IMPACT OF MATERNAL HIV INFECTION AND HAART ON PLASMA IMMUNOGLOBULINS AND CYTOKINE SECRETION BY B AND T LYMPHOCYTE

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Supervisor:
Prof. Charles C Onyenekwe

2017

CERTIFICATION

This to certify that this dissertation titled: **"**Impact of maternal HIV infection and HARRT on plasma immunoglobulins and cytokine secretion by B and T lymphocyte**"** being submitted to the Department of Medical Laboratory Science, Faculty of Health Sciences and Technology, Nnamdi Azikiwe University Awka, Nnewi campus was carried out by **Ewenighi Obianuju Chinwe**, Registration number 2011347005F under the supervision of Prof. C. C Onyenekwe.

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APPROVAL PAGE

This work **"Impact of maternal HIV infection and HAART on plasma immunoglobulins and cytokine secretion by B and T lymphocyte"** presented **Ewenighi Obianuju Chinwe** (Reg. No: **2011347005F**) has been accepted and approved for the award of DOCTOR OF PHILOSOPHY DEGREE (Ph.D.) at Nnamdi Azikiwe University Awka.

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Dean SPGS Signature Date

DEDICATION

This work is dedicated to God Almighty and to all mothers and children going through the travails of HIV infection.

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LIST OF ABBREVIATIONS

ART – Antiretroviral therapy

ARV – Antiretroviral

CON A - Concanavalin A

HAART - Highly active antiretroviral therapies

HIV - Human immunodeficiency virus

IFN- γ – Interferon gamma

IL10 – Interleukin 10

IL2 – Interleukin 2

IL4 – Interleukin 4

MTCT - Mother-to-child transmission

NVP- Niverapin

PBMC - Peripheral blood mononuclear cells

PCR – Polymerase chain reaction

PHA - Phytohemaglutinin

PLHIV - People living with HIV

PMTCT - Prevention from mother to child transmission of HIV

PW – Pokeweed

TH1 – T helper cell class 1

TH2 – T helper cell class 2

TNF-α – Tissue necrosis factor alpha

UNAIDS – United nations programme on HIV/AIDS

WHO – World Health Organization

ABSTRACT

Human immunodeficiency virus (HIV) infection weakens the immunity of an individual; reduces lymphocyte population, alters cytokine secretion and impairs B and T cell function. The physiological status of pregnant women predisposes them to HIV progression hence the use of HAART for suppressing HIV replication, multiplication and restoration of the immune system. One of the ways to access the immune system is by invitro stimulation assay which specifically determines lymphocyte function without interference from other immune cells. This study evaluated impact of maternal HIV infection and HAART on plasma immunoglobulins and cytokine secretion by B and T lymphocyte. A total of 207 pregnant women aged (30 ± 4) years were recruited for the study using simple random sampling technique. The subjects were divided into HIV infected HAART treated (n = 122), HIV infected HAART naïve (n = 13) and HIV uninfected (n = 72) pregnant women as control. The study design was a case controlled study. Invitro stimulation of harvested B and T lymphocytes were performed using mitogens: concanavalin A (con A), phytohemaglutinin (PHA) and pokeweed (PW). Cytokine levels (interleukin 2- IL2, tissue necrosis factor alpha-TNF-α, interferon gamma-IFN- γ, interleukin 4 - IL4, interleukin 10- IL10) were measured in the supernatant of B and T lymphocyte using cytokine magnetic bead panel. However, plasma concentrations of Immunoglobulin A (IgA), Immunoglobulin M (IgM), Immunoglobulin G (IgG) were also analyzed using enzyme linked immunosorbent assay (ELISA). Column analysis (t-test, one-way ANOVA- Bonferroni's Multiple Comparison Test) and Mann-Whitney test were done using Graph Pad Prison 5. Significant values were set at $P < 0.05$. HIV infected HAART treated pregnant women showed significantly low levels of IFN-γ, IL-10 by con A, PW and PHA stimulation; and IL-4 by con A stimulation. Secretions of TNF-α and IL-2 using con A, PW and PHA stimulation were similar in supernatants of B and T lymphocyte culture of HIV infected HAART treated and control pregnant mothers. Trimester categorization revealed significantly low IL-10 secretions throughout 1st, 2nd and 3rd trimester in HIV infected HAART treated pregnant mothers. Secretions of TNF-α, IL-2 and IL-4 were similar in both normal and HIV infected HAART treated pregnant mothers at 1st. 2nd and 3rd trimester. There was significantly low IFN-γ secretion by PW and PHA stimulations in HIV infected HAART treated pregnant mothers at 1st trimester. TNF- α showed significantly ($P = 0.02$; 0.03) low level by PHA stimulation as pregnancy progressed to 3rd trimester. HAART treated pregnant women showed significantly ($P < 0.0001$) higher IgG but significantly ($P < 0.0001$) low IgM levels compared to control pregnant women. There was no significant difference in plasma IgA amongst the groups ($P>0.05$). Plasma IgG was significantly higher ($P<0.0005$) in 1st and 3rd trimesters in HAART treated pregnant women compared to control pregnant women. The findings in this study showed that the low secretions of IFN-γ, IL-4, IL-10 observed among HIV infected HAART treated pregnant mothers were normalized as pregnancy progressed to term but for IL-10 which remained significantly low throughout 1st, 2nd and 3rd trimester. The consistently lowered IL-10 level throughout the trimesters is an indication of possible role of this regulatory cytokine in HIV infection.

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

The major means of human immunodeficiency virus (HIV) transmission in sub Saharan Africa is by unprotected heterosexual intercourse which accounts for 80% of infection. Mother To Child Transmission (MTCT) accounts for 10% and other means accounts for the remaining 10% (WHO, 2010). Since the first case of AIDS was reported in a 13-year old girl in Nigeria in 1986, the epidemic has persisted with national HIV sero-prevalence rate of 1.8% in 1991, 5.8% in 2001, 4.4% in 2005, 4.6% in 2008, 4.1% in 2010 and currently at 3.1% in 2014 antenatal survey, the number of new infections remains unacceptably high (UNAIDS, 2014).

Globally, Nigeria also contributes the highest number of vertically transmitted childhood HIV infections, accounting for 30% of the global burden. The latter is partly due to the large number of people living with HIV (PLHIV) (3.4 million) in Nigeria of whom 57% are women (Federal Ministry of Health Nigeria, 2013). Most of these women do not know that they are HIV positive and therefore do not receive intervention during pregnancy, labour and breastfeeding to prevent vertical transmission of HIV to their infants. Prevention from Mother to Child Transmission (PMTCT) was established to prevent the 10% of MTCT (Federal Ministry of Health Nigeria, 2013). In Nigeria, PMTCT services started in 11 pilot tertiary institutions in 2002 with less than 1% coverage. The number of PMTCT sites has increased to 5,622 out of the 22,726 public sector health facilities available in the country (WHO, 2010). To achieve the goal of eliminating MTCT, at least 90% of HIV-infected women should have access to comprehensive PMTCT services including ARV prophylaxis during pregnancy and the breastfeeding period. Between 2006 and 2013 the number of HIV positive pregnant women who received ARVs to reduce the risk of MTCT increased from 13,000 to 58,000 in 2013 (UNAIDS, 2014). However, this was still only 27% of the 244,000 HIV-infected women who were estimated to have been pregnant in 2013 (Federal Ministry of Health Nigeria, 2013). The current 2013 PMTCT protocol in Nigeria otherwise known as “Test and Treat Approach” allows every HIV infected pregnant mother to be placed on triple combination therapy regardless of the CD4+T-cell count value through

pregnancy, labour, delivery, breastfeeding and thereafter. This is summarized in two headings:(i) providing lifelong ART to all pregnant and breastfeeding women living with HIV regardless of CD4 count or clinical stage or (ii) providing ART (ARV drugs) for pregnant and breastfeeding women with HIV during the mother-to-child transmission risk period and then continuing lifelong ART for those women eligible for treatment for their own health. The post-partum mothers breastfeed their baby for 8 months to 1year; the first 6 months is exclusive breast feeding while the next 6 months is complementary (a combination of breast milk, water and food). The baby receives niverapin (NVP) syrup (< 2.5kg = 1ml, > 2.5kg = 1.5ml) from birth till 6 weeks of age and then septrin until weaned. Detection of viral antigen using polymerase chain reaction (PCR) is done on the baby at six (6) weeks of age and six (6) weeks after the baby is weaned ((WHO Consolidated guidelines, 2013).

The successfulness of PMTCT lies in the level of immune strength of the mother. HIV weakens the immune strength of the pregnant mother through increase in HIV viremia, decrease in CD4+ cell counts, decrease in neutrophil phagocytosis, reduction of lymphocyte transformation, enhancement of Th1/Th2 shift in cytokine production and decrease in immunoglobulin A, G and M (Clerici et al, 2000; Müller et al, 2002; Pacheco et al, 2006; Onyenekwe et al 2010; Ifeanyichukwu et al 2010; Kolte, 2013). A successful pregnancy is characterized by an increase in Th2 cytokines and suppression of Th1 cytokine production. A Th1 to Th2 cytokine shift is also observed in the disease progression of HIV infection (Simona Fiore et al 2006). Highly active antiretroviral therapy (HAART) suppresses HIV viremia, increases CD4+ cell counts, increases neutrophil phagocyte, enhances lymphocyte transformation thus counteracts the Th1 to Th2 shift in cytokine production and increases immunoglobulin A, G and M (Onyenekwe et al 2010; Ifeanyichukwu et al 2010; Kolte, 2013). Hence, there is need to monitor the changes in immune responses of HIV infected mother on HAART through labour, delivery and breasting feeding.

The immune strength of the HIV pregnant mother determines if the exposed child gets infected with the virus or not. Again, the breast milk of the mother contains antibodies (immunologlobulins) that would have naturally boosted the immune system of the baby prior to the time they are able to produce their own antibody. If the mother's immune strength is boosted, the breast milk is also boosted but the reverse is the case and such scenario gives the baby a very

high chance of contacting the HIV virus from the mother during the recommended breastfeeding. HAART use in pregnancy is the standard care for women and has resulted in the decline of MTCT rate to below 1–2% (European Collaborative Study (a), 2005; and European Collaborative Study (b), 2005).

To determine the subject's immune response, lymphocyte stimulation assay was done. This is because of its specificity to assess lymphocyte function in cytokine stimulation. Lymphocyte (B cell, T cell) amidst other immune cells secrets cytokine which plays a major role in humoral and cell mediated immunity. Assay of cytokine directly from whole blood (plasma/serum) does not display specific immune response of the lymphocyte because cytokine in the plasma could be secreted by lymphocyte, neutrophil, monocyte/macrophage, liver, brain or endocrine organ. Therefore, peripheral blood mononuclear cells (PBMC) isolation and lymphocyte (T cell and B cell) stimulation allow for a description of T cell responses without the influence of other whole blood components. Cytokines are primarily produced by immune system but many other organs (liver, brain, endocrine gland) produce cytokines to influence immune response.

Lymphocyte proliferation assay can be done with antigens or mitogens; here mitogens; concanavalin A, phytohemaglutinin and pokeweed were used. These mitogens provide strong stimuli that are not antigen specific, and usually do not discriminate as well as antigens in reflecting different levels of immunodeficiency. Some normal individuals may not respond to a given antigen but almost everyone's lymphocytes can be stimulated to proliferate nonspecifically by stimulating them in vitro with mitogens. PHA and con A stimulates T cell while PWM was used to stimulate both B cell and T cell. It is assumed that there exist some individual differences in response to phytohemaglutinin, pokeweed and concanavalin A. The stimulatory effect of concanavalin A is slow and can last for days while Phytohemagglutinin has the potential to induce closer contacts between adjacent cell membranes. Combined use of these mitogens was to compare the mitotic abilities of the mitogens on different individuals.

1.2 Statement of problem

HIV infection impaires immunity and causes death in women of reproductive age thereby rendering the children motherless. Over 90% of the HIV infection in children is acquired through

mother-to-child transmission (MTCT) and this results in their poor health or death within the first two years of life where PMTCT services are lacking. Transmission of HIV infection in children has become a critical health problem that threatens to undermine the positive impact of child survival strategies in Nigeria and African continents. The cost and care for HIV infected children and perhaps the motherless amongst them place a heavy burden on families, communities, health care system, society and Nigeria at large. Overall, there is a geographic disparity in the PMTCT coverage. A strategy to increase national focus on all the high burden states is therefore required to turn around the tide of the HIV epidemic among children in Nigeria.

1.3 Significance of the study

Until now, the impact of HIV-1 infection and/or of anti-retroviral drugs on maternal TH1/TH2 cytokine expression patterns and plasma immunoglobulins at different stages of gestation has not been evaluated clearly and extensively. The immunologic methods applied in this work are to assess the immunologic strength and response of the HIV infected mother as this plays a major role in PMTCT. This study also took into consideration the immune strength/profile of the infected pregnant women at different stages of pregnancy of gestations like first, second and third trimesters. An accurate understanding of the timing of HIV-1 transmission from mother to fetus is very important for the design of intervention strategies.

1.4 Aim and Objectives

Aim: Impact of maternal HIV infection and HAART on plasma immunoglobulins and cytokine secretion by B and T lymphocyte.

Objectives:

i) To evaluate plasma immunoglobulin (A, G, M) levels among HIV infected HAART Treated, HIV infected HAART naive and HIV uninfected pregnant women.

ii) To evaluate IL-2, IFN-γ, TNF-α, IL-4 and IL-10 after B and T lymphocyte stimulations with concanavalin-A, pokeweed and phytohemaglutinin among HIV infected HAART Treated, HIV infected HAART naive and HIV uninfected pregnant women.

iii) To assess the relationship between the immunological parameters and gestational age (1st, 2nd and 3rd trimesters respectively) of HAART Treated HIV infected and HIV uninfected pregnant women.

1.5 Research questions:

1. Does HIV infection and HAART use alter serum Immunoglobulin A, G, M levels in pregnant women?
2. Does HIV infection and HAART use alter cytokine secretion by B and T lymphocyte?
3. Does gestational age affect immunoglobulin levels and cytokine secretion by B and T lymphocyte?

1.6 Hypothesis:

1. Ho: HIV infection and HAART do not alter serum Immunoglobulin A, G, M levels in pregnant women.

   Ha: HIV infection and HAART alter serum Immunoglobulin A, G, M levels in pregnant women.

2. Ho: HIV infection and HAART do not alter cytokine secretion by B and T lymphocyte.

   Ha: HIV infection and HAART alter cytokine secretion by B and T lymphocytes.

3. Ho: Gestational age does not affect immunoglobulin A, G, M levels and cytokine secretion by B and T lymphocyte in pregnant women.

   Ha: Gestational age does not affect immunoglobulin A, G, M levels and cytokine secretion by B and T lymphocyte in pregnant women.

CHAPTER TWO

LITERATURE REVIEW

2.1 IMMUNE SYSTEM

The immune system is the collection of cells, tissues and molecules that protects the body from numerous pathogenic microbes and toxins in our environment. This defense against microbes has been divided into two general types of reactions: reactions of innate immunity and reactions of adaptive immunity. Thus, innate and adaptive immunity can be thought of as two equally important aspects of the immune system. Each aspect differs with respect to how quickly it responds and for how long it responds to pathogens, its central effector cell types and its specificity for different classes of microbes (Janeway et al, 2001).

2.2 TYPES OF IMMUNE SYSTEM:

2.2.1 Innate/non-specific immunity or nonspecific immunity is the natural resistances with which a person is born. It provides resistances through several physical, chemical and cellular approaches. Innate immunity consists of cells and proteins that are always present and ready to mobilize and fight microbes at the site of infection. The main components of the innate immune system are 1) physical epithelial barriers, 2) phagocytic leukocytes, 3) dendritic cells, 4) a special type of lymphocyte called a natural killer (NK) cell, and 5) circulating plasma proteins (see figure 2.1). Microbes first encounter the epithelial layers, physical barriers that line skin and mucous membranes. Subsequent general defenses include secreted chemical signals (cytokines), antimicrobial substances, fever, and phagocytic activity associated with the inflammatory responses. The phagocytes express cell surface receptors that can bind and respond to common molecular patterns expressed on the surface of invading microbes. Through these approaches, innate immunity can prevent the colonization, entry and spread of microbes (Flajnik et al, 2010). Innate immune response can be summarized thus:

- Skin - a great physical barrier, like a waterproof wall.
- Mucus – sticky, germs get stuck in it, it also has antibody in it.

