PROFILES OF PROGESTERONE, SYSTEMIC CYTOKINES AND BLOOD LYMPHOCYTE LEVELS DURING PREGNANCY IN WOMEN LIVING WITH HUMAN IMMUNODEFICIENCY VIRUS INFECTION IN WESTERN KENYA

By

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A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCES AND TECHNOLOGY (MEDICAL IMMUNOLOGY)

SCHOOL OF PUBLIC HEALTH AND COMMUNITY DEVELOPMENT

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DECLARATION

I, Stanslaus Kiilu Musyoki, declare that the work presented herein is my original work and has not been presented for the award of any degree in any other university or institution.

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ACKNOWLEDGEMENTS

I acknowledge my research mentors, Prof. Collins Ouma, Prof. Simeon Mining and Dr. Kiprotich Chelimo, who provided generous support in the form of time and guidance for this research. The Academic Model Providing Accessible Treatment and Healthcare (AMPATH) and Moi Teaching and Referral Hospital (M.T.R.H) as institutions for allowing me to use their facilities. Not forgetting the immense effort by Dr. Wilfred Emonyi, who ensured that all challenges during laboratory analysis, including procurement of reagents, was sorted out. I also wish to appreciate the study participants who made it possible to achieve the research objectives by their cooperation and providing their samples for analysis. Finally, my appreciation goes to all those who could have contributed indirectly or directly to the success of this research but not mentioned.

DEDICATION

I dedicate this work to my entire family members for their continual support and encouragement.

ABSTRACT

The impact of HIV infection on progesterone, systemic cytokines, and lymphocyte levels in advancing pregnancy remains unclear despite the reported abortions among HIV-infected women. Thus, the main objective of this study was to determine and compare the profiles of progesterone, systemic cytokines and blood lymphocyte counts in advancing pregnancies of HIV-infected and HIV-non-infected women population. In a longitudinal cohort study, 44 HIV-infected and 44 HIV-non-infected pregnant women were consecutively recruited at Academic Model Providing Accessible Treatment and Healthcare (AMPATH) and Moi Teaching and Referral Hospital (M.T.R.H) in Western Kenya. Progesterone was analyzed using Enzyme Linked Immuno-Sorbent Assay (ELISA) while flow cytometry method was used to analyze cytokines and lymphocytes. Categorical variables were analyzed using Pearson's Chi-Square test. The changes and differences in the outcomes were assessed using repeated measures analysis of variance and Wilcoxon two-sample tests, respectively. The mean change in progesterone during the second, and the third trimesters were significant (P<0.0001) compared to that of the first trimester in both groups. Among the HIV-positive women, significant mean change in IL-2 [in the second trimester (P=0.036) compared to first; in third trimester (P=0.003) compared to second trimester]; IL-6 [in the second trimester (P=0.029) compared to first trimester] and IL-4 [in the third trimester, (P=0.022) compared to second trimester] were different from what was observed among the HIV-negative women. The mean change in IFN-γ, TNF, and IL-10 were similar when compared between the two groups. Among the HIV-positive women, significant changes were observed in $CD8^+$ [in the third trimester compared to the first (P=0.005) and second (P=0.007) compared to the first trimester]. Similar mean changes in $CD3^+$, $CD4^+$, $CD19^+$, and $CD56/16^{+,}$ was observed when compared between the two groups. In conclusion, the present study demonstrates that, HIV infection attributes to changes in progesterone, cytokines and lymphocytes as pregnancy advances. The present study recommends that, alongside $CD4^+$ and $CD8^+$ count progesterone, T_H1/T_H2 cytokines, $CD19^+$ and $CD56/16^+$ should be monitored during pregnancy among the HIV-infected women. The present findings are significant in that they provide a baseline for possible use of cytokines in supplementation therapy. Future research should focus and explore on the succinct roles of the endocrine response in regard to other reproductive hormones, other cytokines not considered in the present study and the use of animal model to understand dynamics of lymphocyte counts without anti-retroviral treatment during pregnancy in HIV-infected women.

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ABBREVIATION AND ACRONYMS

The following abbreviations and acronyms have been used:

AIDS- Acquired Immunodeficiency Syndrome

AMPATH-Academic Model Providing Access to Healthcare

ANC-Ante-Natal Clinics

APC- Allophycocyanin

ART-Antiretroviral Therapy

ARV- Anti-retrovirus

BD-Becton – Dickinson

CBA-Cytometric Bead Array

CD-Cluster of Differentiation

CI- Confidence Interval

DR- Doctor

EDTA-Ethylenediamine Tetra Acetic Acid

EFV- efavirenz

ERC- Ethical Review Committee

FACS -Fluorescent Activated Cell Sorting

FITC- Fluorescein Isothiocyanate

HAART -Highly Active Antiretroviral Therapy

HIV-Human Immunodeficiency Virus

H_o -Null hypothesis

IFN-Interferon

IL-Interleukin

IQR- Interquartile Range

MCH-Maternal and Child Health Care

ML-Micro Litre

MM^3- Millimetres cubed

MTRH-Moi Teaching and Referral Hospital

N/A-Not Applicable

NG- Nano-Grams

NK-Natural Killer Cell

PE-Phycoerythrin

PerCP- Peridinin Chlorophyll Protein

SEM- Standard Error of the Mean

TDF- Tenofovir DF

T_H1-T helper type 1

T_H2-T helper type 2

TNF-Tumor Necrosis Factor

TM- Trade Mark

µL-Micro liter

USA- United States of America

DEFINITION OF OPERATIONAL TERMS

AMPATH: Academic Model for Providing access to Healthcare (AMPATH) is the largest HIV and AIDS treatment and care Programme in Kenya located in Western Kenya.

Immune system: This is a network that secures the host from both internal threats (such as cell transformation i.e. malignancy) and external (such as fungi bacteria and viruses).

$CD3^+$: This is a marker for all T-lymphocytes. The present study evaluated $CD3^+$ to show how the total T cell population is affected by HIV infection during pregnancy.

$CD4^+$: These are lymphocytes that signal other cells by producing cytokines to mount an immune response. They are also called T helper cells, and they are depleted in HIV-infected persons.

$CD8^+$: These are lymphocytes that destroy infected cells by intracellular pathogens, e.g., HIV.

$CD19^+$: This is a marker for the B- lymphocytes. The present study evaluated $CD19^+$ to designate humoral immune response.

$CD56/16^+$: These are markers which designate Natural Killer cell.

Change in Variables: This is how the outcomes become different either positively or negatively as pregnancy advanced.

Compensation: This is an experimental setup for multicolor assays in flow cytometry for complex data to ensure proper visualization and analysis.

Differences in Variables: This is how the measured outcomes are dissimilar between the two groups i.e. higher or lower.

Human Immunodeficiency Virus (HIV): This is a retrovirus which is transmissible and would induce suppression of the immune system resulting to AIDS.

Immune Deficiency: This is the incapacitation of components immune system resulting in susceptibility to diseases that otherwise will not happen with an intact immune system. Otherwise, the persons are Immuno-compromised.

Enzyme-Linked Immunosorbent Assay (ELISA): This is a test that detects antibodies which indicate that the individual is infected by a particular pathogen, e.g., HIV.

Flow Cytometry: This is a technique that uses the principle of sorting and counting different types of cells. Fluorescent markers label the cells.

Highly Active Antiretroviral Therapy (HAART): It is the treatment administered against HIV, which may include a combination of drugs to reduce the virus load.

Progesterone: This is a reproductive hormone that maintains pregnancy. The present study measured progesterone since it has an influence on systemic cytokines and lymphocytes.

Systemic cytokines: Systemic cytokines are proteins that are important in communication in the immune system. They may be involved in mounting or immune-regulating the immune response. The present study analyzed serum T_H1 (IL-2, TNF, and IFN-γ) and T_H2 (IL-4, IL-5, IL-10) cytokines. Total TNF was analyzed but not the variants of TNF.

Trends of Variables: The general direction in which the outcomes would occur, e.g., increasing or decreasing as pregnancy advanced

Western Kenya: The present study defines western Kenya as the region that includes the former Nyanza, Western, and Rift Valley provinces. These areas form the referral area for MTRH/AMPATH the study site.

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CHAPTER ONE

INTRODUCTION

1.1: Background

Although the proportion of HIV-infected population in Kenya has reduced, women remain the most infected in the general population with reports that, about 7 and 4 % of women and men respectively being infected with HIV in 2012 (Waruiru *et al*., 2014). Complicated pregnancies and abortions have also been reported among women with low progesterone levels (Bosch *et al*., 2010) and the HIV-infected pregnant women (Ticconi *et al*., 2003). Pregnancy and Human Immunodeficiency Virus (HIV) prompt varied immune responses in the human immune system (Sauce, Elbim, & Appay, 2013).

During pregnancy, progesterone, systemic cytokines and lymphocyte responses have a vital role in the success or failure of gestation (Aagaard-Tillery, Silver, & Dalton, 2006; Tuckey, 2005). During pregnancy and HIV infections the immune system is altered to create tolerance towards paternal antigens on the cells of the fetus and to destroy HIV respectively. The changes during pregnancy are induced by hormones produced to sustain a pregnancy, whose blood levels increase as pregnancy advances. These hormones include; Human chorionic gonadotropin hormone, Human placental lactogen, estrogen, and progesterone. Among these hormones, progesterone is the key hormone that maintains pregnancy and has a significant impact on immune response (Zen *et al*., 2010). The increase in progesterone levels during pregnancy is thought to be partly responsible for an immunosuppression seen during pregnancy (Druckmann & Druckmann, 2005). It is, therefore, important in maintaining and enhancing the success of fetus survival during a healthy pregnancy by suppressing the immunity mediated by T-cells (Piccinni, Scaletti, Maggi, & Romagnani, 2000).

Progesterone also plays a significant role in determining the pattern of cytokines produced by the T-cells and how they are distributed during pregnancy (Piccinni *et al*., 2000) and has been noted. Progesterone has effects on thymus involution, can block development of T-cells, and also suppress T_H1 cell proliferation promoting T_H2 cell proliferation and development (Pantaleo *et al*., 2013). Progesterone thus remains the most crucial reproductive hormone during pregnancy. The impact of HIV on the profiles of blood progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women is unknown. As such, the present study determined and compared the profiles of blood progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in, Western Kenya.

Cytokines are secreted proteins which are involved in growth, differentiation, and activation to regulate and determine whether the immune response is allergic, antibody or cell-mediated (Borish & Steinke, 2003). During pregnancy, a shift towards T_H2 cytokines is enhanced with the suppression of T_H1 response. The T_H1 type of cytokines includes interleukin-2 (IL-2), gamma interferon, IL-12 and tumor necrosis factor, while the T_H2 type of cytokines includes IL-4, IL-5, IL-6, IL-10, and IL-13 (Spellberg & Edwards, 2001). Among these T_H1 and T_H2 type of Cytokines, pregnancy, and HIV infections independently have been shown to affect mainly INF-γ, TNF, IL-6, IL-10, IL-4 and thus the choice of these cytokines in the present study. A successful gravid is characterized by an increased levels of T_H2 (anti-inflammatory) cytokines mainly interleukin-10 (IL-10), IL-4 and IL-6 and inhibition of T_H1 (pro- inflammatory) cytokine mainly interferon-gamma (IFN-γ), IL-2 and Tumor Necrosis Factor (TNF) (Kidd, 2003; Piccinni *et al*., 2000). The anti-inflammatory cytokines are associated with humoral immune response, which relies on B-cells and the use of antibodies while pro-inflammatory responses are associated with T-cell-mediated immune response in which Cytotoxic T-cells ($CD8^+$) are increased to fight infections such as HIV (Galli *et al*., 2001; Kidd,

2003). Immune response in early HIV infections, on the contrary, is characterized by increased levels of inflammatory cytokines, which activate a cell-mediated immune response to kill the virally infected cells (Nixon & Landay, 2010). Thus, systemic cytokines paradigm is relevant to both pregnancy and HIV infections. However, the delicate immunologic balance of systemic cytokines in co-joint cases of HIV infection and pregnancy and the impact of HIV on these cytokines remain unknown. The present study thus determined and compared the profiles of systemic cytokines (IL-2, IFN-γ, TNF, IL-10, IL-6, and IL-4) levels in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in, Western Kenya.

Lymphocytes [T ($CD3^+$) cell subsets ($CD4^+$ & $CD8^+$), B ($CD19^+$), and natural killer ($CD56/CD16^+$) cells] are white blood cells which vary in function and determine the type of the immune response and effector mechanisms of an immune response (Caligiuri, 2008; Moore, Moore, & Toews, 2001). Regarding lymphocyte immune response during pregnancy, T-cell mediated immunity is altered such that T_H2 responses that enhance B-cell (humoral) immune response is predominant (Zen *et al*., 2010). Natural Killer cell activity is also suppressed during pregnancy (Tang, Alfirevic, & Quenby, 2011). Pregnancy and HIV affect all the lymphocytes, and thus the current study chose $CD3^+$, $CD4^+$, $CD4^+$, $CD19^+$ and $CD56/16^+$ markers to represent all categories of lymphocytes. T-cell subsets ($CD4^+$ and $CD8^+$) during HIV infections show a marked reduction of $CD4^+$ T-lymphocytes mainly as a result of the cellular destruction by the high turnover of specific activated $CD8^+$ cytotoxic T-cells (Sousa, Carneiro, Meier-Schellersheim, Grossman, & Victorino, 2002). T-helper ($CD4^+$) cell count may be used in concert with $CD8^+$ cell count to enhance the accuracy with which HIV infections can be monitored. HIV infection and pregnancy are two conditions, which may result to perturbation in the count of lymphocytes especially T-lymphocytes (Druckmann & Druckmann, 2005; Sauce *et al*., 2013). However, inconsistent results have been reported concerning lymphocyte counts during

pregnancy and HIV infections (Temmerman *et al*., 1995; Sauce *et al*., 2013). Therefore, the dynamics of lymphocytes in pregnancy and how they are affected by HIV infection are not clear. As such, the present study determined and compared the profiles of lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD56/16^+$) blood counts in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in, Western Kenya.

Progesterone-Cytokine-T-cell network during pregnancies thus exist (Arck, Hansen, Jericevic, Piccinni, & Szekeres-Bartho, 2007; Druckmann & Druckmann, 2005). Progesterone plays a major role in determining the lymphocytes and cytokines pattern and their distribution during pregnancy. A shift in maternal immunological repertoire in cases of HIV infection may disturb the power of the network, and this forms the basis of the present study. Despite the reported cases of complications and abortions in previous studies among the HIV-infected pregnant women, the change in progesterone, systemic cytokines and lymphocyte that can be attributed to HIV infections during pregnancy remains unknown. The present study was therefore designed to evaluate the impact of HIV infection on immune events that could explain the success or possible failure of pregnancy.

1.2: Statement of the problem

Although the proportion of HIV-infected population in Kenya has reduced, women remain the most infected in the general population with reports that, about 7 and 4 % of women and men respectively being infected with HIV in 2012. Complicated pregnancies and abortions have also been reported among women with low progesterone levels and the HIV-infected pregnant women. Despite these, remarkably little is known about the change in progesterone and immune events attributable to HIV infection during pregnancy. It, therefore, remains unknown if HIV infection contributes to low progesterone and if it affects systemic cytokines and lymphocytes during pregnancy. Thus the

present study aimed to determine and compare the profiles of progesterone, T_H1/T_H2 systemic cytokines and blood lymphocytes in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in Western Kenya.

1.3: Study objectives

1.3.1: General objective

To describe and analyze the profiles of progesterone, systemic cytokines and lymphocytes blood levels in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in Western Kenya.

1.3.2: Specific objectives

1. To determine and compare the profiles of progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in Western Kenya.

2. To establish the profiles of T_H1 (IL-2, IFN-γ, TNF) and T_H2 (IL-10, IL-6, IL-4) type of systemic cytokines levels in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in Western Kenya.

3. To analyze the profiles of blood lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD56/16^+$) counts in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in Western Kenya.

1.3.3: Research hypotheses

1. **H_0:** There is no difference in the profiles of progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected among women attending AMPATH/MTRH Centre in Western Kenya.

2. **H_0:** There is no difference in the profiles of T_H1(IL-2, IFN-γ, TNF) and T_H2 (IL-10, IL-6, IL-4) type of systemic cytokines levels in advancing pregnancy of HIV-infected and HIV-non-infected among women attending AMPATH/MTRH in Western Kenya.

3. **H_0:** There is no difference in the profiles of blood lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD56/16^+$) blood counts in advancing pregnancy of HIV-infected and HIV-non-infected among women attending AMPATH/MTRH Centre in Western Kenya.

1.4: Significance of the study

Based on the present study findings, the study indicates that alongside $CD4^+$ and $CD8^+$ counts, progesterone, T_H1 and T_H2 systemic cytokines and $CD19^+$ counts should be included in the routine monitoring of HIV-infected pregnant women during pregnancy. Altogether these immune parameters would guide the clinicians in the clinical care of HIV-infected pregnant women. These findings also provide baseline information for future interventions and studies for possible use of cytokines in supplementation therapy as a means of enhancing immune responses in HIV-infected pregnant women.

CHAPTER TWO

LITERATURE REVIEW

2.1: Immune responses in pregnancy and HIV infections

Previous studies have suggested a progesterone-cytokine-T cell network during pregnancy (Arck *et al*., 2007; Druckmann & Druckmann, 2005). Progesterone promotes T_H2 cell development hence increasing the production of anti-inflammatory cytokines (Pantaleo *et al*., 2013).

The fetal-placental unit produces T_H2 cytokines to avoid damaging T_H1 mediated immune responses (Bowen, Chamley, Keelan, & Mitchell, 2002). Thus, the type of cytokine produced during pregnancy determines the success or failure of gestation. Cytokines produced during pregnancy favors antibody production (mediated by T_H2 cytokines) over T-cell responses (mediated by T_H1 cytokines) (Conde-Agudelo, Villar, & Lindheimer, 2008). Regarding this, it remains a fact that cell-mediated responses are suppressed during pregnancy, as an effect of suppressive mechanisms of cytokines produced by regulatory T-cells that favor the survival of the fetus in successful pregnancies (Østensen, Förger, & Villiger, 2006; Sasaki *et al*., 2004). Furthermore, it has been shown that failure to regulate cytokine production during pregnancy has been associated with abortions mediated by maternal NK and T_H1 cells (Wilczyński, 2006).

During the asymptomatic period of HIV infection, the levels of T_H1 cytokines remain high (Galli *et al*., 2001) and patients have no clinical problems except for possible lymphadenopathy. In HIV infections, T-cells are responsive to the negative regulation by Tregs both spontaneously and upon stimulation (Terzieva *et al*., 2009). Interleukin-2 is a cytokine majorly secreted by $CD4^+$ T-cells, and it is dominant during HIV infections. Interleukin-2 has also been known to induce the production of

INF-γ, IL-4, and TNF (Sutton *et al*., 2004). It induces the activation and proliferation of T and B, as well as enhancement of the activity of the natural killer cell. A previous study has indicated that IL-6 has been associated with HIV disease progression (Shive *et al*., 2012). In this scenario, a shift in the maternal immunological repertoire towards T_H1 cytokines in HIV infections may disturb the balance of the progesterone-cytokine-T cell network, which may cause complications and abortions during pregnancy.

Regarding the effect of ARV on the profiles measured, a previous study indicates that progesterone levels have been reported to be comparable in HIV patients on antiretroviral drugs and those not yet on drugs (Cu-Uvin *et al*., 2000; Ogundahunsi *et al*., 2011). In another study, it was observed that $CD4^+$ counts are affected by use of simplified once-daily regimen ARV, the therapy given to the HIV-infected pregnant women, but only after 12 months (Palacios *et al*., 2009). The present study was however carried out within the nine months of pregnancy, a time before the effects of ARVs could be established. There are no other studies that indicate the effect of ARV on systemic cytokines, $CD8^+$, $CD19^+$, $CD56/16^+$, the other variables measured in the present study. Therefore the use of ARV by the HIV-infected pregnant women is a limitation of the present study since it has been reported to improve the health of HIV-infected persons.

It is known that progesterone, 1L-2, IFN-γ, TNF, IL-6, IL-10, IL-4, T-cells ($CD4^+$, $CD8^+$), B-cells ($CD19^+$) and NK ($CD56/16^+$) cells have a paramount role and immune feedback in both pregnancy and HIV infection independently (Aagaard-Tillery, Silver, & Dalton, 2006; Tuckey, 2005). However, the impact of HIV infections on these key immune parameters as pregnancy advances remained unknown despite the reported cases of abortion among the HIV–infected pregnant women. Most of the previous studies did not consider the HIV-infected women and none of them investigated T_H1 and T_H2 (IL-2, INF-γ, TNF, IL-6, IL-10, and IL-4), T-cells ($CD4^+$, $CD8^+$), B-cells ($CD19^+$) and NK

(CD56/16^{+}) cells, all-inclusive. Therefore, the current study determined and compared profiles of progesterone, T_H1 (1L-2, IFN-γ, TNF) and T_H2 (IL-6, IL-10, IL-4), and lymphocytes [T (CD3^{+}) cells, T cell subsets (CD4^{+}, CD8^{+}), B (CD19^{+}) cells and NK (CD56/16^{+}) cells] between HIV-infected and non-infected women attending AMPATH Centre in Eldoret, Western Kenya.

2.2: Progesterone levels in pregnancy and HIV infections

During pregnancy, progesterone is increasingly produced and as a result several changes occur in the immune system of a pregnant woman to secure the fetus and at the same time maintain a maternal defense against pathogens (Arck *et al*., 2007; Druckmann & Druckmann, 2005). After conception, the *Corpus leuteum* produces and maintains high levels of progesterone in the first 12 weeks of pregnancy to sustain a pregnancy through the first trimester (Beltman, Lonergan, Diskin, Roche, & Crowe, 2009). Any significant drop in progesterone levels during the first weeks of pregnancy may result in miscarriage (Bosch *et al*., 2010). At around 12 weeks of healthy pregnancy, the placenta begins to produce progesterone in place of the *Corpus leuteum* (Tuckey, 2005). During pregnancy ,the placenta continues to produce progesterone, and the levels rise to just before delivery (Tuckey, 2005).

Approximately 70% decrease in progesterone production was noted in HIV-1-infected cell cultures compared to controls in a study which investigated HIV infection of human placenta (Amirhessami-Aghili & Spector, 1991). However, this study did not determine blood progesterone levels in advancing pregnancy, used in the routine monitoring of progesterone. Complicated pregnancies and abortions have also been reported among women with low progesterone levels (Bosch *et al*., 2010). The studies reviewed did not consider doing a follow-up on the study participants and were unable to show if HIV infection could affect the production of progesterone during pregnancy. It, therefore,

remained unknown if co-joint cases of pregnancy and HIV infections would present with low progesterone levels during pregnancy compared to HIV-non-infection and pregnancy. As such, the current study determined and compared the profiles of progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in Western Kenya.

