+}CD154^{+}$ T cell markers and HIV VL was measured using Pearson correlation (r) whereby the relationship was demonstrated for VL versus $CD4^{+}CD69^{+}$ markers (r=0.701) and p

value <0.0001, VL versus $CD8^+CD69^+$ markers (r=0.5356) and p value <0.0001 and VL versus $CD8^+CD154^+$ markers (r=0.4963) and p value <0.0001. HIV VL was not associated with $CD4^+CD154^+$ markers(r=0.05498, p-value 0.59). $CD4^+CD69^+$ expression was highly associated with HIV VL compare to others(r=0.7) (figure 4.3.1)

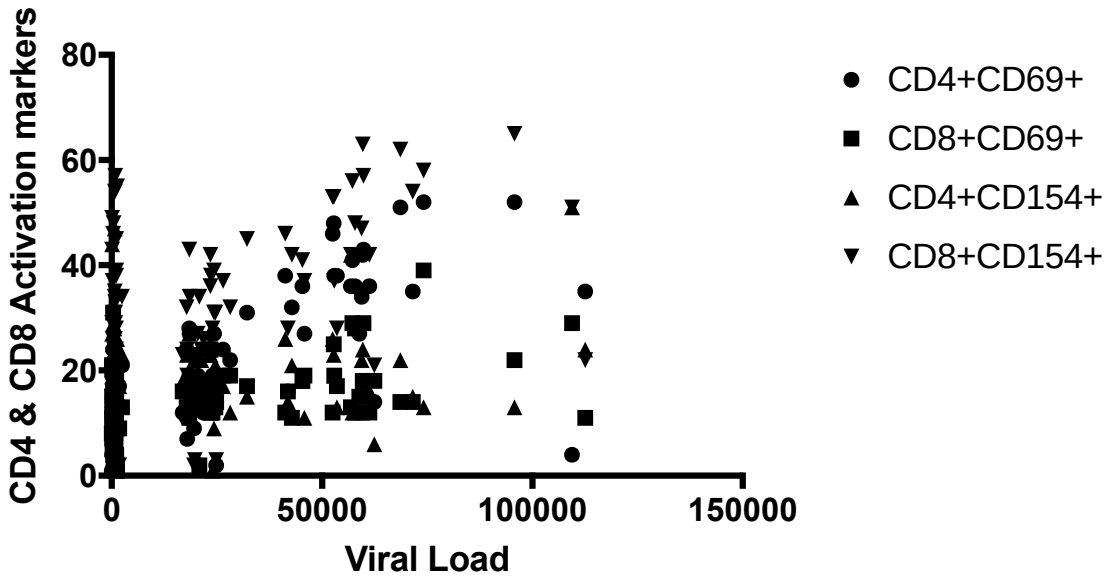


Figure 4.6.1: Correlation of T cell activated markers and HIV VL using Pearson's correlation test

Table 4.6.1 Summary pearsons correlation for HIV vl and T cell activated penotypes.

	Viral Load vs. CD4+CD69+	Viral Load vs. CD8+CD69+	Viral Load vs. CD4+CD154+	Viral Load vs. CD8+CD154+
Pearson r				
r	0.701	0.5356	0.05498	0.4963
95% confidence interval	0.5817 to 0.7908	0.3745 to 0.6654	-0.1482 to 0.2537	0.3276 to 0.6344
P value				
P (two-tailed)	<0.0001	<0.0001	0.5967	<0.0001

4.6.2 Relationship between cytokine and HIV VL before and after HAART.

The linear dependence of IL-2, IL-4, IL-6, IL-10, and TNF-α and IFN-γ and HIV_VL were assessed using Pearson correlation (r). The positive relationship was observed for HIV_VL and IL-2 **(**r= 0.4148 p value <0.0001), HIV_VL and IL-6 (r= 0.8164 p value <0.0001), HIV_VL and IL-10 (r= 0.8667 p value <0.0001) while HIV_VL was associated negatively with TNF (r=-0.3483 p value 0.0005) and IFN (r= -0.4835 p value <0.0001) (figure 4.3.2.1 and 4.3.2.2). IL-4 did not show statistically significant relationship with HIV VL (r= 0.0839 p value = 0.41)

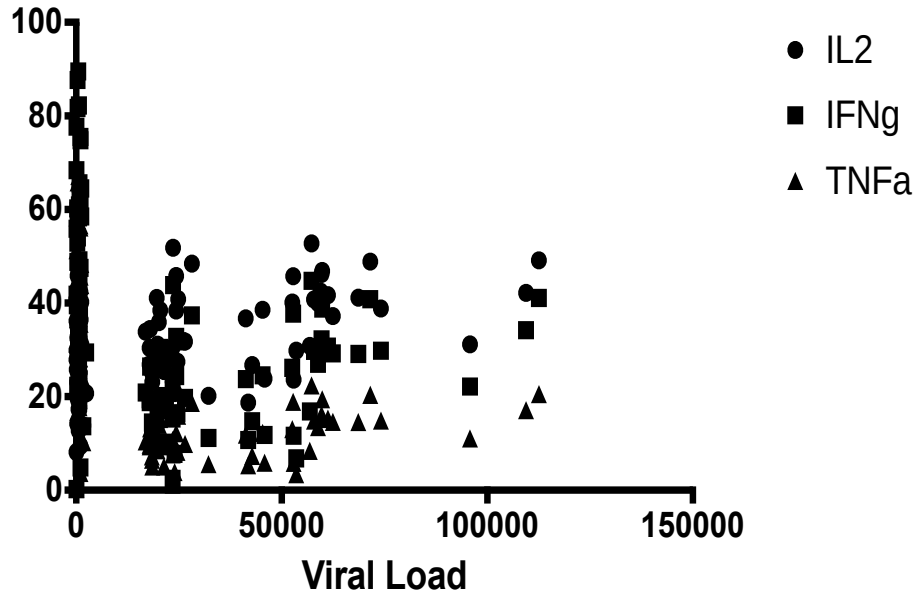


Figure 4.6.2.1 Relationship between Pro inflammatory cytokine and HIV _VL before and after HAART using Pearson's correlation test