- Cilia – hairs that pass debris up throat and out to the nostrils.
- Lysosyme - an enzyme present in tears that breaks down bacteria.
- Phagocytes – various cells that scavenge up and engulf cell debris.
- Commensal bacteria- Non-harmful bacteria on skin and gut that leave little or no room for harmful bacteria to attach, and limited nutrients for them to grow.
- Acid - in stomach and urine, make it hard for any germs to survive.
- Fever – elevates the temperature making it difficult for infectious agents to survive.

Figure 2. 1: Innate immune reactions generally occur much sooner and are less sustained than adaptive immune reactions. Source: (Flajnik et al, 2010).

2.2.2 Adaptive/specific immunity: The adaptive immune system, on the other hand, is called into action against pathogens that are able to evade or overcome innate immune defenses. Components of the adaptive immune system are normally silent; however, when activated, these components "adapt" to the presence of infectious agents by activating, proliferating, and creating potent mechanisms for neutralizing or eliminating the microbes. Adaptive immune response is highly specific for a particular pathogen and improves with each successive encounter. In effect the adaptive immune system 'remembers' the infection agent and can prevent it from causing disease later. For example, diseases such as measles and diphtheria induce adaptive immunity which generates lifelong immunity following infection. The two key features of the adaptive immune response are thus **specificity and memory**. Immune memory is retained by B-cells and T-cells. Responses by B-cells are humoral, responses by T-cells are called cellular. This gives rise to two types of adaptive immune responses: humoral and cell mediated immune response.

2.3 TWO TYPES OF ADAPTIVE IMMUNE RESPONSES

2.3.1 Humoral immunity: Mediated by antibodies produced by B lymphocytes. Humoral immunity is mediated by secreted antibodies, produced in the cells of the B lymphocyte lineage (B-cell). It is also called the *antibody-mediated beta cullularis immune system* and mediated by macromolecules (as opposed to cell-mediated immunity) found in extracellular fluids such as secreted antibodies, complement proteins and certain antimicrobial peptides. Humoral immunity is so named because it involves substances found in the humours, or body fluids. Secreted antibodies bind to antigens on the surfaces of invading microbes, which flags them for destruction. Humoral immunity refers to antibody production, and all the accessory processes that accompany it including: TH2 activation and cytokine production, germinal center formation and isotype switching, affinity maturation and memory cell generation. It also refers to the effector functions of antibodies, which include pathogen and toxin neutralization, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination (Janeway et al, 2001).

2.3.2 Cell-mediated immunity: mediated by T lymphocytes. Cell-mediated immunity is an immune response that does not involve antibodies but rather involves the activation of *macrophages* and *natural killer cells*, the production of *antigen-specific cytotoxic T-lymphocytes*, and the release of various *cytokines* in response to an antigen (Uematsu & Akira, 2007). Cellular immunity protects the body by:

- activating antigen-specific cytotoxic T-lymphocytes that are able to destroy body cells displaying epitopes (fragments) of foreign antigen on their surface, such as virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens;
- activating macrophages and natural killer cells, enabling them to destroy intracellular pathogens; and
- Stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive immune responses and innate immune responses.

Cell-mediated immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in defending against fungi, protozoans, cancers, and intracellular bacteria [Uematsu & Akira, 2007].

2.4 PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Peripheral blood mononuclear cells (PBMCs) are group of blood cells having a round nucleus (as opposed to a lobed nucleus) (Delves et al, 2011) whereas erythrocytes and platelets have no nuclei, and neutrophils, basophils, and eosinophils have multi-lobed nuclei. When peripheral whole blood is drawn for human immune system studies, it is often processed to remove red blood cells by density gradient centrifugation. Most commonly this method uses Ficollpaque/histopaque, a solution of high molecular weight sucrose polymers (hydrophilic polysaccharide). Ficoll separates whole blood into four fractions above and below the density of 1.077g/ml from top to bottom; **plasma, PBMC, ficoll** and **red cell** at bottom comprising polymorphonuclear cells (such as neutrophils and eosinophils) and erythrocytes. (Delves et al, 2011).

Peripheral blood mononuclear cells (PBMC) are the populations of immune cells that remain at the less dense, upper interface of the Ficoll layer, often referred to as the buffy coat forming under a layer of plasma and are the cells collected when the Ficoll fractionation method is used. Erythrocytes (red blood cells) and polymorphonuclear cells (PMNs) which include neutrophils and eosinophils are generally removed during this fractionation as they are denser than 1.077g/ml. Basophils, however can be greater or less dense then 1.077g/ml and thus may be present to a small degree in the less dense PBMC fraction.

2.4.1 COMPONENTS OF PBMC

PBMCs include **lymphocytes** (T cells, B cells, and NK cells), **monocytes/macrophages, and dendritic cells**. In humans, the frequencies of these populations vary across individuals (figure 2.2). Lymphocytes are typically in the range of 70 – 90% of PBMCs, monocytes range from 10 – 30% of PBMCs, while dendritic cells are rare, being only 1 – 2% of PBMCs. The frequencies of cell types within the lymphocyte population include 70 – 85% CD3+ T cells (45 – 70% of PBMC), 5 – 20% B cells (up to 15% of PBMC), and 5 – 20% NK cells (up to 15% of PBMC) (Delves et al, 2011).

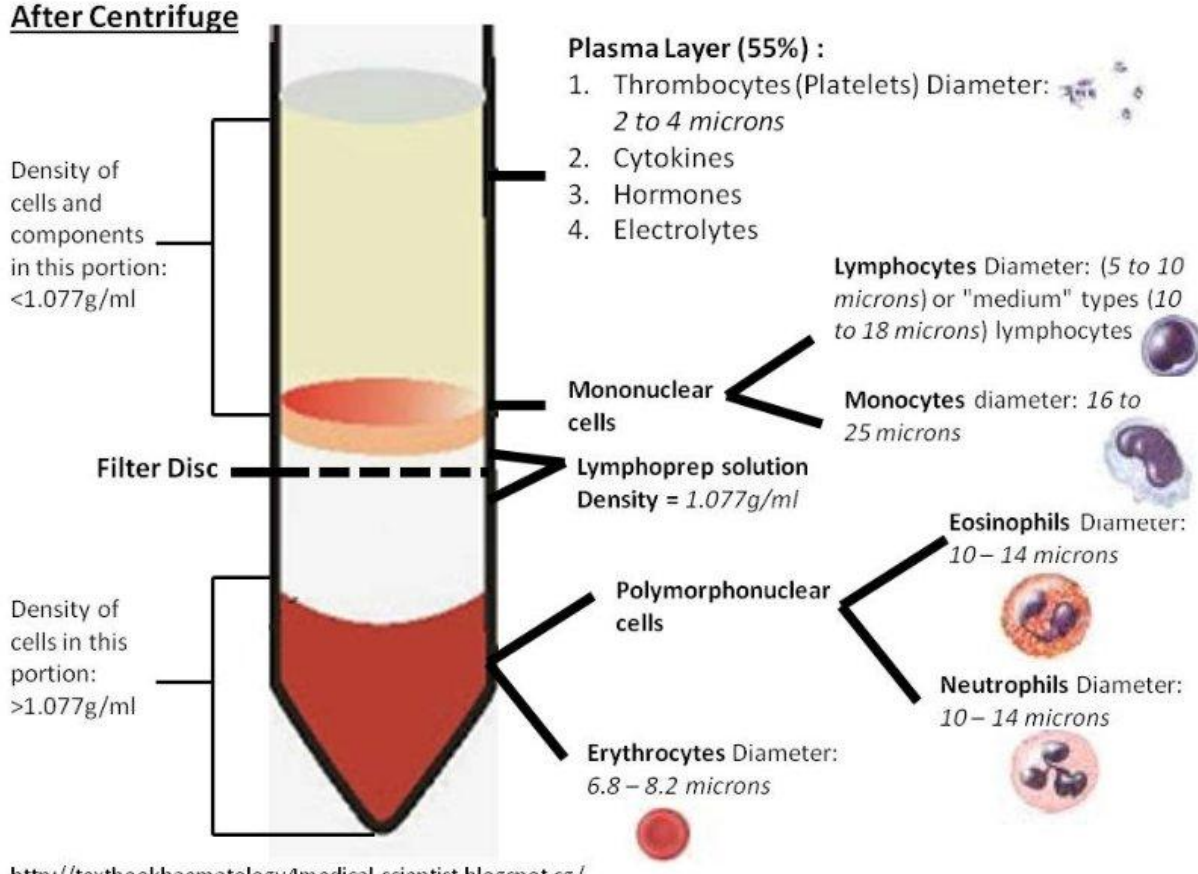


Figure 2.2: T cell population in PBMC (textbookhaematology4medical-scientist.blogspot.sg/) retrieved on 20th December 2017.

The **CD3+** compartment is composed of **CD4 T cells** (25 – 60% of PBMC) and **CD8 T cells** (5 – 30% of PBMC), in a roughly 2:1 ratio. Both CD4 and CD8 T cells can be further subsetted into naïve, the antigen-experienced central memory, effector memory, and effector subtypes that exist in resting or activated states. Multiple markers can be used to identify these compartments to varying similarities and thus the frequencies reported by people using different markers may vary (Delves et al, 2011).

CD4 T cells are known as helper T cells and can be further classified into various functional subtypes based on the expression profiles of specific cytokines, surface markers, or transcription factors. These include regulatory T cells, TH1, TH2, and TH17 cells as well as other described subpopulations such as TH9, follicular helper, and TR1 types. (Andrea, 2012).

2.5 CYTOKINES

Cytokines also called lymphokines or monokines are proteins made by the cells of immune system usually durng cell activation. They are a category of signaling molecules that mediate and regulate immunity, inflammation and hematopoiesis. Cytokines are primarily produced by immune system but many other organs (liver, brain, endocrine gland) produce cytokines to influence immune response. Cytokines are a large group of proteins, peptides or glycoproteins that are secreted by specific cells of immune system. Cytokines are small proteins (< 30 kDa) and similar to hormones and growth factors. Cytokines as soluble factors act as messenger on such cells and promote activity focused on regulation and control immune response or elimination of virus of viruses and other pathogen (Whilton & Micheal, 1996). Cytokines are produced throughout the body by cells of diverse embryological origin (Horst Ibelgaufts, 2013). Cytokine is a general name; other names are defined based on their presumed function, cell of secretion, or target of action. For example:

- Lymphokines- cytokines made by lymphocytes
- Interleukins – are cytokines made by one leukocyte and act on other leukocytes.
- Chemokines -are cytokines with chemotactic activities.

- Monokines – cytokines made by monocytes

Cytokines may act on the cells that secrete them (autocrine action example IL-2 for T cell activation), on nearby cells (paracrine action example T Cell Help for B cell), or in some instances on distant cells through blood stream (endocrine action example inflammatory cytokines). Cytokines control the type and duration of the immune response**.**

2.6 TH1 CYTOKINES

Cytokines as a mediator of adaptive immunity play a major role in the adaptive immune system. Cytokines control the type and duration of the immune response**.** Th1 lymphocytes are critical in the cellular immune response and they play an important role in host defense systems for intracellular microbial agents and viruses. TH1 cell promoting factors include IFNγ, IL-12 (p70) (Hsieh et al, 1993; Whilton & Micheal, 1996) and the activation of the transcription factors STAT1 and STAT4. The expression of the Interleukin-12 receptor β2-chain (IL-12Rβ2) is required for Th1 cellular differentiation since it allows for the responsiveness to IL-12 on the Th1 cells. IL-12R activation increases IFNγ expression through STAT1 signals to induce the Th1 master regulator T-bet. This further increases IFNγ expression while reduces IL-4 mediated suppression of IFN-γ (Hsieh et al, 1993). Th1 cells are the primary source for the inflammatory cytokines lFNγ, IL-2, and TNFβ (LTα) (Kelso, 1993; Whilton & Micheal, 1996; Casssatella, 2003).

Functions: Th1 cells which produce interferon (IFN)-gamma, interleukin (IL)-2 and tumor necrosis factor (TNF)-β, evoke cell-mediated immunity and phagocyte-dependent inflammation. Th1 cytokines stimulate macrophages, lymphocytes, and PMNs in the destruction of bacterial pathogens. These cytokines also help foster the development of cytotoxic lymphocytes (CTL & NK cells) that are responsible for the cell-mediated immune response against viruses and tumor cells. Due to the central role of Th1 cells in immune system, over activation or misdirected activation also makes them key players in Th1-dominant autoimmune diseases such as multiple sclerosis, type-1 diabetes, rheumatoid arthritis, and delayed-type hypersensitivity responses. Further, Th1-dominated responses are involved in the pathogenesis of organ-specific autoimmune disorders, Crohn's disease, sarcoidosis, acute kidney allograft rejection, and some unexplained recurrent abortions (Whilton & Micheal, 1996; Romagnani, 2000; Fiore et al, 2006).

TH1 cytokine includes:

IL-2: Stimulates growth, differentiation, and survival of antigen-selected cytotoxic T cells. Necessary for T cell memory, T-cell development, and self / non-self-recognition. Promotes the differentiation of certain immature T cells into regulatory T cells. Promotes the differentiation of T cells into effector T cells and into memory T cells.

IFNγ

- Activates macrophages and inhibits Th2 lymphocyte proliferation
- Stimulates B cells to produce receptors that enhance the attachment of microbes to phagocytes

TNFα

- A general, potent, and pleiotropic immune activator and regulator of immune cell function

TNFβ/LTα

- Activates neutrophils to enhance their microbial killing activity during phagocytosis

IL-12

- Differentiates naive T cells into Th1 cells. It is known as a T cell-stimulating/promoting factor, which can stimulate the growth and function of T cells. It stimulates the production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from T cells and natural killer (NK) cells, and reduces IL-4 mediated suppression of IFN-γ.

2.7 TH2 CYTOKINES

Th2 cells mediate the activation and maintenance of the humoral, or antibody-mediated, immune response against extracellular parasites, bacteria, allergens, and toxins. Th2 cells mediate these functions by producing various cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, and IL-17E (IL-25) that are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses. These cytokines also counteract the Th1 responses that allow for the Th2 responsiveness to IL-4. IL-4 signals through STAT6 to up-regulate GATA3 expression, the master regulator of Th2 cell differentiation. Repression of this activity results in the development failure of IL-4 producing cells. IL-4 also suppresses Th1 and Th17 cell responses through the up-regulation of transcriptional repressor(s) of IFNγ and IL-17 production.

Function: Th2 cells, which produce IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, IL-24, evoke strong antibody responses (including those of the IgE class) and eosinophil accumulation, but inhibit several functions of phagocytic cells (phagocyte-independent inflammation) (Whilton & Micheal, 1996; Romagnani, 2000). Th2 cytokines have effects on many cell types in the body as the cytokine receptors are widely expressed on numerous cell types. Th2 cells stimulate and recruit specialized subsets of immune cells, such as eosinophils and basophils, to the site of infection or in response to allergens or toxin leading to tissue eosinophilia and mast cell hyperplasia. They induce mucus production, goblet cell metaplasia, and airway hyper-responsiveness. Th2 cells also control the regulation of B cell class-switching to IgE. Because of their influence on the production of antibodies and allergic responses, over activation of Th2 cells appears to be responsible for the exacerbation of allergies (Type-1, immediate hypersensitivity reactions), autoimmune reactions such as chronic graft-versus-host disease, progressive systemic sclerosis, and systemic lupus erythematosus. Additionally, Th2 cells are also known to be responsible for the development of asthma and other allergic inflammatory diseases. Interestingly, Th2 cells also produce the growth factor amphiregulin and IL-24 which have anti-tumor effects (Whilton & Micheal, 1996; Romagnani, 2000). Allergen-specific Th2 responses are responsible for atopic disorders (hereditary allergy) in genetically susceptible individuals. Further, Th2-dominated responses play a pathogenic role in both progressive

systemic sclerosis and cryptogenic fibrosing alveolitis, and favor a more rapid evolution of HIV infection towards the full-blown disease (Whilton & Micheal, 1996; Romagnani, 2000).

TH2 Cytokines includes:

Amphiregulin: An EGF family member growth factor with anti-tumor effects

IL-3: Assists in the recruitment and maintenance of basophils into lymphoid tissues in response to infection

IL-4: Inhibits the proliferation and differentiation of Th1 cells

Stimulates B cell (TH2) proliferation and maturation into plasma cells

Regulates the class switching of antibodies

Increases IgE production

IL-5

- Attracts and activates eosinophils

IL-6

- Critical role in B cell maturation into IgG secreting cells
- Plays a significant role in inflammation and autoimmunity

IL-10

IL-10 is a cytokine with multiple, pleiotropic, effects in immunoregulation and inflammation. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. IL-10 can block NF-κB activity, and is involved in the regulation of the JAK-STAT signaling pathway. Inhibits secretion of various cytokines by Th1 cells, macrophages, and dendritic cells. It induces IFNγ and CD8+ T cell dependent anti-tumor immunity.