2.3: Systemic cytokines in pregnancy and HIV infections

Pregnancy is known to contribute to the systemic change in cytokines toward T_H2 dominance (Orsi & Tribe, 2008). Some cytokines produced in pregnancy are beneficial for the foetal growth: IL-4 and IL-10, where others seem deleterious: IL-2, IFN-γ, and TNF (Pantaleo *et al*., 2013; Piccinni *et al*., 2000; Roncarolo *et al*., 2006). Interleukin-4 and IL-10 suppress T_H1 responses (Sasaki *et al*., 2004). The success of pregnancy seems to be linked with the secretion of IL-10 and IL-4, and both cytokines inhibit the production and activity of T_H1 cells (Saito, Nakashima, Shima, & Ito, 2010). In previous study levels of IL-10 were shown to be low during early pregnancy and markedly increased in last days of pregnancy and post-partum (Bento *et al*., 2009). In this study, IL-10 and IL-4 were also shown to promote progesterone production in healthy pregnancies. In a different study IL-6 and TNF have been observed to be increased in cases of spontaneous abortions (Udenze, Amadi, Awolola, & Makwe, 2015). Pregnancy is also associated with low levels of IFN-γ (Laresgoiti-Servitje, Gómez-lópez, & Olson, 2010). Despite this knowledge on the critical role of IL-10, IL-4, IL-6 and TNF and how they are affected by pregnancy, no study has shown how HIV infection could impact these cytokines during pregnancy. It, therefore, remained unknown if HIV infection is attributed to increasing or decrease of IL-10, IL-4, IL-6 and TNF as pregnancy advanced.

Change in the T_H1 and T_H2 cytokines production during HIV infection may affect the prognosis of HIV disease (Sukwit *et al*., 2001). Predominance in high T_H1 responses in early HIV infection is

responsible for effective control of the virus (Johnston & Fauci, 2007). During the advanced stages of HIV infection, production of T_H1 cytokines is decreased whereas secretion of T_H2 cytokines is increased (Sterling *et al*., 2001). This change in cytokines contributes to the pathogenesis of the disease leading to AIDS.

In HIV-infection, pro-inflammatory, and regulatory cytokines increase with higher T-regulatory cells, indicating that inflammation and immunoregulation have a common pathophysiologic origin in HIV infections (Richardson & Weinberg, 2011). Regarding HIV-infected women during pregnancy, higher levels of pro-inflammatory cytokines were noted in late pregnancy which may have contributed to an increased chance of maternal-fetal death (Richardson & Weinberg, 2011). A previous study observed that there is little difference in IL-2 between the HIV-infected patients and normal healthy volunteers (Alonso, Resino, Bellón, & Muñoz-Fernández, 2000). However, a different study on pregnant women showed no significant difference in levels of the production of intracellular IL-2 between HIV-infected and HIV-non-infected expectant mothers (Sutton *et al*., 2004). However, these studies did not do a follow-up on pregnant women to understand the longitudinal changes that occur across trimesters of pregnancy.

Furthermore, other studies indicate that both pregnancy and HIV infection are biased towards a T_H2-type response by T-cells (Alonso *et al*., 2000). Another study has also reported that HIV-infected pregnant women have a significantly increased risk of complicated pregnancy and abortions (Ticconi *et al*., 2003). Despite the reported complications and abortions among the HIV-infected pregnant women, the impact of HIV infection on systemic cytokines as pregnancy advances remains unknown. Cytokines could be a cause of abortion and could also be used as a supplementation therapy as a means of enhancing immune responses in HIV-infected pregnant women, and thus it was critical to determine how they are affected by HIV infection during pregnancy. The highlighted

studies did not measure and compare the profiles of systemic cytokines in advancing pregnancy of HIV-infected and non-infected women to understand their dynamics clearly. Hence, the current study established the profiles of T_H1 (IL-2, IFN-γ, TNF) and T_H2 (IL-10, IL-6, IL-4) type of systemic cytokines levels in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in Western Kenya.

2.4: Lymphocytes in pregnancy and HIV infections

Varied results have been observed regarding the lymphocyte counts during pregnancy and HIV infections. Some studies have reported that there is no alteration in the $CD4^+$ and $CD8^+$ T-cell levels during pregnancy (Van Benthem *et al*., 2002; Tuomala *et al*., 1997; Burns *et al*., 1996), while others have clearly shown that there are change in $CD4^+$ and $CD8^+$ T counts during pregnancy (Chama, Morrupa, Abja, & Kayode, 2009; Ono *et al*., 2008). In regards to HIV infections, the marked depletion of $CD4^+$ T-lymphocytes has been shown to result from the cellular destruction by specific activated $CD8^+$ cytotoxic T-cells (Sousa *et al*., 2002). The decline rate of $CD4^+$ T-cell count in HIV infection can vary such that some patients may have a quick depletion of $CD4^+$ counts for some months while others may experience relatively stable $CD4^+$ counts for years (Lindgren *et al*., 1996).

Among the HIV-infected pregnant women, a previous study observed that $CD4^+$ counts decrease steadily during pregnancy and after birth among HIV-positive women (Burns *et al*., 1996). In this study, the percent $CD8^+$ counts increased at or near childbirth and reduced to the first trimester after delivery in both HIV-negative and HIV-positive women. Another study has also shown HIV-infected pregnant women to have low $CD4^+$ and elevated $CD8^+$ compared to HIV-negative pregnant but remained stable throughout pregnancy and after delivery in both groups of women (Mikyas *et al*., 1997). Other observational studies on HIV-infected women and men, found that women who became

pregnant had better health in comparison to those who did not become pregnant (Sterling *et al*., 2001). Although the pregnant women in the study were younger and had higher initial $CD4^+$ counts than the non-pregnant women, their health remained better even after pregnancy. Additionally, women with repeated pregnancies during the study tended to have better immune status than did women with only one pregnancy. A study in Kenya did not support an additive effect of HIV and pregnancy on the immune response as determined by $CD4^+$ and $CD8^+$ counts (Temmerman *et al*., 1995). The percentage of $CD4^+$ T-cells was lower after delivery than in pregnancy, in both HIV-positive and negative women; however this was different for $CD8^+$ counts. Absolute $CD8^+$ Counts and percentages were observed to be significantly higher after delivery than in pregnancy.

In B-cells, $CD19^+$ has been indicated to be crucial for B-cell activation by the T-cell-dependent mechanisms of antigens and also the maturation of the activated cells or their selection into becoming B-memory-cells (Rickert, Rajewsky, & Roes, 1995) and therefore an important marker for B-cell and humoral immune responses (Krop, Shaffer, Fearon, & Schlissel, 1996) . B-cells and the antibodies they produce have been evaluated and shown to have a crucial role in pregnancy success (Muzzio, Zenclussen, & Jensen, 2013). Regulatory B-cells, which is a subset of B-cells having powerful tolerance functions, expand during pregnancy (Muzzio, Zygmunt, & Jensen, 2014). However, another study showed almost no change in B-cells during pregnancy (Kühnert, Strohmeier, Stegmüller, & Halberstadt, 1998).

Natural Killer cells ($CD56/16^+$) cells play an important immune role in HIV disease and pregnancy (McKenna, 2012). Natural Killer cells which predominate in blood circulation have lower levels of cytokine production, but with an increased cytotoxicity (Moretta, Marcenaro, Parolini, Ferlazzo, & Moretta, 2008) thus their suppression partly maintain the pregnancy to term. A decrease in absolute counts of $CD56/16^+$ in the third trimester of gestation have also been shown (Mahmoud *et al*., 2001).

Natural Killer cell cytotoxicity has also been observed to decrease in the decidua of normal early pregnancies (Ho, Chao, Chen, Yang, & Huang, 1996) with low NK cell numbers (Ono *et al*., 2008). Lower blood NK cell percentages have been shown to occur in women without recurrent miscarriages than in women with recurrent miscarriages (Seshadri & Sunkara, 2014).

Although research on lymphocytes in HIV infection and pregnancy has become more pronounced as per the highlighted literature, no study has measured all lymphocytes inclusively. The majority of the studies compared HIV-infected pregnant women to HIV-infected non-pregnant women and never considered the HIV-non-infected pregnant women in advancing pregnancy. A comparison of lymphocyte counts between HIV-infected pregnant and HIV-non-infected pregnant give a better understanding of the impact of HIV on lymphocyte counts as the pregnancy progressed. Despite the reported complications and abortions among the HIV-infected pregnant women, the highlighted studies did not clearly indicate which arm of immunity dominates among the HIV-infected women since they did not consider all the lymphocytes in the studies. It, therefore, remained unclear how HIV infection would affect total lymphocytes counts as pregnancy advances considering all arms of the immune response in a single study. Thus, the current study determined, compared and reported the profiles of blood lymphocytes ($CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, and $CD56/16^+$) counts in advancing pregnancy of HIV-infected and HIV-non-infected women attending AMPATH/MTRH Centre in Western Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1: The study site

The study participants were recruited at the Academic Model Providing Accessible Treatment, and Health (AMPATH) and the Moi Teaching and Referral Hospital (MTRH) situated in Eldoret town. Moi Teaching and Referral Hospital is the second largest hospital in Kenya and serves referral patients from Western Kenya. Academic Model Providing Accessible Treatment and Healthcare is under MTRH and is the largest HIV and AIDS treatment and care programme in Kenya. At AMPATH about 130,000 HIV-infected persons have so far been enrolled into care; running services in 19 sub-counties of Western Kenya (Appendix I) (http://kenya.usaid.gov/Programmes/health/83).

At AMPATH clinics, HIV-positive pregnant women are enrolled. The $CD4^+$ and $CD8^+$ T counts are routinely determined at baseline and repeated every six months for all patients regardless of ARV use. Follow-up visits are then arranged, and data is collected on intercurrent symptoms. Patients who do not meet criteria for initiation of ARV are seen at every three months to reassess their clinical status; patients who meet criteria are provided with ARV and then followed monthly for the rest of their life. To prevent mother to child transmission of HIV, part of their clinical care, all HIV-infected pregnant women with $CD4^+$ cell count equal or greater than 500 cells/μL are started on a simplified once-daily regimen of tenofovir DF (TDF), lamivudine (3TC), and efavirenz (EFV) (EFV600mg+3TC300mg+TDF300mg).

Laboratory analysis was performed at the AMPATH Reference Laboratory, which is fully equipped to perform diagnostics in support of HIV care and management. The facilities were adequate to perform sample analysis for the present study.

3.2: The study design

The present study employed a longitudinal and laboratory-based cohort study design. In this study, data on the profiles of progesterone, systemic cytokines and lymphocytes levels in advancing pregnancy of HIV-infected women were measured and compared to HIV-non-infected women.

3.2.1: The study population

The study evaluated adult asymptomatic HIV-infected pregnant women, who are free from acute untreated infection, and antiretroviral-naïve at the beginning of the study. As controls, the study also included healthy HIV-non-infected pregnant women.

3.2.2: Sample size determination

A powered sample size to answer objectives of the study was determined under some assumptions. The study assumed that the profiles of progesterone, cytokines, and lymphocytes in the two groups are consistent across time. The study further assumed that the two groups had the same variance. That is, the sum of the between-subject variance and the within-subject variance were the same for the two groups and correlation of the repeated measures was thus taken as 0.5. It was assumed that the sample size that would detect a difference in the study variables between the two groups would also answer the question of whether the two groups are the same or different. The sample size was 90% powered and determined under the assumption that the standard deviation was 100 units, and the difference in the change in the variables between the two groups was 50 units leading to the

medium effect size of 0.5 (Cohen, 1988). Under these assumptions, the sample for the study was determined using the formula given below (Hedeker, Gibbons, & Waternaux, 1999).

$$n = \frac{2 \times \left(Z_{1-\alpha/2} + Z_{\beta} \right)^2 \left(1 + (r-1)\rho\right)}{r \times \delta^2}$$

Where: $\delta = \left(\frac{\mu_N - \mu_P}{\sigma} \right)$ is the effect size, μ_N and μ_P are the assumed mean change in the study variables for the HIV-positive pregnant women and the HIV-negative pregnant women respectively, σ is the standard deviation, r is the number of time points, ρ is the assumed correlation between the repeated measures, $Z_{1-\alpha/2}$ and Z_{β} are the $(1-\alpha/2)\times 100\%$ and the $(1-\beta)\times 100\%$ percentiles of the standard normal distribution under the type I and type II errors respectively. Type I error was taken to be 5%.

When the sample size formula was substituted it was as follows:-

$$n = \frac{2*[1.96 + 1.282]^2\,[1 + (3-1)0.5]}{3*(0.5)^2}$$

After adjusting for an attrition rate of five percent, 44 participants per group was reached. Thus the participants required for the study was 88.

3.2.2.1: Inclusion criteria

i. Asymptomatic HIV-infected pregnant women for the study group and healthy HIV-non-infected pregnant women as controls.

ii. $CD4^+$ cell count greater than or equal to 500 cells/μL.

iii. Aged equal or above 18 years.

3.2.2.2: Exclusion criteria

i. Women with any complications in pregnancy (previous or present).
ii. Active infection other than HIV as defined by fever ≥38°C at the time of recruitment or receipt of acute therapy for infection within 14 days before the visit.
iii. Patients with symptoms or medical report of other infections e.g. tumors, *Diabetic Mellitus*, pneumonia, influenza, herpes, autoimmunity or evidence of acute infection and untreated medical illness.
iv. Participants who frequently abuse drugs, e.g., smoking, alcohol, etc.

3.3: The Study procedures

3.3.1: Pilot study

A pilot study was conducted at the study site before committing to the main study to evaluate the feasibility, equipment and methods of the study. The participants used in the pre-test were not included in the main study. The sample size for the pilot survey was 10% (9) of 88 sample size of the study (Connelly, 2008). Since pilot trials have small sample sizes, statistical significance focus on estimation, and confidence intervals other than 95% confidence intervals, such as 85% - 75%, is best used for the estimation. Statistical significance for the pilot study was determined using Bayesian inference methods where a confidence interval of 80% was utilized for the estimation (Gelman *et al*., 2013). The result gave a Confidence interval = ±4.09%. The confidence interval is set to be between 3 and 5 %.

3.3.2: Validity and reliability of research instruments

Data collection included the face-to-face interview to capture the demographics and laboratory blood analysis for progesterone, cytokines and lymphocyte counts. Qualified and trained research assistants collected data to ensure accuracy in sample collection, analysis, and reporting. Validity and reliability of laboratory-based research depend on the quality assurance and control of the laboratory analysis procedures and equipment. The validity of the results was thus ensured by both external quality control and internal quality control systems for the sample analysis and instruments used in this study. Manufacturer's instructions were also followed. Tests re-test technique, and the use of authenticated standards and controls were used to ensure the accuracy of the results.

3.3.3: Recruitment of study participants and sample collection

In a prospective cohort study between June 2013 and June 2014, 44 adult asymptomatic HIV-infected pregnant women were recruited from the HIV care outpatient clinics at AMPATH Centre. Forty-four healthy HIV-non-infected pregnant women matched by age, parity, gestational age and marital status were also recruited from MTRH-Mother and Child Health clinics (MCH) as controls. All participants in the study were antiretroviral (ARV) therapy naïve before recruitment to avoid any previous effects by ARV given before pregnancy. A trained qualified medical/clinical officer took a complete physical examination and medical history at enrollment point. Face-to-face, guided interview protocol (Appendix II) was used to enable exclusion and inclusion of the participants and to obtain the demographic data. Information collected from the records and face-to-face interview included age, parity, gestational age, marital status and medical history of the study participants.

During the discussion, eligibility was assessed; if eligibility was confirmed, then the purpose, procedures, and risks and benefits of the study were discussed with the participant.

The prospective participants were given adequate opportunities to discuss and contemplate their participation. Participants were also given time to ask questions and to have all concerns addressed. Throughout the study, free and informed consent process was exercised. Participants who passed the eligibility criteria were recruited. A consecutive sampling technique was used to recruit those who met the inclusion criteria until the required sample size was achieved. A specimen (~5 ml) of blood from each of the study participants was collected aseptically by venipuncture into sterile EDTA (EthyleneDiamine-Tetraacetic Acid) VACUTAINER® blood collection tube (lavender top) and plain VACUTAINER® blood collection tube (~2.5 ml in each tube) after signing an informed and voluntary consent form (Appendix III). Immediately after blood collection, Aliquots (200 µL) were separated for laboratory analysis.

3.3.4: Laboratory analyses

Progesterone levels, systemic cytokines and lymphocyte counts were measured by trimester in advancing pregnancy of each study participant. The first blood sample for laboratory analysis from the participants was collected at the time of recruitment during the first trimester. The second blood sample collection and laboratory analysis were done after 12 weeks, a time in the second trimester. The third and final blood sample collection and laboratory analysis were also performed 12 weeks after the second blood sample collection, a time in their third trimester.

3.3.4.1: Progesterone assay

Enzyme Linked Immunosorbent Assay (Appendix IV) method was used to analyze progesterone levels. Human^{TM} progesterone kit (Human Diagnostics Company, Wiesbaden, Germany) was used to analyze the samples. Blood serum was used for this procedure. A blood sample collected in the plain tubes was used for progesterone analysis. The sample was allowed to clot and form serum, the liquid part formed above the clot, which was used for this analysis. If the blood samples were not analyzed immediately, they were refrigerated at 2-8°C for a maximum period of five days. Before performing the assay, samples, standards, controls and reagents i.e. conjugate; substrate and buffer were also brought to room temperature (24+/-4°C). The diluted buffer was also stored at room temperature (24+/-4°C). Standards of increasing concentrations were used to construct standard curves and to determine the unknowns, which were the participants' samples. Controls of known concentration were provided together with the kit to ensure the accuracy of the results. The reagent kit provided the wash buffer, standards, controls, conjugate (horseradish peroxidase), substrate (3,3´,5,5´-tetramethylbenzidine) and stop solution (hydrogen peroxide). The desired number of microtiter wells in the holder was secured first before the procedure.

In the procedure, 25 µL of each standard, controls and samples was dispensed into appropriate wells and incubated for 5 minutes at 24+/-4°C. After incubation, 200 µL of enzyme conjugate was then added to each well and mixed thoroughly for 10 seconds. Then the mixture was incubated at 24+/-4°C for 60 minutes. The contents of the wells were shaken briskly after the incubation and rinsed three times with the prepared wash solution (400 µL per well). The wells were floated on absorbent paper to remove residuals. Then 200 µL of the substrate reagent was added to all the wells and then incubated again at 24+/-4°C for about 15 minutes. To each well 100, µL of stop solution was added to stop the enzymatic reaction. Finally, the absorbance for each test was determined at 450±10 nm

with ELx800 Absorbance Microplate ELISA Reader (BioTek Instruments, Winooski, USA). The absorbance of the tests was done within 10 minutes after stopping the enzymatic reaction. The results were calculated automatically in the microplate ELISA reader using a 4-parameter logistics curve fit.

3.3.4.2: Cytokines and lymphocytes immunophenotyping

Quantitative analysis of systemic cytokines levels and lymphocyte counts was done using FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The systemic cytokines were analyzed using BD™ Cytometric Bead Array (CBA) Technology (BD Biosciences, San Jose, CA, USA). This type of assay captures the cytokines with beads of known fluorescence and size, enabling it to detect using FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The present study used The BD™ CBA Human T_H1/T_H2 Cytokine Kit II to quantitatively measure IL-2, IL-4, IL-6, IL-10, total TNF (but not variants), and IFN-γ levels in a single sample. The detection reagent is composed of phycoerythrin (PE)-conjugated antibodies, which give a fluorescence indicating the amount of bound analyte. During the procedure, the analyte is sandwiched between the capture bead and the detection reagent (capture bead+ analyte + detection reagent). Six beads coated with specific capture beads for IL-2, IL-4, IL-6, IL-10, total TNF (but not variants), and IFN-γ with identified fluorescence intensities when mixed form a bead array that is resolved using flow cytometer in the PE channel. The concentrations of the cytokines are revealed by the intensity of each PE-fluorescence of sandwich complexes. The reagents contained in the kit included; cytokine capture beads, cytometer setup beads, PE detection reagent, cytokine standards, PE positive control detector, wash buffer, assay diluent, and serum enhancement buffer. The cytokines kit components were stored at 2 to 80°C.

In preparation for the analysis, fresh standards were prepared by reconstituting them with 2.0 mL of assay diluent and were allowed to stand for at least 15 minutes at room temperature (24+/-4°C). Eight tube serial dilutions were then performed to achieve different concentrations of the standards. Ten (10) μL aliquot of each capture bead, for each cytokine to be analyzed was then mixed and vortexed thoroughly. The mixed capture beads were then centrifuged at 200g for 5 minutes. Then the supernatant was carefully aspirated and discarded. The capture beads pellet was then suspended in an equal volume to amount discarded of serum enhancement buffer, vortexed thoroughly and incubated at room temperature (24+/-4°C) for 30 minutes and protected from light. Compensation for the instrument was performed using the cytometer setup beads.

In performing the cytokines assay procedure, the prepared mixed capture beads were well vortexed and 50 μL of the beads added to all assay tubes. Fifty (50) μL of the prepared standard dilutions were then added to the control assay tubes. Fifty (50) μL of each unknown samples were also added to appropriately labeled sample tubes into all assay tubes, 50 μL of cytokine phycoerythrin (PE) detection reagent was then added and incubated for 3 hours at room temperature (24+4°C), protected from light; wrapped in aluminum foil Then after the incubation, one mL of wash buffer was added to each assay tube and centrifuged at 200*g* for 5 minutes. Carefully the supernatant was then aspirated and discarded from each assay tube. Then 300 μL of wash buffer was added to each assay tube to resuspend the bead pellet. The test assays were then acquired on the flow cytometer using BD Cell Quest™ software (BD Biosciences, San Jose, CA, USA) and analyzed using FCAP Array software (BD Biosciences, San Jose, CA, USA). The limit detection of IL-4, IL-6, IL-10, IL-2, TNF and IFN-γ were 2.6, 3.0, 2.8, 2.6, 2.8 and 7.1 pg/ml, respectively. T_H1 and T_H2 cytokine overall procedure and incubation times are as shown in Appendix V.

Immunophenotyping of $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD56/16^+$ counts was done using a BD Multitest (BD Biosciences, San Jose, CA, USA) method. MultiTEST method is based on fluorescence triggering, allowing gating of the fluorescence to have the lymphocyte population, which reduces contamination of nucleated red blood cells or unlysed cells in the gate. The absolute number (cells/µL) of the cells in the blood sample was determined by comparing bead events with the cellular events. The reagents were stored at 2–8°C. The reagents included; CaliBRITEs, FACS Lysing Solution, TruCOUNT Controls and Lysable whole blood control. The samples were processed by FACS Lyse /No wash technique. Using the TruCOUNT tubes the blood samples for $CD3^+$, $CD4^+$ and $CD8^+$ counts were stained using BD Multitest $CD3^+/CD8^+/CD45^+/CD4^+$ reagent (BD Biosciences, San Jose, CA, USA) a four parameter T-cell flow cytometry panel with antibodies ($CD3^+$ fluorescein isothiocyanate (FITC), $CD8^+$ phycoerythrin (PE), $CD45^+$ peridinin chlorophyll protein (PerCP) and $CD4^+$ Allophycocyanin (APC). Using the TruCOUNT tubes the blood samples for $CD19^+$ and $CD16/CD56^+$ were stained using the BD Multitest $CD3^+/CD16/CD56^+/CD45^+/CD19^+$ reagent (BD Biosciences, San Jose, CA, USA), a four-color direct immunofluorescence reagent kit with antibodies ($CD3^+$ fluorescein isothiocyanate (FITC), $CD56/16^+$ phycoerythrin (PE), $CD45^+$ peridinin chlorophyll protein (PerCP) and $CD19^+$ Allophycocyanin (APC). These samples were stained within 48 hours after specimen collection and analyzed within 24 hours after staining. Lymphocyte immunophenotyping procedure and incubation times are as shown in Appendix VI.