Table 4.6.2.1 Summary pearsons correlation for HIV VL and proinflamatory cytokines

	Viral Load vs. IL2	Viral Load vs. IFN-γ	Viral Load vs. TNF-α
Pearson r			
r	0.4148	-0.3483	-0.4835
95% confidence interval	0.2327 to 0.5688	-0.5138 to -0.1579	-0.6242 to -0.3124
P value			
	<0.0001	0.0005	<0.0001

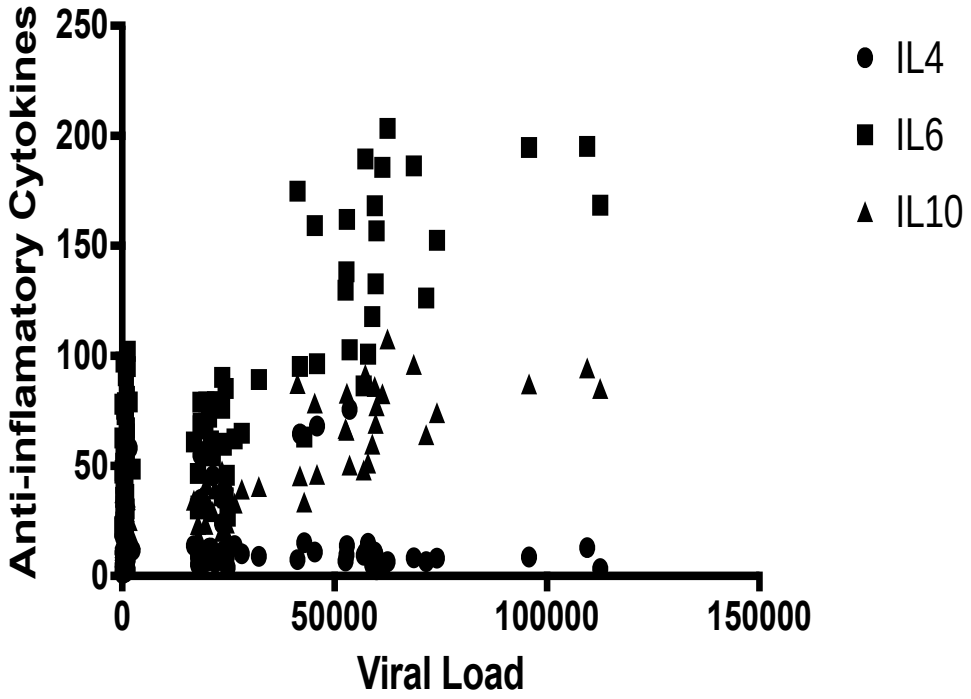


Figure 4.6.2.2 Relationship between anti-inflammatory cytokine and HIV _VL before and after HAART using Pearson's correlation test

Table 4.6.2.2 Summary pearsons correlation analysis for HIV VL and antiinflamatory cytokine

	Viral Load vs. IL4	Viral Load vs. IL6	Viral Load vs. IL10
Pearson r			
r	0.08392	0.8164	0.8667
95% confidence interval	-0.1196 to 0.2807	0.7359 to 0.8741	0.8059 to 0.9094
P value			
P (two-tailed)	0.4188	<0.0001	<0.0001

CHAPTER FIVE: DISCUSSION

During this study, IL-2; IL-4; IL-6; IL-10; IFN-γ; TNF-α plasma levels and peripheral T cell activation markers (CD69 and CD154) expression were determined to assess changes and relationship between cytokines, activation markers expression and HIV VL for one hundred HIV^+ individuals at initiation and at month six of HAART treatment. The study found statistically significant differences in plasma cytokines levels and peripheral T cell activation markers at initiation and after six months of HAART. A positive relationship was found between IL-10, IL-6 and HIV VL. CD69 expression on $CD4^+$ and $CD8^+$ T cell demonstrated a better correlation with HIV VL at initiation and after 6 months of HAART than CD154 expression on the same T cell.

In this study, Il-4; IL-6 and IL-10 were high at initiation of HAART and tended to reduce following HAART at 6 months. The same statistically significant changes observed between HAART naïve, after six months and heath controls groups in progressive manner. These data supported the facts that HAART reduce partially chronic inflammation and restore immune system in HIV infected /AIDS patients following successful treatment and highlight on mechanisms by which HIV infection causes inflammation (IL-6) and immune modulation (IL-10) via inhibition of pro-inflammatory cytokines, the mechanism that HIV uses to profit host cell cycle for its replication; latency or reservoir (Gori et al., 2016; Maharaj et al., 2017; Slim & Saling, 2016; Lucie et. al, 2013;Vandergeeten et al., 2012; Z. Wang, Shang, & Jiang, 2017). This Study findings concurred with other study urged that for HIV to survive, it directly subvert specific immune responses by IL-10 and other anti inflammatory cytokines induction (Gori et al., 2016; Maharaj et al., 2017; Slim & Saling, 2016) and

single cells study which showed that high frequency of circulating of $CD4^{+}$T cell from HIV infected /AIDS patients produced more of IL-10 compared to HIV naïve. The same results were highlighted in HIV infected /AIDS individuals with rapid HIV infection progression and in active HIV viral replication cases (Ostrowski et al., 2001).

Pro-inflammatory cytokines, IL-2; IFN-γ and TNF-α demonstrated statistically significant changes compared their levels at initiation and after six months of HAART. IL-2; IFN-γ and TNF-α plasma levels were low at initiation and tended to increase after six months of HAART. These data showed that antiviral mechanisms increase with HAART treatment and support the idea that HIV inhibit normal release of IFN-γ and TNF-α for host colonization. Both the anti-inflammatory and pro-inflammatory findings support the research findings that had demonstrated a shift from Th-1 into Th-2 cytokines during HIV/AIDS and the trend from Th-2 immune milieu in active HIV infection to Th-1 immune milieu following successful HAART. (Osakwe et al., 2010; Subauste et al., 2017; Vandergeeten et al., 2012; Vandergeeten et al., 2013). However, other study reported an increased level and relationship between IL-2, IFN-γ and IL-10 with high plasma HIV VL but high IL-10 was indicator of a depressed immune system following HAART initiation in the study participants (de Medeiros et al., 2016; Huang et al., 2016; Turk et al., 2018).