IL-13

- Stimulates B-cell production of IgE
- Attracts basophils and mediates the release of granules
- Triggers mast cells to release granules

IL-17E (IL-25)

- Co-mediates production IL-4, IL-5 and IL-13

IL-31

- Implicated in inflammatory responses in the skin
- Recruitment of PMNs, monocytes, and T cells to the sites of infection

In excess, Th2 responses will counteract the Th1 mediated microbicidal action. The optimal scenario would therefore seem to be that humans should produce a well-balanced Th1 and Th2 response, suited to the immune challenge. Many researchers regard allergy as a Th2 weighted imbalance, and recently immunologists have been investigating ways to redirect allergic Th2 responses in favour of Th1 responses to try to reduce the incidence of atopy (Whilton & Micheal, 1996; Gereda et al, 2000; Romagnani, 2000). An additional strategy is being used to prevent the onset of disease; this involves the study of pregnancy and early postnatal life. Both of these states are chiefly viewed as Th2 phenomena (to reduce the risk of miscarriage, a strong Th2 response is necessary to modify the Th1 cellular response in utero). The fetus can switch on an immune response early in pregnancy, and because pregnancy is chiefly a Th2 situation, babies tend to be born with Th2 biased immune responses. These can be switched off rapidly postnatally under the influence of microbiological exposure or can be enhanced by early exposure to allergens. It is also hypothesised that those who go on to develop full blown allergies may be those who are born with a generally weaker Th1 response, although it is now apparent that babies with allergies produce weak Th1 and Th2 responses (Whilton & Micheal, 1996; Gereda et al, 2000).

2.8 PROPERTIES OF CYTOKINE

1. Pleiotropy: Same cytokine has different effects on cells; can be activating or inhibiting, example: IL-10 can be inhibitory to macrophages and Th1 cells yet activating for Th2 cells and B cells. This is called pleiotropism (Nicola, 1994).

2. Redundancy: Multiple cytokines exert similar actions. This phenomenon is largely due to multiple cytokines utilizing common receptor subunits and common intracellular cell signalling molecules/pathways (Kelso, 1993). Different cytokines have similar functions (IL-2 and IL-4 induce B cell proliferation)

3. Synergy: Both IL-2 or IL-4 activate CTLLs in vitro. (CTLLs are cytokine dependent cell line). Maximal proliferation obtained when both IL-2 and IL-4 added. Both cytokines need to be blocked in order to inhibit growth. Multiple cytokines work together to do the same thing [INF-gamma and TNF work together to increase expression of MHC-1 on many cells] (Bartee & McFadden, 2013).

4. Antagonism: Different cytokines work against each other (IFNγ activates macrophages and IL-10 inhibits macrophage activation).

2.9 MITOGEN

Mitogen lectins (proteins) from plant that induces blast transformation; DNA, RNA, and protein synthesis; and proliferation of lymphocytes; e.g., concanavalin A, phytohemagglutinin, pokeweed or lipopolysaccharide. Mitogenesis is the induction (triggering) of mitosis, typically via a mitogen. Mitogens trigger signal transduction pathways in which mitogen-activated protein kinase (MAPK) is involved, leading to mitosis. B cells can enter mitosis when they encounter an antigen matching their immunoglobulin. Mitogens are often used to stimulate lymphocytes and therefore assess immune function (Miller-Keane, 2003). Mitogens are not only lectins such as phytohemagglutinins, pokeweed and concanavalin A, but also substances from streptococci (associated with streptolysin S) and from strains of α-toxin-producing staphylococci eg Lipopolysaccharide (Miller-Keane, 2003).

Phytohaemagglutinin (PHA) is a lectin that has carbohydrate-binding specificity for a complex oligosaccharide containing galactose, N-acetylglucosamine, and mannose. It agglutinates most mammalian red blood cell types. PHA has multiple effects on cell metabolism; it induces mitosis triggering cell division in T lymphocytes, and affects the cell membrane in regard to transport and permeability to proteins (Goldstein & Poretz 2012). Concanavalin A (ConA) is also a lectin and binds mainly internal and nonreducing terminal α-D-mannosyl and α-D-glucosyl groups (Goldstein & Poretz 2012). ConA is known to stimulate mouse T-cell subsets (four functionally distinct T cell populations) and at least one subset of human T cells (Dwyer & Johnson, 1981). Pokeweed mitogen is a lectin from the pokeweed (Phytolacca americana). It is mitogenic for both T and B cells. PWM is a T and B cell dependent mitogen, and very weak T-mitogen. Both PHA and ConA (among lectins) are better to test T-cells. Poke weed mitogen (PWM), a lectin purified from *Phytolacca americana* is frequently used as a B cell-specific stimulus to trigger proliferation and immunoglobulin secretion. It has been shown that highly purified PWM preparations failed to induce B cell proliferation. By contrast, commercially available PWM preparations with B cell activity contained Toll-like receptor (TLR) ligands such as TLR2-active lipoproteins, lipopolysaccharide and DNA of bacterial origin. These microbial substances contribute to the stimulatory activity of PWM. B cell mitogenicity of PWM preparations results from synergistic activity of the poke weed lectin and microbial TLR ligands present in the PWM preparations (Bekeredjian-Ding *et al*., 2012).

The most commonly used mitogens in clinical laboratory medicine are:

Name	**Acts upon T cells?**	**Acts upon B cells?**
Phytohaemagglutinin (PHA)	yes	no
Concanavalin A (conA)	yes	no
Lipopolysaccharide (LPS)	No	yes
Pokeweed mitogen (PWM)	Yes	yes

1. Pokeweed mitogen -

Poke weed mitogen (PWM), a lectin purified from *Phytolacca americana* is frequently used as a B cell-specific stimulus to trigger proliferation and immunoglobulin secretion, though it produces B and T lymphocyte blastogenesis. Bekeredjian-Ding et al, (2012) investigated the mechanisms underlying the B cell stimulatory capacity of PWM. Strikingly, they observed that highly purified PWM preparations failed to induce B cell proliferation. By contrast, commercially available PWM preparations with B cell activity contained Toll-like receptor (TLR) ligands such as TLR2-active lipoproteins, lipopolysaccharide and DNA of bacterial origin. They showed that these microbial substances contribute to the stimulatory activity of PWM. Additional experimental data highlighted the capacity of PWM to enable B cell activation by immunostimulatory DNA. PWM activation of immunoglobulin secretion requires T cells. PWM induce IgG requires accessory cells as well as T cells and B cells and that PWM is likely to stimulate via more than one receptor (Bekeredjian-Ding et al, 2012)

2. Phytohemaglutinin PHA - phaseolus vulgaris is a lectin. Broadly, lectins bind to sugar/carbohydrate moities attached to proteins. Each lectin recognizes a very specific sugar group - orientation on the peptide, length and composition. As implied by its name, phytohemagglutinin was found to clump (agglutinate) erythrocytes (red blood cells). This function is due to the fact that PHA can recognize glycosylated proteins on the surface of red blood cells, and can bridge two or more cells, forming clumps. It causes severe allergy by switching on a runaway meitogenic event. The specific sites of PHA: T-cell interaction still seem to be poorly defined although some literature suggests that PHA initiates activating signals via the CD2 receptor. Some suggest that PHA and IL 2, monocyte, IL6 needed to be present together for the proliferation of T cells to occur.

Lymphocytes become activated upon ligation of their antigen receptors (T cell receptor - TCR - for T cells, and the B cell receptor - BCR or immunoglobulin - for B cells) and subsequent downstream activation. The normal way a lymphocyte is activated is by engaging a cognate antigen, either presented on an antigen presenting cell or in solution. The engagement of the antigen receptor leads to clustering of many surface proteins on the lymphocyte. This clustering

is crucial to bring all the signaling components into close enough proximity to propagate the signal. Therefore, anything that will cluster the surface receptors will lead to signaling, as the lymphocyte can't really distinguish between clustering due to meeting cognate antigen from clustering due to antibody ligation (as in anti-CD3 stimulation of T cells) or lectin- mediated agglutination. As the specificity of lymphocytes in primary culture is largely unknown, immunologists and others use tools to get them to proliferate in the absence of antigen, such as antibodies and PHA or ConA. These nonspecific means of stimulation lead to polyclonal expansion of the cells in culture, regardless of their antigen specificity.

n/b PHA binds more strongly to cells than does CONA. Much smaller quantities of PHA than of CON A suffix for maximum stimulation. Mitogenic activity of PHA used in this work is 5 µg/ML while CON is 70µg/ML.

3. Concanavalin A - is a mitogen derived from canavalia Ensiformis type IV. It functions as a lectin. It acts upon T cell and thus produces T lymphocyte blastogenesis. ConA stimulates many different cells and promote aggregation of some cell types (e.g.red blood cells). Concanavalin A interacts with diverse receptors containing mannose carbohydrates, notably rhodopsin, blood group markers, insulin-receptor, the Immunoglobulins and the carcino-embryonary antigen (CEA). It also interacts with lipoproteins. it is a lectin and likely to bind pathogen recognition receptors on innate immune cells (an example of a classical lectin receptor is the mannose receptor, which is expressed on the surface of monocytes). Concanavalin A (ConA) is a lectin (carbohydrate-binding protein) originally extracted from the jack-bean, *Canavalia ensiformis*. It binds specifically to certain structures found in various sugars, glycoproteins, and glycolipids α-D-mannosyl and α-D-glucosyl groups. ConA is a plant mitogen, It has the D_2 symmetry. Its tertiary structure has been elucidated, and the molecular basis of its interactions with metals as well as its affinity for the sugars mannose and glucose are well known. ConA strongly agglutinates erythrocytes irrespective of blood-group, and various cancerous cells.

ConA can also initiate cell division (mitogenesis) principally acting on T-lymphocytes, by stimulating the energy metabolism of thymocytes within seconds of exposure. PHA and ConA

bind and cross-link components of the T cell receptor, and their ability to activate T cells is dependent on expression of the T cell receptor.

4. Lipopolysaccharide (LPS) toxin from gram-negative bacteria is thymus-independent. They may directly activate B cells, regardless of their antigenic specificity.

LPS actually DOES NOT activate human B cells. However, it can activate murine B cells that express TLR4 unlike human B cells. In contrast to mouse B-cells, human B-cells express neither TLR4 nor CD14, the two canonical ligands for Gram– bacteria LPS, and are therefore unresponsive to LPS." (Garraud et al, 2012).

Plasma cells are terminally differentiated and, therefore, cannot undergo mitosis. Memory B cells can proliferate to produce more memory cells or plasma B cells. This is how the mitogen works, that is, by inducing mitosis in memory B cells to cause them to divide, with some becoming plasma cells.

2.10 HUMAN IMMUNO VIRUS (HIV)

Is either of two retroviruses that contain two single-strand linear RNA molecules per virion and reverse transcriptase (RNA to DNA), transcribes its RNA into a cDNA provirus that is then incorporated into the host cell, infect and destroy helper T cells of the immune system causing the marked reduction in their numbers that is diagnostic of AIDS (American Heritage, 2005). Acquired immune deficiency syndrome (AIDS) is a disease of the human immune system caused by infection with human immunodeficiency virus (HIV). (Sepkowitz, 2001)

Acquired immunodeficiency syndrome (AIDS) can also be defined in terms of either a $CD4^+$ T cell count below 200 cells per µL or the occurrence of specific diseases in association with an HIV infection (Del Rio & Curran, 2010). In the absence of specific treatment, around half of people infected with HIV develop AIDS within ten years (Del Rio & Curran, 2010). . The most common initial conditions that alert to the presence of AIDS are pneumocystis pneumonia (40%), cachexia in the form of HIV wasting syndrome (20%) and esophageal candidiasis. Other common signs include recurring respiratory tract infections (Del Rio & Curran, 2010). . Opportunistic infections may be caused by Kaposi's sarcoma, Burkitt's lymphoma, primary

central nervous system lymphoma, and cervical cancer (Vogel et al, 2010) bacteria, viruses, fungi and parasites that are normally controlled by the immune system (Holmes et al, 2003)

2.11 HIV TYPES

HIV is a highly variable virus which mutates very readily. This means there are many different strains of HIV, even within the body of a single infected person. Based on genetic similarities, the numerous virus strains may be classified into types, groups and subtypes. There are two types of HIV: HIV-1 and HIV-2. Both types are transmitted by sexual contact, through blood, and from mother to child, and they appear to cause clinically indistinguishable AIDS. However, it seems that HIV-2 is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV-2. Worldwide, the predominant virus is HIV-1, and generally when people refer to HIV without specifying the type of virus they will be referring to HIV-1. The relatively uncommon HIV-2 type is concentrated in West Africa and is rarely found elsewhere (Peeters et al, 2001; Peeters & Courgnaud, 2002). The strains of HIV-1 can be classified into four groups: the "major" group M, the "outlier" group O and two new groups, N and P. These four groups may represent four separate introductions of simian immunodeficiency virus into humans. (Peeters et al, 2001; Peeters & Courgnaud, 2002). Group O appears to be restricted to west-central Africa. Group N is a strain discovered in 1998 in Cameroon and extremely rare. In 2009 a new strain closely relating to gorilla simian immunodeficiency virus was discovered in a Cameroonian woman. It was designated HIV-1 group P (Plantier et al, 2009). More than 90 percent of HIV-1 infections belong to HIV-1 group M. Within group M there are known to be at least nine genetically distinct **subtypes** (or clades) of HIV-1. These are subtypes A, B, C, D, F, G, H, J and K. (WHO, 2011)

Occasionally, two viruses of different subtypes can meet in the cell of an infected person and mix together their genetic material to create a new hybrid virus (a process similar to sexual reproduction, and sometimes called "viral sex") (Burke, 1997). Many of these new strains do not survive for long, but those that infect more than one person are known as "circulating recombinant forms" or **CRFs**. For example, the CRF A/B is a mixture of subtypes A and B.

2.12 HIV STRUCTURE

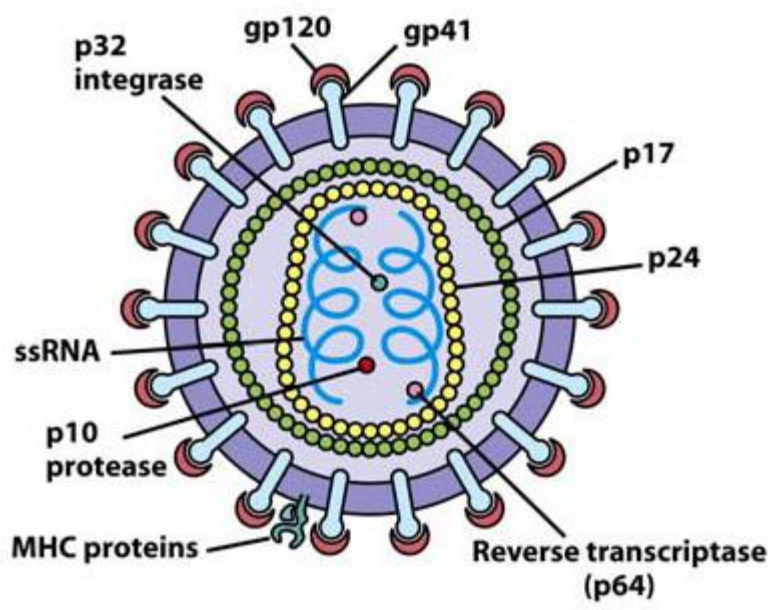


Figure 2.3: The HIV viral structure. Source: Thomas K. Kuby. "Immunology." New York: W.H. Freeman and Company; 2007.

Basic structure of the virus

1. **The viral envelope**: the outer coat of the virus consists of two layers of lipids; different proteins are embedded in the viral envelope, forming "spikes" consisting of the outer glycoprotein (gp) 120 and the transmembrane gp41. The lipid membrane is borrowed from the host cell during the budding process (formation of new particles). Gp120 is needed to attach to the host cell, and gp41 is critical for the cell fusion process (figure 2.3).

2. **The HIV matrix proteins:** consisting of the p17 protein, lie between the envelope and core (figure 2.3).

3. **The viral core:** contains the viral capsule protein p24 which surrounds two single strands of HIV RNA and the enzymes needed for HIV replication, such as reverse transcriptase, protease, ribonuclease, and integrase; out of the nine virus genes, there are three, namely **gag, pol and env**, that contain the information needed to make structural proteins for new virus particles. p24 is used in the context of early diagnosis of HIV infection in children; during the synthesis of the virus, many proteins get lost and can therefore be detected in the plasma (figure 2.3).