During the procedure and the initial stage, all tubes were labeled. Then 20µL of the MultiTEST $CD3^+/CD8^+/CD45^+/CD4^+$ or $CD3^+/CD16CD56^+/CD45^+/CD19^+$ fluorochromes-labelled antibodies were added into the bottom of the tubes. Fifty (50) µl of well-mixed whole blood sample was then added, vortexed and then incubated for 15 minutes at room temperature (24+/-4°C). The cells were then lysed with 450 µl of BD FACS lyse reagent and incubated again for another 15 minutes. BD-

Multiset™ Software (BD Biosciences, San Jose, CA, USA), which is an application for analyzing multicolor flow cytometry data, was used for sample acquisition.

For the machine set up, the cytometer was equipped with 635-nm and 488-nm lasers and was able to detect both forward and side light scatter. The machine was also set on a four-color fluorescence with detectable emissions in four ranges: 515–545 nm, 562–607 nm, >650 nm and 652–668 nm. The cytometer could threshold or discriminate by the use of the >650-nm channel. By use of BD calibrite™ beads the photomultiplier tube voltages and fluorescence compensation were set, and instrument sensitivity checked before use. A single-stained compensation control tube for each color was also set up, and the compensation controls were kept within the linear range of each detector. Set up of the compensation controls was done for each experiment. The data files were then acquired and immediately analyzed.

3.4: Data management and analyses

All the data was collected on standard case report forms (Appendix VII), a tool developed for this study. The standard report forms were identified by the participants' study number. Data was analyzed using STATA version 13 (StataCorp, Timberlake, United Kingdom). Normality assumption of the data was assessed using Shapiro-Wilks test. Residual checks only showed a mild violation of the normality assumption and homogeneity of variance. The test for categorical variables in the demographics was done using Pearson's Chi-Square test and summarized as frequencies and their corresponding percentages.

To show the trends of progesterone, systemic cytokines and lymphocytes, line graphs commonly known as the spaghetti plots were used. The graphs were stratified by the groups of the study participants. The change in progesterone, systemic cytokines and lymphocytes were assessed using

repeated measures regression models. The interaction effect was also included in the analysis to assess the change by the group over time. The repeated measures model used for all the outcomes was of the form;

$$y_{ijt} = \alpha_0 + \alpha_1 G_{ij} + \alpha_2 T_{it} + \alpha_3 G_{ij} T_{it} + \varepsilon_{ijt} \qquad Equation(1)$$

Where y_{ijt} – is the outcome, measured for person in group j at time t
G – is the group i.e. HIV status,
T – is the time i.e. trimester
GT- is the interaction between the group and time i.e. HIV status and trimester
ε_{ijt} – is the random person by time effect, ~ N (0,1)

Equation 1 is the repeated measures analysis of variance. The results in the change in progesterone, systemic cytokines and lymphocyte counts were then summarized as means alongside the corresponding 95% confidence Intervals (95% CI) and presented using tables.

Test for differences in progesterone, systemic cytokines and lymphocyte between the study groups was done using Wilcoxon two-sample test. The results in the differences in progesterone, systemic cytokines, and lymphocyte counts were then summarized as means alongside the corresponding 95% confidence Intervals (95% CI) and presented using tables. *P*-values ≤ 0.05 were considered statistically significant.

3.5: Ethical considerations

Ethical approval (Appendix VIII) was obtained from Moi Teaching and Referral Hospital Ethical Review Committee (ERC) for research on human participants (#000915) before initiation of this study. Research permissions were also sought from Maseno University and AMPATH (Appendices IX and X, respectively). Also, written informed consent was obtained from the study participants, and they could withdraw any time from the study with no consequences as to access to medical services. Care was taken to ensure that no psychological, social or legal harm was implicated to the

participants and hence confidentiality was ensured throughout the study period. The study thus ensured the use of study codes only on data documents, proper disposal of study data or documents, data documents were securely stored within locked locations and security codes to computerized records were assigned. Access to information about individual participants was only restricted to the researcher.

CHAPTER FOUR

RESULTS

4.1: The demographic characteristics of the study participants

The demographic characteristics of the study participants at recruitment were collected and summarized (Table 4.1). Forty-four HIV-positive and an equal number of HIV-negative participants were used as the study participants. The median age of the study participants was 28 (IQR: 25-31) years. Thirty-six participants (41%) were at parity one, 37 (42%) were at parity two, 12 (14%) were at parity three and 3 (3%) were at parity four. The median gestation age of the study participants was 8 (7-9) weeks at the first trimester. The median age for the HIV-positive [29 (IQR: 26-31)] participants was comparable (P=0.053) to the HIV-negative [27 (IQR: 24-30)] participants. Similarly, the differences in parity and gestational age between the HIV-positive and the HIV-negative participants were comparable (P=0.058 and P=0.147, respectively). The proportion of the married (or single) among participants among the HIV-negative [39 (89%)] was also comparable (P=0.156) to the proportion of the married (or single) among the HIV-positive [34 (77%)] participants.

Table 4.1: The demographic characteristics of the study participants

Variable	**Levels**	**HIV-positive, (n=44, 50%)**	**HIV-negative, (n=44, 50%)**	**Overall, (n=88,100%)**	***P*-Value**
		n(%) or median(IQR)	**n (%)**	**n (%)**	
Age		29 (26-31)	27 (24-30)	28 (25-31)	0.053
Parity	One	12 (27%)	24 (55%)	36 (41%)	0.058
	Two	23 (52%)	14 (32%)	37 (42%)	
	Three	7 (16%)	5 (11%)	12 (14%)	
	Four	2 (5%)	1 (2%)	3 (3%)	
Marital status	Married	34 (77%)	39 (89%)	73 (83%)	0.156
	Single	10 (23%)	5 (11%)	15 (17%)	
Gestation Age		8 (7-8)	8 (7-10)	8 (7-9)	0.147

Forty-four HIV-positive and forty-four HIV-negative participants were used as the study participants. The *P*-values for the demographic characteristics were determined using Pearson's Chi-Square test. The differences in age, parity, gestation age and marital were comparable between the two groups.

4.2: The profiles of progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women

To show the trends of progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women, the mean progesterone levels in the first, second and third trimesters of advancing pregnancy of both groups were illustrated using a line graph (Figure 4.1). Among the HIV-positive and HIV-negative participants in the first, the second and the third trimesters, the mean progesterone levels were 21.5, 49.3, 111.6 ng/ml, and 25.8, 55.9, 123.2 ng/ml, respectively. Both groups demonstrated an increasing trend of progesterone as pregnancy advanced.

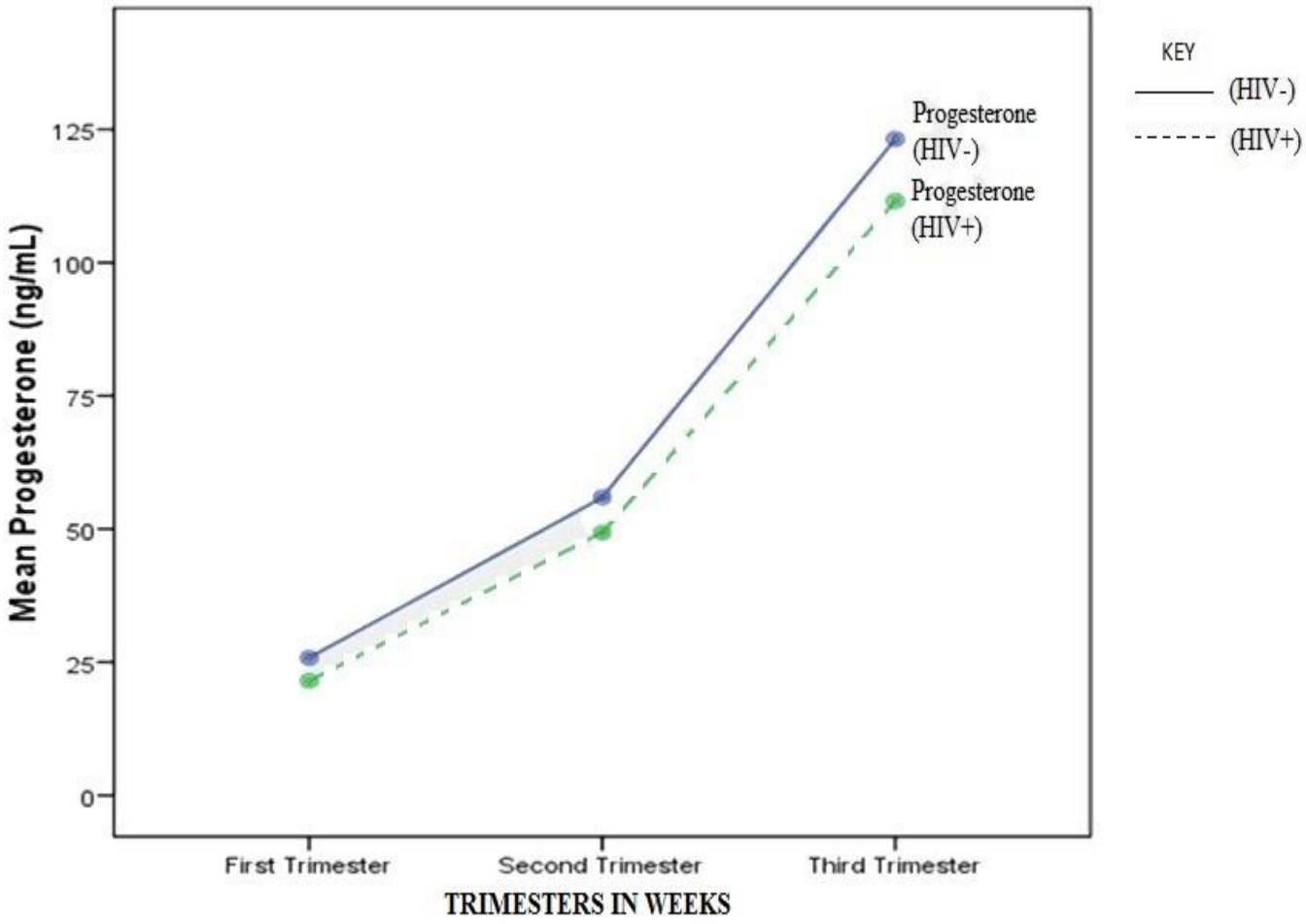


Figure 4.1: The trends of progesterone in advancing pregnancy stratified by HIV status.
Among the HIV-positive and HIV-negative participants, the mean progesterone levels increased as pregnancy advanced.

To determine the change in progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women, the mean change in progesterone levels were measured at second trimester compared to the first trimester and in the third trimester compared to the second and the first trimester of advancing pregnancy in both groups (Table 4.2). Among the HIV-positive, the mean change in progesterone during the second, 62.3 (95% CI: 59.0, 65.5) ng/ml, and the third, 90.1 (95% CI: 86.9, 93.3) ng/ml, trimesters, were higher (P<0.0001) compared to that of the first trimester, 27.8 (95% CI: 24.6, 31.1) ng/ml. The mean change in progesterone for the third trimester was also higher (P<0.0001) compared to that of the second trimester, 62.3 (95% CI: 59.0, 65.5) ng/ml.

Likewise, among the HIV-negative, the mean change in progesterone during the second, 67.3 (95% CI: 64.1, 70.5) ng/ml and the third trimesters, 97.4 (95% CI: 94.1, 100.6) ng/ml, were higher (*P*<0.0001) than that of the first trimester, 30 (95% CI: 26.8, 33.3) ng/ml. The mean change in progesterone for the third trimester 97.4 (95% CI: 94.1, 100.6) ng/ml was also higher (*P*<0.0001) compared to that of the second trimester 67.3 (95% CI: 64.1, 70.5) ng/ml.

Table 4.2: The change in progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women

	HIV-Positive (n=44)		**HIV-negative (n=44)**	
	Mean Change (95% CI) ***P*-value**		**Mean Change (95% CI)** ***P*-value**	
Trimester	**1**	**2**	**1**	**2**
2	27.8 (24.6, 31.1) ***P*<0.0001**	N/A	30.1 (26.8, 33.3) ***P*<0.0001**	N/A
3	90.1 (86.9, 93.3) ***P*<0.0001**	62.3 (59.0, 65.5) ***P*<0.0001**	97.4 (94.1, 100.6) ***P*<0.0001**	67.3 (64.1, 70.5) ***P*<0.0001**

Changes in progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women were measured using repeated measures analysis of variance. The mean change in progesterone levels during the second, and the third trimesters were significant (*P*<0.0001) compared to that of the first trimester in both groups. The mean change in progesterone for the third trimester was also higher (*P*<0.0001) compared to that of the second trimester. N/A=Not applicable.

To determine the differences in progesterone levels between the two groups across the trimesters of pregnancy, the mean differences in progesterone levels between HIV-infected and HIV-non-infected women were measured during the first trimester, second and third trimesters of advancing pregnancy (Table 4.3).

The mean difference of progesterone levels between HIV-infected and HIV-non-infected women observed were significant in all trimesters of pregnancy i.e. first trimester (-4.3 (95% CI: -8.2, -0.5) ng/ml, *P*=0.028)], second trimester [-6.6 (95% CI: -10.3, -3.0) ng/ml, *P*=0.001] and third trimester [-11.6 (95% CI: -17.2, -6.1) ng/ml, *P*=0.0001]. Since gestational age can influence the differences in progesterone levels, the differences in gestational age between the two groups in all trimesters were also assessed to confirm if the difference of progesterone levels was influenced by the age of the pregnancy (Table 4.3). The findings reveal that there was comparable mean difference between the two groups in gestational age in the first trimester [-0.7 (95% CI: -1.4, 0.1) ng/ml, *P*=0.079], second trimester [-0.6 (95% CI: -1.4, 0.1) ng/ml, *P*=0.110] and third trimester [-0.9 (95% CI: -1.8, 0) ng/ml, *P*=0.058] weeks of pregnancy.

Table 4.3: The differences in progesterone in advancing pregnancy between the HIV-infected and the HIV-non-infected women

Variable		**HIV-positive (n=44)**	**HIV-negative (n=44)**	**Difference (95% CI)**	
	Trimester	**Mean ± SEM**	**Mean± SEM**	**(Mean HIV+) - (Mean HIV-)**	***P*-Value**
Progesterone	1	21.5± 1.3	25.8± 1.5	-4.3(-8.2, -0.5)	**0.028**
	2	49.3± 1.5	55.9± 1.0	-6.6(-10.3, -3.0)	**0.001**
	3	111.6±1.7	123.2±2.2	-11.6(-17.2, -6.1)	**0.0001**
Gestation Age	1	7.7± 0.2	8.4± 0.3	-0.7(-1.4, 0.1)	0.079
	2	19.8±0.2	20.4±0.3	-0.6(-1.4, 0.1)	0.110
	3	31.5±0.3	32.4±0.3	-0.9(-1.8, 0)	0.058

Test for differences in progesterone between the study groups was done using Wilcoxon two-sample test. The mean difference of progesterone between HIV-infected and HIV-non-infected women observed were significant in all trimesters [first; *P*=0.028, second; *P*=0.001 and third; *P*=0.0001] of pregnancy.

4.3: The profiles of T_H1 (IL-2, IFN-γ, TNF) and T_H2 (IL-10, IL-6, IL-4) type of systemic cytokines levels in advancing pregnancy of HIV-infected and HIV-non-infected women

The present study present results for the profiles of T_H1 (IL-2, IFN-γ, TNF) and T_H2 (IL-10, IL-6, IL-4) type of systemic cytokines levels in advancing pregnancy of HIV-infected and HIV-non-infected women. To demonstrate the trends of T_H1 type cytokines levels, the means of IL-2, IFN-γ, and TNF in the first, second and third trimesters of advancing pregnancy were presented graphically in Figure 4.2. Among the HIV-positive and HIV-negative participants, the mean IL-2 levels for the first, second and third trimesters were 4.2, 3.6, 4.4 pg/ml and 3.6, 3.3, 3.0 pg/ml, respectively. Unlike the HIV-negative participants, which showed a decrease to the second trimester and remained same in

the third trimester, the HIV-positive demonstrated a decline in the second trimester and then an increase in the third trimester.

Among the HIV-positive participants, the trend of IFN-γ levels during the first, second and third trimesters declined as pregnancy advanced that is, 4.5, 2.5, and 2.2 pg/ml, respectively. Among the HIV-negative participants, the trend of IFN-γ levels during the first, the second and the third trimesters was also decreasing as pregnancy advanced that is, 2.7, 1.8, and 1.2 pg/ml, respectively. Among the HIV-positive and HIV–negative participants, the mean TNF levels for the first, second and third trimesters were 7.3, 5.2, 5.5 pg/ml and 6.7, 5.4, and 3.7, respectively. A declining trend is noted for both groups; however, a slight increase in the mean TNF levels was observed among the HIV-positive pregnant women in the third trimester.

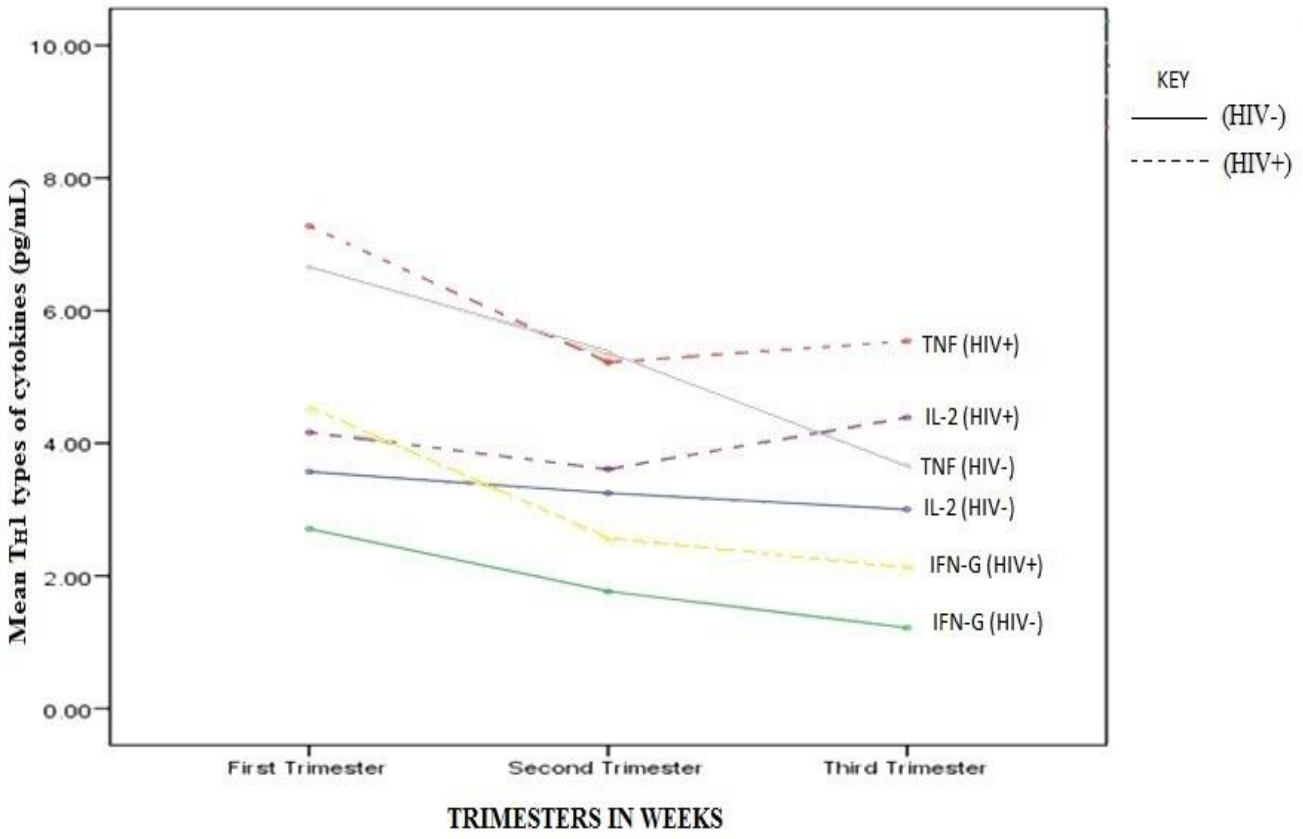


Figure 4.2: The trends of the T_H1 type of cytokine levels in advancing pregnancy stratified by HIV status.

The trends of the T_H1 type of cytokines were inconsistent in both groups. Unlike the HIV-negative participants, which showed a decreasing trend, the HIV-positive demonstrated a decline of IL-2 levels in the second trimester and then an increase in the third trimester. Among the HIV-positive participants, the trend of IFN-γ levels during the first, second and third trimesters declined as pregnancy advanced. Among the HIV-negative participants, the trend of IFN-γ levels during the first, the second and the third trimesters was also decreasing as the pregnancy progressed. A declining trend in TNF levels is noted for both groups; however, a slight increase in mean TNF levels was observed among the HIV-positive pregnant women in the third trimester.

To show the trends of T_H2 type cytokine levels in advancing pregnancy of HIV-infected and HIV-non-infected women, the means of 1L-10, IL6 and IL-4 in the first trimester, the second and third trimesters of advancing pregnancy of both groups were illustrated using a line graph as shown in Figure 4.3. Among the HIV-positive and HIV-negative participants, the mean IL-10 levels for the first, second and third trimesters were 2.9, 3.1, and 3.4 pg/ml, and 1.4, 2.1, and 2.2 pg/ml, respectively. An increasing trend of IL-10 levels was observed in both groups as pregnancy

advanced. Among the HIV-positive and HIV-negative participants, the mean IL-6 levels for the first, second and third trimesters were 4.4, 5.0, 4.8 pg/ml and 4.3, 3.7, 3.7 pg/ml, respectively. Among the HIV-positive and HIV-negative participants, the mean IL-4 levels for the first, second and third trimesters were 2.6, 6.2, 5.1 pg/ml and1.9, 3.9, 4.5 pg/ml, respectively. Both groups demonstrated an increasing trend in the second trimester and decline in the third trimester.