This study has demonstrated statistically significant changes in CD69 and CD154 expression on both CD4 and CD8 T cell following HAART. The high percentage expression of CD69 and CD154 was noticed during an active phase of HIV infection (before HAART), compared to their expression after six months of HAART and not all T cell that expressed CD69 express further CD154. These data meant that T cell

stimulation and activation state is high before HAART initiation compared to six months after HAART but also not all T cell that receive first signal of activation from TCR get at state of full activation and expression of CD154. These data concur with findings from other studies that demonstrated that HIV inhibits induction of CD154 through CD40-CD154 signal transduction and not via T cell receptor ligation with HIV gp120 as it was hypothesized before (Carlos et al., 2017; Subauste et al., 2004).

$CD4^{+}CD69^{+}$, $CD8^{+}CD69^{+}$; $CD8^{+}CD154^{+}$ T cell showed positive relationship with HIV VL at initiation and after six months of HAART while $CD8^{+}CD154^{+}$ T cell did not shaw relationship with HIV VL. The finding meant that T cell activation made it more permissible to HIV infection and replication, which in turn increases HIV VL. These findings highlight on other study report that had reported relationship between T cell activated markers expression and HIV VL using Foxp3, HLA DR (Lubanga et al., 2016; Wu et al., 2017).

Cytokines and T cell activation level were high in HIV patients (both HAART naïve and after six months of HAART) compared to HIV naïve population (controls). These finding may explain the persistent immune activation and chronic inflammation in HIV infection even in controlled HIV VL and concurred with other study reports that had hypothesized that HIV infection influence immunosenescence (Nasi et al., 2017; Sokoya & Rossouw, 2017)

In this study, sex and age were not found to be a factor that influences neither cytokines plasma level nor T cell activation markers expression before and after six months of HAART treatment. Thus, cytokines and T cell activation markers may be a potential good biomarker. Similar findings were demonstrated in other studies whereby HLA-DR and or CD38 expression or plasma cytokines in HIV^{+} patients

above 18 years old, treatment naïve and after 6 months were not influenced by sex nor by age (Fricke et al., 2012; Song et al., 2011; Turk et al., 2018). Although it was not statistically significant with ANOVA test (P value > 0.05) and not objective of this study, the data showed the highest expression (CD69 and CD154) for both $CD4^+$ and $CD8^+$ T cell and highest plasma cytokines levels (IL-6, IL-10) as well data over lap at initiation and after 6 months of HAART in 25-44 years old subgroup. These findings concurred with other study reports that reported persistent immune activation and chronic inflammation, premature aging and exhausted T cell phenotypes in HIV/AIDS even after virological success following HIV therapy (Nasi et al., 2017)

CHAPTER SIX:CONCLUSION AND RECMENDATION

6.1 Conclusion

1. Untreated HIV/AID is characterized by high level of IL- 4, IL-6, IL-10 and lower level of IFN-γ and TNF-α plasma level. Effective HAART reestablish immune homeostasis and IL- 4, IL-6, IL-10 get reduced while IFN-γ and TNF-α get increasing. Although HAART reestablish immune homeostasis, is not comparable to HIV/AID naïve population.

2. CD69 and CD154 are more expressed on both T in untreated HIV/AID patients. Although it get reduced with effective HAART, it is comparable to HIV/AID naïve population. CD69 and CD154 express differently.

3. IL-10, IL-6 plasma level and CD69 expression on T increase together with HIVVL and IFN-γ and TNF-α decrease while HIVVL is increasing.

6.2 Recommendations

1. The study recommended that IL-10, IL-6 plasma level, CD69 expression level on T cell to be considered as an alternative biomarker for assessment of hallmark of immune activation and inflammation in HIV infection.
2. The study recommended T cell culture and T cell challenging with HIV specific antigens to study the underling mechanisms that are behind deferent expression of CD69 and CD154 in HIV/AIDS.

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APPENDICES

Appendix 1: Revised Who Steps Questionnaire

PARTICIPANT ID:
DATE:

STUDY TITLE: **PLASMA CYTOKINES LEVELS, ACTIVATED T CELL MARKERSS AND VIRAL LOAD IN HUMAN IMMUNODEFICIENCY VIRUS POSITIVE PATIENTS IN RWANDA**

CONSCENT HAS BEEN OBTAINED: YES NO

Contact Information if possible:
_____

DEMOGRAPHIC INFORMATION:
CODE

1. SEX: Male Female C1

2. AGE: C2

3. EDUCATION LEVEL C3
Incomplete primary Complete primary Secondary and above

4. MARITAL STATUS C4
Single Married Others

NUTRITION INFORMATION:

5 MEALS / DAY C5
One /day two/ day three/day other… Specify

6 FRUITS VEGETABLE/WEEK
C6
None > once/week

BEHAVIOURAL INFORMATIONS:

7 SMOKING STATUS C7
Ever Smoked Current Smoker

8 SEXUAL PRACTICES
C8
Use of condom: Yes/NO Partner: one/ more

Sexual orientation: Homosexual Heterosexual Bisexual

9 ALCOHOL CONSUMPTION C9

None One/ Week > One/ Week

HIV SPECIFIC INFORMATION

10 BASELINE CD4+ T CELL COUNT:

C10

<200cells/µl 200cells/µl -350cells/µl >350 cells/µl

11 TYPE OF cART FIRST LINE IN USE

C11

12 ADHERENCE LEVEL TO cART

C12

Good Bad

Average

13 RECENT SERIOUS INFECTION

C13

1……..

2 ……..