2.13 Viral structural proteins

- gag (group-specific antigen) codes for the precursor gag polyprotein which is processed by viral protease during maturation to MA (matrix protein, p17); CA (capsid protein, p24); SP1 (spacer peptide 1, p2); NC (nucleocapsid protein, p7); SP2 (spacer peptide 2, p1) and P6 protein (Steven, 1994).

- pol codes for viral enzymes reverse transcriptase (RT) and RNase H, integrase (IN), and HIV protease (PR) (Votteler & Schubert, 2008). HIV protease is required to cleave the precursor Gag polyprotein to produce structural proteins, RT is required to transcribe DNA from RNA template, and IN is necessary to integrate the double-stranded viral DNA into the host genome (Mushahwar, 2007).

- env (for "envelope") codes for gp160, which is cleaved by a host protease, furin, within the endoplasmic reticulum of the host cell. The post-translational processing produces a surface glycoprotein, gp120 or SU, which attaches to the CD4 receptors present on

lymphocytes, and gp41 or TM, which embeds in the viral envelope to enable the virus to attach to and fuse with target cells (Steven, 1994; Mushahwar, 2007).

2.13.1 Essential regulatory elements

- *tat* (HIV trans-activator) plays an important role in regulating the reverse transcription of viral genome RNA, ensuring efficient synthesis of viral mRNAs and regulating the release of virions from infected cells. (Votteler & Schubert, 2008). Tat is expressed as 72-amino acid one-exon Tat as well as the 86-101 amino-acid two-exon Tat, and plays an important role early in HIV infection. Tat (14-15kDa) binds to the bulged genomic RNA stem-loop secondary structure near the 5' LTR region forming the trans-activation response element (TAR) (Mushahwar, 2007; Votteler & Schubert, 2008).
- *rev* (regulator of expression of virion proteins): The Rev protein binds to the viral genome via an arginine-rich RNA-binding motif that also acts as a NLS (nuclear localization signals), required for the transport of Rev to the nucleus from cytosol during viral replication (Votteler & Schubert, 2008). Rev recognizes a complex stem-loop structure of the mRNA *env* located in the intron separating coding exon of Tat and Rev, known as the HIV Rev response element (RRE) (Mushahwar, 2007; Votteler & Schubert, 2008). Rev is important for the synthesis of major viral proteins and is hence essential for viral replication.

2.13.2 Accessory regulatory proteins

- *vpr* (lentivirus protein R): Vpr is a virion-associated, nucleocytoplasmic shuttling regulatory protein (Votteler & Schubert, 2008). It is believed to play an important role in replication of the virus, specifically, nuclear import of the preintegration complex. Vpr also appears to cause its host cells to arrest their cell cycle in the G2 phase. This arrest activates the host DNA repair machinery which may enable integration of the viral DNA.[5] HIV-2 and SIV encode an additional Vpr related protein called Vpx which functions in association with Vpr (Votteler & Schubert, 2008).
- *vif* - Vif is a highly conserved, 23 kDa phosphoprotein important for the infectivity of HIV-1 virions depending on the cell type (Montagnier, 1999]. HIV-1 has been found to

require Vif to synthesize infectious viruses in lymphocytes, macrophages, and certain human cell lines. It does not appear to require Vif for the same process in HeLa cells or COS cells, among others (Votteler & Schubert, 2008).

- *nef*- Nef, negative factor, is a N-terminal myristoylated membrane-associated phosphoprotein. It is involved in multiple functions during the replication cycle of the virus. It is believed to play an important role in cell apoptosis and increase in virus infectivity (Votteler & Schubert, 2008).
- *vpu* (Virus protein U) - Vpu is specific to HIV-1. It is a class I oligomeric integral membrane phosphoprotein with numerous biological functions. Vpu is involved in CD4 degradation involving the ubiquitin proteasome pathway as well as in the successful release of virions from infected cells (Mushahwar, 2007; Votteler & Schubert, 2008].
- *tev*: This gene is only present in a few HIV-1 isolates. It is a fusion of parts of the *tat*, *env*, and *rev* genes, and codes for a protein with some of the properties of tat, but little or none of the properties of rev (Benko et al, 1990).

2.14 HIV replication cycle:

1. **Entry- Binding and fusion**

Proteins, called envelope proteins, embedded in the outer membrane of the HIV virion bind to receptors on the surface of target cells. T-cells (white blood cells) have CD4 and CCR5 receptors to which HIV can bind. Binding of the HIV envelope protein to CD4 and CCR5 allows the HIV-1 outer membrane to fuse with the cell’s outer membrane and the contents of the virus particle to enter the cell (figure 2.4).

2. Reverse Transcription

The genetic material of the virus is in the form of RNA, or ribonucleic acid. There are two strands of RNA in each HIV-1 virus particle. An enzyme known as reverse transcriptase initiates the formation of one double-stranded molecule of viral DNA (deoxyribonucleic acid) by copying the sequence of the RNA strands contained in the virus particle (figure 2.4).

3. Integration

The viral DNA enters the nucleus of the host and becomes integrated into the host's DNA. An enzyme called integrase is key in this process. Once the viral DNA has integrated into the cell's DNA, the cell is infected for the remainder of its life. The integrated viral DNA is now referred to as a **provirus** (figure 2.4).

4. Transcription

The provirus DNA serves as a template for the creation of new viral RNA via a process known as **transcription**. The host cell's own machinery that is normally used for the transcription of human genes is used by the virus to create new viral RNA molecules. The newly formed viral RNA moves out of the infected cell's nucleus (figure 2.4).

5. Translation

The viral RNA carries code for the synthesis of viral proteins and enzymes. The code is translated into long chains of amino-acids, known as polypeptide chains, which fold to form the protein and enzyme components of new virus particles (figure 2.4).

6. Assembly

Components that are required to build new virus particles, namely viral proteins, enzymes and genetic material (viral RNA) move to the cell's outer membrane where they accumulate and assemble in the form of a bud. A variety of host cell proteins are recruited to assist in virus assembly (figure 2.4).

7. Release (budding) and Maturation

Host-cell proteins cut the virus bud from the cell's outer membrane, thereby releasing a new virus particle. During and after assembly and release, a viral enzyme called protease cuts the HIV polypeptide chains at several positions, in a process called maturation, to make the finished components of the new, infectious, virus particle. A single infected cell can release many new HIV particles which move on to infect other cells in various parts of the body, where the viral life cycle is repeated. The infected cells are eventually destroyed (figure 2.4).

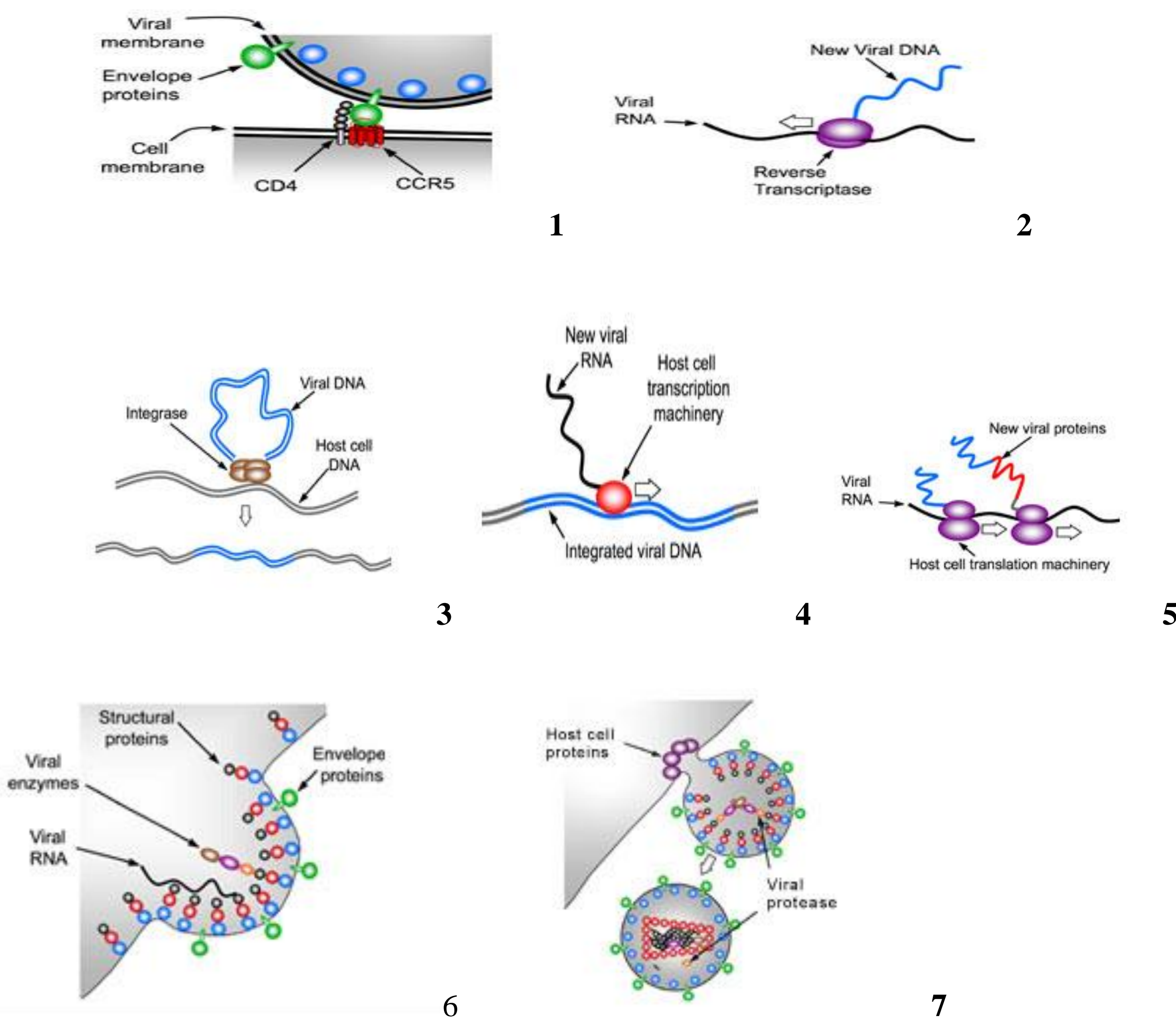


Figure 2.4 showing stages of HIV replication cycle

2.15 IMMUNOGLOBULINS

Immunoglobulins are heterodimeric proteins composed of 2 heavy and 2 light chains. They can be separated functionally into variable domains that bind antigens and constant domains that specify effector functions, such as activation of complement or binding to Fc receptors. The variable domains are created by means of a complex series of gene rearrangement events and can then be subjected to somatic hypermutation after exposure to antigen to allow affinity maturation. Each variable domain can be split into 3 regions of sequence variability termed the complementarity-determining regions (CDRs) and 4 regions of relatively constant sequence termed the framework regions. The 3 CDRs of the heavy chain are paired with the 3 CDRs of the light chain to form the antigen-binding site, as classically defined. The constant domains of the heavy chain can be switched to allow altered effector function while maintaining antigen specificity (Schroeder & Cavacini, 2010).

2.15.1 Structural elements

Immunoglobulins belong to the eponymous immunoglobulin superfamily (IgSF) (Torres et al, 2008). They consist of 2 heavy (H) and 2 light (L) chains (Fig ?), where the L chain can consist of either a κ or a λ chain. Each component chain contains one NH2-terminal variable (V) IgSF domain and 1 or more COOH-terminal constant (C) IgSF domains, each of which consists of 2 sandwiched β-pleated sheets pinned together by a disulfide bridge between 2 conserved cysteine residues (Williams & Barclay, 1988). Each V or C domain consists of approximately 110 to 130 amino acids, averaging 12,000 to 13,000 kd. Both immunoglobulin L chains contain only 1 C domain, whereas immunoglobulin H chains contain either 3 or 4 such domains. H chains with 3 C domains tend to include a spacer hinge region between the first (CH1) and second (CH2) domains (figure 2.5). A typical L chain will thus mass approximately 25 kd, and a 3 C domain Cγ H chain with its hinge will mass approximately 55 kd. Considerable variability is allowed to the amino acids that populate the external surface of the IgSF domain and to the loops that link the β strands. These solvent-exposed surfaces offer multiple targets for docking with other molecules. The ability to identify component parts of the antigen independently of the rest makes it possible for the B cell to discriminate between 2 closely related antigens, each of which can be

viewed as a collection of epitopes. It also permits the same antibody to bind divergent antigens that share equivalent or similar epitopes, a phenomenon referred to as cross-reactivity (Williams & Barclay, 1988).

2.15.2 Antigen recognition and the Fab

Early studies of immunoglobulin structure were facilitated by the use of enzymes to fragment IgG molecules. Papain digests IgG into 2 Fab fragments, each of which can bind antigen, and a single Fc fragment. Pepsin splits IgG into an Fc fragment and a single dimeric $F(ab)_2$ that can cross-link, as well as bind, antigens. The Fab contains 1 complete L chain in its entirety and the V and C_H1 portion of 1 H chain (Fig ?). The Fab can be further divided into a variable fragment (Fv) composed of the V_H and V_L domains, and a constant fragment composed of the C_L and C_H1 domains. Single Fv fragments can be genetically engineered to recapitulate the monovalent antigen-binding characteristics of the original parent antibody (Smith et al, 2004). Intriguingly, a subset of antibodies in a minority of species - camelids (Hamers-Casterman et al, 1993) and nurse shark (Roux et al, 1998), lack light chains entirely and use only the heavy chain for antigen binding. Although these unusual variants are not found in human subjects, there are a number of ongoing attempts to humanize these types of antibodies for therapeutic and diagnostic purposes (Vincke et al, 2009). Figure 2.5.

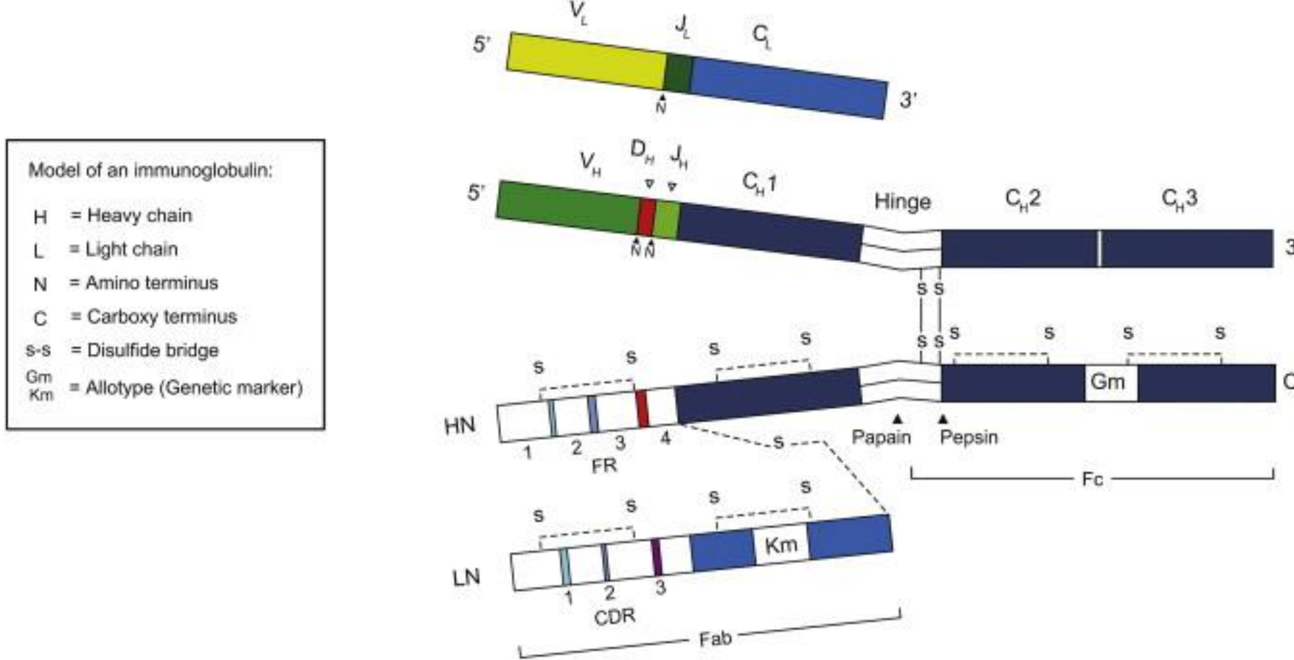


Figure 2.5: Two-dimensional model of an IgG molecule. The H and L chains at the top deconstruct the antibody at a nucleotide level. The chains at the bottom deconstruct the protein sequence. See the text for further details. (Torres et al, 2008).