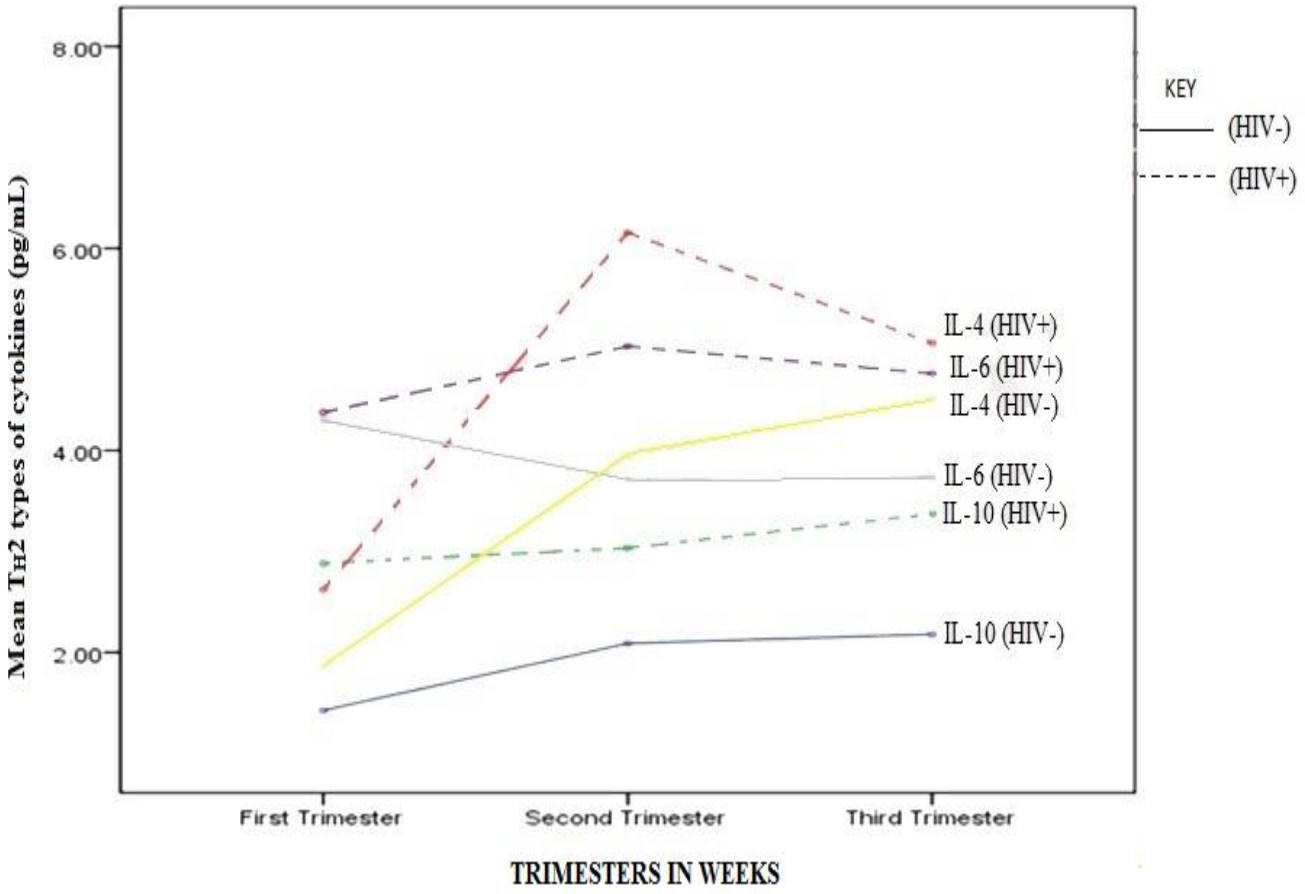


Figure 4.3: The trends of the T_H2 type of cytokine levels in advancing pregnancy stratified by HIV status.

The trends of the T_H2 type of cytokines were inconsistent in both groups. An increasing trend of IL-10 levels was observed in both groups as pregnancy advanced. Among the HIV-positive, there was an increasing trend of IL-6 levels in the second trimester and a decrease in the third trimester. This was opposite among the HIV–negative participants were a decreasing trend is noted in the second trimester and an increase in the third trimester. Both groups demonstrated an increase in the second trimester and decline in the third trimester of IL-4 levels.

To determine the change in cytokines in advancing pregnancy of both groups, the mean change in T_H1 (IL-2, IFN-γ, TNF) and T_H2 (, 1L-10, IL6, IL-4) cytokines were measured at second trimester compared to the first trimester and in the third trimester compared to the second and the first trimester of advancing pregnancy in both groups (Table 4.4).

Among the HIV-positive the mean change in IL-2 levels during the second trimester was lower (*P*=0.036) compared to that of the first trimester, -0.6 (95% CI: -1.1, -0.1) pg/ml. The mean change in IL-2 levels during the third trimester was higher (*P*=0.003) and comparable (*P*=0.399) to that of the second 0.8 (95% CI: 0.3, 1.3) and first 0.2 (95% CI: -0.3, 0.7) pg/ml, trimesters, respectively. Among the HIV-negative participants, the mean change in IL-2 levels during the second trimester was comparable (*P*=0.226) to that of the first trimester, -0.3 (95% CI: -0.8, 0.2) pg/ml. The mean change in IL-2 levels for the third trimester was lower (*P*=0.032) and comparable (*P*=0.348) to that of the first, -0.6 (95% CI: -1.1, -0.1) pg/ml and second, -0.2 (95% CI: -0.8, 0.3) pg/ml trimesters, respectively.

Among the HIV-positive, the mean change in IFN-γ in the second trimesters was lower (*P*<0.0001) compared to that of the first trimester -2.0 (95% CI: -2.7, -1.2) pg/ml. The mean change in IFN-γ levels for the third trimester was also lower (*P*<0.0001) compared to that of the first trimester, -2.3 (95% CI: --3.1, -1.6) pg/ml, but comparable (*P*=0.313) to that of the second trimester, -0.4 (95% CI: -1.2, 0.4) pg/ml. Among the HIV-negative, the mean change in IFN-γ during the second trimester was lower (*P*=0.015) compared to that of the first trimester, -0.9 (95% CI: -1.7, -0.2) pg/ml. The mean change in IFN-γ for the third trimester was lower (*P*<0.0001) and comparable (*P*=0.156) to that of the first trimester, -1.5 (95% CI: -2.3, -0.7) pg/ml and second trimester -0.6 (95% CI: -1.3, 0.2) pg/ml, respectively.

Among the HIV-positive, the mean change in TNF levels during the second trimester was lower (P<0.0001) compared to the first trimester, -2.1 (95% CI: -3.1, -1.0) pg/ml. The mean change in TNF levels for the third trimester was also lower (P=0.001) compared to that of the first trimester, -1.7 (95% CI: -2.8, -0.7) pg/ml and comparable (P=0.551) to that of the second trimester, 0.3 (95% CI: -0.7, 1.4) pg/ml. Among the HIV-negative, the mean change in TNF during the second trimester was lower (P=0.016) compared to that of the first trimester, -1.3 (-2.4, -0.2) pg/ml. The mean change in TNF for the third trimester was also lower compared to that of the first trimester, [-3.0 (95% CI: -4.0, -1.9) pg/ml, P<0.0001] and the second trimester, [-1.7 (95% CI: -2.7, -0.6 pg/ml), P=0.002] respectively.

Among the HIV-positive, the mean change in IL-10 levels in the second trimesters was comparable (P=0.554) to that of the first trimester, 0.2 (95% CI: -0.4, 0.7) pg/ml. The mean change in IL-10 levels for the third trimester was also comparable to that of the first trimester, [0.5 (95% CI: -0.1, 1.0 pg/ml), P=0.082] and that of the second trimester, [0.3 (95% CI: -0.2, 0.9) pg/ml, P=0.258]. Among the HIV-negative, the mean change in IL-10 levels during the second trimester was higher (P=0.019) than that of the first trimester, 0.7 (95% CI: 0.1, 1.2) pg/ml. The mean change in IL-10 levels for the third trimester, was higher (P=0.008) compared to that of first trimester 0.8 (95% CI: 0.2, 1.3) pg/ml but comparable (P=0.745) to that of the second trimester, 0.1 (95% CI: -0.5, 0.6) pg/ml.

Among the HIV-positive, the mean change in IL-6 during the second was higher (P=0.029) compared to the change in the first trimesters, 0.7 (95% CI: 0.1, 1.2) pg/ml. The mean change in IL-6 for the third trimester was comparable to that of the first trimester, [0.4 (95% CI: -0.2, 1.0) pg/ml, P=0.202], and second trimester, [-0.3 (95% CI: -0.9, 0.3) pg/ml, P=0.367], respectively. Among the HIV-negative, the mean change in IL-6 in the third trimester was comparable to those of first [-0.6 (-

1.2, 0.01) pg/ml, P=0.066] and second [0.02 (-0.6, 0.6) pg/ml, P=0.941] trimester in advancing pregnancy.

Among the HIV-positive, the mean change in IL-4 levels in the second trimester was higher (P<0.0001) compared to that of the first trimester 3.5 (95% CI: 2.6, 4.5) pg/ml. The mean change in IL-4 levels for the third trimester was also higher (P<0.0001) than that of the first trimester, 2.4 (95% CI: 1.5, 3.4) pg/ml, but lower (P=0.022) compared to that of the second trimester, -1.1 (95% CI: -2.0, -0.2) pg/ml. Among the HIV-negative, the mean change in IL-4 levels during the second trimester was higher (P<0.0001) than that of the first trimester, 2.1 (95% CI: 1.1, 3.0) pg/ml. The mean change in IL-4 during the third trimester was also higher (P<0.0001) compared to that of the first trimester 2.7 (95% CI: 1.7, 3.6) pg/ml and comparable (P=0.234) to that of the second trimester, 0.6 (95% CI: -0.4, 1.5) pg/ml.

Table 4.4: The change in T_H1 and T_H2 type of cytokines in advancing pregnancy of HIV-infected and HIV-non-infected women

		HIV-Positive (n=44)		HIV-negative (n=44)	
Cytokine		**Mean change (95% CI)** ***P*- value**		**Mean change (95% CI)** ***P*- value**	
	Trimester	**1**	**2**	**1**	**2**
IL-2	**2**	-0.6(-1.1, -0.03) ***P*=0.036**	N/A	-0.3(-0.8, 0.2) *P*=0.226	N/A
	3	0.2(-0.3, 0.7) *P*=0.399	0.8(0.3, 1.3) ***P*=0.003**	-0.6(-1.1, -0.1) ***P*=0.032**	-0.2(-0.8, 0.3) *P*=0.348
IFN-γ	**2**	-2.0(-2.7, -1.2) ***P*<0.0001**	N/A	-0.9(-1.7, -0.2) ***P*=0.015**	N/A
	3	-2.3(-3.1, -1.6) ***P*<0.0001**	-0.4(-1.2, 0.4) *P*=0.313	-1.5(-2.3, -0.7) ***P*<0.0001**	-0.6(-1.3, 0.2) *P*=0.156
TNF	**2**	-2.1(-3.1, -1.0) ***P*<0.0001**	N/A	-1.3(-2.4, -0.2) ***P*=0.016**	N/A
	3	-1.7(-2.8, -0.7) ***P*=0.001**	0.3(-0.7, 1.4) *P*=0.551	-3.0(-4.0, -1.9) ***P*<0.0001**	-1.7(-2.7, -0.6) ***P*=0.002**
IL-10	**2**	0.2(-0.4, 0.7) *P*=0.554	N/A	0.7(0.1, 1.2) ***P*=0.019**	N/A
	3	0.5(-0.1, 1.0) *P*=0.082	0.3(-0.2, 0.9) *P*=0.258	0.8(0.2, 1.3) ***P*=0.008**	0.1(-0.5, 0.6) *P*=0.745
IL-6	**2**	0.7 (0.1, 1.2) ***P*=0.029**	N/A	-0.6(-1.2, 0.01) ***P*=0.056**	N/A
	3	0.4 (-0.2, 1.0) *P*=0.202	-0.3 (-0.9, 0.3) *P*=0.367	-0.6(-1.2, 0.01) *P*=0.066	0.02 (-0.6, 0.6) *P*=0.941
IL-4	**2**	3.5(2.6, 4.5) ***P*<0.0001**	N/A	2.1(1.1, 3.0) ***P*<0.0001**	N/A
	3	2.4(1.5, 3.4) ***P*<0.0001**	-1.1(-2.0, -0.2) ***P*=0.022**	2.7(1.7, 3.6) ***P*<0.0001**	0.6(-0.4, 1.5) *P*=0.234

Changes in systemic cytokines levels in advancing pregnancy of HIV-infected and HIV-non-infected women were measured using repeated measures analysis of variance. Among the HIV-positive women, significant change in IL-2 [in the second trimester (*P*=0.003) compared to first and in the third trimester (*P*=0.003) compared to the second trimester], IL-6 [in the second trimester (*P*=0.029) compared to the first trimester] and IL-4 [in the 3rd trimester, (*P*=0.022) compared to the second trimester] were different from what was observed among the HIV-negative women. N/A=Not applicable.

In order to determine the difference in systemic cytokines across the trimesters of pregnancy between the two groups, the mean difference in IL-2, IFN-γ, TNF, 1L-10, IL6 and IL-4 levels were also measured at first trimester, and thereafter in the second and third trimesters of advancing pregnancy (Table 4.5). There were significant differences in the mean IFN-γ levels between HIV-positive and HIV-negative pregnant women in the first trimester [1.8 (95% CI: (0.4, 3.2) pg/ml, *P*=0.013)] and the third trimester [1 (95% CI: 0.1, 1.7) pg/ml, *P*=0.021]. The two groups were comparable (*P*=0.113) in the second trimester 0.7 (95% CI: (-0.2, 1.7) pg/ml. The mean difference of TNF between HV-infected and HIV-non-infected were comparable in all trimesters [first, 0.6 (95% CI: -2.5, 3.4) pg/ml, *P*= 0.694, second, -0.2 (95% CI: -2.7, 2.3) pg/ml, *P*=0.917 and third, 1.8 (95% CI: -0.4, 4.1) pg/ml, *P*=0.099). The mean difference of IL-2 and IL-6 levels between HIV-positive and HIV-negative pregnant women were also comparable in all trimesters of pregnancy, first trimester { [0.6 (95% CI: -1.0, 2.2) pg/ml, *P*=0.467] and [0.1 (95% CI: -1.4, 1.6) pg/ml, *P*=0.908]}, second trimester {[0.3 (95% CI: -1.0, 1.8) pg/ml, *P*=0.608] and [1.3 (95% CI: -0.1, 2.8) pg/ml, *P*=0.078]} and third trimester {[1.4 (95% CI: -0.1, 2.9) pg/ml, *P*=0.075] and [1.1 (95% CI -0.3, 2.3) pg/ml, *P*=0.120]}, respectively. The mean difference in IL-10 levels between the HIV-positive and the HIV-negative participants was significant in all trimesters, trimester one [1.5 (95% CI: 0.4, 2.5) pg/ml, *P*=0.008], trimester two [1.0 (95% CI: 0.2, 1.9) pg/ml, *P*=0.019], and trimester three [1.2 (95% CI: 0.3, 2.1) pg/ml, *P*=0.009]. The differences in mean IL-4 levels between the HIV-positive and the HIV-negative participants were only significant (*P*=0.017) in the second trimester 2.3 (95% CI: 0.4, 4.0) pg/ml.

Table 4.5: The differences in $T_H1$and T_H2 type of cytokines between HIV-infected and HIV-non-infected women in advancing pregnancy

Variable		HIV-positive (n=44)	HIV-negative (n=44)	Difference (95% CI)	
	Trimester	Mean ± SEM	Mean ± SEM	(Mean HIV+) - (Mean HIV-)	*P*-Value
IFN-γ	1	4.5± 0.7	2.7± 0.2	1.8(0.4, 3.2)	**0.013**
	2	2.5± 0.4	1.8± 0.2	0.7(-0.2, 1.7)	0.113
	3	2.2± 0.3	1.2± 0.2	1(0.1, 1.7)	**0.021**
TNF	1	7.3± 1.0	6.7± 1.2	0.6(-2.5, 3.4)	0.694
	2	5.2± 0.8	5.4± 1.0	-0.2(-2.7, 2.3)	0.917
	3	5.5± 0.9	3.7±0.7	1.8(-0.4, 4.1)	0.099
IL-2	1	4.2± 0.7	3.6±0.4	0.6(-1.0, 2.2)	0.467
	2	3.6± 0.6	3.3±0.4	0.3(-1.0, 1.8)	0.608
	3	4.4± 0.7	3.0±0.4	1.4(-0.1, 2.9)	0.075
IL-4	1	2.6±0.6	1.9±0.3	0.7(-0.6, 2.1)	0.267
	2	6.2±0.7	3.9±0.6	2.3(0.4, 4.0)	**0.017**
	3	5.1±0.6	4.5±0.5	0.6(1.1, 2.2)	0.509
IL-6	1	4.4±0.6	4.3±0.4	0.1(-1.4, 1.6)	0.908
	2	5.0±0.6	3.7±0.4	1.3(-0.1, 2.8)	0.078
	3	4.8±0.5	3.7±0.4	1.1(-0.3, 2.3)	0.120
IL-10	1	2.9±0.5	1.4±0.2	1.5(0.4, 2.5)	**0.008**
	2	3.1±0.3	2.1±0.2	1.0(0.2, 1.9)	**0.019**
	3	3.4±0.4	2.2±0.2	1.2(0.3, 2.1)	**0.009**

Test for differences in systemic cytokines between the study groups was done using Wilcoxon two-sample test. Significant differences between HIV-positive and HIV-negative of IFN-γ [in the first trimester (*P*=0.013) and third trimester (*P*=0.021)], IL-4 [in the second trimester (*P*=0.017)] and IL-10 [in first trimester (*P*= 0.008), second trimester (*P*=0.019), and third trimester (*P*=0.009)] levels were observed.

4.4: The profiles of lymphocyte counts in advancing pregnancy of HIV-infected and HIV-non-infected women

To show trends of T-lymphocytes ($CD3^+$) and its cell subset counts ($CD4^+$ and $CD8^+$), the mean $CD3^+$, $CD4^+$, and $CD8^+$ counts were measured during the first, second and third trimesters of advancing pregnancy and presented graphically in Figure 4.4. Among the HIV-positive and HIV-negative participants, the $CD3^+$ counts for the first, second and third trimesters were 1571, 1531, 1676 cells per mm^3 and 1253, 1138, 1202 cells per mm^3, respectively. In both groups, $CD3^+$ counts were observed to decline in the second trimester and increase in the third trimester.

Among the HIV-positive and HIV-negative participants, the $CD4^+$ cell count for the first, second and third trimesters were 634, 594, 586 cells per mm^3 and 637, 608, 654 cells per mm^3 respectively. The results indicate a decreasing trend of $CD4^+$ counts among the HIV-positive but a decrease and a rise in the third trimester of the HIV–negative participants. Among the HIV-positive and HIV-negative participants, the $CD8^+$ cell count for the first, second and third trimesters were 875, 877, 949 cells per mm^3 and 515, 474, 475 cells per mm^3, respectively. Unlike the HIV-negative participants who indicated a slight decrease, the HIV-positive participants showed a general increase of $CD8^+$ cell count across trimesters of pregnancy.

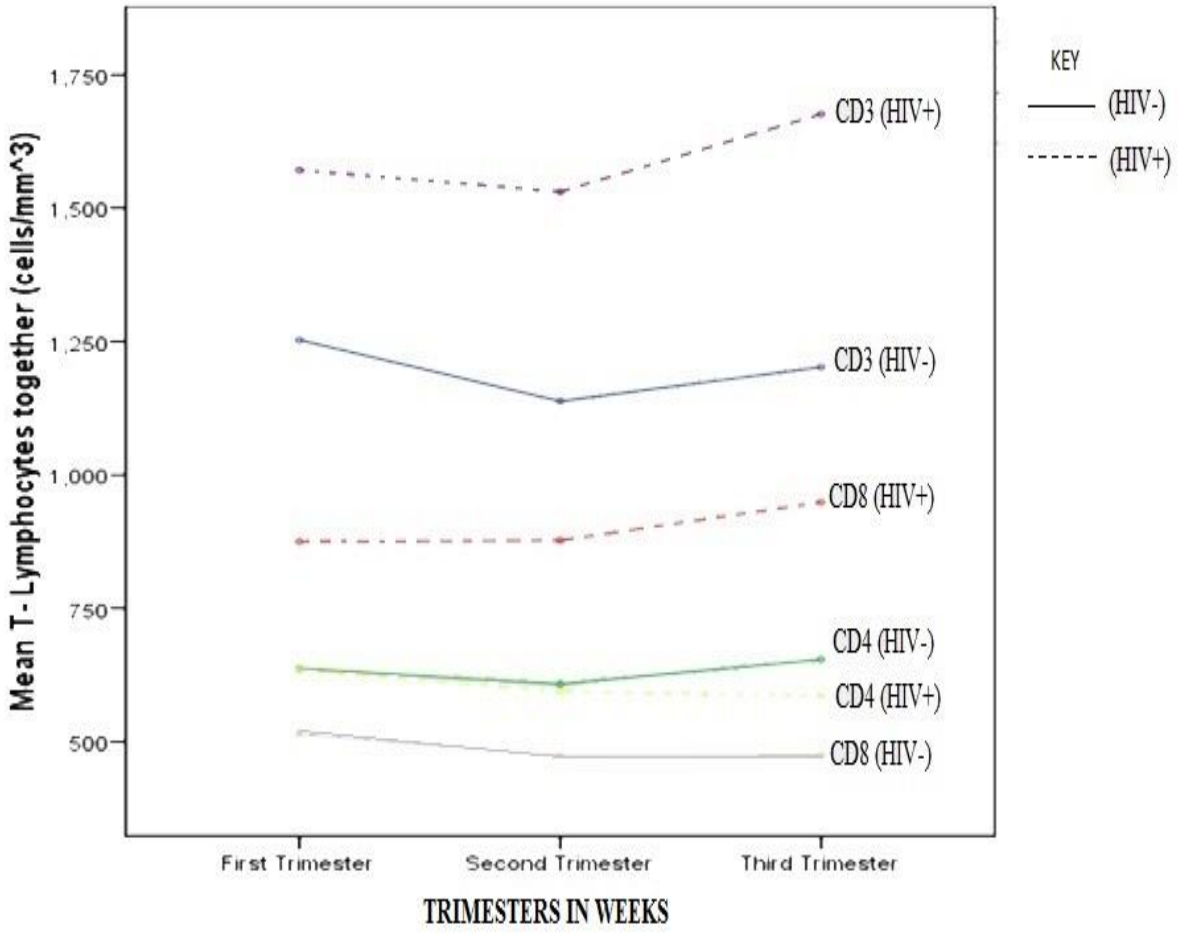


Figure 4.4: The trends of T-lymphocytes ($CD3^+$) and its subset ($CD4^+$ and $CD8^+$) counts in advancing pregnancy stratified by HIV status

The study observed inconsistent trends of T-lymphocytes in both groups. In both HIV-infected and non-infected $CD3^+$ counts were observed to decline in the second trimester and increase in the third trimester. The results indicate a decreasing trend of $CD4^+$ counts among the HIV-positive but a decrease and a rise in the third trimester of the HIV–negative participants. Unlike the HIV-negative participants who indicated a slight decline, the HIV-positive participants showed an increase in $CD8^+$ cell count across trimesters of pregnancy.

To show the trends of B-lymphocytes ($CD19^+$), the mean $CD19^+$ counts were measured during the first, second and third trimesters of advancing pregnancy and presented graphically in Figure 4.5. Among the HIV-positive and HIV-negative participants, the mean $CD19^+$ cell count for the first, second and third trimesters were 169, 201, 177 cells per mm3 and 198, 225, 226 cells per mm^3,

respectively. The mean $CD19^+$ cell count seems to increase in advancing pregnancy in both groups, though a decrease is noted in the third trimester among the HIV-positive women.