3……

APPENDIX 2: INFORMED CONSENT

MOI UNIVERSITY COLLEGE OF HEALTH SCIENCES / MOI TEACHING AND REFERRAL HOSPITAL

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC) INFORMED CONSENT FORM (ICF)

STUDY TITLE: PLASMA CYTOKINES LEVELS, ACTIVATED T CELL MARKERSS AND VIRAL LOAD IN HUMAN IMMUNODEFICIENCY VIRUS POSITIVE PATIENTS IN RWANDA

Name of Principal Investigator(s): Jean Pierre NGANGALI

Name of Organization: NATIONAL REFERENCE LABORATORY
Kigali_ Rwanda
P.O. BOX 4sixsix8
Kigali

Name of Sponsor: INTRA ACP MOBILITY SCHEME

Informed Consent Form for: HIV positive patients above 18 attending Kigali University referral and teaching Hospital (CHUK), Ruhengeli referral hospital, Gihundwe provincial hospital, Rwamagana provincial hospital and Kabutare hospital HIV clinic for HIV testing and or HIV care

This Informed Consent Form has two parts:

- Information Sheet (to share information about the study with you)
- Certificate of Consent (for signatures if you choose to participate) you will be given a copy of the signed Informed Consent Form

PART I: Information Sheet

Introduction

You are being asked to take part in a research study. This information is provided to tell you about the study. Please read this form carefully. You will be given a chance to ask questions. If you decide to be in the study, you will be given a copy of this consent form for your records.

Taking part in this research study is voluntary. You may choose not to take part in the study. You could still receive other treatments. Saying no will not affect your rights to health care or services. You are also free to withdraw from this study at any time. If after data collection you choose to quit, you can request that the information provided by you be destroyed under supervision- and thus not used in the research study. You will be notified if new information becomes available about the risks or benefits of this research. Then you can decide if you want to stay in the study

Purpose of the study

The purpose of the study is to find out whether cytokines and T cell activation markers levels may fit as immunological biomarker for HIV infection monitoring and care.

Type of Research Project/Intervention

The research consists in measuring TNF-α, IFN-γ, IL-2, IL6, IL-10, T cell activated markerss and HIV viral load for both clients at the start of the cART treatment and for whom are on cART for six months. 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 and used to measure the above-mentioned parameters.

Why have I been identified to participate in this study?

This study is on HIV positive patients above 18 years old and participation is based on random selection so everyone has equal chance of participating if they are wiling

How long will the study last?

You will be in this study for only 1 day.

What will happen to me during the study?

If you accept to participate in this study, you will be asked to answer 12 questions, on designed questionnaire in form of an interview. Those questions are about your age, sex, educational level, smoking status, marital status and alcohol consuptional status. The rest of the information on the other questions will be obtained from your file such as your current and baseline and or current CD4 count, baseline and current HIV viral load, type of ARV medication and duration of therapy.

And then after, venous blood sample (8 ml) will be taken and used to measure TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10, T cell activated markers and HIV viral load **What side effects or risks I can expect from being in the study?**

A little pain may occur while drawing blood and small swelling at the site of the needle prick, although swelling is less likely to occur.

Are there benefits to taking part in the study?

Yes, as you are taking part in this study, you are helping in development and acquisition of new knowledge and new biomarkers for better HIV care.

Reimbursements:

There will be no reimbursement for participation.

Who do I call if I have questions about the study?

Questions about the study: Jean Pierre NGANGALI, Tel: +2fifty7885975six8 or +2fifty7385975six8

Questions about your rights as a research subject: You may contact Institutional Review Ethics Committee chairperson Dr Jean Baptiste MAZARATI

P. O. Box 4sixsix8 , Telephone : +2fifty78830980 Kigali_RWANDA

Will the information I provide be kept private?

All reasonable efforts will be made to keep your protected information (private and confidential. Protected Information is information that is, or has been, collected or maintained and can be linked back to you. Using or sharing ("disclosure") of such information must follow National privacy guidelines. By signing the consent document for this study, you are giving permission ("authorization") for the uses and disclosures of your personal information. A decision to take part in this research means that you agree to let the research team use and share your Protected Information as described below.

As part of the study, Jean Pierre NGANGALI and his study team may share the results of your laboratory tests. They may also share portions of your medical record, with the groups named below:

Rwanda National Ethics Committee,
The Institutional Review and Ethics Committee,
National privacy regulations may not apply to these groups; however, they have their own policies and guidelines to assure that all reasonable efforts will be made to keep your personal information private and confidential.
The sponsor may give your personal health information, not containing your name, to others or use it for research purposes other than those listed in this form. In handling your personal information, the sponsor, Jean Pierre NGANGALI and associated staff will keep your information in strict confidence, and shall comply with any and all applicable laws regarding the confidentiality of such information.
The study results will be retained in your research record for at least six years after the study is completed. At that time, the research information not already in your medical record will be stored for a period of five years under a password encrypted computer..
Any research information entered into your medical record will be kept indefinitely.
Unless otherwise indicated, this permission to use or share your Personal Information does not have an expiration date. If you decide to withdraw your permission, we ask that you contact Jean Pierre NGANGALI in writing and let him know that you are withdrawing your permission. The mailing address is Rwanda National Ethics Committee, P.O BOX 4sixsix8 Kigali_Rwanda. At that time, we will stop further collection of any information about you. However, the health information collected before this withdrawal may continue to be used for the purposes of reporting and research quality.
You have the right to see and copy your personal information related to the research study for as long as the study doctor or research institution holds this information. However, to ensure the scientific quality of the research study, you will not be able to review some of your research information until after the research study has been completed.
Your treatment, payment or enrollment in any health plans or eligibility for benefits will not be affected if you decide not to take part. You will receive a copy of this form after it is signed.

PART II: Consent of Subject:

I have read or have had read to me the description of the research study. The investigator or his/her representative has explained the study to me and has answered all of the questions I have at this time. I have been told of the potential risks, discomforts and as well as the possible benefits of the study. I freely volunteer to take part in this study.