2.16 FIVE MAJOR TYPES OF IMMUNOGLOBULINS

There are 5 main classes of heavy chain constant domains. Each class defines the **IgM, IgG, IgA, IgD, and IgE isotypes**. IgG can be split into 4 subclasses, IgG1, IgG2, IgG3, and IgG4, each with its own biologic properties, and IgA can similarly be split into IgA1 and IgA2.

1. **IgA**

IgA antibodies are found in areas of the body such the nose, breathing passages, digestive tract, ears, eyes, and vagina. IgA antibodies protect body surfaces that are exposed to outside foreign substances. This type of antibody is also found in saliva, tears, and blood. About 10% to 15% of the antibodies present in the body are IgA antibodies. A small number of people do not make IgA antibodies. IgA serum levels tend to be higher than IgM levels but considerably lower than IgG levels. Conversely, IgA levels are much higher than IgG levels at mucosal surfaces and in secretions, including saliva and breast milk (Woof & Mestecky, 2005). In particular, IgA can contribute up to 50% of the protein in **colostrum, the "first milk"** given to the neonate by the mother. Although generally a monomer in the serum, IgA at the mucosa, termed secretory IgA (sIgA), is a dimer (sometimes trimer and tetramer) associated with a J-chain and another polypeptide chain, the secretory component. There are 2 subclasses of IgA, IgA1 and IgA2, with structures that differ mainly in their hinge regions. IgA1 has a longer hinge region with a duplicated stretch of amino acids that is lacking in IgA2. This elongated hinge region increases the sensitivity of IgA1 to bacterial proteases in spite of partial protection by glycans. Such increased protection against protease digestion might explain why IgA2 predominates in many mucosal secretions, such as the genital tract, whereas more than 90% of serum IgA is in the form of IgA1.

IgA is critical at protecting mucosal surfaces from toxins, viruses, and bacteria by means of direct neutralization or prevention of binding to the mucosal surface. Intracellular IgA might also be important in preventing bacterial or viral infection, pathogenesis, or both. The polymeric nature of sIgA might be particularly important. For example, polymeric IgA (pIgA) is more effective than monomeric IgA at preventing Clostridium difficile toxin A–induced damage to epithelial cells (Stubbe et al, 2000). Specific bacteria can be trapped by the glycans on IgA. It has

been proposed that sIgA might also act as a potentiator of the immune response in intestinal tissue by means of uptake of antigen to dendritic cells (Corthesy, 2007).

2. **IgG.**

IgG (IgG1 > IgG2 > IgG3 > IgG4) is the predominant isotype found in the body. It has the longest serum half-life of all immunoglobulin isotypes. It is also the most extensively studied class of immunoglobulins. IgG antibodies are found in all body fluids. They are the smallest but most common antibody (75% to 80%) of all the antibodies in the body. They activate complement cascade, involved in transplacental transport and participate in the secondary immune response. IgG antibodies are very important in fighting bacterial and viral infections. IgG antibodies are the only type of antibody that can cross the placenta in a pregnant woman to help protect her baby (fetus). IgG antibodies also contribute directly to an immune response, including neutralization of toxins and viruses. Here again, IgG subclass affects the outcome of this interaction. In subjects with HIV, it has been shown that IgG3 antibodies can be more effective at neutralizing virus than IgG1 antibodies, presumably through an increase in antibody flexibility, improving antibody access or inducing changes in the oligomer structure of the virus (Cavacini et al, 2003; Yeh et al, 2006].

3. **IgM.**

IgM is the first immunoglobulin expressed during B-cell development. IgM antibodies are about 5% to 10% of all the antibodies in the body. Naive B cells express monomeric IgM on their surface and associate with CD79a and CD79b, polypeptide chains that participate in IgM cell signaling. IgM functions by opsonizing (coating) antigen for destruction and fixing complement. The pentameric nature of the antibody renders it very efficient in this process. IgM antibodies are associated with a primary immune response and are frequently used to diagnose acute exposure to an immunogen or pathogen. These relatively low-affinity IgM antibodies are also called natural antibodies. Some of these natural antibodies not only participate as a first line of defense but also play a role in immunoregulation (Boes, 2000). IgM antibodies are the largest antibody, they are found in blood and lymph. They also cause other immune system cells to destroy foreign substances.

4. **IgE.**

IgE antibodies are found in the lungs, skin, and mucous membranes. They cause the body to react against foreign substances such as pollen, fungus spores, and animal dander. They are involved in allergic reactions to milk, some medicines, and some poisons. IgE antibody levels are often high in people with allergies. Although IgE is present at the lowest serum concentration and has the shortest half-life, IgE is a very potent immunoglobulin. It is associated with hypersensitivity and allergic reactions, as well as the response to parasitic worm infections. IgE binds with extremely high affinity to FcεRI, which is expressed on mast cells, basophils, Langerhans cells, and eosinophils. Circulating IgE upregulates FcεR expression on these cells. The combination of strong binding and upregulation of FcεR expression contributes to the remarkable potency of this immunoglobulin.

Recently, there has been the development of anti-IgE antibodies as therapy for allergy and asthma (Chang et al, 2007). Antibodies are designed to target free IgE, as well as B cells with membrane-bound IgE, but not IgE bound to FcεR because the latter would stimulate degranulation and the release of inflammatory mediators. IgE has a much lower affinity for FcεRII, or CD23, which is expressed both on the same cells as FcεRI and on B cells, natural killer cells, and platelets.

5. **IgD.**

IgD antibodies are found in small amounts in the tissues that line the belly or chest. They have a short serum half-life, which can be attributed to the sensitivity of the molecule, with the hinge region in particular, to proteolysis. The function of circulating IgD is unclear because it is not known to participate in the major antibody effector mechanisms. Circulating IgD can react with specific bacterial proteins, such as the IgD-binding protein of Moraxella catarrhalis, independently of the variable regions of the antibody (Riesbeck & Nordstrom, 2006). The binding of these bacterial proteins to the constant region of IgD results in B-cell stimulation and activation. Although the membrane-bound form of IgD has been more extensively studied, even here its function remains poorly understood. Similar to IgM, membrane-bound IgD is associated with CD79a and CD79b for signaling. IgD is expressed on the membranes of B cells when they

leave the bone marrow and populate secondary lymphoid organs. Most IgD+ B cells also coexpress IgM, and both participate in B-cell receptor signaling through CD79a and CD79b. IgD can replace IgM and vice versa on IgD+IgM+ B cells. It has been proposed that membrane-bound IgD regulates B-cell fate at specific developmental stages through changes in activation status (Geisberger et al, 2006).

2.16.1 Light chains are proteins produced by plasma cells. They are used to assemble the heavy chain constant domains of immunoglobulins (Ig). There are two types of light chain in humans (as in other mammals),

1. **kappa (κ) chain**, encoded by the immunoglobulin kappa locus (IGK@) on chromosome 2
2. **lambda (λ) chain**, encoded by the immunoglobulin lambda locus (IGL@) on chromosome 22

Each immunoglobulin class expresses only one class of light chain. Once set, light chain class remains fixed for the life of the B lymphocyte. In a healthy individual, the total kappa to lambda ratio is roughly 3:1 in serum (measuring intact whole antibodies) or 1:1.5 if measuring free light chains, with a highly divergent ratio indicative of neoplasm. The exact normal ratio of kappa to lambda, according to a novel polyclonal free light chain assay, ranges from 0.26 to 1.65 (Katzman et al, 2001). Both the kappa and the lambda chains can increase proportionately, maintaining a normal ratio. This is usually indicative of something other than a blood cell dyscrasia, such as kidney disease. Ig light chains produced in neoplastic plasma cells, such as in multiple myeloma, are called **Bence Jones proteins**. Increased levels of free Ig light chains have also been detected in various inflammatory diseases. It is important to note that, in contrast to increased levels in lymphoma subjects, these Ig light chains are polyclonal. Recent studies have shown that these Ig light chains can bind to mast cells and, using their ability to bind antigen, facilitate activation of these mast cells (Redegeld et al, 2002). Activation of mast cells results in the release of various pro-inflammatory mediators which are believed to contribute to the development of the inflammatory disease. Recent studies have shown that Ig light chains not only activate mast cells but also dorsal root ganglia (Rijnierse et al, 2009) and neutrophils

(Braber et al, 2012), expanding their possible role as mediators in inflammatory disease. Serum free light chain (SFLC) testing is ordered to help detect, diagnose, and monitor plasma cell disorders (dyscrasias), including multiple myeloma and primary amyloidosis, and to monitor the effectiveness of treatment (Rijnierse et al, 2009; Braber et al, 2012).

2.17 LYMPHOCYTE

In human, adults lymphocytes make up roughly 20 to 40 percent of the total number of white blood cells. They are agranulocytes (no granules). They are found in the circulation and also are concentrated in central lymphoid organs and tissues, such as the spleen, tonsils, and lymph nodes, where the initial immune response is likely to occur. They include **natural killer cells (NK cells)** (which function in cell-mediated, cytotoxic innate immunity), **T cells** (for cell-mediated, cytotoxic adaptive immunity), and **B cells** (for humoral, antibody-driven adaptive immunity). They are the main type of cell found in lymph, which prompted the name lymphocyte.

2.17.1 Three main classification of lymphocyte

1 & 2. T cells and B cells

Both originate from stem cells in the bone marrow and are initially similar in appearance. Some lymphocytes migrate to the thymus, where they mature into T cells; others remain in the bone marrow, where—in humans—they develop into B cells. Most lymphocytes are short-lived, with an average life span of a week to a few months, but a few live for years, providing a pool of long-lived T and B cells. T cells (thymus cells) and B cells [bursa-derived cells**]** are the major cellular components of the adaptive immune response. T cells are involved in cell-mediated immunity, whereas B cells are primarily responsible for humoral immunity (relating to antibodies). The process of B-cell maturation was elucidated in birds and the B most likely means "bursa-derived" referring to the bursa of Fabricius "B Cell". However, in humans (who do not have that organ), the bone marrow makes B cells, and the B can serve as a reminder of bone marrow. (Merriam-Webster Dictionary, 2011).

The function of T cells and B cells is to recognize specific “non-self” antigens through receptor molecules on their surfaces during a process known as antigen presentation. Once they have identified an invader, the cells generate specific responses that are tailored to maximally eliminate specific pathogens or pathogen-infected cells. B cells respond to pathogens by producing large quantities of antibodies which then neutralize foreign objects like bacteria and viruses. In response to pathogens some T cells, called **T helper cells**, produce cytokines that direct the immune response, while other T cells, called **cytotoxic T cell**s, produce toxic granules that contain powerful enzymes which induce the death of pathogen-infected cells. Following activation, B cells and T cells leave a lasting legacy of the antigens they have encountered, in the form of **memory cells**. Throughout the lifetime of an animal these memory cells will “remember” each specific pathogen encountered, and are able to mount a strong and rapid response if the pathogen is detected again.

3. Natural killer cells

NK cells are a part of the innate immune system and play a major role in defending the host from both tumors and virally infected cells. NK cells distinguish infected cells and tumors from normal and uninfected cells by recognizing changes of a surface molecule called MHC (major histocompatibility complex) class I. NK cells are activated in response to a family of cytokines called interferons. Activated NK cells release cytotoxic (cell-killing) granules which then destroy the altered cells (Janeway et al, 2001). They were named "natural killer cells" because of the initial notion that they do not require prior activation in order to kill cells which are missing MHC class I.

Table 2.1: Typical recognition markers for lymphocytes (Berrington et al, 2005)

CLASS	FUNCTION	PROPORTION	PHENOTYPIC MARKER(S)
NK cells	Lysis of virally infected cells and tumour cells	7% (2-13%)	CD16 CD56 but not CD3
Helper T cells	Release cytokines and growth factors that regulate other immune cells	46% (28-59%)	TCRαβ, CD3 and CD4
Cytotoxic T cells	Lysis of virally infected cells, tumour cells and allografts	19% (13-32%)	TCRαβ, CD3 and CD8
γδ T cells	Immunoregulation and cytotoxicity	5% (2%-8%)	TCRγδ and CD3
B cells	Secretion of antibodies	23% (18-47%)	MHC class II, CD19 and CD21

In the circulatory system they move from lymph node to lymph node. This contrasts with macrophages, which are rather stationary in the nodes.

2.18 FACTORS AFFECTING MOTHER-TO-CHILD TRANSMISSION OF HIV-1

Transmission from mother-to-child of HIV is affected by a number of factors. These include: viral, maternal, obstetrical, fetal and infant factors.

2.18.1 Viral factors

1. Viral load

Transmission is increased in the presence of high levels of maternal viraemia. Clinical observations of increased transmission in these situations, such as in advanced disease and at the time of seroconversion, are supported by the presence of high levels of p24 antigenaemia (European Collaborative Study, 1992; Mayaux et al, 1995]. With the development of new techniques for the measurement of the virus, such as quantitative Polymerase Chain Reaction (PCR) DNA and RNA, an association has been shown between the maternal viral load and the risk of transmission from mother to child, more than half of the women with viral loads of >50 000 RNA copies per ml at the time of delivery have been shown to transmit the virus (Fang et al, 1995; Thea et al, 1997). A New York study showed a mean viral load of 16000 RNA copies/ml in transmitters and 6600 RNA copies /ml in non-transmitters (Thea et al, 1997). Women in this study with measurable viral loads were almost six times more likely to transmit than those in whom the virus was undetectable, after controlling for the CD4+ count. In a French study, transmission rates increased with increasing viral load: 12% in those with less than 1000 copies/ml compared with 29% in those with more than 10 000 copies/ml (Mayaux et al, 1997). Few studies have shown a threshold viral load for transmission and it appears that it can occur at low viral levels, for reasons which are not well understood, but which probably reflect the multiple influences acting on mother-to-child transmission (Fang et al, 1995; Koup et al, 1996; Thea et al, 1997). The local viral load in cervico-vaginal secretions and in breast milk may also be an important determinant of transmission risk intrapartum and through breastfeeding (Loussert-Ajaka et al, 1995). HIV-1 levels in these fluids have been shown in most studies to be correlated with CD4+ count and plasma viral load (Nduati et al, 1995). The presence of sexually transmitted diseases or other causes of inflammation, vitamin A deficiency and local immune response may affect viral shedding. In Rwanda, postnatal transmission was associated with the

presence of HIV-1 infected cells in breast milk (Van de Perre et al, 1992). Viral load in HIV pregnant women receiving Triple combination of ART has shown to be low or undetectable (Eliane Borges-Almeida et al, 2011).

2. **Viral genotype and phenotype**

A number of HIV-1 sub-types or clade groups have been identified, with differing geographical distributions (Boswell et al, 1995). There is little evidence on the effect of sub-type on infection or transmission, although some studies have shown an increased in-vitro ability of sub-type E to infect epithelial cells from the vagina and cervix (John & Kreiss 1996). The subtype may affect the cell tropism of the virus, and in turn the infectivity, in-utero, through genital infection or in breast milk. Most studies on viral variants in mothers and children have demonstrated that the strains in the infant are a distinct subset of maternal virus, although the major maternal variant has also been shown to be transmitted (Cavaco-Silva et al, 1998). Different viral phenotypes show differing tissue tropism. Macrophage-tropic non-syncytium-inducing (NSI) viral isolates appear to be preferentially transmitted to children even when the dominant maternal strains are syncytium inducing (SI) (John & Kreiss 1996). There may be a difference in disease progression for the child related to the viral strain. High virus isolates have been associated with transmitting mothers whereas low virus isolates were associated with non-transmitting mothers (Colognesi et al, 1997; De Rossi et al, 1997). Increased strain diversity in the mother may theoretically influence the rate of transmission. Repeated exposure to different viral strains through pregnancy, occurring through unprotected intercourse may be the mechanism responsible for the observed increase in transmission in these cases (Bulterys & Goedert et al, 1995).

2.18.2 Maternal factors

1. **Maternal immunological status**

Transmission from mother to child is more likely with decreased maternal immune status, reflected by low CD4+ counts, low CD4+ percentages or high CD4+/CD8 ratios (Kolte, 2013). These in turn may be markers for higher viral loads, as opposed to risk factors in themselves, although an interaction between viral load and immune response may be present. In the European Collaborative Study (ECS), there was an increased risk of mother-to-child

transmission where maternal CD4+ counts were below 700/mm3. Transmission increased almost linearly in this study with decreasing CD4+ counts [European Collaborative Study 1996]. Several other studies have noted similar associations (Wood, 1996; Pitt et al, 1997). In the WITS study, the association between low CD4+ percentages and transmission was only seen in women without persistently positive viral cultures. Where there was at least one negative culture and high CD4+ cell percentages, transmission rates were in the range of 1-4% (Pitt et al, 1997). There have been conflicting results about the role of neutralizing antibodies in preventing transmission. Some studies have shown that high levels of maternal neutralizing antibody are associated with lower rates of transmission, while in others no association was observed (Husson et al, 1995). Women who transmit in-utero may have lower levels of autologous neutralizing antibody than those who do not transmit, or those women where transmission occurs intrapartum (Bryson, 1996). Antibody to the V3 loop of HIV-1 envelope gp120 has not been shown to be protective; neither do antibody-dependent cellular cytotoxicity (ADCC) antibodies do not appear to be protective. One report has correlated maternal antibodies to the carboxy region of the gp41 envelope glycoprotein with lack of vertical transmission (Ugen et al, 1997). Infection through breastfeeding has been associated with a lack of IgM and IgA in breast milk (Van de Perre et al, 1992; Nduati et al, 1995).