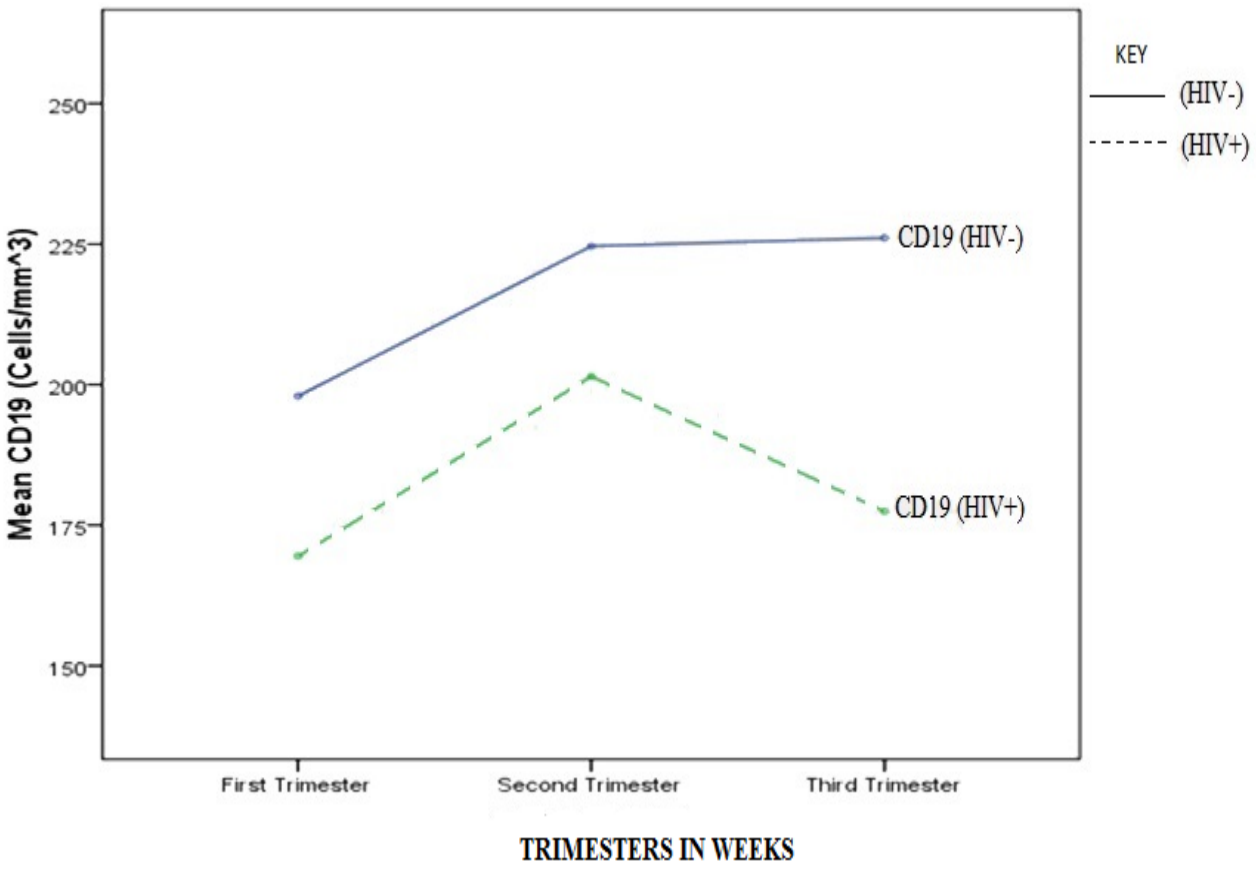


Figure 4.5: The trends of $CD19^+$ counts in advancing pregnancy stratified by HIV status
The mean $CD19^+$ cell count increased in advancing pregnancy among the HIV-negative, while HIV-positive women showed an increase in the second trimester with a decrease is noted in the third trimester among the HIV-positive women.

To demonstrate the trends of Natural Killer ($CD56/16^+$) counts, the mean $CD56/16^+$ counts were measured during the first, second and third trimesters of advancing pregnancy and presented graphically in Figure 4.6. Among the HIV-positive and HIV-negative participants, the mean $CD56/16^+$ counts for the first, second and third trimesters were 237, 215, 217 cells per mm^3 and 234, 194, 183 cells per mm^3, respectively. These results demonstrate a decreasing trend in the mean $CD56/16^+$ counts as pregnancy advanced in both groups.

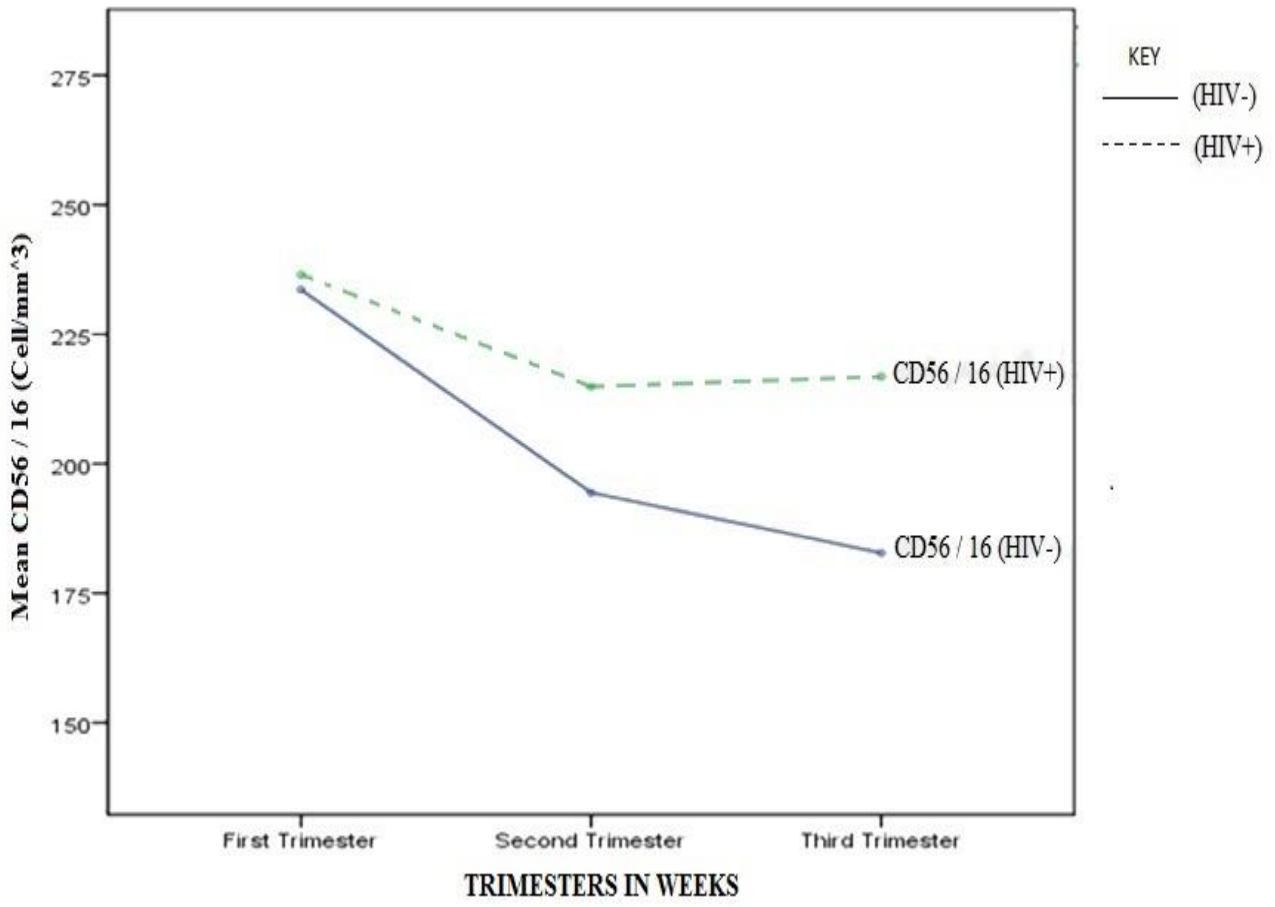


Figure 4.6: The trends of CD56/16$^+$ counts in advancing pregnancy stratified by HIV status
The trends of CD56/16$^+$ counts in advancing pregnancy demonstrate a decreasing trend as pregnancy advanced in both groups.

To determine the change in lymphocyte counts in advancing pregnancy of both groups, the mean change in CD3$^+$, CD4$^+$, CD8$^+$, CD19$^+$, CD56/CD16$^+$ were measured at second trimester compared to the first trimester and in the third trimester compared to the second and the first trimester of advancing pregnancy in both groups (Table 4.6).

Among the HIV-positive, the mean change in CD3$^+$ counts during the second trimester was comparable (P=0.099) to that of the first trimesters -41 (95% CI: -89, 8) cells per mm^3. However, there were higher mean change in CD3$^+$ counts in the third trimester compared to those of the second trimester [146 (95% CI: 98, 194), (P<0.0001)] and first trimester [105 (95% CI: 57, 153) P<0.0001]

cells per mm^3. Among the HIV-negative participants the mean change in $CD3^+$ counts during the second trimester was lower ($P<0.0001$) compared to that of the first trimester, -115 (95% CI: -163, -67) cells per mm^3. Likewise, the mean change in $CD3^+$ counts for the third trimester was also lower ($P=0.038$) compared to that of the first trimester, -51 (95% CI: -99, -3) cells per mm^3 but higher ($P=0.009$) compared to that of the second trimester, 64 (95% CI: 16, 112) cells per mm^3.

Among the HIV-positive participants, the mean change in $CD4^+$ counts in the second compared to the first trimesters was lower [-40 (95% CI: -55, -25), $P<0.0001$] cells per mm3. There was a comparable ($P=0.324$) mean change in $CD4^+$ counts in the third trimester compared to the second trimester -8 (95% CI: -23, -8) cells per mm^3. The mean change in $CD4^+$ counts in the third trimester was also lower ($P<0.0001$) compared to the first trimester, -48 (95% CI: -63, -33) cells per mm^3. The HIV-negative participants showed a lower ($P<0.0001$) mean change in $CD4^+$ counts during the second trimester compared to that of the first trimester, -29 (95% CI: -44, 14) cells per mm^3. The mean change in $CD4^+$ counts for the third trimester was higher compared to that of the first trimester, [17 (95% CI: 2, 32,) cells per mm^3 $P=0.031$] cells per mm3 and second trimester, [46 [(95% CI: 30, 61) cells per mm^3, $P<0.0001$] among the HIV-negative participants.

Among the HIV-positive participants, the mean change in $CD8^+$ counts in the second trimester compared to the first trimesters was comparable [2 (95% CI: -49, 45) cells per mm^3, ($P=0.927$)]. The mean change in $CD8^+$ counts for the third trimester was also higher relative to those of the first ($P=0.005$) and the second ($P=0.007$) trimesters, the mean changes being 74 (95% CI: 22, 126), and 71 (95% CI: 20, 123) cells per mm^3, respectively. Among the HIV-negative participants, comparable ($P=0.122$) mean change in $CD8^+$ counts in the second trimester to that of the first trimester, -41 (95% CI: -92, 11) cells per mm^3 were observed. The mean change in $CD8^+$ counts for the third

trimester was also comparable to that of the first trimester, [-40 (95% CI: -92, 12) cells per mm^3, P=0.231] and the second trimester [1 (95% CI: 30, 61) cells per mm^3, P=0.974].

Among the HIV-positive, the mean change in CD19+ in the second trimester compared to that of the first trimester 32 (95% CI: 18, 46) cells per mm^3, was higher (P<0.0001). The mean change in $CD19^+$ for the third trimester was comparable (P=0.274) to that of the first trimester, 8 (95% CI: -6, 22) cells per mm^3 but lower (P=0.001) to that of the second trimester, -24 (95% CI: -38, -10) cells per mm^3. Among the HIV-negative, the mean change in $CD19^+$ during the second trimester was higher (P<0.0001) to that of the first trimester, 27 (95% CI: 12, 41) cells per mm^3. The mean change in $CD19^+$ for the third trimester was also higher (P<0.0001) to that of the first trimester, 28 (95% CI: 13, 42) cells per mm^3 and comparable (P=0.842) to that of the second trimester, 1 (95% CI: -31, 8) cells per mm^3.

Among the HIV-positive, the mean change in $CD56/16^+$ counts in the second trimester compared to the first trimester was lower [-22 (95% CI: -41, 2) cells per mm^3, P=0.028]. The mean change in $CD56/16^+$ counts for the third trimester was lower (P=0.045) to that of the first trimester, -20 (95% CI: -39, 0) cells per mm^3, but comparable (P=0.846) to that of the second trimester, 2 (95% CI: -17, 21) cells per mm^3. Among HIV-negative participants, the mean change in $CD56/16^+$ counts during the second trimester was lower (P<0.0001) to that of the first trimester, -39 (95% CI: -58, -20) cells per mm3. The mean change in $CD56/16^+$ counts for the third trimester was also lower (P<0.0001) and comparable (P=0.236) to those of the first trimester, -51 (95% CI: -70, -32) cells per mm^3 and the second trimester (-12 (95% CI: -31, 8), cells per mm^3, respectively.

Table 4.6: The change in lymphocyte counts in advancing pregnancy of HIV-infected and HIV-non-infected women

		HIV-Positive (n=44)		HIV-negative (n=44)	
Lymphocyte		**Mean change (95% CI) *P*-value**		**Mean change (95% CI) *P*-value**	
	Trimester	**1**	**2**	**1**	**2**
CD3$^+$	**2**	-41(-89, 8) *P*=0.099	N/A	-115(-163, -67) ***P*≤0.001**	N/A
	3	105(57, 153) ***P*<0.0001**	146(98, 194) ***P*≤0.001**	-51(-99, -3) ***P*=0.038**	64(16, 112) ***P*=0.009**
CD4$^+$	**2**	-40(-55, -25) ***P*<0.0001**	N/A	-29(-44, 14) *P*≤0.001	N/A
	3	-48(-63, -33) ***P*<0.0001**	-8(-23, 8) *P*=0.324	17(2, 32) ***P*=0.031**	46(30, 61) ***P*<0.0001**
CD8$^+$	**2**	2(-49, 45) *P*=0.927	N/A	-41(-92, 11) *P*=0.122	N/A
	3	74(22, 126) ***P*=0.005**	71(20, 123) ***P*=0.007**	-40(-92, 12) *P*=0.231	1(-51, 53) *P*=0.974
CD19$^+$	**2**	32(18, 46) ***P*<0.0001**		27(12, 41) ***P*<0.0001**	
	3	8(-6, 22) *P*=0.274	-24(-38, -10) ***P*=0.001**	28(13, 42) ***P*<0.0001**	1(-13, 16) *P*=0.842
CD56/16$^+$	**2**	-22(-41, -2) ***P*=0.028**	N/A	-39(-58, -20) ***P*<0.0001**	
	3	-20(-39, 0) ***P*=0.045**	2(-17, 21) *P*=0.846	-51(-70, -32) ***P*<0.0001**	-12(-31, 8) *P*=0.236

Change in lymphocyte counts in advancing pregnancy of HIV-infected and HIV-non-infected women were measured using repeated measures analysis of variance. Among the HIV-positive, significant changes were only observed in CD8$^+$ [in the third trimester compared to the first (*P*=0.005) and second trimester (*P*=0.007) compared to first] different from the HIV-negative group. N/A=Not applicable.

To determine the mean differences in lymphocyte counts between the two groups in advancing pregnancy, the mean differences in CD3$^+$, CD4$^+$, CD8$^+$, CD19$^+$, CD56/CD16$^+$ between HIV-positive and HIV-negative participants were measured during the first trimester and after that in the second and third trimesters of advancing pregnancy (Table 4.7). The mean differences in CD3$^+$ cell count between HIV-positive and HIV-negative were significant in all trimesters of pregnancy, third trimester, [474 (95% CI: 386, 562) cells per mm^3, *P*<0.0001], second trimester [393 (95% CI: 293,

492) cells per mm^3, $P<0.0001$] and first trimester, [318 (95% CI: 216, 421) cells per mm^3, $P<0.0001$].

The mean difference in $CD4^+$ cell count between HIV-positive and HIV-negative was only significant in the third trimester [-68 (95% CI: 1-6, 30) cells per mm^3, $P=0.001$]. In the second trimester, [-15 (95% CI: 54, 25) cells per mm^3, $P=0.464$] and the first trimester, [-3 (95% CI: -43, 36), $P=0.864$] cells per mm^3 the mean differences in $CD4^+$ counts was comparable. The mean differences in $CD8^+$ counts between HIV-positive and HIV-negative were significant in all trimesters i.e. the third trimester [474 (95% CI: 400,548) cells per mm^3, $P<0.0001$], the second trimester [404 (95% CI: 314, 493) cells per mm^3, $P<0.0001$] and the first trimester, [360 (95% CI: 274, 447) cells per mm^3, $P<0.0001$]. The mean differences in $CD19^+$ counts between HIV-positive and HIV-negative were also significant in all trimesters, the third trimester [-49 (95% CI: -68,-30) cells per mm^3, $P<0.0001$], the second trimester [-24 [(95% CI: -42,-4) cells per mm^3, $P=0.021$] and first trimester [-29 (95% CI: -47,-10) cells per mm^3, $P=0.003$] cells per mm^3. Regarding $CD56/16^+$, the mean differences between the HIV-positive and the HIV-negative participants were comparable in the three trimesters. However, the differences kept increasing, [3 (95% CI: -28, 44) cells per mm^3, $P=0.888$], 21[(95% CI: -17, 58) cells per mm^3, $P=0.280$] and 34 [(95% CI: -8, 76) cells per mm^3, $P=0.114$] cells per mm^3 in the first, second through to the third trimester, respectively.

Table 4.7: The differences in lymphocyte counts between HIV-infected and HIV-non-infected women in Advancing Pregnancy

Variable.		HIV-positive (n=44)	HIV-negative (n=44)	Difference (95% CI)	
	Trimester	Mean ± SEM	Mean ± SEM	(Mean HIV+) - (Mean HIV-)	*P*-Value
$CD3^+$	1	1571 ±26.4	1253 ±44.5	318(216, 421)	**<0.0001**
	2	1531 ±29.7	1138 ±4.6	393(293, 492)	**<0.0001**
	3	1676 ±21.4	1202 ±38.6	474(386, 562)	**<0.0001**
$CD4^+$	1	634 ±12.3	637 ±15.9	-3(-43, 36)	0.864
	2	594 ±11.8	608 ±15.9	-15(-54, 25)	0.464
	3	586 ±11.1	654 ±15.6	-68(1-6, 30)	**0.001**
$CD8^+$	1	875 ±26.2	515 ±34.7	360(274, 447)	**<0.0001**
	2	877 ±34.5	474 ±29.2	404(314, 493)	**<0.0001**
	3	949 ±25.3	475 ±27.7	474(400, 548)	**<0.0001**
$CD19^+$	1	169 ±5	198 ±7.7	-29(-47, -10)	**0.003**
	2	201 ±6.8	225 ±7.2	-24(-42, -4)	**0.021**
	3	177 ±3.6	226 ±8.9	-49(-68, -30)	**<0.0001**
$CD56/16^+$	1	237 ±12.9	234 ±16.2	3(-28, 44)	0.888
	2	215 ±10.2	194 ±15.9	21(-17, 58)	0.280
	3	217 ±9.2	183 ±19.2	34(-8, 76)	0.114

Test for differences in lymphocyte counts between the study groups was done using Wilcoxon two-sample test. Significant differences between HIV-positive and HIV-negative of $CD3^+$, $CD8^+$ (*P*<0.0001 in both cases in all trimesters), $CD19^+$ [first; *P*=0.003, second; *P*=0.021 and third; *P*<0.0001] and $CD4^+$ (in the third trimester, *P*=0.001) were observed.

CHAPTER FIVE

DISCUSSION

The present study was designed to demonstrate the profiles of progesterone, systemic cytokines and blood lymphocyte levels during pregnancy in HIV-infected and HIV-non-infected women. During pregnancy, progesterone, systemic cytokines and lymphocyte responses have a vital role in the success or failure of pregnancy (Aagaard-Tillery *et al.*, 2006; Tuckey, 2005). On this basis, the present study provides insight into the profiles of progesterone levels, systemic cytokines and lymphocyte counts observed in cohorts of HIV-infected pregnant women compared to HIV-non-infected pregnant women in Western Kenya. In the current study, demographic data regarding age, parity, gestational age, and marital status were comparable in both groups. Thus demographic characteristics of the study participants may not have contributed to the differences observed in the present study findings.

5.1.: The profiles of progesterone levels in advancing pregnancy of HIV-infected and HIV-non-infected women

The increasing trend of progesterone observed in the present study is comparable to previous studies which showed that progesterone remain significantly high and increases as pregnancy advances in HIV-negative populations (Arck *et al*., 2007; Di Renzo *et al*., 2005; Piekorz, Gingras, Hoffmeyer, Ihle, & Weinstein, 2005), though these studies did not consider the HIV-positive cases. The current finding and the reviewed previous studies, thus support the hypothesis that progesterone continuously increase in advancing pregnancy. The continued increase of progesterone in advancing pregnancy irrespective of the HIV status could be explained partly by the fact that, progesterone is a

key reproductive hormone during pregnancy produced to enhance the success of pregnancy (Di Renzo *et al*., 2005). Progesterone is known to relax the uterus during pregnancy, which enables the retention of the fetus by inhibition of contraction-related protein (Beltman *et al*., 2009; Zakar & Mesiano, 2011). Endocrine status has been observed to be responsible in at least part of the induction of immune-modulation among other mechanisms in advancing pregnancy (Muzzio *et al*., 2014). However, it remains to be determined whether additional roles and mechanisms exist for progesterone during pregnancy and whether or not there is any effect post-partum in both HIV-positive and HIV-negative groups.

The current study also demonstrated significant lower progesterone levels in all trimesters of pregnancy among the HIV-positive women compared to HIV-negative women. These results are consistent with those observed in a previous study (Amirhessami-Aghili & Spector, 1991), which showed that HIV infection in human placenta leads to a decrease of up to seventy percent (70%) in the production of progesterone in HIV-infected cell cultures compared to HIV-non-infected. Potential clinical consequences of such differences in progesterone levels could lead to complications and adverse birth outcomes of pregnancies associated with HIV-infection (Amirhessami-Aghili & Spector, 1991; Ticconi *et al*., 2003). However, in contrast to these earlier studies, the present study did not report any complication during pregnancy among the HIV-infected women compared to HIV-negative women despite the low progesterone levels reported. The mechanisms under which HIV infection can concomitantly lead to a reduction in progesterone production in pregnancy remains to be determined. Regarding the significantly lower mean change in progesterone levels in all trimesters of pregnancy among the HIV-positive compared to the HIV-negative pregnant women, this is the first study that has demonstrated this pattern in both HIV-positive and HIV-negative pregnant women across the trimesters. This aspect makes the present

study unique and an addition to the existing knowledge. The observed significant changes in progesterone between trimesters of pregnancy are a clear indication that pregnancy can only survive if there is a significant increase of progesterone as pregnancy advances.

The observed results on progesterone indicate that progesterone is continuously being produced and would increase as pregnancy advances despite HIV infection. However, there is also an indication that HIV infection may reduce its production during pregnancy as shown by the lower levels among the HIV-infected women compared to HIV-non-infected women. Although abortions were not reported in the present study, the current findings suggest that advanced cases of HIV-infection could result to much lower progesterone levels during pregnancy due to their deteriorated health status. All HIV-positive women were asymptomatic cases and received ARV treatment upon enrollment which may have improved the health of HIV-infected persons hence reducing chances of any abortion. Therefore, it would be prudent to monitor progesterone levels during pregnancy to avoid possible complications or abortions among the HIV-infected pregnant women. Additional studies, focusing on the succinct roles of the endocrine response especially in HIV infection during pregnancy thus, remain to be explored further.