_____ _____ _____
Signature of subject/thumbprint Date

_____ _____ _____
Printed name of Investigator Signature of Investigator Date

APPENDIX 3: INFORMED CONSENT FORM/

Urupapuro Rugaragaza Kwemera Kugira Uruhare Mu Bushakashatsi Ku Bushake

MOI UNIVERSITY COLLEGE OF HEALTH SCIENCES / MOI TEACHING AND REFERRAL HOSPITAL
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC) INFORMED CONSENT FORM (ICF)

INYITO Y'UBUSHAKASHATSI: PLASMA CYTOKINES LEVELS, ACTIVATED T CELL MARKERSS AND VIRAL LOAD IN HUMAN IMMUNODEFICIENCY VIRUS POSITIVE PATIENTS IN RWANDA

Nitwa Jean Pierre NGANGALI ndi umunyeshuri muri kaminuza ya Moi University, mu ishami ry'ubuvuzi ho mu gihugu cya Kenya. Ndimo gukora ubushakashatsi bugamije gupima uko ubwirinzi bwa muntu buboneka mu maraso bungana kandi bwunganirana mu bantu babana n'ubwandu bw'agakoko gatera SIDA. Ibyo bipimo ni IL-10, IL-2, IL-4, IL-6, IFN-γ, TNF- α, HIV viral load na T cell activation markerss. Ibi bizapimwa mu maraso y'uwemeye kubushake kugira uruhare mu bushakashatsi mbere yo gutangira imiti ndetse nabamaze amezi atandatu batangiye iyi miti igabanya ubwandu bw'agakoko gatera SIDA. Ibisubizo bizagirwa ibanga hagati yanjye nyirabyo. 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 nabamaze atandatu (six) umuntu afata

iyo miti. Hashobora kubaho ububabare buke cyane mu gihe amaraso aza kuba afatwa ariko ubwo bubabare buramara igihe gito 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. Wemerewe kumbaza ikibazo icyo ari cyo cyose igihe icyaricyo cyose kandi ni uburenganzira bwawe kubona ibisobanuro

Murakoze cyane

……..Itariki……
……

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

Mugize ikibazo mwambaza kuri telefoni ifite nomero: 07885975six8 no kuri E-Mail: ngangalijeanpierre@gmail.com

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

Dr Jean Baptiste MAZARATI at 0788309807, Umuyobozi mukuru

APPENDIX 4: IMMUNOPHENOTYPING PROCEDURE.

SOP for Immunophenotyping Principle

Flow cytometry can be applied to study the expression of surface antigens on leukocytes and other cells. Furthermore, intracellular antigens can be detected after permeabilization of the cells. A defined set of fluorochrome-conjugated monoclonal antibodies allows analysis of several human specimens such as peripheral blood, bone marrow and lymph nodes for the presence of an aberrant population fitting the definition of leukemia or lymphoma.

1. Background

A flow cytometer uses one or more lasers to evaluate the size (forward light scatter), granulation (sideward light scatter) and emitted fluorescence on a single cell basis. The fluorochromes FITC, PE, PerCP, PerCP Cy5.5 and APC are commonly applied in four-color flow cytometry. These fuorochromes are coupled to antibodies specific antigens present on the surface or in the cytoplasm of a cell. Immunophenotyping using four-color flow cytometry serves an adequate platform to combine markers for maturation and lineage identification. By using a combination of different fluorochrome-conjugated monoclonal antibodies for antigens that define a particular subpopulation (e.g. neutrophils, B or T cell) or a maturational stage of a defined subpopulation (progenitors or mature cells), abnormal proliferation of or lack of certain subpopulations can be evaluated. In this way, malignanT cell in leukemia and lymphoma may be distinguished from a normal background.

3. Abbreviations

APC: Allo Phyco Cyanin.

FACS: Fluorescence Activated Cell Sorter

FCS: Fetal Calf Serum (or fetal bovine serum (FBS))

FSC Forward light SCatter

FITC: Fluorescent IsoThioCyanate.

FL: Fluorescence signal (-1, -2, -3, and -4 indicate the four detectors for emission wavelengths)

PBS: Phosphate Buffered Saline.

PE: PhycoErythrin.

PerCP: Peridinyl Chlorophyllin.

PerCP-Cy5.5: Peridinyl ChloroPhyllin coupled to the dye Cy5.5 (a tandem dye;

Note: tandem dyes are less stable than single dyes).

SSC Side SCatter light.

4. Materials

4.1 Patient material.

Screening of human specimens by flow cytometry can be performed on all single cell suspensions from peripheral blood, bone marrow, lymph nodes, fine needle aspirates, pleural fluid, cerebrospinal fluid and so on. Different specimens will need different work-up procedures before immunophenotyping can be performed (as described in Methods-section).

4.2 Reagents

- Wash buffer: prepare PBS with 1% FCS (store up to 1 week at 4ºC)
- FACS lysing solution*:* prepare 1*FACS lysing solution according to manufacturer's instructions (dilute the concentrate 10 times with (sterile) distilled water); the prepared solution can be stored at 4ºC for one week.

4.3 Antibodies

Antibodies not limited to the following may be applied in the four-color flow cytometry immunophenotyping procedure as long as fruorochrome may be excited by red or blue LASER:

CD45-APC BD Biosciences

CD3-FITC BD Biosciences

CD3-PerCP-Cy5.5 BD Biosciences

CD4-PE BD Biosciences

CD5-FITC BD Biosciences

CD10-PE BD Biosciences

CD19-PerCP-Cy5.5 BD Biosciences

CD33-PE BD Biosciences

CD38-FITC BD Biosciences

CD71-FITC BD Biosciences

CD79a-PE BD

CD117-PE

CD138-PERCP

HLA-DR-PERCP

CD20-FITC

CD13-PE

TDT-FITC

CD3six-PE

FoxP3-PE

CD45RO-APC

Anti-kappa light chain F (ab) 2-PE BD Biosciences

Anti-lambda light chain-PE BD Biosciences.

APPENDIX A describes which cells can be recognized with which antibodies and their pitfalls in leukemia and lymphoma immunophenotyping.