It was observed that among HIV positive non ARV treated pregnant women, immunoglobulin (Ig) G levels declined gradually throughout pregnancy until delivery, and increased in the 6 month post-partum period while total IgM and IgA levels remained stable throughout pregnancy. In the same manner, mean CD4 and CD8 cell counts declined to a low level 6 months before delivery, increased gradually until delivery and rose sharply to a peak level 3 months post-partum. In contrast, CD4 and CD8 percentages were stable during pregnancy, and increased slightly thereafter. These findings suggest that pregnancy does not accelerate HIV progression, but in view of the intrinsic variability in serial CD4 counts, caution should be exercised when assessing changes in immunological markers in individual pregnant women (European Collaborative Study, 1997).

2. **Maternal nutritional factors**

Serum vitamin A levels in HIV-1 positive mothers have been correlated with the risk of transmission in a Malawi study. The mean vitamin A level in those mothers who transmitted virus to their children was significantly lower than in those who did not transmit. Women with vitamin A levels below 1.4 umol/l had a 4.4-fold increased risk of transmission, which dropped with increasing vitamin A levels (Semba et al, 1994). One US study showed no relationship between low vitamin A levels and transmission (Burger et al, 1997), while another cohort study did show a correlation (Greenberg et al, 1997). The result of randomised controlled trials in Malawi, South Africa, Tanzania, and Zimbabwe between 1995 and 2005 found no evidence that vitamin A supplementation has an effect on the risk of MTCT of HIV. However, antenatal vitamin A supplementation significantly improved birth weight but there was no evidence of an effect on preterm births, stillbirths, deaths by 24 months, postpartum CD4 levels, and maternal death (Shey Wiysonge et al, 2011). Vitamin A was shown to improve birth weight but has no evidence to prevent MTCT (Kongnyuy et al, 2009). Alternatively, low vitamin A levels may be a marker for other deficiencies or behavioral factors, which influence transmission. Other micronutrients have been suggested as having a possible role, including zinc and selenium.

3. **Behavioural factors**

Several behavioral factors have been associated with an increased rate of transmission from Mother-to-child. These include cigarette smoking and maternal hard drug use. Research has associated cigarette smoking with premature rupture of membrane and increased vertical transmission of HIV-1 in infants (Burns et al, 1994; Turner et al, 1997).The use of hard drug has also been associated with increased MTCT (Matheson et al, 1997). Unprotected sexual intercourse during pregnancy has been linked to an increased risk of mother-to-child transmission. A transmission rate of 30% was shown in women who had more than 80 episodes of unprotected sex during pregnancy compared with 9.1% in those with no unprotected intercourse (Matheson et al, 1996). A similar association is suggested in two African studies (Lallemant et al, 1994). This may be due to an increased concentration or strain diversity of HIV-1, or the effect of cervical or vaginal inflammation or abrasions. The presence of sexually transmitted disease during pregnancy has been correlated with increased risk of transmission

(Mandelbrot et al, 1996), and STDs have been shown to increase viral shedding in cervico-vaginal secretions (Ghys et al, 1997).

4. Placental factors

Placental factors have been implicated in transmission of the virus from mother to child (Temmerman et al, 1995; Shearer et al, 1997). Placental infection with HIV-1 has been reported and Hofbauer cells and possibly trophoblasts express CD4+ and are thus susceptible to infection (Douglas & King, 1992). An association between increased transmission and the presence of chorioamnionitis was described early in the epidemic. Other placental infections and non-infectious conditions such as abruptio placentae have also been implicated (Boyer et al, 1994). Breaks in the placental surface can occur at any stage of pregnancy and may be related to transmission, although the significance of these may, in turn, depend upon the maternal viral load (Burton et al, 1996). Smoking and drug use, both associated with increased transmission, may exert this effect through placental disruption (Mofenson, 1997). In areas of high malaria prevalence, infection of the placenta is common in pregnancy. Placental P. falciparum infestation has been associated with poorer survival in infants born to HIV-1 positive mothers in Malawi, which may represent increased transmission rates (Bloland et al, 1995) and with higher rates of transmission from mother to child in Kenya (Nahlen et al, 1998).

2.18.3 Obstetric factors

With the majority of mother-to-child transmission occurring at the time of labour and delivery, obstetric factors are important determinants of transmission. Suggested mechanisms for intrapartum transmission of HIV-1 include direct skin and mucous membrane contact between the infant and maternal cervico-vaginal secretions during labour, ingestion of virus from these secretions, and ascending infection to the amniotic fluid (Mandelbrot et al, 1996; Reggy et al, 1997). HIV-1 in cervico-vaginal secretions may be raised four-fold during pregnancy (Henin et al, 1993). The higher rate of infection in first-born twins may be due to longer exposure of the infants to infected secretions (Duliege et al, 1995). Several obstetric factors have been implicated, although results are not consistent across studies with regard to the relative importance of different obstetric factors. In the French perinatal cohort study, preterm delivery,

intrapartum haemorrhage and obstetric procedures were related to transmission risk (Mandelbrot et al, 1996). Other factors such as the use of fetal scalp electrodes, episiotomy, vaginal tears and operative delivery have been implicated in some studies but not in others (Datta et al, 1994; European Collaborative Study, 1996). The duration of labour does not appear to be as important as the duration of rupture of membranes (Pitt et al, 1997). Prolonged rupture of membranes has been associated with increased risk of transmission in a number of studies and is an important risk factor (Biggar et al, 1996; Mandelbrot et al, 1996). In an American study, duration of ruptured membranes of over four hours nearly doubled the risk of infection, regardless of the eventual mode of delivery (European Collaborative Study, 1994). Delivery by elective cesarean section (ECS), cesarean section prior to labor and rupture of membranes is associated with a lower rate of vertical transmission of HIV compared with other modes of delivery. The efficacy of ECS among women receiving combination antiretroviral therapy or among women with low viral loads is unknown (Ann, 2000). In France, women who received long-course antiretroviral treatment in pregnancy and had an elective caesarean section had a transmission rate of less than 1% (Mandelbrot et al, 1998). International Perinatal HIV Group suggests that elective cesarean section reduces the risk of transmission of HIV-1 from mother to child independently of the effects of treatment with zidovudine (International Perinatal HIV Group, 1999).

2.18.4 Fetal factors

Fetal genetic factors may play a part in transmission. Little is known about the role of genetic factors such as the CCR-5 delta32 deletion and HLA compatibility of mother and infant in the determination of transmission risk (Luscher et al, 1998; Mangano et al, 1998; Misrahi et al, 1998). Concordance between infant and maternal HLA has been associated with increased risk of transmission (MacDonald et al, 1998). Preterm infants have higher reported rates of transmission of HIV-1 in several studies (Datta et al, 1994). Women with low CD4+ counts are more likely to have preterm deliveries, which may influence this finding. The higher rates of infection seen in first-born twins have been widely reported and have formed part of the evidence for the role of intrapartum transmission (Goedert, 1997). This effect is more pronounced in vaginally delivered twins, where a two fold increase in infection is seen in first born twins than second born, but is also present in twins delivered by caesarean section (Duliege et al, 1995).

Other fetal factors may include co-infection with other pathogens, fetal nutrition and fetal immune status (Steihm, 1996).

2.18.5 Infant factors

Before now, breastfeeding is responsible for a high proportion of mother-to-child transmission in developing countries, this no longer the case. Breastfeeding is now recommended for HIV positive mothers both in high income countries and low income countries (WHO, 2013). Even in high-income countries, breastfed babies are less likely to acquire childhood illnesses than those given replacement foods (Bahl, 2005). However, the risk of HIV infection means that replacement feeding should always be given if it is feasible and safe to do so. In some countries, including the UK and the USA, there is a possibility that HIV positive mothers who choose to breastfeed may be charged with child endangerment if they persist. Nevertheless the UK Department of Health advises that*:* Under exceptional circumstances, and after seeking expert professional advice on reducing the risk of transmission of HIV through breastfeeding, a highly informed and motivated mother might be assisted to breastfeed. (WHO, 2013).

A study in 2012 found that HIV-neutralising antibodies are released by some B cells that are present in breast milk. A further study in 2013 identified a particular protein in breast milk, named as TNC, that actively inhibits HIV (Duke Medicine, 2013). This could explain why mother-to-child transmission of HIV does not occur more often than it does (Friedman 2012). Breast milk also contains nutrients, agents and antibodies that protect the infant from the risk of childhood diseases such as diarrhea. Without being breastfed, an infant runs the risk of becoming seriously ill with diseases other than HIV. Where treatment for them is limited or inaccessible, an infant's health can be compromised. Similarly, unsafe and unreliable replacement feeding when clean water and resources are unavailable can also be a danger to an infant's health. Breastfeeding is therefore highly recommended in low- and middle-income countries. All children born to HIV-positive mothers should be tested for HIV to determine their status. If an infant is discovered to be HIV positive, mothers are encouraged to exclusively breastfeed for the first 6 months and continue breastfeeding while mixed feeding for up to 2 years (WHO, 2010').

2.19 HIV EFFECT ON PREGNANCY

Pregnant women are considered to be a special population group due to their specific susceptibility to some infectious diseases because of the unique 'immunological' condition caused by pregnancy. During normal pregnancy, the human decidua contains a high number of immune cells, such as macrophages, natural killer (NK) cells and regulatory T cells (Treg) (Aluvihare et al, 2004; Zenclussen, 2005). Seventy percent of decidual leukocytes are NK cells, 20–25% are macrophages and 1.7% are dendritic cells (Mor et al, 2006). From the adaptive immune system, B cells are absent, but T lymphocytes constitute about 3–10% of the decidual immune cells (Wicherek et al, 2009). HIV infection in pregnancy is associated with a complex pattern of changes in the hemopoietic and the immune systems, resulting in abnormalities of peripheral blood (PB) counts and changes in T and B lymphocytes (Borges-Almeida et al, 2011). Decrease of T helper and increase of cytotoxic lymphocytes, profound changes in the cytokine profile and a variety of B lymphocyte abnormalities have been repeatedly described (Clerici et al, 2000; Müller et al, 2002; Pacheco et al, 2006; Onyenekwe et al, 2010; Ifeanyichukwu et al, 2011; Kolte, 2013).

A successful pregnancy is characterised by an increase in Th2 cytokines and suppression of Th1 cytokine production. A Th1 to Th2 cytokine shift is also observed in the disease progression of HIV infection. Highly active antiretroviral therapy (HAART) suppresses HIV viremia, increases CD4+ cell counts and counteracts the Th1 to Th2 shift (Fiore et al, 2006).

Fiore et al (2006) found out that immunomodulation induced by HAART with increased IL-2 (TH1) and decreased IL-10 (TH2). HAART use and IL-10-Env slopes were not significantly associated with prematurity risk, but each unit increase in IL-2-PHA slope was associated with an 8% increased risk of premature delivery. HAART use in pregnancy provides significant benefits in delaying HIV disease progression and reducing the risk of mother-to-child-transmission, but may be counterproductive in terms of successful pregnancy outcome.

2.20 ANTIRETROVIRAL THERAPY IN THE PREVENTION OF MOTHER-TO-CHILD TRANSMISSION OF HIV

Mother-to-child transmission (MTCT) of HIV-1 has been significantly reduced with the use of antiretroviral therapies, resulting in an increased number of HIV-exposed uninfected infants. (Mandelbrot et al, 2001; Newell et al, 2007; Sachdeva et al, 2008; Thorne et al, 2009). Infants in the clinical trial were followed to 18 months to determine their HIV infection status; result showed that transmission rate is consistent with that observed in the larger clinical trial cohort study (Gray, 2000; Kuhn et al, 2001b). The ineffectiveness of these trials has brought about this current triple combination therapy. The development and successful implementation of prevention of mother-to-child transmission (PMTCT) programs has reduced the risk of perinatal transmission of HIV-1 from 25-30% to less than 1% (Mandelbrot et al, 2001; Müller et al 2002; European Collaborative Study, 2005; Pascheco et al 2006; Newell et al 2007; Sachdeva et al 2008; Thorne et al, 2009) and have shown to prolong life and improves quality of life (Mocroft et al, 2003; Porter et al, 2003).

Recent finding on the use of HAART by HIV positive pregnant women has proved 100% non-transmittance of the virus. The study of Borges-Almeida et al, on mother-child pairs from HAART treated HIV+ mothers and HIV-uninfected mothers were studied showed that after one-year follow-up, none of the exposed infants became seropositive for HIV (Borges-Almeida et al, 2011). It is known that the use of highly active antiretroviral therapy (HAART) reduces viral replication, increases CD4+ T cell counts and decrease immune activation and apoptosis in HIV-infected individuals (Gougeon et al, 1999). The recent introduction of highly active antiretroviral therapy (HAART) in HIV-infected pregnant women has led to low and undetectable viral load and drastically decreased vertical transmission of HIV (Borges Almeida et al, 2011).

HIV-1 mother-to-child transmission (MTCT) occurs mainly at three stages, pregnancy (prepartum), labor/delivery (intrapartum) and breastfeeding (postpartum). Several factors like complex pattern of changes in the hemopoietic and the immune systems resulting in abnormalities of peripheral blood (PB) counts and changes in T and B lymphocytes, low CD4+ lymphocyte counts, high viral load, impaired immune response, advanced disease status, non-adherence to drug, smoking and abusing drugs (Borges-Almeida et al, 2011), decrease of T

helper and increase of cytotoxic lymphocytes, profound changes in the cytokine profile and a variety of B lymphocyte abnormalities (Clerici et al, 2000; Müller et al, 2002; Pacheco et al, 2006;), Th1 to Th2 cytokine shift (Simona et al, 2006) have been implicated in an increased risk of HIV-1 MTCT. However, long term antiretroviral therapy (ARV) is able to restore, at least in part, the immune function (Müller et al, 2002; Pacheco et al, 2006; Newell et al, 2007; Sachdeva et al, 2008). The assessment of pregnancy complications in HIV-positive women and changes in the rates of such complications over 11 years by Reitter et al, showed that there was a significant reduction in the rate of preterm deliveries and an increase in the vaginal delivery rate, reflecting changes in treatment policies in the same period and the availability of more effective antiretroviral therapy options. The rates of complications such as GDM, pre-eclampsia, preterm contractions, PROM and postnatal complications were stable over the 11 years, but were still increased compared with the general population (Reitter et al, 2014).

In HIV-infected women, co-infections that target the placenta, fetal membranes, genital tract, and breast tissue, as well as systemic maternal and infant infections, have been shown to increase the risk for mother-to-child transmission of HIV (MTCT). Active co-infection stimulates the release of cytokines and inflammatory agents that enhance HIV replication locally or systemically and increase tissue permeability, which weakens natural defenses to MTCT. HAART use in pregnancy has shown to increase IL-2 (Th1) and decreased IL-10(Th2) and hence provides significant benefits in delaying HIV disease progression and reducing the risk of mother-to-child-transmission but may be counterproductive in terms of successful pregnancy outcome (Simona et al, 2006).

To prevent mother to child transmission of HIV, Nigerian government in 2013 adopted the "Lifelong ART for all pregnant and breastfeeding women living with HIV" by WHO. This refers to the approach in which all pregnant women living with HIV receive a triple-drug ART regimen regardless of CD4 count or clinical stage, both for their own health and to prevent vertical HIV transmission and for additional HIV prevention benefits. Infants of mothers who are receiving ART and are breastfeeding should receive six weeks of infant prophylaxis with daily Niverapin (NVP) and then continues with Septrin until 6 wks after weaned. If infants are receiving replacement feeding, they should be given four to six weeks of infant prophylaxis with daily

NVP (or twice-daily AZT). Infant prophylaxis should begin at birth or when HIV exposure is recognized postpartum (WHO, 2013).