5. 2: The profiles of T_H1 (IL-2, IFN-γ, TNF) and T_H2 (IL-10, IL-6, IL-4) type of blood cytokine levels in advancing pregnancy of HIV-infected and HIV-non-infected women

The current study present data on profiles of systemic cytokine levels in co-joint cases of HIV infection and pregnancy compared to HIV-non-infected pregnant women. In line with the current study , a decline in the production of IL-2 in healthy pregnancy was observed in a previous study with its lowest levels observed in the third trimester (Marzi *et al*., 1996). Another different study on pregnant women also concurred with the present study and showed no significant difference in levels

in the production of intracellular IL-2 between HIV-infected and HIV-non-infected pregnant women (Sutton *et al*., 2004). In contrast to the present study a difference in IL-2 between the HIV-infected patients and normal healthy volunteers (Alonso *et al*., 2000) was observed. However, these studies did not follow up the participants and did *in vitro* analysis which different from the present study. In the present study and in respect to mean change in IL-2, no other study has shown such findings, and thus this is a great addition to the existing knowledge and calls for more studies to confirm the present findings. Regarding HIV-infected women during pregnancy, an increase of IL-2 in the third trimester was observed in the present study. This result is supported by a previous study that higher levels of inflammatory cytokines were noted in late pregnancy which contributed to an increased chance of maternal-fetal death (Richardson & Weinberg, 2011). However, the present study did not report any abortion.

The decline noted in IL-2, an inflammatory cytokine, in both groups is an indication that pregnancy independently without HIV infection suppresses its production. However, an increase of IL-2 among the HIV-infected in the third trimester observed in the present study could be explained by the fact that HIV infection is an inflammatory condition and thus the increase IL-2 production. Interleukin -2 remain the primary inflammatory cytokine and therefore any major increase would affect pregnancy and could result in complicated pregnancy. Therefore, monitoring the changes that would occur among the HIV-infected pregnant women would be helpful to clinicians to manage possible complications during pregnancy.

The present study demonstrates a decline of IFN-γ as pregnancy advanced. In support of this finding a previous study also indicated that hormones produced during pregnancy are associated with low levels of IFN-γ (Laresgoiti-Servitje *et al*., 2010). HIV infection, on the other hand, is an IL-2 inflammatory phenomenon and according to a previous study, it has been shown to stimulate IFN-γ

production by $CD8^+$ T-cells (Lai, Lin, Liao, Tang, & Chen, 2009). This information is supported by the present study, which observed higher IFN-γ among the HIV-infected compared to the HIV-non-infected women. Also in line with the present study, a previous study noted that proliferative T-cells producing IL-2 and IFN-γ were robust in response to HIV infection (Alatrakchi *et al*., 2006). In contrast to the present study, another previous study observed that IFN-γ production was lower in polyclonally activated T-cell cultures from HIV-infected pregnant compared to controls (Hygino *et al*., 2012).

The significant higher IFN-γ levels among the HIV-positive pregnant women at first trimester compared to the HIV-negative group indicate that HIV infection could have initiated a T_H1-mediated immune response to counteract the infection. The observed higher levels of IFN-γ levels among the HIV-positive pregnant women compared to the HIV-negative, support the fact that HIV infection is an inflammatory disease which is associated with an increase in IFN-γ production with or without pregnancy. Higher IFN-γ levels during the first trimester in both groups, with a decline as pregnancy advances could be explained by the fact that IFN-γ is produced in early pregnancy for proper implantation of fetus (Murphy *et al*., 2009) and suppression thereafter by hormones produced in pregnancy (Laresgoiti-Servitje *et al*., 2010) to avoid pregnancy loss (Murphy *et al*., 2009). Previous studies related to the present study were only keen on the levels of IFN-γ without going to the details of the changes between trimesters. This aspect makes the current study unique and an addition to the existing knowledge. It has been shown that IFN-γ over-activity could affect immune-regulatory systems in pregnancy (Wilczyński *et al*., 2003) resulting in loss of pregnancy (Murphy *et al*., 2009). However the present study did not report any loss of pregnancy and this could be attributed to the fact that HIV-infected women were on ARV treatment. It is on this basis and the higher levels observed in the present findings that regular monitoring of IFN-γ would be of great importance to

clinicians to ensure minimal complications or abortions occur during pregnancy among the HIV-infected women. These results also offer a baseline data on HIV-positive pregnant women regarding IFN-γ for future studies surrounding this cytokine.

Higher TNF levels among the HIV-infected women in the present study was supported by a previous investigation which observed that TNF production by antigen-activated peripheral blood mononuclear cells was also higher in HIV-infected pregnant women than in controls (Alonso *et al*., 2000). This result is also supported by other previous studies that indicate that HIV proteins target TNF receptor signaling, resulting in apoptosis of T-cells (Herbein & Khan, 2008) and that TNF is crucial in causing inflammation, the hallmark of HIV infections (Kumar, Abbas, & Herbein, 2013). About the present study and as highlighted in the previous studies, pregnancy, and HIV infection independently influence TNF production.

The TNF decline in advancing pregnancy compared to the first trimester in both groups observed in the current study imply that pregnancy in HIV-infected or HIV-non-infected women has a suppressive effect on TNF. A previous study noted that high levels of TNF resulted in abortion (Makhseed *et al*., 2000) hence this trend could be a normalcy to maintain pregnancy. A previous study has shown that progesterone inhibits HIV replication during pregnancy by reducing TNF levels, a phenomenon essential for viral replication (Muñoz *et al*., 2007) thus the decrease of TNF seems to be subject to increase of progesterone in advancing pregnancy in both groups. In addition to this explanation, $CD19^{+}$ have also been reported to increase in pregnancy and strongly down-regulate TNF production (Rolle *et al*., 2013).

A previous study has indicated that anti-TNF during pregnancy are promising in reducing abortions. However it was noted that it should be avoided at the time of conception (Verstappen, King, Watson,

Symmons, & Hyrich, 2011). Similarly, another study suggested that the placenta is very sensitive to pro-inflammatory signals especially at conception and that TNF could be a good target for managing risks to the fetus during pregnancy (Carpentier, Dingman, & Palmer, 2011). The present study findings, therefore, indicate that TNF is a medically important cytokine that should be monitored and possibly be controlled during pregnancy among the HIV-infected women. The monitoring of TNF during pregnancy would assist in the timely management and reduce incidences of complicated pregnancies or abortion due to TNF. Since inflammation is a hallmark of HIV infection, the utilization of TNF inhibitors may be significant in managing HIV-infected pregnant women and ultimately lead to successful pregnancy. The present study thus provides a good baseline for future studies of possible therapeutic strategies in the management of the HIV-infected women regarding TNF and other cytokines. Furthermore, the detailed understanding of T_H1 cytokines activity that may cause complicated pregnancy or abortion would form a major base for therapeutic strategies for a possible blockade.

The current study demonstrates an increasing trend of IL-10 levels in advancing pregnancies with or without HIV infection in both groups. This result is in line with a previous study that noted that the success of healthy pregnancy is associated with the production of IL-10 (Saito *et al*., 2010). Levels of IL-10 were shown to be low during early pregnancy and markedly increased in last days of pregnancy and post-partum (Bento *et al*., 2009). To explain the high levels in both groups, a previous study has indicated that IL-10 down-regulates inflammatory activities (Ouyang, Rutz, Crellin, Valdez, & Hymowitz, 2011) due to HIV infection and pregnancy in all trimesters (Okun & Coussons-Read, 2007). In other related studies in support of the present findings, it was observed that there was a significantly higher *in vitro* production of IL-10 by peripheral blood mononuclear cells during pregnancy than 2 years after pregnancy (Amoudruz *et al*., 2006) and higher levels in

early pregnancy (Pantaleo *et al*., 2013) and last trimester of pregnancy (Doria *et al*., 2004) and at delivery (Makhseed *et al*., 2000). In support of the present findings and in the context of co-joint cases of HIV and pregnancy, higher levels of IL-10 were produced by activated T-cell cultures from HIV-infected pregnant women compared to their controls and were shown to control HIV replication (Bento et al., 2009). Similarly in another study, higher production of IL-10 levels was obtained in T-cell cultures from pregnant women following the addition of HIV antigens (Hygino *et al*., 2012). A distinct emergence of IL-10-producing CD8+ T-cells, which is HIV-specific has also observed in a previous study (Clutton *et al*., 2013). This information offers a good explanation for the present study finding which found higher and continuous increase of IL-10 among the HIV-infected women. However, the highlighted studies did not present results on mean change in IL-10 as pregnancy advanced. This explains the uniqueness of the present study.

The secretion of high levels of IL-10, therefore, seems to be vital in enhancing the success of pregnancy in both cases of HIV-infected and HIV-non-infected women. The higher IL-10 levels among the HIV-infected pregnant women appear to be as a result of the synergic effect of both pregnancy and HIV infection. These findings on IL-10 are a clear indication that for pregnancy to be successful in cases of HIV infection or not, then significant high production of IL-10 is paramount. On this basis, IL-10 seem promising as a therapeutic option in the management of the HIV-infected pregnant women presenting with significantly elevated levels of abortive T_H1 cytokines. The persistently high levels of IL-10 would be utilized in progressive dysfunction of the immune response attributed to HIV infection. It is also on this basis that IL-10 monitoring during pregnancy is important to be sure that there is an environment that is conducive to foetal survival especially among the HIV-infected pregnant women. Lower levels of it would indicate a risk of complications during pregnancy.

Similar to the present study findings, higher IL-6 levels have been reported in HIV-infections (Á. H. Borges *et al*., 2015). A previous study also observed that depletion of HIV-specific CD8+ T-cells led to the up-regulation of IL-6 production (Clutton *et al*., 2013). In line with the present study and to explain this, a previous study observed higher IL-6 in HIV infection and was attributed to be a cause of inflammation (A. H. Borges *et al*., 2014). According to a previous study, IL-6 increased in the third trimester of gestation during pregnancy which contrasts the present study in the reference to HIV-non-infected women (Doria *et al*., 2004). In concurrence to the present study, with respect to pregnant HIV-non-infected women, a previous study showed significantly higher spontaneous *in vitro* production of IL-6 by peripheral blood mononuclear cells during pregnancy than two years postpartum (Amoudruz *et al*., 2006). Significantly higher concentrations of IL-6 were also observed at normal delivery in a healthy pregnancy in another study (Makhseed *et al*., 2000). A previous study has indicated that high IL-6 has been associated with HIV disease progression risk (Shive *et al*., 2012). The present study did not observe adverse progression among the HIV-infected women; however this literature is an important indication of the important role of the IL-6. The reason for not reporting adverse progression of the HIV-infected in the present study could be attributed to the fact that the participants were on ARV.

The present study observed that IL-6 levels were comparable in most of the analysis; however, an indication of HIV perturbation was HIV infection as shown by opposite trends when the two study groups were compared. Only a few studies have been conducted regarding IL-6 during pregnancy, and none has shown the mean changes as pregnancy advanced and the possible effects of higher IL-6 among the HIV-infected pregnant women. In spite of this, IL-6 understanding has important consequences for therapeutic strategies since IL-6 has been identified as an acute inflammatory

cytokine. Interleukin-6 is also a known B-cell differentiation factor, and thus its important roles can never be ignored.

The success of healthy pregnancy has been shown to be associated with the production of IL-4 (Saito *et al*., 2010). In support of this, the present study observed an increase in IL-4 as pregnancy advanced, which can be explained by a previous study that reported that progesterone promote its production (Piccinni *et al*., 2000). Interleukin-4 start to be produced in early gestation since it could act as a potent inhibitor of inflammatory responses (Pantaleo *et al*., 2013). The receptors for IL-4 are required for differentiation of T-helper cells into the T_H2 (Liao, Lin, Wang, Li, & Leonard, 2011) and thus plays a critical role as an anti-inflammatory cytokine during pregnancy thereby providing a conducive environment for pregnancy (Makhseed *et al*., 2000). Interleukin-4 alone has been indicated to have the ability to activate all T_H2 effector activities (Fallon *et al*., 2002). These statements largely support and explain the observed results in the present study, which shows an increase of IL-4 in advancing pregnancy of both HIV-infected and HIV-non-infected women. However, an earlier study contrasts this observation in that IL-4 is not important for fetal survival even during allogeneic pregnancy (Fallon *et al*., 2002). No study has investigated IL-4 in respect to co-joint cases of HIV infection and pregnancy and also none has reported the mean change in IL-4 as pregnancy advanced. The significant higher IL-4 levels in the second trimester among the HIV-infected women display that its anti-inflammatory role remain vital during co-joint cases of pregnancy, and HIV infection and synergy of the conditions came into play during this trimester. Therefore IL-4 has been shown to be an important anti-inflammatory cytokine, which could be a potential therapeutic target to manage HIV-infected women presenting with the destructive T_H1 type of cytokines during pregnancy.

On Overall, the current findings on systemic cytokines concur with a previous study (Richardson & Weinberg, 2011) which observed higher plasma concentrations of T_H1 (known inflammatory) cytokines throughout pregnancy in HIV-infected compared with HIV-non-infected pregnant women. The higher blood levels of the T_H1 type of cytokines could be due to the pathophysiology of HIV infection which tends to produce inflammatory cytokines during primary infection (Galli *et al*., 2001). In both groups, a shift towards the T_H2 type of cytokines was also observed. This result is explained by the fact that progesterone induce anti-inflammatory phenomenon with T_H2 cytokines switch in pregnancy (Arck *et al*., 2007). T_H2 cytokines provide a conducive environment for successful implantation and pregnancy (Keelan *et al*., 2003; Laird *et al*., 2003) and at birth (Keelan *et al*., 2003). Higher levels of T_H1 cytokines have been observed in cases of abortion as compared to normal pregnancy, indicating a shift towards T_H2 in normal pregnancy and a T_H1-shift in unexplained abortions (Raghupathy *et al*., 2000). Therefore, most studies including the current study support the hypothesis that normal pregnancy is an anti-inflammatory bias. Since both HIV infection and pregnancy have been shown to affect T_H1 and T_H2 systemic cytokine, it would be critical to monitor them during pregnancy.

The alterations of systemic cytokine patterns in pregnancy in part are related to endocrine changes with progesterone as a potent modulator of cytokine expression (Østensen *et al*., 2006). The overall decrease in pro-inflammatory cytokines and increase in anti-inflammatory cytokines as pregnancy advances reported by the present study and a recent study (Denney *et al*., 2011) supports the current concepts of immune-regulation for the success of pregnancy. Importantly, the current study present data on mean change in T_H1 and T_H2 type of cytokine, which is not reported in the previous studies. Therefore this makes the current study unique and fills a gap in the existing knowledge on the impact of HIV infection on T_H1 and T_H2 cytokines during pregnancy. The present study investigated on T_H1

and T_H2 type of cytokines only and thus future studies should focus on a more expansive experiment that can provide a better understanding of the dynamic profiles of other cytokines not considered in the present study.

5.3: The profiles of lymphocyte counts in advancing pregnancy of HIV-infected and HIV-non-infected women.

Pregnancy is reported as a physiologically immuno-compromised state. Thus any alterations in lymphocyte cell types may occur in advancing pregnancy (Santner-Nanan *et al*., 2013). The present study results were comparable to a previous study of pregnant women without HIV infection in which it was observed that $CD3^+$ counts were slightly elevated (Kühnert *et al*., 1998) indicating that pregnancy independently stimulates T-cell production. The higher turnover of $CD3^+$ counts among the HIV-positive pregnant women could be due to the fact that HIV infection leads to activation of T-cells ($CD3^+$) cells hence proliferation and increase in total $CD3^+$ numbers compared to the HIV-non-infected pregnant women. The effector T-lymphocytes ($CD3^+$) have been shown to have important roles in immune responses and consist of T-helper ($CD4^+$) and cytotoxic T-cells ($CD8^+$) (Dong, Martinez, & T, 2010).

Comparable findings to the present study in regard to higher $CD4^+$ counts in the first trimester than in the later parts of pregnancy among the HIV-non-infected women were also observed in a previous study (Chama *et al*., 2009). Thus, these observations show that there is a slight fall in the mean $CD4^+$ count in normal pregnancy without HIV infection. Similar to the present study in the context of HIV-infected pregnant women, in asymptomatic HIV infection, low absolute counts of $CD4^+$ were reported (Brettle *et al*., 1995), however, the researchers explained that the observed results were primarily related to change in the other components of the white cell count as there were no changes

in overall $CD4^+$ percent. In contrast to the present study, a previous study observed that pregnancy has no significant effect on $CD4^+$ counts (Van Benthem *et al*. 2002). In another study, HIV-negative pregnant women revealed a decreasing trend but different from the present study, they had lower $CD4^+$ counts than HIV-positive pregnant women in all trimesters of pregnancy (Ekwempu, Ekwempu, Ikeh, Olabode, & Agaba, 2012). In regards to HIV-positive women, the characteristic lower $CD4^+$ counts is as a consequence of cellular destruction by specific activated cytotoxic $CD8^+$ T-cells (Sousa *et al*., 2002). This would mean that HIV infection can interfere with the $CD4^+$ counts, the main parameter used in monitoring health status of HIV-infected person. The monitoring of $CD8^+$ counts has also come in handy to monitor the level of immune activation, which would influence the depletion rate of the $CD4^+$ in HIV-infected persons. In a general view of immunological change in $CD4^+$ cell count in both HIV-infected and non- infected women, the present study and majority of previous studies seems to support that pregnancy reduces $CD4^+$ counts irrespective of the HIV status.

In line with the present study a previous study has reported that $CD4^+$ count dropped during pregnancy while $CD8^+$ count was consistently higher in HIV-positive than HIV-negative women (Ono *et al*., 2008). The present findings and some of the previous studies in regard to T-cells subsets ($CD4^+$ and $CD8^+$) imply that both HIV and pregnancy affect both $CD4^+$ and $CD8^+$ T-cells since significant alterations are observed in both groups. Higher $CD8^+$ counts among the HIV-positive pregnant women compared to HIV-negative pregnant women is best explained by T-cell homeostasis resulting from HIV infection (McMichael & Rowland-Jones, 2001). The alterations of T-lymphocyte ($CD4^+$ and $CD8^+$) is as a result of immune-regulatory mechanisms during pregnancy (Wilczyński *et al*., 2003) and immune activation in HIV infection (Sousa *et al*., 2002). In contrast and partly in support of the current study, another study observed an increase of both $CD4^+$ and $CD8^+$ counts in

HIV-negative pregnant women while in HIV-infected pregnant women, $CD4^+$ counts were low and $CD8^+$ counts were higher compared to HIV-negative pregnant and non-pregnant women (Mikyas *et al*., 1997). Also in contrast to the present study, other previous studies observed stable mean $CD4^+$ and $CD8^+$ Counts throughout pregnancy (Burns *et al*., 1996; Tuomala *et al*., 1997). Some more studies have reported that there is no alteration in the $CD4^+$ and $CD8^+$ T-cell levels during pregnancy (Van Benthem *et al*., 2002; Tuomala *et al*., 1997; Burns *et al*., 1996), while others have clearly shown that there are change in $CD4^+$ and $CD8^+$ T counts during pregnancy (Chama *et al*., 2009; Ono *et al*., 2008). Despite the fact that researches on T-lymphocytes remain much pronounced, no study was able to investigate the mean changes as pregnancy advanced. The present study thus has contributed to the existing knowledge regarding the mean change in T-lymphocytes that occur per trimester as pregnancy advances.

The present study findings on $CD4^+$ and $CD8^+$ counts have confirmed some of the previous studies. However, majority of the reviewed studies did not longitudinally follow the participants and some did not also consider HIV-infected pregnant women. The follow-up and the comparison of these $CD4^+$ and $CD8^+$ counts between HIV-infected and HIV-non-infected pregnant women give the strength for the present study compared to the previous studies. Immunity to HIV is $CD4^+$ and $CD8^+$ T cell dependent. The $CD4^+$ and $CD8^+$ counts thus remain relevant in HIV infection and pregnancy which explains why these counts should continue to be monitored and utilized in the management of HIV-infected pregnant women.

Similar to the present study, regulatory B-cells, a subset of B-cells with strong tolerance roles, were shown to expand during pregnancy (Muzzio *et al*., 2014). However, an earlier study showed almost no change in $CD19^+$ during pregnancy (Kühnert *et al*., 1998). The $CD19^+$ marker has been indicated to be of crucial importance for B-cell activation and proliferation by T-cell-dependent mechanisms

and activated cells selection into becoming B-memory-cells (Rickert *et al*., 1995) and therefore an important marker for B-cells and humoral immune responses (Krop *et al*., 1996). In another study, $CD19^+$ and the antibodies they produce have been evaluated and shown to have a crucial role in pregnancy success (Muzzio *et al*., 2013) thus giving a clear understanding of the increasing trend of $CD19^+$ counts in the current study. In the reviewed literature no study has evaluated $CD19^+$ among the HIV-infected pregnant women and followed them longitudinally. This gives the present study strength, and the current findings add to the existing knowledge on the impact of HIV infection on B-cell immune responses during pregnancy. The higher $CD19^+$ counts among the HIV-infected compared to HIV-non-infected pregnant women in all trimesters indicate that pregnancy with or without HIV infection influences B-cell immune response. Therefore, the present study findings suggest that $CD19^+$ should be monitored alongside $CD4^+$ and $CD8^+$ counts in the management of HIV-infected pregnant women. Counts of $CD19^+$ would show the competence of humoral arm of the immune response, which would be more helpful to the clinician for better management of the HIV-infected pregnant women.

Concerning Natural Killer ($CD56/16^+$) cells, the present study results demonstrate that pregnancy with or without HIV infection suppresses the production of these cells which otherwise could affect local immune regulatory mechanisms in pregnancy (Wilczyński *et al*., 2003). This general suppression of $CD56/16^+$ is largely because of the fact that progesterone inhibits the activity and turnover of NK cells by blocking their degranulation and suppressing transformation of NK cells into destructive lymphokine activated killer cells (Van Nieuwenhoven, Heineman, & Faas, 2003) otherwise reported in recurrent spontaneous abortions (Emmer *et al*., 2000). Natural Killer cells which predominate in blood circulation demonstrate lower production of cytokines, with an increased cytotoxicity (Moretta *et al*., 2008) thus their suppression partly maintains pregnancy.

Another study on Kuwait women, in agreement with the present findings, also observed a decrease in absolute counts of $CD56/16^{+}$ in the third trimester of pregnancy and a general decline in pregnancy (Mahmoud *et al*., 2001). No previous study has contrasted the current findings. A decrease in absolute counts of $CD56/16^{+}$ in the third trimester of pregnancy have also been shown in a previous study (Mahmoud *et al*., 2001). Lower blood counts of $CD56/16^{+}$ percentages have been shown to occur in women without recurrent miscarriages than in women with recurrent miscarriages (Seshadri & Sunkara, 2014). No previous study has reported the mean change in these cells and followed HIV-infected pregnant women as pregnancy advanced hence making a new insight to the existing knowledge.

Regarding HIV infection during pregnancy, the current results indicate that HIV infection does not necessarily lead in higher levels of $CD56/16^{+}$ as shown by the comparable differences in all trimesters. The ARV treatment to the HIV-infected pregnant women in the present study could have contributed to this finding. However, this remains to be determined if ARV has any effect on counts of $CD56/16^{+}$ as pregnancy advances. Although, the present study indicate that there is no difference of $CD56/16^{+}$ counts between HIV-infected and HIV-non-infected women the role of $CD56/16^{+}$ in abortion cannot be assumed.