4.4 Other equipment.

FACS Calibur flow cytometer and accompanying software (CellQuestPro)

Pipettes

Pipetting tips

Tubes

Protective Equipment …

5. Methods

5.1 Preparation of samples.

It is preferable to process all samples within 24 hours. Cerobrospinal fluid should be processed within 1 hour. Cells from fine needle aspirations can be preserved somewhat longer when culture medium with glucose (e.g. RPMI) and some protein (1-5% FCS) is added. This medium should be washed away before antibody staining.
Note, that every patient material should be considered potentially contagious/infectious/hazardous. Local precautions should be strictly followed.

- Cells from peripheral blood and bone marrow aspirates can be stained without a preparation step. However, there are some exceptions:
    1. The cell concentration is expected to be very high. Please dilute a small amount of the sample with wash buffer+protein. A cell concentration of >>20.10^{six}/ml will result in distribution of the antibodies over a too large amount of cells resulting in weak fluorescent signals.
    2. The sample needs to be analyzed for B cell lymphoma. Immunoglobulins present in the blood or bone marrow plasma will catch the anti-kappa and anti-lambda immunoglobulin light chain antibodies, thereby leaving the B

cells only weakly stained or even negative. Please wash the blood and bone marrow sample at least three times with wash buffer+protein before staining with antibodies (2000rpm, 5-7 minutes, mild speed of brake). Resuspend (and if applicable dilute) the sample in wash buffer+protein.

When a "washed sample" is to be stained and analyzed the next day cells can be preserved when culture medium with glucose (e.g. RPMI) and some protein (1-5% FCS) is added at a 1:1 ratio with the sample size. This medium should be washed away before antibody staining.

- Lymph nodes should be transferred into single cell suspensions according to the local protocol (please use protein-rich wash buffer (PBS+1%FCS ore PBS+0.1% human or bovine serum albumin) to keep the cells as viable as possible).
- Cells from **fine needle-aspirates and cerebrospinal fluid** (and other fluids) might contain only few cells and might therefore need to be concentrated by a centrifugation step: 2000 rpm, 5 min. After centrifugation, discard the supernatant and resuspend the cells in wash buffer+protein. If the cell pellet is rather contaminated with red blood cells, it might be considered to perform a red blood cell lysing step using FACS lyse solution (10 min, room temperature).

5.2 Antibody staining.

Which antibody combinations are tested depends on the query and the results of a first screening.

The currently proposed antibody mixtures are in table 1.

Tube	FL1 - FITC	FL2 - PE	FL3 – PerCP	FL4 – APC
1				**CD45**
2	**CD38**	**CD10**	**CD19**	**CD45**
3	**CD5**	**CD33/MPO**	**CD3**	**CD45**
4	**kappa**	**lambda**	**CD19**	CD45
5	**CD71**	MPO	Cyt CD3	**CD45**
Six	**(Cyto) CD3**	**CD4**	**surface CD3**	**CD45**

Note: Tubes 1-5 are single step stainings; tube six is a performed in a first-and-second step fashion (sixA and sixB, see appendices B and/or C).

First screening:

1. Leukemia? Then stain screening tubes 1, 2 and 3.
2. Lymphoma? Stain tubes 1, 2, 3 and 4 (note to pre-wash the sample to remove plasma immunoglobulins)
3. Leukemia or lymphoma? Stain tubes 1, 2, 3 and 4 (note to pre-wash the sample to remove plasma immunoglobulins)

Optional continuation after first screening results:

1. screening tubes 1, 2 and 3 show accumulation of mature B cells ($CD19^{+}$, $CD45^{bright}$):

Stain tube 4 in addition (can be dealt with the next day (<24h.); note to pre-wash the sample to remove plasma)

2. screening tubes 1, 2 and 3 show accumulation of $CD45^{dim}$ cells with low to intermediate SSC without expression of CD19 (B cell) and CD33 (myeloid).

Please stain tube six to confirm or exclude immature T cell (surface CD3 $^{negative\ -}$, intracellular $CD3^{+}$). Note that these cells might be $CD10^{+}$ which fits the common-T-ALL markers. If intracellular CD3 is negative and the SSC is rather intermediate, this leukemia might be a $CD33^{negative}$ acute myeloid leukemia

3. screening tubes 1, 2 and 3 show accumulation of $CD45^{negative}$ cells with low to intermediate SSC without expression of CD19 (B cell), CD33 (myeloid).

Please check stain tube 5 and six in addition (can be dealt with the next day (<24h.)). $CD71^{bright}$ expression in tube 5 confirms erythroid lineage of $CD45^{negative}$ cells BUT

REMEMBER SOME ALL AND AML LOSS CD45 AND HIGHLY EXPRESS CD71

. Otherwise, surface CD3 ^{negative} in combination with intracellular $CD3^+$ confirms presence of immature T cell and thus T-ALL

Note: $CD45^{negative}$ cells may also be metastasized carcinoma/solid tumor cells (no other markers positive) or malignant plasma cells ($CD38^{very\ bright}$)

4. screening tubes 1, 2 and 3 show accumulation of mature T cell ($CD5^+$ or surface $CD3^+$, $CD45^{bright}$)

Stain in addition your multiset /multitest CD3/CD4/CD8/CD45 tube to analyze CD4/CD8 ratio to analyze the predominance of $CD4^+$ or $CD8^+$ population (can be dealt with the next day (<24h.))

Examples of these results and other diagnoses can be found in *APPENDIX D*

The actual antibody staining for surface antigens is performed as follows:

- Put as many FACS tubes in a rack as needed
- Label these tubes by the name or abbreviation of the name of the patient and add the number of the tube (1, 2 and 3 or 1, 2, 3, 4, etc.)
- Put the appropriate amount of fluorescently labeled antibodies in the corresponding tubes according to the scheme in APPENDIX B. Add wash buffer+protein to balance the volume in each tube to 20µl (see APPENDIX B).

Note: when antibody cocktails have been pre-aliquoted according to APPENDIX C, please put 20µl of this pre-aliquoted mixture in the corresponding tubes

- Add 30µl of cell sample, mix gently.