Niverapin (NVP) has been used during pregnancy, both as a part of combination regimens to treat maternal HIV infection and for perinatal chemoprophylaxis to reduce mother-to-child transmission. The drug has several characteristics that make it attractive for use in perinatal HIV chemoprophylaxis. These characteristics include potent, rapid antiviral activity; rapid absorption across the gastrointestinal tract, placenta and blood brain barrier; a long half-life after a single dose (median 61 to 66 h in pregnant women and 45 to 64 h in infants); availability in both tablet and suspension formulations; and a relatively inexpensive cost (Van-Leeuwen et al, 2003).

2.21 IMMUNOLOGICAL ABNORMALITIES IN HIV-EXPOSED UNINFECTED NEWBORNS

Mother-to-child transmission (MTCT) of HIV-1 has been significantly reduced with the use of antiretroviral therapies, resulting in an increased number of HIV-exposed uninfected infants. Children born to HIV infected mothers are exposed intra-utero to several drugs and cytokines that can modify the developing immune system, and influence the newborn's immune response to infections and vaccines. However, some studies showed immunological abnormalities among HIV exposed uninfected infants (Eliane Borges-Almeida et al, 2011). Several changes in peripheral blood (PB) counts and T CD4+ and CD8+ lymphocytes have been detected in HIV-exposed uninfected newborns and attributed to alterations in maternal cytokine profile caused by the HIV infection as well as by the ARV treatment (Clerici et al, 2000; Nielsen et al, 2001; Bunders et al, 2005; Carniel et al, 2008; Ono et al, 2008; Eliane Borges-Almeida, et al 2011; Kolte, 2013).

Control over HIV replication can be independently achieved by pharmacological or immunologic means, HAART is associated with weaker HIV-specific and -non-specific immune responses (Clerici et al, 2000). Hematologic and immunologic functions examined in 19 HIV-negative infants of HIV-positive mothers and 19 control infants of HIV-negative mothers showed lower naive CD4 counts, lower red blood cell counts and reduced thymic output in infants of HIV-positive mothers compared with controls. This finding suggests impairment of

progenitor cell function (Nielsen et al, 2001). In uninfected infants born to HIV+ women, several immunological abnormalities were found, related to the residual maternal immune changes induced by the HIV infection and those associated with antiretroviral treatment. Maternal smoking was associated to changes in cord CD3/CD4 lymphocytes and maternal hard drug abuse was associated with more pronounced changes in the cord B cell line. Maternal age, smoking, weeks of gestation and child's gender had no relation to birth weight (Borges-Almeida et al, 2011)

Cardoso et al, (2013) observed a dysfunctional innate immune response in HIV-1-infected mothers and newborns. The activation of intracellular Toll-like receptors (TLR7/TLR8) could restore defective cytokine secretion by myeloid dendritic cells (mDCs), but not by plasmacytoid dendritic cells (pDCs). Whether defective pDC activation during pregnancy prevents over activation of the immune system or is due to signaling defects has yet to be clarified. Applying this knowledge of the innate immune response of HIV-infected mothers and newborns could contribute to new formulations for drugs or the development of novel vaccination strategies.

The immunological response of formula fed exposed uninfected infants from HIV pregnant mothers on HAART by Ono *et al*, (2008) showed a lower percentage of naive T cells and higher percentages of central memory T cells among exposed uninfected infants (EU) than control unexposed neonates. At 12 months, EU infants still had higher mean fluorescence intensity of CD38 on T cells. Despite effective maternal virologic control at delivery, HIV-exposed uninfected children were born with lower levels of naive T cells. Immune activation was present at birth and remained until at least 12 months of age, suggesting that in-utero exposure to HIV causes subtle immune abnormalities (Ono et al, 2008). Miles et al, demonstrated that infants born to HIV positive mothers had reduced CD4 counts and increased markers of differentiation (CCR7(-) CD45RA(-) CD27(-)) and senescence (CD57, PD-1) and these resulted in their inability to develop immunological memory. At 2 weeks of age, maternal HIV status had no discernable effect on the infants' CD4 counts, interferon (IFN)-γ release, but by 10 weeks of age, there was reduced antigen-specific proliferation and the CD4 count of seroreverters had dropped to levels characteristic of HIV-positive African infants of equivalent age and well below those typical of HIV-negative infants (Miles *et al*, 2010).

An increase in B lymphocytes, especially the CD19/CD5+ ones, was observed in cord blood of HIV-exposed newborns. Children of HIV+ hard drug using mothers had also an increase of immature B-cells, nevertheless, none of these children had HIV at the end of the follow-up (Eliane Borges-Almeida et al, 2011). Findings have indicated alterations in immunoglobulin levels in uninfected children born to HIV-infected women, suggesting that fetal exposure to a chronically activated maternal immune system is associated with an altered humoral response (Ono *et al*, 2008; Madeleine et al, 2010; Cardoso *et al*, 2013).

2.22 IMMUNOLOGICAL ABNORMALITIES IN HIV-EXPOSED UNINFECTED NEWBORNS NORMALIZES AT LATER AGE

Nevertheless, some studies had demonstrated that the immunological abnormality observed among HIV-exposed uninfected newborns normalizes/stabilizes at later age of their lives (Bunders et al, 2005; Kolte, 2013). In a prospective controlled study by Borges-Almeida et al 2011, 36 mother-child pairs from HIV+ mothers and 15 HIV-uninfected mothers were studied. After one-year follow-up none of the exposed infants became seropositive for HIV (Eliane Borges-Almeida et al, 2011). Cell proliferation among HIV exposed uninfected infants separated into three groups (E1, aged 6.1–8.8 months; E2, aged 9.1–17.1 months and E3 aged 18.1–26.3 months) showed no difference neither for BCG nor for Phytohemagglutinin (PHA)-stimulated cultures. The unexposed infants showed higher TNF-α concentration in cultures with BCG and PHA in comparison with E1 group. T-lymphocyte subpopulations also showed differences, with the youngest HIV-exposed groups (E1 and E2) showing a predominant proliferation of $CD4^{+}$T cells in cultures with BCG, whereas E3 and UE groups had a robust $\gamma\delta^{+}$ T-cell expansion. There was lower IFN-γ concentration in the samples from E1 group in comparison with all of the other groups. The study showed a delay in immune system maturation of HIV-exposed uninfected infants, nevertheless the immunological changes normalized with increase in age as shown among E3 and UE groups having a robust $\gamma\delta^{+}$ T-cell expansion (Bunders et al, 2005)

HIV exposed uninfected children were shown to have reduced thymic size compared with children born to HIV-negative mothers, but no evidence of impaired thymic function, immune regulation, or antibody vaccination response was detected, suggesting that no qualitative immune deficits persist in HIV-EU children beyond infancy (Kolte, 2013). Immunological abnormalities

detected in HIV-EU infants are recovered at 15 months of age, and even if diminished in size, thymic function is normalized at this age (Kolte, 2013).

2.23 IMMUNOLOGICAL ABNORMALITY IN HIV INFECTED PREGNANT WOMEN RESTORED WITH HAART

During pregnancy, establishing fetal-maternal tolerance is essential to pregnancy success. HIV-infected women display different immunological profiles from HIV-negative women and this immune unbalance may interfere with the prevention of fetal rejection and may partly explain the increased risk of abortion in HIV-infected women (Kolte, 2013). The study on responsiveness of myeloid dendritic cells (mDCs) and plasmacytoid DCs (pDCs) to Toll-like receptors (TLRs) that are associated with the antiviral response reveals that peripheral blood mononuclear cells (PBMCs) from HIV-1-infected mothers and CB were defective in TNF-α production after activation by TLR2, TLR5, TLR3 and TLR7. However, the TNF-α response was preserved after TLR7/8 (CL097) stimulation, mainly in the neonatal cells. Furthermore, only CL097 activation was able to induce IL-10 and IFN-α secretion in both maternal and CB cells in the infected group. An increase in IFN-α secretion was observed in CL097-treated CB from HIV-infected mothers compared with control mothers. The function of both mDCs and pDCs was markedly compromised in the HIV-infected group, and although TLR7/TLR8 activation overcame the impairment in TNF-α secretion by mDCs, such stimulation was unable to reverse the dysfunctional type I IFN response by pDCs in the HIV-infected samples. The findings highlight the dysfunction of innate immunity in HIV-infected mother-newborn pairs. The activation of the TLR7/8 pathway could function as an adjuvant to improve maternal-neonatal innate immunity (Cardoso et al, 2013).

Restoration of immunological functions in HAART treated infected pregnant women has been studied. Larger thymic size was associated with higher CD4 counts and higher thymic output while abundant thymic tissue seemed to have broader immunological repertoires among HIV infected pregnant women treated with HAART (Kolte, 2013). However, follow-up of this study for 5 years showed that thymic restoration primarily lies within the first two years of HAART. The use of recombinant human growth hormone (rhGH) stimulated thymopoiesis as expressed by thymic size, density, and output strongly supporting the assumption that rhGH possesses the

potential to stimulate the ageing thymus, holding promise as a future means to complete CD4 restoration and renew the T- cell receptor (TCR) repertoire in subjects who respond insufficiently to HAART (Kolte, 2013).

The studies of (Kolte, 2013) also demonstrate increased levels of Tregs in HIV-infected subjects despite long-term treatment with HAART, suppressed viral loads, and normalized CD4 counts and immune activation suggesting that Tregs expand irreversibly in HIV-infection independently of viral load, CD4 depletion or level of immune activation in HAART subjects.

2.24 BREASTFEEDING RECOMMENDATIONS FOR INFANTS OF HIV POSITIVE MOTHERS BY W.H.O 2013

A) In high income countries

National health agencies and the WHO 2013 guidelines recommend that HIV-positive mothers in high income countries:

- Avoid breastfeeding: risk of HIV transmission is far greater than the risk of replacement feeding
- Replacement feed: the only infant feeding method that does not expose an infant to HIV
- Administer HIV treatment: provide infants with 4-6 weeks of once-daily nevirapine (NVP) or twice-daily zidovudine (AZT). (WHO, 2013) 'Global update on HIV treatment 2013: Results, Impact and Opportunities'.

Replacement feeding means giving a baby commercial infant formula (prepared from powder and boiling water) or home-modified animal milk (boiled with added water, sugar and micronutrients) instead of breast milk. In regions of the world where clean water and facilities are available; it is usually promoted as the only option.

Even in high-income countries, breastfed babies are less likely to acquire childhood illnesses than those given replacement foods (Rajiv et al, 2005). However, the risk of HIV infection means that replacement feeding should always be given if it is feasible and safe to do so. In some countries, including the UK and the USA, there is a possibility that HIV positive mothers who choose to breastfeed may be charged with child endangerment if they persist. Nevertheless, the

UK Department of Health advises that*:* Under exceptional circumstances, and after seeking expert professional advice on reducing the risk of transmission of HIV through breastfeeding, a highly informed and motivated mother might be assisted to breastfeed.

B) In low and middle income countries.

National health agencies and the WHO 2013 guidelines recommend that HIV-positive mothers in low-income countries: breastfeed exclusively:

- if there is little access to clean water, sanitation and health services
- continue breastfeeding: for 6 months, then introduce complementary foods and wean baby at 12 months provided the breastfeeding mother is on HAART
- administer HIV treatment: provide infant with once-daily nevirapine (NVP) for 6 weeks.

Supporting the advice to breastfeed in combination with HAART, the use of HAART has greatly reduced HIV transmission from breastfeeding mother to infant. HAART resulted in lower breast milk HIV-1 RNA than ZDV/NVP; however, ZDV/NVP yielded comparable breast milk HIV-1 RNA levels in the first 2 weeks' post-partum. Breast milk HIV-1 RNA remained suppressed in the ZDV/NVP despite increased plasma HIV-1 levels, which might reflect local drug effects or compartmentalization (Michael Chung et al, 2008).

Breast milk itself offers some protective effect on the baby. A study in 2012 found that HIV-neutralising antibodies are released by some B cells that are present in breast milk (Braibant et al, 2013). A protein in breast milk called Tenascin-C or TNC that neutralizes HIV and may protect babies from acquiring HIV from their infected mothers has been identified for the first time by researchers at Duke Medicine (Duke University Medical Center, 2013). Barbra Richardson et al found that 69% of hiv positive lactating mothers in nairobi women had detectable IFN-γ responses from cells in breast milk positively correlated with breast milk viral load, a higher IFN-γ response correlated with higher viral load. However, there was no correlation between the maternal IFN-γ response and detection of an IFN-γ response in uninfected infants, suggesting that protection is occurring in the breast milk rather than being transferred from the mother to the infant. In earlier studies, the team showed that breast milk viral load positively correlates with the development of an IFN-γ response in exposed infants.

Detection of cytokines in breast milk associated with the cellular immune response (MIP-1α, MIP-1β, RANTES, and SDF-1) positively correlated with the strength of the maternal IFN-γ response. This study demonstrates that high levels of HIV-specific immune cells in breast milk positively correlate with the prevention of mother-to-child transmission, and that the broader this response is, the better the protection (Lohman-Payne, 2012). Breast milk also contains nutrients, agents and antibodies that protect the infant from the risk of childhood diseases such as diarrhea. Without being breastfed, an infant runs the risk of becoming seriously ill with diseases other than HIV. Where treatment for infant is limited or inaccessible, an infant's health can be compromised. Similarly, unsafe and unreliable replacement feeding when clean water and resources are unavailable can also be a danger to an infant's health. Breastfeeding is therefore highly recommended in low- and middle-income countries. All children born to HIV-positive mothers should be tested for HIV to determine their status. If an infant is discovered to be HIV positive, mothers are encouraged to exclusively breastfeed for the first 6 months and continue breastfeeding while mixed feeding for up to 2 years (WHO, 2010).

New results from the Breastfeeding, Antiretrovirals, and Nutrition (BAN) study done in Lilongwe, Malawi, by Denise Jamieson et al, revealed that limitation of breastfeeding duration is not suitable for HIV-infected women and their infants. Mothers' compliance with early weaning resulted in significant increases in infant morbidity, growth faltering, and death (Jamieson et al, 2012). Several independent groups (Kafulafula et al, 2010; Kuhn et al, 2010; Taha et al, 2011) have previously reported adverse outcomes in HIV-exposed infants whose mothers had been encouraged to reduce the duration of breastfeeding.

Breastfeeding remains a common practice in parts of the world where the burden of HIV is highest and the fewest alternative feeding options exist. World Health Organization (WHO) advises that most HIV-infected African women breastfeed their infants because of poverty, unavailability of appropriate replacement foods and high risk of infectious disease morbidity and mortality. This is in contrast to industrialised countries with readily available infant formula, lower risk of infections and better access to health care. The guideline also states that ARV treatment should be provided for all pregnant and breastfeeding women living with HIV for life, previously known as Option B+. Countries who do not have the resources to provide lifelong

ARVs to all pregnant women, should offer treatment to mothers living with HIV until one week after they finish breastfeeding, previously known as Option B. After this point, a mother will be assessed to see if she is eligible for treatment for her own health going forward (WO, 2013). Studies have shown that antiretroviral drugs given to the mother through pregnancy, labour and breastfeeding (Thior et al, 2006; Palombi et al, 2007; Kilewo et al. 2009; Peltier et al, 2009; Chasela et al, 2010; Shapiro et al, 2010; Thomas et al, 2011) or infant (Kumwenda et al, 2008; SWEN, 2008; Chasela et al, 2010; Omer & SWEN, 2011) for up to 6 months after delivery reduce HIV transmission to the breastfed infant. On the basis of this emerging evidence, WHO now recommends antiretroviral prophylaxis for the mother or infant throughout breastfeeding The 2013"Lifelong ART for all pregnant and breastfeeding women living with HIV" by WHO recommends that post-partum mothers breastfeed their baby for 8 months to 1year; the first 6 months is exclusive breast feeding while the next 6 months is complementally (a combination of breast milk, water and food). The baby receives NVP syrup from birth till 6 wks of age and then septrin until weaned. Detection of viral antigen using polymerase chain reaction (PCR) is done on the baby at six (6) weeks of age and six (6) weeks after the baby is weaned.

The increasing success of prevention of mother-to-child HIV transmission programmes means that in Africa, very large numbers of HIV-exposed, uninfected (HIV-EU) children are being born. There is some evidence that HIV-EU African children are at increased risk of mortality, morbidity and slower early growth than their HIV-unexposed counterparts (Filteau, 2009). A likely major cause of this impaired health is less exposure to breast milk as mothers are either less able to breastfeed or stop breastfeeding early to protect their infant from HIV infection. Thus the recent PMTCT guideline advocates 6months exclusive breastfeeding and 4-6 months complementary feeding. Treatment of mothers and/or their infants with antiretroviral drugs is a strategy that has been employed for several decades to reduce HIV transmission through pregnancy and delivery, but the effect of ART treatment of HIV positive breastfeeding mothers and prophylaxis on the exposed child is the most recent field of study in PMTCT (WHO, 2013). The recent WHO guideline on PMTCT is to attain specific health sector goals in the Global Health Sector Strategy on HIV/AIDS 2011–2015 and the Global Plan towards the elimination of new HIV infections among children by 2015 and keeping their mothers alive.