Taken together, the lymphocytes remain important and relevant in the immune responses attributed to HIV and pregnancy. Unlike the previous studies highlighted, the present study has been able to determine and compare all the lymphocytes inclusive, to have a clear picture in the form of immune response that dominates in co-joint cases of HIV-infection and pregnancy. The present study has confirmed that pregnancy with or without HIV-infection influences B-cell immune response with adequate suppression of cell-mediated immunity in cases of HIV-infected pregnant women on ARV. The current study provides baseline data for future similar studies that would provide an in-depth

exploration for a better understanding of the complex interaction of HIV, pregnancy and ARV treatment.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSION, AND RECOMMENDATIONS

6.1: Summary of findings

1. The present study observed an increasing trend of progesterone as pregnancy advanced in both groups. The mean change in progesterone during the second, and the third trimesters were significant compared to that of the first trimester in both groups. The mean differences in progesterone between HIV-positive and HIV-negative participants observed were significant in all trimesters of pregnancy.
2. The trends of the cytokines were inconsistent in both groups. Among the HIV-positive women, significant mean change in IL-2 [in the second trimester compared to first and in the third trimester compared to second trimester], IL-6 [in the second trimester compared to first trimester] and IL-4 [in the 3^{rd} trimester, compared to second trimester] were observed different from what was observed among the HIV-negative women. The mean change in IFN-γ, TNF and IL-10 were similar when compared between the two groups. Significant differences between HIV-positive and HIV-negative of IFN-γ [in the first trimester and third trimester], IL-4 [in the second trimester] and IL-10 [in the first trimester, second trimester and third trimester] levels were observed. The mean difference of TNF, IL-2, and IL-6 between HV-infected and HIV-non-infected were comparable in all trimesters.
3. The lymphocytes also showed inconsistent trends in both groups. Among the HIV-positive, significant mean changes were observed in $CD8^+$ [in the third trimester compared to the first and second trimester compared to first] different from the HIV-negative group. Otherwise, the present study observed similar mean change in $CD3^+$, $CD4^+$, $CD19^+$, and $CD56/16^+$ when

compared between the two groups. Significant differences between HIV-positive and HIV-negative of $CD3^+$ (in all trimesters), $CD8^+$ (in all trimesters), $CD19^+$ (in all trimesters) and $CD4^+$ (in the third trimester) were observed. Otherwise, the mean differences in $CD56/16^+$ between HIV-positive and HIV-negative participants were comparable in the three trimesters.

6.2: Conclusions

1. The present study demonstrates an increasing trend of progesterone levels, with significant mean changes in all trimesters of pregnancy in both groups. However, significantly lower levels were demonstrated among the HIV-infected women compared to the HIV-non-infected women.
2. Despite the inconsistent trends and similar results reported for IFN-γ, TNF, and IL-10 when compared between the two groups, HIV-infected pregnant women present with higher levels of IFN-γ, IL-10 and IL-4 with a significant change in IL-2, IL-6 and IL-4 and non-significant mean change in IL-10 different from the HIV-negative group. The mean difference of TNF, IL-2, and IL-6 between HIV-infected and HIV-non-infected are comparable during pregnancy.
3. Similarly, the present study demonstrates inconsistent trends in the lymphocytes as pregnancy advanced in both groups. However, HIV-infected women show significant mean change in $CD8^+$ and non-significant change in $CD3^+$, $CD4^{+,}$ and $CD19^+$ different from HIV-non-infected women with higher levels of $CD3^+$ and $CD8^+$ T cell subset and lower $CD4^+$ and $CD19^+$ counts during pregnancy. Otherwise, comparable differences in $CD56/16^+$ are evident in all trimesters. The study also presents similar mean change in $CD3^+$, $CD4^+$, $CD19^+$, and $CD56/16^+$ when compared between the two groups.

6.3: Policy recommendations

The study recommends:

1. For a regular check on the progesterone levels during pregnancy among the HIV-infected women.
2. That T_H1 and T_H2 type of cytokines should also be considered in routine monitoring of the HIV-infected women.
3. That $CD19^+$ and $CD56/16^+$ counts should be included in the routine tests alongside $CD4^+$ and $CD8^+$ cell count.

6.4. Recommendations for future research work

1. Future research should focus on the succinct roles of the endocrine response regarding other reproductive hormones especially in HIV infection and pregnancy.
2. A more expansive experiment could provide a better understanding of the dynamic profiles of other cytokines not considered in the present study.
3. Future studies should consider using an animal model to understand the profiles of lymphocyte counts without anti-retroviral treatment during pregnancy in HIV-infected women. The present study did not control for antiretroviral use among the HIV-infected because of patients clinical benefit and ethical issues for human participants.

REFERENCES

Aagaard-Tillery, K. M., Silver, R., & Dalton, J. (2006). Immunology of normal pregnancy. *Seminars in Fetal and Neonatal Medicine*, *11*, 279–295.

Alatrakchi, N., Damond, F., Matheron, S., Beretta-Tempelhoff, S., Campa, P., Carcelain, G., & Autran, B. (2006). Proliferative, IFNgamma and IL-2-producing T-cell responses to HIV-2 in untreated HIV-2 infection. *AIDS (London, England)*, *20*(1), 29–34.

Alonso, R., Resino, S., Bellón, J. M., & Muñoz-Fernández, M. A. (2000). Antiretroviral treatment induces a shift to type-2 cytokine responses in HIV-1 infected pregnant women. *European Cytokine Network*, *11*, 647–653.

Amirhessami-Aghili, N., & Spector, S. A. (1991). Human immunodeficiency virus type 1 infection of human placenta: potential route for fetal infection. *J. Virol.*, *65*, 2231–2236.

Amoudruz, P., Minang, J. T., Sundström, Y., Nilsson, C., Lilja, G., Troye-Blomberg, M., & Sverremark-Ekström, E. (2006). Pregnancy, but not the allergic status, influences spontaneous and induced interleukin-1β (IL-1β), IL-6, IL-10 and IL-12 responses. *Immunology*, *119*, 18–26.

Arck, P., Hansen, P. J., Jericevic, B. M., Piccinni, M. P., & Szekeres-Bartho, J. (2007). Progesterone during pregnancy: Endocrine-immune cross talk in Mammalian Species and the role of stress. *American Journal of Reproductive Immunology*.

Beltman, M. E., Lonergan, P., Diskin, M. G., Roche, J. F., & Crowe, M. A. (2009). Effect of progesterone supplementation in the first week post conception on embryo survival in beef heifers. *Theriogenology*, *71*, 1173–1179.

Bento, C. A. M., Hygino, J., Andrade, R. M., Saramago, C. S. M., Silva, R. G., Silva, A. A. L., & Andrade, A. F. B. (2009). IL-10-secreting T cells from HIV-infected pregnant women downregulate HIV-1 replication: effect enhanced by antiretroviral treatment.

AIDS (London, England), *23*, 9–18.

Borges, A. H., O'Connor, J. L., Phillips, A. N., Rönsholt, F. F., Pett, S., Vjecha, M. J., & Lundgren, J. D. (2014). Determinants of IL-6 levels during HIV infection. *Journal of the International AIDS Society*, *17*(4 Suppl 3), 19482.

Borges, Á. H., O'Connor, J. L., Phillips, A. N., Rönsholt, F. F., Pett, S., Vjecha, M. J., & Lundgren, J. D. (2015). Factors Associated with Plasma IL-6 Levels during HIV Infection. *Journal of Infectious Diseases*, *212*(4), 585–595.

Borish, L. C., & Steinke, J. W. (2003). 2. Cytokines and chemokines. *The Journal of Allergy and Clinical Immunology*, *111*(2 Suppl), S460–S475.

Bosch, E., Labarta, E., Crespo, J., Simón, C., Remohí, J., Jenkins, J., & Pellicer, A. (2010). Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for in vitro fertilization: Analysis of over 4000 cycles. *Human Reproduction*, *25*, 2092–2100.

Bowen, J. M., Chamley, L., Keelan, J. A., & Mitchell, M. D. (2002). Cytokines of the placenta and extra-placental membranes: Roles and regulation during human pregnancy and parturition. *Placenta*.

Brettle, R. P., Raab, G. M., Ross, A., Fielding, K. L., Gore, S. M., & Bird, A. G. (1995). HIV infection in women: immunological markers and the influence of pregnancy. *AIDS (London, England)*, *9*, 1177–1184.

Burns, D. N., Nourjah, P., Minkoff, H., Korelitz, J., Biggar, R. J., Landesman, S., & Nugent, R. P. (1996). Changes in CD4+ and CD8+ cell levels during pregnancy and post partum in women seropositive and seronegative for human immunodeficiency virus-1. *American Journal of Obstetrics and Gynecology*, *174*, 1461–1468.

Caligiuri, M. A. (2008). Human natural killer cells. *Blood*, *112*, 461–469.

Carpentier, P. A., Dingman, A. L., & Palmer, T. D. (2011). Placental TNF-?? signaling in illness-induced complications of pregnancy. *American Journal of Pathology*, *178*(6), 2802–2810.

Chama, C. M., Morrupa, J. Y., Abja, U. A., & Kayode, A. (2009). Normal CD4 T-lymphocyte baseline in healthy HIV-negative pregnant women. *Journal of Obstetrics and Gynaecology : The Journal of the Institute of Obstetrics and Gynaecology*, *29*, 702–704.

Clutton, G., Yang, H., Hancock, G., Sande, N., Holloway, C., Angus, B., & Dorrell, L. (2013). Emergence of a distinct HIV-specific IL-10-producing CD8+ T-cell subset with immunomodulatory functions during chronic HIV-1 infection. *European Journal of Immunology*, *43*(11), 2875–2885.

Cohen, J. (1988). Statistical power analysis for the behavioral sciences. *Statistical Power Analysis for the Behavioral Sciences*.

Conde-Agudelo, A., Villar, J., & Lindheimer, M. (2008). Maternal infection and risk of preeclampsia: systematic review and metaanalysis. *Am J Obstet Gynecol*, *198*, 7–22.

Connelly, L. M. (2008). Pilot studies. *Medsurg Nursing*, *17*(6), 411–412.

Cu-Uvin, S., Wright, D. J., Anderson, D., Kovacs, A., Watts, D. H., Cohn, J., & Reichelderfer, P. S. (2000). Hormonal levels among HIV-1-seropositive women compared with high-risk HIV-seronegative women during the menstrual cycle. Women's Health Study (WHS) 001 and WHS 001a Study Team. *Journal of Women's Health & Gender-Based Medicine*, *9*(8), 857–863.

Denney, J. M., Nelson, E. L., Wadhwa, P. D., Waters, T. P., Mathew, L., Chung, E. K., & Culhane, J. F. (2011). Longitudinal modulation of immune system cytokine profile during pregnancy. *Cytokine*, *53*, 170–177.

Di Renzo, G. C., Mattei, A., Gojnic, M., & Gerli, S. (2005). Progesterone and pregnancy. *Current Opinion in Obstetrics & Gynecology*, *17*, 598–600.

Dong, C., Martinez, G. J., & T, C. (2010). T cells : the usual subsets. *Most*, *54*(1), 77030–77030.

Doria, A., Ghirardello, A., Iaccarino, L., Zampieri, S., Punzi, L., Tarricone, E., & Cutolo, M. (2004). Pregnancy, cytokines, and disease activity in systemic lupus erythematosus. *Arthritis and Rheumatism*, *51*, 989–995.

Druckmann, R., & Druckmann, M. A. (2005). Progesterone and the immunology of pregnancy. In *Journal of Steroid Biochemistry and Molecular Biology* (Vol. 97, pp. 389–396).

Ekwempu, A. I., Ekwempu, C. C., Ikeh, E., Olabode, A., & Agaba, E. (2012). Comparison of CD4 Cell Counts in Pregnant HIV-Seropositive and HIV-Seronegative Nigerian Women. *Laboratory Medicine*.

Emmer, P. M., Nelen, W. L., Steegers, E. A., Hendriks, J. C., Veerhoek, M., & Joosten, I. (2000). Peripheral natural killer cytotoxicity and CD56(pos)CD16(pos) cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. *Human Reproduction (Oxford, England)*, *15*, 1163–1169.

Fallon, P. G., Jolin, H. E., Smith, P., Emson, C. L., Townsend, M. J., Fallon, R., & McKenzie, A. N. J. (2002). IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity*, *17*, 7–17.

Galli, G., Annunziato, F., Cosmi, L., Manetti, R., Maggi, E., & Romagnani, S. (2001). Th1 and Th2 responses, HIV-1 coreceptors, and HIV-1 infection. *Journal of Biological Regulators and Homeostatic Agents*, *15*, 308–313.

Gelman, A., Carlin, J. B., Stern, H. S., Dunson, D. B., Vehtari, A., & Rubin, D. B. (2013).

Bayesian Data Analysis, Third Edition (Chapman & Hall/CRC Texts in Statistical Science). CRC Press.

Hedeker, D., Gibbons, R. D., & Waternaux, C. (1999). Sample Size Estimation for Longitudinal Designs with Attrition: Comparing Time-Related Contrasts Between Two Groups. *Journal of Educational and Behavioral Statistics*.

Herbein, G., & Khan, K. A. (2008). Is HIV infection a TNF receptor signalling-driven disease? *Trends in Immunology*, *29*(2), 61–67.

Ho, H. N., Chao, K. H., Chen, C. K., Yang, Y. S., & Huang, S. C. (1996). Activation status of T and NK cells in the endometrium throughout menstrual cycle and normal and abnormal early pregnancy. *Human Immunology*, *49*, 130–136.

Hygino, J., Vieira, M. M., Kasahara, T. M., Xavier, L. F., Blanco, B., Guillermo, L. V. C., & Bento, C. A. M. (2012). The impact of pregnancy on the HIV-1-specific T cell function in infected pregnant women. *Clinical Immunology*, *145*, 177–188.

Johnston, M. I., & Fauci, A. S. (2007). An HIV vaccine--evolving concepts. *The New England Journal of Medicine*, *356*, 2073–2081.

Keelan, J. A., Blumenstein, M., Helliwell, R. J. A., Sato, T. A., Marvin, K. W., & Mitchell, M. D. (2003). Cytokines, prostaglandins and parturition - A review. *Placenta*.

Kidd, P. (2003). Th1/Th2 balance: The hypothesis, its limitations, and implications for health and disease. *Alternative Medicine Review*.

Krop, I., Shaffer, A. L., Fearon, D. T., & Schlissel, M. S. (1996). The signaling activity of murine CD19 is regulated during cell development. *Journal of Immunology (Baltimore, Md. : 1950)*, *157*, 48–56.

Kühnert, M., Strohmeier, R., Stegmüller, M., & Halberstadt, E. (1998). Changes in lymphocyte subsets during normal pregnancy. *European Journal of Obstetrics*

Gynecology and Reproductive Biology, *76*, 147–151.

Kumar, A., Abbas, W., & Herbein, G. (2013). TNF and TNF receptor superfamily members in HIV infection: New cellular targets for therapy? *Mediators of Inflammation*.

Lai, Y. P., Lin, C. C., Liao, W. J., Tang, C. Y., & Chen, S. C. (2009). CD4+ T cell-derived IL-2 signals during early priming advances primary CD8+ T cell responses. *PLoS ONE*, *4*.

Laird, S. M., Tuckerman, E. M., Cork, B. A., Linjawi, S., Blakemore, A. I. F., & Li, T. C. (2003). A review of immune cells and molecules in women with recurrent miscarriage. *Human Reproduction Update*.

Laresgoiti-Servitje, E., Gómez-lópez, N., & Olson, D. M. (2010). An immunological insight into the origins of pre-eclampsia. *Human Reproduction Update*.

Liao, W., Lin, J.-X., Wang, L., Li, P., & Leonard, W. J. (2011). Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nature Immunology*, *12*, 551–559.

Lindgren, S., Martin, C., Anzén, B., Strand, H., Bredberg-Rådén, U., & Ehrnst, A. (1996). Pattern of HIV viraemia and CD4 levels in relation to pregnancy in HIV-1 infected women. *Scandinavian Journal of Infectious Diseases*, *28*, 425–433.

Mahmoud, F., Abul, H., Omu, A., Al-Rayes, S., Haines, D., & Whaley, K. (2001). Pregnancy-associated changes in peripheral blood lymphocyte subpopulations in normal Kuwaiti women. *Gynecologic and Obstetric Investigation*, *52*, 232–236.

Makhseed, M., Raghupathy, R., Azizieh, F., Farhat, R., Hassan, N., & Bandar, A. (2000). Circulating cytokines and CD30 in normal human pregnancy and recurrent spontaneous abortions. *Human Reproduction (Oxford, England)*, *15*, 2011–2017.

Marzi, M., Vigano, A., Trabattoni, D., Villa, M. L., Salvaggio, A., Clerici, E., & Clerici, M.

(1996). Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy. *Clinical and Experimental Immunology*, *106*(1), 127–133.

McKenna, D. (2012). NK cells (CD3 & CD19 depleted apheresis product with overnight stimulation in IL-2). *Blood Transfusion*, *10*, s67.

McMichael, A. J., & Rowland-Jones, S. L. (2001). Cellular immune responses to HIV. *Nature*, *410*, 980–987.

Mikyas, Y., Aziz, N., Harawa, N., Gorre, M., Neagos, N., Nogueira, M., & Plaeger, S. (1997). Immunologic activation during pregnancy: Serial measurement of lymphocyte phenotype and serum activation molecules in HIV-infected and uninfected women. *Journal of Reproductive Immunology*, *33*, 157–170.

Moore, B. B., Moore, T. A., & Toews, G. B. (2001). Role of T- and B-lymphocytes in pulmonary host defences. *European Respiratory Journal*, *18*(5), 846–856.

Moretta, A., Marcenaro, E., Parolini, S., Ferlazzo, G., & Moretta, L. (2008). NK cells at the interface between innate and adaptive immunity. *Cell Death and Differentiation*, *15*, 226–233.

Muñoz, L. D., Serramía, M. J., Fresno, M., Muñoz-Fernández, M. A., Munoz, L. D. A., Serramia, M. J., & Munoz-Fernandez, M. A. (2007). Progesterone inhibits HIV-1 replication in human trophoblast cells through inhibition of autocrine tumor necrosis factor secretion. *Journal of Infectious Diseases*, *195*, 1294–1302.

Murphy, S. P., Tayade, C., Ashkar, A. A., Hatta, K., Zhang, J., & Croy, B. A. (2009). Interferon gamma in successful pregnancies. *Biology of Reproduction*, *80*(5), 848–859.

Muzzio, D., Zenclussen, A. C., & Jensen, F. (2013). The role of B cells in pregnancy: the good and the bad. *American Journal of Reproductive Immunology (New York, N.Y. :*

1989), *69*, 408–12.

Muzzio, D., Zygmunt, M., & Jensen, F. (2014). The role of pregnancy-associated hormones in the development and function of regulatory B cells. *Frontiers in Endocrinology*.

Nixon, D. E., & Landay, A. L. (2010). Biomarkers of immune dysfunction in HIV. *Current Opinion in HIV and AIDS*, *5*(6), 498–503.

Ogundahunsi, O. A., Ogundipe, M. O., Akinola, N. O., Soyinka, O. O., Odewabi, A. O., & Oyegunle V A. (2011). The effect of HIV and antiretroviral therapy on fertility hormones in amenorrheic HIV-positive women. *African Scientist*, *11*(3), 143–146.

Okun, M. L., & Coussons-Read, M. E. (2007). Sleep disruption during pregnancy: How does it influence serum cytokines? *Journal of Reproductive Immunology*, *73*, 158–165.

Ono, E., Dos Santos, A. M. N., Machado, D. M., Succi, R. C. D. M., Amed, A. M., Salomão, R., & De Moraes-Pinto, M. I. (2008). Immunologic features of HIV-1-infected women on HAART at delivery. *Cytometry Part B - Clinical Cytometry*, *74*, 236–243.

Orsi, N. M., & Tribe, R. M. (2008). Cytokine Networks and the regulation of uterine function in pregnancy and parturition. In *Journal of Neuroendocrinology* (Vol. 20, pp. 462–469).

Østensen, M., Förger, F., & Villiger, P. M. (2006). Cytokines and pregnancy in rheumatic disease. In *Annals of the New York Academy of Sciences* (Vol. 1069, pp. 353–363).

Ouyang, W., Rutz, S., Crellin, N. K., Valdez, P. A., & Hymowitz, S. G. (2011). Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual Review of Immunology*, *29*, 71–109.

Palacios, R., Hidalgo, C., Ríos, M. J., Rivero, A., Muñoz, L., Lozano, F., & Santos, J. (2009). Effectiveness and safety of simplification from tenofovir-lamivudine (TDF-3TC) to tenofovir-emtricitabine (TDF-FTC) co-formulation (Truvada®) in virologically suppressed HIV-infected patients on HAART. *European Journal of Clinical*

Microbiology and Infectious Diseases, *28*(4), 399–402.

Pantaleo, M., Piccinno, M., Roncetti, M., Mutinati, M., Rizzo, A., & Sciorsci, R. L. (2013). Evaluation of serum concentrations of interleukin (IL)-4, IL-10, and IL-12 during pregnancy in bitches. *Theriogenology*, *79*, 970–973.

Piccinni, M. P., Scaletti, C., Maggi, E., & Romagnani, S. (2000). Role of hormone-controlled Th1- and Th2-type cytokines in successful pregnancy. *Journal of Neuroimmunology*, *109*, 30–33.

Piekorz, R. P., Gingras, S., Hoffmeyer, A., Ihle, J. N., & Weinstein, Y. (2005). Regulation of progesterone levels during pregnancy and parturition by signal transducer and activator of transcription 5 and 20alpha-hydroxysteroid dehydrogenase. *Molecular Endocrinology (Baltimore, Md.)*, *19*, 431–440.

Raghupathy, R., Makhseed, M., Azizieh, F., Omu, a, Gupta, M., & Farhat, R. (2000). Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. *Human Reproduction (Oxford, England)*, *15*(3), 713–718.

Richardson, K., & Weinberg, A. (2011). Dynamics of regulatory T-cells during pregnancy: Effect of HIV infection and correlations with other immune parameters. *PLoS ONE*, *6*.

Rickert, R. C., Rajewsky, K., & Roes, J. (1995). Impairment of T-cell-dependent B-cell responses and B-1 cell development in CD19-deficient mice. *Nature*, *376*, 352–355.

Rolle, L., Memarzadeh Tehran, M., Morell-García, A., Raeva, Y., Schumacher, A., Hartig, R., & Zenclussen, A. C. (2013). Cutting edge: IL-10-producing regulatory B cells in early human pregnancy. *American Journal of Reproductive Immunology (New York, N.Y. : 1989)*.

Roncarolo, M. G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K., & Levings, M.

K. (2006). Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews*.

Saito, S., Nakashima, A., Shima, T., & Ito, M. (2010). Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy. *American Journal of Reproductive Immunology*.

Santner-Nanan, B., Straubinger, K., Hsu, P., Parnell, G., Tang, B., Xu, B., & Nanan, R. (2013). Fetal-maternal alignment of regulatory T cells correlates with IL-10 and Bcl-2 upregulation in pregnancy. *Journal of Immunology (Baltimore, Md. : 1950)*, *191*, 145–53.

Sasaki, Y., Sakai, M., Miyazaki, S., Higuma, S., Shiozaki, A., & Saito, S. (2004). Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. *Molecular Human Reproduction*, *10*, 347–353.

Sauce, D., Elbim, C., & Appay, V. (2013). Monitoring cellular immune markers in HIV infection: from activation to exhaustion. *Current Opinion in HIV and AIDS*, *8*, 125–31.

Seshadri, S., & Sunkara, S. K. (2014). Natural killer cells in female infertility and recurrent miscarriage: a systematic review and meta-analysis. *Human Reproduction Update*, *20*(3), 429–38.