Note: droplets of the sample that are not at the bottom of the tube, can be removed by absorbing these in the cotton wool of an ear stick

- Incubate for a minimum of 15 minutes (max. 45 minutes) at room temperature in the dark
- Add 1ml of 1*FACS lysing solution to remove the erythrocytes
- incubate for 10 minutes at room temperature in the dark
- add 2 ml of wash buffer+protein (1fifty0rpm, 5 min)
- When the cell pellet is rather “red” please discard the supernatant and repeat the lysing step; otherwise discard the supernatant and resuspend the cell pellet in approximately 300µl. When necessary an additional wash step can be performed.
- The stained cells are now ready for analysis on the flow cytometer; continue at *Acquisition of data* (see below).

5.3 Acquisition of data.

1. Open the Acquisition and Analysis template "*Leukemia copy*" (May be change this name…right mouse click …then see menu …rename) on the desktop of the computer next to the FACS Calibur flow cytometer by double mouse-click. To acquire data in this template all plots are put in the acquisition and analysis mode (can be adjusted using the "*format dot plot/density plot*" feature in the menu "*plots*": The "*inspector*" dash board appears.
2. Go to menu "*Acquire*"; select "*connect to flow cytometer*". A browser will appear in which data folders and sample identification can be specified.

3. First, go to menu "Cytometer"; select "*instrument settings*"; click "*open*" and find FACSstation/BD files/instrument setting files; click on *calib files* (this is a calibration file); click in the dashboard on "*set*" and then "*done*". These calibration settings are now uploaded in the system.

4. Go to menu "Cytometer"; select "*Dectectors/Amps*"; in this dashbord FSC and SSC can be adjusted to standardize interpretation of data. Change FSC from 2.00 towards approximately 2.40 and change SSC from 400 to approximately 480 (Check on Calibration report). Leave this dashboard in the lower pcART of the screen; it will be used later on.
5. Go back to menu "*Acquire*"; and select "*acquisition and storage*"; here you can choose how many events (cells) are acquired and saved (standard 100,000 of gate R3, please adjust once and save the template again in the menu "File", then it's okay) all data should be saved. Moreover you select which parameters are measured and stored (Choose parameter p1: FSC, parameter p2: SSC, parameter p3: FL1 (FITC), parameter p4: FL2 (PE), parameter p5: FL3 (PerCP), parameter p7: FL4 (APC).
6. In this menu "*Acquire*", select "*counters*". This dashboard enables you to view the acquisition rate (it should not exceed 3000 events/s) and to check if the total amount of events to be acquired is nearly achieved or achievable in low cellular specimens. So, leave this dashboard somewhere below on the screen.

7. Make a new folder for the patient's data using the "*browser*" menu of the acquisition control (approximately first line in this box: *directory*). The data should be stored xxx… place to be defined by you sometimes this browser is hidden between the templates; then move your template a bit and you will find it on the screen. The folder name should contain the acquisition date and the Patients name (To be designed by you… YYMMDD NAME). If necessary change the file number to 1 in the line below. Type the patient's name in the box that states "*sample ID*". This name will be the file name that is stored in the patient's data folder: NAME.001, NAME.002 and so on.
8. If necessary load the preset antibody panel into the template (small arrow close above were the filenames are visible in the *browser* dash board. Use the right mouse click to access the preset panels. If applicable, a panel can be adjusted in the "*Acquire*" menu: "*Edit panels*". After adjusting your panel lay-out always re-upload your new panel lay-out as described above; only then the adjustments will be visible.
9. Put the tube indicator arrow in front of tube 1 (should be also NAME.001)
10. Check the "set up" mode box in the acquisition control dashboard. Put the first tube of the panel the flow cytometer at low speed and stcART acquisition.
11. Using the detector/amps dashboard the FSC and SSC signal can be optimized in such a way that the lymphocytes will fit in the preset gate "LYMPH". Stop acquisition (Press *Pause*, then *Abort*).
12. End the set up mode by unchecking the box and stcART acquisition at a higher flow rate (medium or high); do not exceed 3000 events/s. Acquisition will automatically stop at 100,000 CD45+ cells. Data acquisition can be manually abrogated by pressing *Pause*, then ***SAVE***. This is the operator's decision (too few cells per tube to reach 100,000 or sufficient events for analysis). Using ABORT here, acquired data will be lost.
13. After measuring tube 1, adjust the gates R1 (deselect debris), R3 (CD45 gate), R4 and R5 (upper row left page). They don't need to be perfect; fine-tuning can be done upon analysis.
14. The tube indicator arrow is now in front of tube 2.
15. Acquire data from tube 2. Again, data acquisition will automatically stop at 100,000 CD45+ cells. After measuring tube 2, adjust gate R7 at the upper row, right page (select $CD19^{+}$ cells).

16. The tube indicator arrow is now in front of tube 3. Acquire data from tube 3 and possibly following tubes.
17. When data of another patient should be acquired, stcART anew at **point 7.** Otherwise proceed to the paragraph ANALYSIS OF DATA.

5.4 Analysis of data

Data can be analyzed using the same template as in *acquisition of the data*. To analyze data in this template all plots are put in the acquisition and analysis mode (can be adjusted using the "*format dot plot/density plot*" feature in the menu "*plots*": the "*inspector*" dash board appears).

Template and data can be transferred to another flow cytometer associated computer that has the Cell QuestPro software installed.

Upon analysis the menus "*plots*" (new plots, change data file, next and previous data file), edit (duplicate plots and select all plots), "gates" (gate definitions and colors for subpopulations) and "stats" (statistics) are frequently used. The "*inspector*" dash board (plots menu) can also be used to select or adjust gates and data files in pcARTicular plots.