Shive, C. L., Biancotto, A., Funderburg, N. T., Pilch-Cooper, H. a, Valdez, H., Margolis, L., & Lederman, M. M. (2012). HIV-1 is not a major driver of increased plasma IL-6 levels in chronic HIV-1 disease. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *61*(2), 145–52.

Sousa, A. E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z., & Victorino, R. M. M. (2002). CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *Journal of Immunology (Baltimore, Md. : 1950)*, *169*, 3400–3406.

Spellberg, B., & Edwards, J. E. (2001). Type 1/Type 2 immunity in infectious diseases. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *32*, 76–102.

Sterling, T. R., Vlahov, D., Astemborski, J., Hoover, D. R., Margolick, J. B., & Quinn, T. C. (2001). Initial plasma HIV-1 RNA levels and progression to AIDS in women and men. *The New England Journal of Medicine*, *344*, 720–725.

Sukwit, S., Chuenchitra, T., Samakoses, R., Songprasom, K., Aree, C., Akapirat, S., & Nitayaphan, S. (2001). Flow cytometric detection of intracellular cytokines in peripheral blood of HIV-1 infected Thai children. *Asian Pacific Journal of Allergy and Immunology*, *19*, 107–113.

Sutton, M. Y., Holland, B., Denny, T. N., Garcia, A., Garcia, Z., Stein, D., & Bardeguez, A. D. (2004). Effect of pregnancy and human immunodeficiency virus infection on intracellular interleukin-2 production patterns. *Clinical and Diagnostic Laboratory Immunology*, *11*, 780–785.

Tang, A. W., Alfirevic, Z., & Quenby, S. (2011). Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. *Human Reproduction (Oxford, England)*, *26*(8), 1971–1980.

Temmerman, M., Nagelkerke, N., Bwayo, J., Chomba, E. N., Ndinya-Achola, J., & Piot, P. (1995). HIV-1 and immunological changes during pregnancy: a comparison between HIV-1-seropositive and HIV-1-seronegative women in Nairobi, Kenya. *AIDS (London, England)*, *9*(9), 1057–1060.

Terzieva, V., Popova, D., Kicheva, M., Todorova, Y., Markova, R., Martinova, F., & Yankova, M. (2009). Correlation between the degree of immune activation, production of IL-2 and FOXP3 expression in CD4+CD25+ T regulatory cells in HIV-1 infected

persons under HAART. *International Immunopharmacology*, *9*, 831–836.

Ticconi, C., Mapfumo, M., Dorrucci, M., Naha, N., Tarira, E., Pietropolli, A., & Rezza, G. (2003). Effect of maternal HIV and malaria infection on pregnancy and perinatal outcome in Zimbabwe. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *34*, 289–294.

Tuckey, R. C. (2005). Progesterone synthesis by the human placenta. *Placenta*, *26*(4), 273–81.

Tuomala, R. E., Kalish, L. A., Zorilla, C., Fox, H., Shearer, W., Landay, A., & Burns, D. (1997). Changes in total, CD4+, and CD8+ lymphocytes during pregnancy and 1 year postpartum in human immunodeficiency virus-infected women. *Obstetrics and Gynecology*, *89*, 967–974.

Udenze, I., Amadi, C., Awolola, N., & Makwe, C. C. (2015). The role of cytokines as inflammatory mediators in preeclampsia. *Pan African Medical Journal*, *20*.

van Benthem, B. H. B., Vernazza, P., Coutinho, R. A., & Prins, M. (2002). The impact of pregnancy and menopause on CD4 lymphocyte counts in HIV-infected women. *AIDS (London, England)*, *16*, 919–924.

van Nieuwenhoven, A. L. V., Heineman, M. J., & Faas, M. M. (2003). The immunology of successful pregnancy. *Human Reproduction Update*.

Verstappen, S. M. M., King, Y., Watson, K. D., Symmons, D. P. M., & Hyrich, K. L. (2011). Anti-TNF therapies and pregnancy: outcome of 130 pregnancies in the British Society for Rheumatology Biologics Register. *Annals of the Rheumatic Diseases*, *70*(5), 823–6.

Waruiru, W., Kim, A. A., Kimanga, D. O., Ng’ang'a, J., Schwarcz, S., Kimondo, L., & Maina, W. K. (2014). The Kenya AIDS indicator survey 2012: Rationale, methods, description of participants, and response rates. *Journal of Acquired Immune Deficiency*

Syndromes, *66*(SUPPL. 1), S3–S12.

Wilczyński, J. R. (2006). Immunological Analogy Between Allograft Rejection, Recurrent Abortion and Pre-Eclampsia - the Same Basic Mechanism? *Human Immunology*, *67*, 492–511.

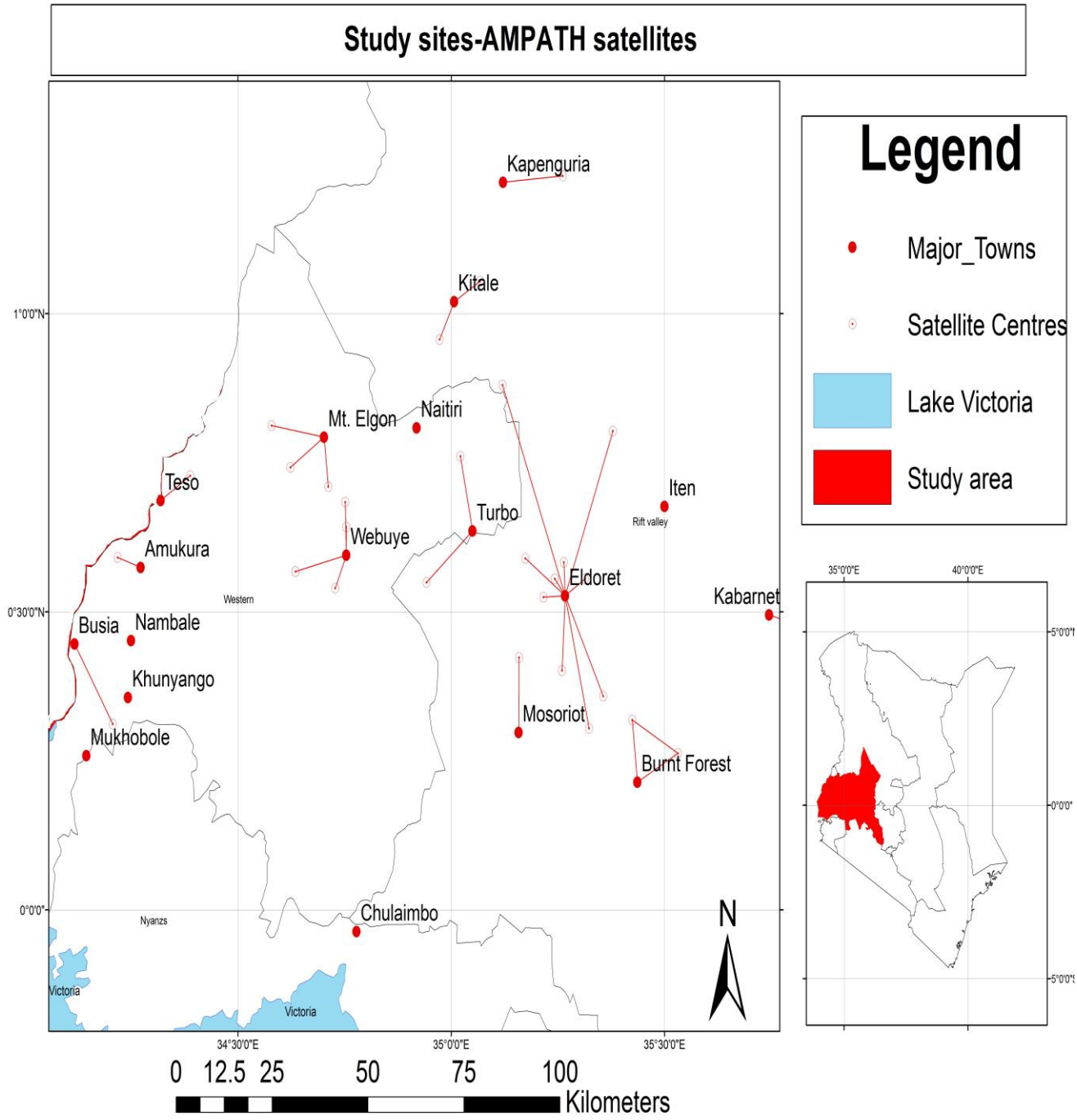
Wilczyński, J. R., Tchórzewski, H., Banasik, M., Głowacka, E., Wieczorek, A., Lewkowicz, P., & Wilczyński, J. (2003). Lymphocyte subset distribution and cytokine secretion in third trimester decidua in normal pregnancy and preeclampsia. *European Journal of Obstetrics Gynecology and Reproductive Biology*, *109*, 8–15.

Zakar, T., & Mesiano, S. (2011). How does progesterone relax the uterus in pregnancy? *The New England Journal of Medicine*, *364*, 972–973.

Zen, M., Ghirardello, A., Iaccarino, L., Tonon, M., Campana, C., Arienti, S., & Doria, A. (2010). Hormones, immune response, and pregnancy in healthy women and SLE patients. *Swiss Medical Weekly*.

APPENDICES

APPENDIX I: AMPATH study sites



APPENDIX II: Sample guideline used to recruit participants

PERSONAL DETAILS

1. Are you willing (and able) to provide an independent written informed consent for enrollment into this study? A) (Yes) b) (no) (qualified medical officers also assess the ability)……..
2. What is your age in years or when where you born? ……
3. What is your current contact?.....
4. What is your marital status? a) (married) b) (single) c)(divorced) d) (window)

PREGNANCY DETAILS

1. Are you pregnant? Answer: a) (yes) b) (no)

   If yes when was your last monthly period?.....
2. Is this your first pregnancy? Yes…….. No……
3. If yes how many times have you become pregnant before?.....
4. Have you ever had a miscarriage or complicated pregnancy? a) (yes) b) (no)

HIV DETAILS

1. Is this the first time to be diagnosed with HIV infection? Answer: a) (yes) b) (no)

If no when did you first test HIV-positive?…….....

2. Have you ever used Anti-retroviral drugs? A) yes b) no

OTHER DETAILS

i. Do you smoke or use any drug substances…….....If yes how frequent in weeks or days…….

ii. Do you suffer or have you suffered from an autoimmune disease, tumor, *Mycobacterium Tuberculosis* (T.B), any acute infection or severe untreated medical illness disease? Answer: a) (yes) b) (no)

If yes state…….....

APPENDIX III: Sample informed consent form

Title: Profiles of progesterone, systemic cytokines, and blood lymphocyte levels during pregnancy in women living with human immunodeficiency virus infection in Western Kenya.

Researcher: Stanslaus Musyoki **Address:** P.O. BOX 3-Eldoret **Phone:** 0721-559-430

Many Thanks for agreeing to be a participant in this study that will take about 18 months. This form outlines the rights as a participant, the aim and the purpose of the study and gives a description of how you will be involved in the study.

The Purposes of this project are: To gain insight and experience in the topic of the profiles of progesterone, systemic cytokines and blood lymphocyte levels during pregnancy in women living with HIV infection in Western Kenya

The methods to be used to collect information: Methods include whole blood cell testing for laboratory variables of interest.

Potential Risks and Discomforts: The only risks associated with the study include; blood drawing which includes pain, discomfort, or bruising. These will be minimized by having only experienced medical personnel perform this procedure.

Potential Benefits to Participants and to Society: The present study results will be available to the caregivers who can then implement. The indirect benefits include increasing knowledge to help provide new interventions in the future.

Confidentiality: All data that is obtained in this study and that will have your identity will only be disclosed with your permission and shall remain confidential. All documents will be kept in a locked

filing cabinet and identified only by code number. Data will be obtained anonymously, and names will not be associated with any data or information.

Contact for concerns about the rights of research participants: Any concerns about your rights as a research participant can be communicated using the office contacts of Research at M.T.R.H through P.O BOX 3-30100 Eldoret.
The information obtained in this study shall be used to write a report. By signing below is an indication that you have agreed to the content of this consent form.
Your signature also indicates that you consent to be a participant in this study and that your information may be put in a confidential and anonymous form and kept for further use after the completion of the present study. You are guaranteed that the following conditions will be met:

1. Your participation in the present study is voluntary.

2. You can withdraw at any stage of the study, for any reason, and without any prejudice, and the information obtained and reports written can be turned over to you. I agree to the terms.

Participant_____ Date _____

Researcher _____ Date _____

APPENDIX IV: Enzyme Linked Immuno-Sorbent Assay (ELISA)

Principle

Specific antibodies are attached covalently to a solid phase support in a microtitre well-allowing antigen e.g., progesterone present in the specimen to bind and then detected by enzyme labeled antibodies and a specific substrate system.

General procedure

i. Read carefully the instruction provided in the kit and followed to the letter.
ii. Use the provided controls as specified and must be included with each test run to ensure valid test results.
iii. The specimens are diluted appropriately, and then added to the solid phase in the microtiter wells and incubated for a specified time and at temperature.
iv. Unbound proteins are then removed by washing the solid phase at least 3 times
v. Add the enzyme conjugate and incubated again as specified in the kit
vi. The Solid phase is again washed to remove excess conjugate
vii. Substrate is then added and the mixture incubated as specified
viii. A color is produced and is measured using an ELISA Reader at the specified wavelength
ix. The results shall then be calculated as instructed in the package insert

APPENDIX V: T_H1 and T_H2 cytokines overall procedure and incubation time

The overall procedure consists of the following steps.

Step	Description
1	Preparation of Human T_H1/T_H2 Cytokine Standards
2	Mixing Human T_H1/T_H2 Cytokine Capture Beads
3	Diluting samples
4	Performing instrument setup with Cytometer Setup Beads (bdbiosciences.com/cbasetup) Note: Can be carried out during the incubation in step 5.
5	Performing the Human Th1/Th2 Cytokine II Assay
6	Acquiring samples (instructions can be found at bdbiosciences.com/cbasetup)
7	Data analysis

T_H1 and T_H2 cytokine procedure and incubation time

Procedure	Incubation time
Preparing standards	15 minutes
Preparing Capture Beads	30 minutes (for Serum/Plasma samples only)
Preparing Cytometer Setup Beads	30 minutes
Performing the assay	3 hours

APPENDIX VI: Lymphocyte immunophenotyping overall procedure and incubation time

The overall procedure consists of the following steps.

Step	Description
1	For each patient sample, label a 12 x 75-mm tube with the sample identification number. For absolute counts, label a TruCOUNT Tube in place of the 12 x 75-mm tube. NOTE: Before use, verify that the TruCOUNT bead pellet is intact and within the metal retainer at the bottom of the tube. If this is not the case, discard the TruCOUNT Tube and replace it with another.
2	Pipette 20 µL of MultiTEST $CD3^+/CD8^+/CD45^+/CD4^+$ or $CD3^+/CD16CD56^+/CD45^+/CD19^+$ reagent into the bottom of the tube. If using a TruCOUNT Tube, pipette just above the stainless steel retainer. Do not touch the pellet.
3	Pipette 50µL of well-mixed, anticoagulated whole blood into the bottom of the tube. NOTE: Avoid smearing blood down the side of the tube. If whole blood remains on the side of the tube, it will not be stained with the reagent. When using a TruCOUNT Tube, accuracy is critical. Use the reverse pipetting technique to pipette sample onto the side of the tube just above the retainer.
4	Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (24+/-4°C) (20–25°C).
5	Add 450 µL 1X FACS Lysing Solution to the tube.
6	Cap the tube and vortex gently to mix. Incubate for 15 minutes in the dark at room temperature (24+/-4°C) (20–25°C). The sample is now ready to be analyzed on the flow cytometer.

APPENDIX VII: Sample standard case report form

PERSONAL DETAILS

Study number:----------Age:----------

Contact----------Marital status:----------

MEDICAL HISTORY

Physical and Clinical assessments……

Gestational age (counting from L.M.P)……

Number of births……

Other medical diagnoses and dates of diagnoses, e.g. *diabetes Mellitus*; .....……

Smoking history/other drugs use,……

Evidence of poor nutrition, stress or lack of exercise……...

MEDICATIONS

All prescribed and non-prescribed drugs (over the counter, herbal, holistic) being taken at the time of the first visit …….

LABORATORY ASSESSMENTS

Pregnancy status……..HIV status…….**.....**

Progesterone levels…….**.....**

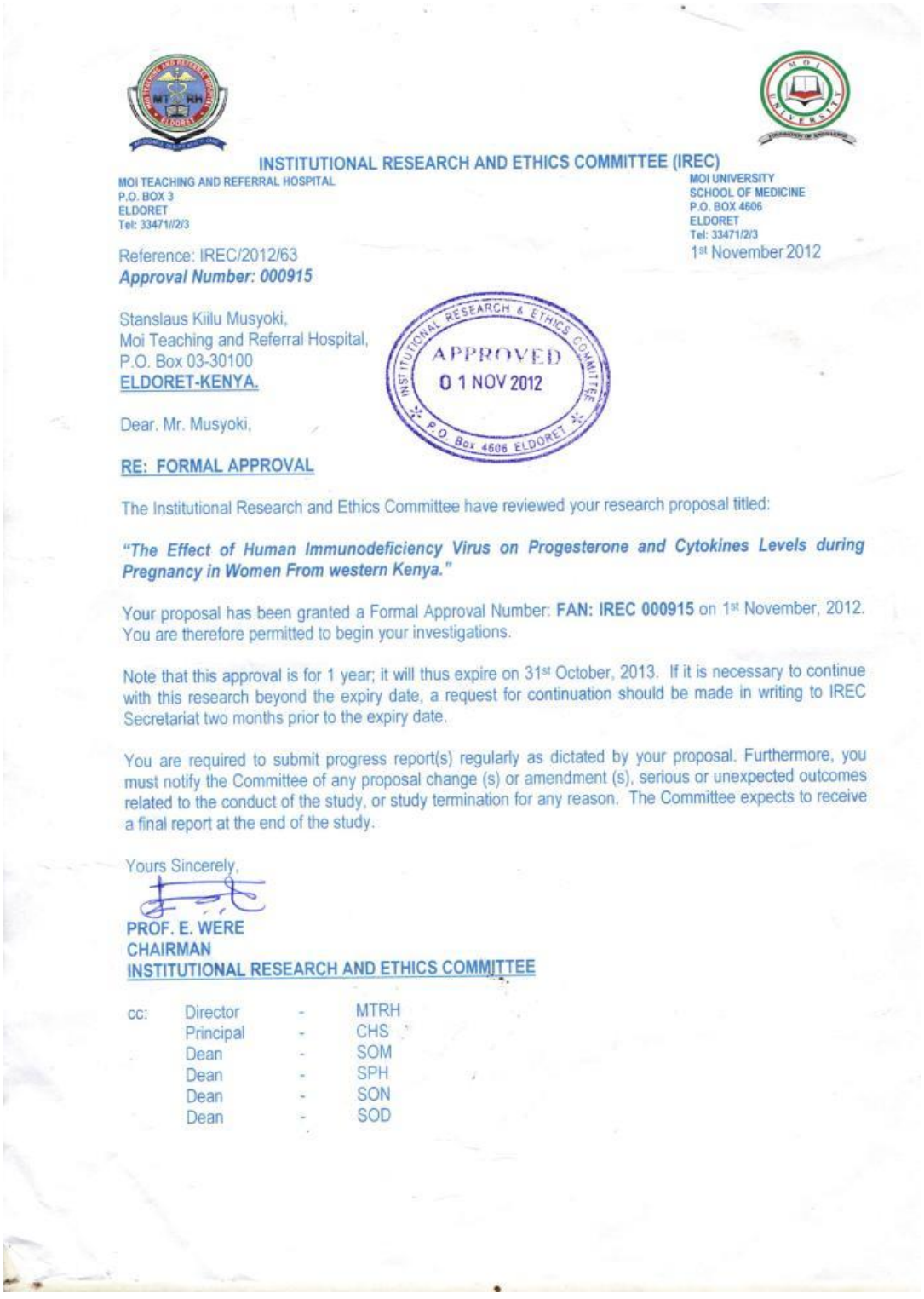
$CD3^+$ count……$CD4^+$ count…….**.....**

$CD8^+$ count……$CD19^+$ count…….**.....**

$CD56/16^+$ count…….**.....**

T_H1 or T_H2 cytokine profile…….**.....**

APPENDIX VIII: Approval letter from Institutional Research and Ethics Committee (IREC)



INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2012/63
Approval Number: 000915

1st November 2012

Stanslaus Kiilu Musyoki,
Moi Teaching and Referral Hospital,
P.O. Box 03-30100
ELDORET-KENYA.

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
APPROVED
0 1 NOV 2012
P.O. Box 4606 ELDORET

Dear. Mr. Musyoki,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee have reviewed your research proposal titled:

"The Effect of Human Immunodeficiency Virus on Progesterone and Cytokines Levels during Pregnancy in Women From western Kenya."

Your proposal has been granted a Formal Approval Number: **FAN: IREC 000915** on 1st November, 2012. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 31st October, 2013. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Yours Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc: Director - MTRH
Principal - CHS
Dean - SOM
Dean - SPH
Dean - SON
Dean - SOD

APPENDIX IX: Approval Letter from School of Graduate Studies, Maseno University

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: PG/PHD/0115/2011

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 3rd June, 2013

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR STANSLAUS KIILU MUSYOKI—PG/PHD/0115/2011

The above named is registered in the Doctor of Philosophy in Biomedical Science and Technology Programme of the School of Public Health and Community Development, Maseno University. This is to confirm that his research proposal titled "The Effect if HIV on Progesterone and Cytokines Levels during Pregnancy in Women from Western Kenya" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

MASENO UNIVERSITY
DIRECTOR
03 JUN 2013
SCHOOL OF GRADUATE STUDIES

Prof. P.O. Owuor
DEAN, SCHOOL OF GRADUATE STUDIES

Maseno University *ISO 9001:2008 Certified*

APPENDIX X: Approval letter from AMPATH, the study site

Academic Model Providing Access To Healthcare

Telephone: 254 53 2033471/2P.O. BOX 4606, ELDORET Fax: 254 53 2060727

RESEARCH

Ref: RES/STUD/07/2013 **May 23, 2013**

To: AMPATH MTRH Clinic: Module 1

In Charge,

RE: PERMISSION TO CONDUCT RESEARCH AT AMPATH

This is to kindly inform you that **Stanslaus Musyoki,** PhD Immunology Student at Maseno University has been granted permission to conduct research at AMPATH Module 1. His study; *"The Effect of Human Immunodeficiency Virus on Progesterone and Cytokines Levels during Pregnancy in Women of Western Kenya"* has been reviewed by IREC and assessed by the Research Office. The supervisors are Prof. S. Mining, Prof. C. Ouma and Dr. K. Chelimo

His research activities should not in any way interfere with the care of patients. This approval does not support access to AMRS data at AMPATH.

The researcher is to submit a final report of his findings to the AMPATH research office.

Should the researcher wish to publish his findings, permission has to be sort from AMPATH Publications Committee. Please contact the Research Office in case of any enquiry regarding this matter.

Thank you,

AMPATH CENTRE RESEARCH 23 MAY 2013 P.O. Box 4606-30100 Eldoret

Jepchirchir Kiplagat

Assistant Program Manager for Research

CC.

- Deputy Chief of Party, Research and Training
- Associate Program Manager, Research and Training