- Select all plots (*edit* menu: *select all*)
- Select first data file from a patient via *plots* menu: *change data file*. Find the corresponding file (.001, **tube 1**) and select *open.* Otherwise use the "*inspector*" dash board (plots menu) and go to *file…select file*.
- In this first data file cell are only stained by CD45 APC. Adjust region R1 in the first plot on page 1 upper left to exclude debris (very low FSC and SSC). The LYMPH region (R2) in this plot is exclusively applied upon acquisition not upon analysis.
- Adjust region R3 in the second plot on page 1 (CD45 vs. SSC) to define $CD45^{+}$ cells (exclude remaining nucleated erythroid cells).
- Adjust region R4 in this plot (CD45 vs. SSC) to define $CD45^{bright}$ lymphocytes.
- Adjust region R5 in this plot (CD45 vs. SSC) to define $CD45^{dim}$ progenitors.
- In the multi-color CD45 vs. SSC dot plot below the density CD45 vs. SSC plot with R3, R4 and R5 regions, $CD45^{bright}$ lymphocytes are now displayed in bright green and $CD45^{dim}$ progenitors in purple.
- In this multicolor plot the mononuclear cell fraction (MNC) can be adjusted to exclude neutrophils.

Note: a dot plot (but not a density plot) can display less or more of the acquired events; adjust using the "*inspector*" dash board: 1, 10, 20, 25, 33, fifty, 100% of events

- Background fluorescence of FL1 (FITC), FL2 (PE) and FL3 (PerCP Cy5.5) is visible in the total analyzed cell population (three plots below text *gate R1*). Background fluorescence of the same FL-signals in the mononuclear cell fraction (thus without neutrophils) is depicted at the bottom row at the first page (left side; three plots below text *gate MNC*).
- Adjust quadrants among the purple progenitor population (gate progenitor G9=R1 and R5) in the two plots utmost right (FL1 vs. FL2 and FL3 vs. FL2) to define back ground fluorescence.. Percentages in quadrants UL+UR+LR should not exceed 2%. This is sometimes difficult to achieve (in case of a lot of specific staining sometimes due to less viable cells in the sample); then the background fluorescence will exceed 2%.
- adjust the gate definition of the two plots below the text CD19+ (middle row, right page) to "G2=lymph" using the "*inspector*" dash board. Lymphocyte events will appear.
- Adjust quadrants among these lymphocytes to define back ground fluorescence of the two plots (FL1 vs. FL2 and FL3 vs. FL2) below the text CD19+ (middle row, right page). Percentages in quadrants UL+UR+LR should not exceed 2%.
- adjust the gate definition of these two plots back to "G8= R7 CD19+" using the "*inspector*" dash board. Events from these plots will disappear again.
- Select all plots and select the next data file (**tube 2**)
- Adjust region R7 in the plot CD19 vs. SSC to include all $CD19^{+}$ cells.
- These cells will be automatically back gated for their CD45 expression in the plot to the right (upper row utmost right plot: CD45 vs. SSC). Mature B cells will be $CD45^{bright}$ or in malignant populations somewhat more dimmish; immature B cells will be $CD45^{dim}$ with often low SSC or even $CD45^{negative}$ in some leukemia. If the latter is the case, please adjust R3 and R4 in the CD45 vs. SSC plot top row left page in such a way that they now include the $CD45^{negative}$ cells.
- Now note the expression of CD38 and CD10 on B cells within gate CD19+ (blue colored B cells: a subpopulation of immature cells might be $CD10^{+}$ but some lymphoma can also be $CD10^{+}$) and that of CD38, CD10 and CD19 in the purple progenitor population (20% or more is considered positive for a pcARTicular marker).

CD10 expression can also be seen on neutrophils (plot CD45 vs. CD10, left page).
CD38 expression can be seen on several cells (plot CD45 vs. CD5, left page), not necessarily aberrant
CD19 is sometimes (weakly) expressed on myeloid progenitors, then (in myeloid leukemia) the SSC is often more intermediate than low

- Select all plots and select the next data file (**tube 3**)
- Note the expression of CD5 and CD33 on B cells within gate CD19+ (blue colored B cells: a small subpopulation of mature B cells can be CD5 positive (<10-20% of mature B cells), immature B cells can express CD33 (often weak).
- Also note the expression of CD5 (T), CD33 (Myeloid) and CD19 (B) in the purple progenitor population (20% or more is considered positive for a particular marker).

A normal progenitor population contains $CD33^{+}CD19^{-}$ myeloid and $CD33^{-}CD19^{+}$ B lymphoid progenitors. The presences of immature T cell are always considered aberrant in a bone marrow sample. Immature T cell might be but don't need to be $CD5^{+}$.
CD5 expression can also be seen on mature T cell (check plot CD45 vs. CD5, left page).
CD33 expression can also be expressed dimly on neutrophils (plot CD45 vs. CD33, left page, gate R1) and very bright on monocytes (plot CD45 vs. CD33, left page, gate MNC excluding the neutrophils).

- When applicable, select all plots and select the next data file (**tube 4**). This tube does not contain CD45. It is used to determine clonality of mature B cells: plot kappa vs. lambda in the CD19+ gate.

Immature B cells will be kappa and lambda negative
Normal mature B cells will be either kappa or lambda positive (a subpopulation of both)
Malignant mature B cells will be either kappa or lambda positive (the expression level can differ per patient). Sometimes normal B cells are still present in a low frequency in the background of the clonal population (some kappa, some lambda positive).

- When applicable, select all plots and select the next data file (**tube 5**). This tube contains CD45 and CD71.

Nucleated erythroid cells will be $CD45^{negative}CD71^{bright}$ (plot CD45 vs. CD71 MNC gate, left page bottom row)
Other proliferating or activated cells can be $CD71^{+}$.
When $CD45^{negative}$ cells are not of erythroid origin (CD71+), they can be $CD45^{negative}$ leukemia or metastasis from solid tumor.

- When applicable, select all plots and select the next data file (**tube six**).

Mature T cell will be $CD45^{bright}$, surface $CD3^{+}$ and intracellular $CD3^{+}$ (MNC gate, left page bottom row, plot CD45 vs. CD3-PerCP-Cy5.5 and plot CD45 vs. cytCD3-FITC).
Part of these mature cells will express CD4 (T-helper cells).
Immature T cell will be $CD45^{dim}$ or $CD45^{negative}$, surface $CD3^{negative}$ and intracellular $CD3^{+}$, some may express CD4.