Before 2010, early weaning was recommended in many programmes to prevent mother-to-child transmission in sub-Saharan Africa. BAN, and others, had recorded that exclusive breastfeeding results in lower risk of HIV transmission than early weaning and non-exclusive breastfeeding during the first few months of life (Coovadia et al, 2007; Kuhn et al, 2007). However, these findings do not mean that all breastfeeding should end when exclusive breastfeeding is no longer developmentally appropriate. After 6 months of age, complementary feeding is the biologically appropriate feeding method and is necessary to protect infants from disease and support adequate growth. Fortunately, the 2010 WHO revision of the infant feeding guidelines for HIV-infected women clearly indicated that breastfeeding should be supported to 12 months and weaning encouraged only once a safe alternative can be provided (WHO, 2010).

The BAN results also establish that early weaning is a poor choice to prevent HIV-1 transmission. In a 48-week follow-up, an effective antiretroviral regimen was used half the period of breastfeeding, the transmission rate by 28 weeks in the infant-nevirapine group (Chasela et al, 2010) is similar to that reported in another study (Coovadia et al, 2012) in which infant nevirapine prophylaxis was continued for 6 months. However, the 28-week transmission rates in the maternal-antiretroviral group (Chasela et al, 2010) are higher than are those reported in Botswana in which transmission, inclusive of intrauterine and intrapartum transmission, occurred in 1·1% of breastfeeding women by 6 months. Maternal antiretroviral therapy was started during pregnancy in the Botswana study (Shapiro et al, 2010) but only during the postnatal period in BAN, (Jamieson et al, 2012) leaving the infant vulnerable to transmission when the regimen had yet to lower maternal viraemia. Infections acquired intrapartum but detectable only after 2 weeks of age and incorrectly attributed to breastfeeding could also have raised the rates. BAN re-emphasizes that breastfeeding is essential for infant survival and wellbeing. Early weaning is neither effective nor safe as an HIV prevention strategy. The BAN results also show that antiretroviral drugs are highly effective in prevention of postnatal HIV-1 transmission and are the only known means to prevent intrauterine and intrapartum transmission. Antiretrovirals could also contribute to improvement of maternal survival, even in women who

do not meet present criteria for treatment. The challenge now is to implement these effective antiretroviral interventions.

2.25 IMMUNE RESPONSE AND PROFILE IN ARV- TREATED AND NON-TREATED PREGNANT WOMEN AND CORD BLOOD

2.25.1 T- Cell profile in pregnant mothers and cord blood:

The differences between subjects in the degree of CD4+ cell recovery upon treatment with highly active antiretroviral therapy (HAART) may in part be due to differences in the supply of naïve CD4+ cells from the thymus. The thymus atrophies with increasing age for which reason the adult thymus was previously assumed to be without function (Kolte, 2013). Larger thymic size was associated with higher CD4 counts and higher thymic output among HIV infected pregnant women on HAART. Subjects with abundant thymic tissue seemed to have broader immunological repertoires, compared with subjects with minimal thymic tissue. The study supports the mounting evidence of a contribution by the adult thymus to immune reconstitution in HIV-infection (Kolte, 2013). In a follow-up study conducted till 5 years of HAART, the importance of the thymus to the rate of cellular restoration was found to primarily lie within the first two years of HAART. The effect of recombinant human growth hormone (rhGH) was then investigated in a randomized, double-blinded placebo controlled trial in 46 adult HIV-infected subjects on HAART. Daily treatment with a low dose of rhGH of 0.7mg for 40 weeks stimulated thymopoiesis as expressed by thymic size, density, and output strongly supporting the assumption that rhGH possesses the potential to stimulate the ageing thymus, holding promise as a future means to complete CD4 restoration and renew the TCR repertoire in subjects who respond insufficiently to HAART (Kolte, 2013).

HIV-infected women, have been found to display different immunological profiles from HIV-negative women, and this immune unbalance may interfere with the prevention of fetal rejection and may partly explain the increased risk of abortion in HIV-infected women. Highly active antiretroviral therapy (HAART) suppresses HIV viremia, increases CD4+ cell counts and counteracts the Th1 to Th2 shift (Simona et al, 2006). CD8 T cell function, lymphocyte surface

phenotype, serum markers of immunologic activation, and viral burden were assessed in 75 HIV-infected pregnant women, including 9 who transmitted infection to their infants. Serial studies during and after pregnancy showed no significant differences in levels of cell-surface or serum activation molecules in transmitting compared to non-transmitting mothers, with the exception of a postpartum increase in tumor necrosis factor alpha in transmitting women. The transmitting women had a median plasma viral load of 65,516 RNA copies/mL at delivery versus 5139 in nontransmitting women (Plaeger et al, 1999).

An increase in B lymphocytes, especially the CD19/CD5+ ones, was observed in cord blood of HIV-exposed newborns. Children of HIV+ hard drug using mothers had also an increase of immature B-cells (Eliane Borges-Almeida et al, 2011). Among HIV positive non ARV treated pregnant women, mean CD4 and CD8 cell counts declined to a low level 6 months before delivery, increased gradually until delivery and rose sharply to a peak level 3 months' post-partum. In contrast, CD4 and CD8 percentages were stable during pregnancy, and increased slightly thereafter. This suggests that pregnancy does not accelerate HIV progression, but in view of the intrinsic variability in serial CD4 counts, caution should be exercised when assessing changes in immunological markers in individual pregnant women (European Collaborative Study, 1997).

T-helper cell responses to HIV have been associated with protection against maternal-infant HIV transmission in the absence of antiretroviral treatment (Kuhn et al, 2001a). In subsequent investigation, Kuhn, et al, (2001b) examined the effect of short-course regimens of zidovudine-lamivudine (ZDV-3TC) given to prevent maternal-infant HIV transmission on the development of T-helper cell responses to HIV and other antigens. HIV-stimulated T-helper cell reactivity in cord blood was detected 10-fold less frequently among those exposed to antiretroviral prophylaxis than among those unexposed. Reductions in HIV-stimulated responses in cord blood occurred despite detectable HIV RNA at delivery among treated women and occurred independent of treatment duration. This suggests that short-course antiretroviral treatment given to prevent maternal-infant HIV transmission may attenuate HIV-stimulated T-cell memory responses in the neonate. T-helper cell reactivity to HIV envelope peptide from infants of HIV seronegative mothers showed no response (Kuhn et al, 2001b). Peripheral blood lymphocyte

(PBL) from HIV untreated pregnant mothers responded to synthetic gp160 envelope (env) peptides. Fetal T cells can be primed to HIV env determinants in utero, suggestive that HIV-specific TH immunity may be protective in newborns, and provide a possible means for identifying newborns that are at risk for HIV infection (Clerici et al, 1993).

2.25.2 Cytokine profile in pregnant mothers and cord blood

Cytokines comprise the interleukins, lymphokines and related regulatory glycoproteins released by cells of the immune system or non-haematopoietic cells, to act as intercellular mediators in the generation of an immune response. Immune profiles of HAART-naïve pregnant women were found to be profoundly different from HAART-treated subjects. Thus: (1) T-cell proliferation to HIV-specific and HIV-unrelated antigens is potent in antiretroviral-naïve but suppressed in HAART-treated individuals; (2) interleukin-(IL)2, IL-12 and IFNγ production is robust in naïve subjects; and (3) a high CCR5/low CXCR4 pattern of HIV coreceptors-specific mRNA is observed in naïve but not in HAART-treated subjects. These data suggest that control over HIV replication can be independently achieved by pharmacological or immunologic means. HAART is associated with weaker HIV-specific and -non-specific immune responses (Clerici et al, 2000).

A Th1 to Th2 cytokine shift is also observed in the disease progression of HIV infection. Highly active antiretroviral therapy (HAART) suppresses HIV viremia, increases CD4+ cell counts and counteracts the Th1 to Th2 shift. Interleukin (IL)-2 (Th1) and IL-10 (Th2) in peripheral blood mononuclear cells (PBMCs) measured three times during pregnancy in 49 HAART treated women showed increased IL-2 and decreased IL-10 and hence provides significant benefits in delaying HIV disease progression and reducing the risk of mother-to-child-transmission but may be counterproductive in terms of successful pregnancy outcome (Simona et al, 2006).

The peripheral blood mononuclear cells (PBMCs) from HIV-1-infected mothers and CB were shown to be defective in TNF-α production after activation by TLR2, TLR5, TLR3 and TLR7. Furthermore, only CL097 activation was able to induce IL-10 and IFN-α secretion in both maternal and CB cells in the infected group. The activation of the TLR7/8 pathway could function as an adjuvant to improve maternal-neonatal innate immunity (Cardoso et al, 2013). Production of IL-2 and IL-12 by PBMCs in non-stimulated as well as stimulated cultures were

similar in both normal and HIV-infected mothers on HAART. HIV+ mothers showed lower values of IL-4 and IL-7, IL-10 in stimulated cultures than the unstimulated and normal control, but higher values of TNFα, IL-2, IL-12 and TNFγ, in stimulated than unstimulated and normal cultures (Eliane Borges-Almeida et al, 2011).

IFN-alpha was detected sporadically in the maternal sera from the groups of transmitters (27%), nontransmitters (21%), and controls (19%). In the cord blood, IFN-alpha was detected on two occasions among transmitters, and on a single occasion in the control group. IFN-beta was absent from both maternal and cord blood in the study group. Although the placental IFNs have an antiviral potential, they are not sufficient to suppress transmission of HIV from mother to infant (Zachar et al, 2000). Cord blood IFN-γamma and IL-10 production after PHA stimulation was found to be greater in HIV infected untreated mothers compared to the controls (Kuhn et al, 2001a). Cord blood mononuclear cells of HIV-exposed newborns produced less IL-4 and IL-7 and more IL-10 and IFN-γ in culture than those of uninfected mothers. Cytokine values in supernatants were similar in infants and their mothers except for IFN-γ and TNF-α lpha that were higher in HIV+ mothers, especially in drug abusing ones (Eliane Borges-Almeida et al, 2011).

Gamma Interferon (IFN-γ) production tended to be lower in HIV infected pregnant women placed on Nvp compared to HIV-uninfected mothers; and HIV-exposed compared to HIV-unexposed infants. In contrast, the median IFN-γ production in response to PHA at birth was higher among HIV-exposed compared to HIV-unexposed infants. IFN-γ production was significantly lower in infants at birth compared to mothers and significantly higher in infants at 6 weeks of age compared to infants at birth (Van Rie et al, 2006).

2.25.3 Immunoglobuline profile:

HIV infection affects B cell function and is associated with increased immunoglobulin levels, including in HIV-infected pregnant women. Immunoglobulin (Ig) G levels declined gradually throughout pregnancy until delivery, and increased in the 6-month post-partum period among HIV positive non ARV treated pregnant women. Total IgM and IgA levels remained stable throughout pregnancy (European Collaborative Study, 1997). Haiti mothers who transmitted infection to their offspring had significantly higher mean concentrations of IgG1 antibodies to

the V3 loop of the primary neutralising domain of the viral envelope (gp 160) than non-transmitters. Concentrations of IgA antibody to this domain were similar in transmitters and non-transmitters (Markham et al, 1994).

Data from children enrolled in the European Paediatric Hepatitis C Virus Network (EPHN) shows that HIV-exposed uninfected children had significantly higher IgG, IgM, and IgA levels than unexposed uninfected children up to at least 24 months. Among HIV-exposed uninfected children, IgG levels from birth until 5 years of age correlated with increased maternal IgG levels. ART exposure in fetal and early neonatal life was associated with lower IgG. These findings indicate alterations in immunoglobulin levels in uninfected children born to HIV-infected women, suggesting that fetal exposure to a chronically activated maternal immune system is associated with an altered humoral response (Bunders et al, 2010).

Majority (16 of 22) of HIV-1-seropositive mothers who delivered uninfected children had IgA antibody to low-molecular-weight HIV-1 polypeptides during pregnancy while only one of those who delivered infected babies had a weak IgA reactivity to HIV-1 during pregnancy. This suggests that the study of IgA may be a diagnostic adjunct to predict the risk of mother-to-child HIV-1 transmission (Re et al, 1992).

CHAPTER THREE

MATERIALS AND METHODS

3.1 STUDY LOCATION

The study was done in two locations. First at PMTCT Clinic of Mother of Christ Specialist, Hospital, Ogui Enugu, Nigeria. In this hospital, subjects were seen, samples collected and the preliminary cell work conducted. The frozen plasma and culture supernatants were then shipped to the second location at Prof. Rong Lijun laboratory, Department of Microbiology and Immunology, University of Illinois at Chicago, USA for further investigations.

3.2 STUDY POPULATION

Test subjects:

HIV positive HAART treated - This group is HIV infected pregnant mothers treated with HAART (highly active anti-retroviral therapy). Mothers in this category were either on Combivire/Nivarapine (Lamovidine + Zidovudine + Nivarapine) or Truvada/Efavirienz (Emtricitabine + Tenofovir + Efavirienz). Subjects were randomly selected from a case controlled study. In this group, a total of 122 subjects were seen at the PMTCT clinic of Mother of Christ Specialist, Hospital, Ogui Enugu. All pregnant mothers on this research were on HAART irrespective of their CD4-T cell count as the recent PMTCT guideline requires. The subjects were grouped into three (3) gestational stages namely: 1st trimester (1-3 months, n = 16), 2nd trimester (4-6 months, n = 52) and 3rd trimester (7-9 months, n = 54). Standard questionnaire for test subjects (see **Appendix I**) was given to each subject after explaining the importance of the research. Their consent was also sought and consent form signed by each subject (see **Appendix II**). The subjects were allowed to participate willingly and withdrawal at any point was granted. The subjects were assured of their safety and privacy throughout the study.

HIV positive HAART naive subjects:

This group is HIV infected pregnant mothers not treated with HAART (highly active anti-retroviral therapy). In this group, a total of 13 subjects were seen. The subjects were not grouped into gestational stages because the number was small and insignificant. The low number seen in

HAART naïve subjects was as a result of unwillingness of the pregnant mothers to willingly submit to free HIV testing and counselling. No antenatal clinic allows compulsory HIV screening until the 9th month. The rule is that HIV test result must be seen before delivery. Standard questionnaire for test subjects (see **Appendix I**) was given to each subject after explaining the aim and objectives of the research. Their consent was also sought and consent form signed by each subject (see **Appendix II**). The subjects were allowed to participate willingly and withdrawal at any point was granted. The subjects were assured of their safety and privacy throughout the study.

Control subjects

A total of seventy-two (72) HIV negative pregnant women were seen at the antenatal clinic of Mother of Christ Specialist Hospital, Ogui Enugu as control subjects. The subjects were grouped into three (3) gestational stages namely: 1st trimester (1-3 months, n = 12), 2nd trimester (4-6 months, n = 35) and 3rd trimester (7-9 months, n = 25). Standard questionnaire for control subjects (see **Appendix I**) was given to each subject after explaining the purpose and benefit of the study. Their consent was also sought after and consent form signed by each subject (see **Appendix II**). The subjects were allowed to participate willingly and withdrawal at any point was granted. The subjects were assured of their safety and privacy throughout the study. For further description of subjects, see **Appendix IX.**

3.3 Inclusion criteria for test subjects

The test subjects included all HIV positive pregnant women on HAART treated and HIV infected HAART naïve pregnant women. Test subjects with normal blood pressure within the range of 100/70-130/80 and non-diabetic were included.

3.3.1 Exclusion criteria for test subjects

HIV positive pregnant women who smoke, alcohol and hard drug abuse were excluded as these factors may cause adverse pregnancy outcomes (though none of the subjects admitted taking hard drugs). Maternal alcohol consumption has shown to suppress IFN regulatory factors which were associated with reduced expression of type I IFN. Maternal alcohol consumption may

facilitate HIV infection and promote vertical transmission of HIV (Mastrogiannis et al, 2014). HIV positive non-pregnant women were excluded. Test subjects with diabetes, gestational diabetes, pre-eclampsia or those whose blood pressure was above 130/80 were excluded.

3.4 Inclusion criteria for control subjects

The control subjects included all HIV uninfected pregnant women. Control subjects with normal blood pressure within the range of 100/70-130/80 and non-diabetic were included.

3.4.1 Exclusion criteria for control subjects

HIV uninfected pregnant women with diabetes, gestational diabetes, pre-eclampsia or those whose blood pressure was above 130/80 were excluded. HIV uninfected pregnant women who smoke, alcohol and hard drug abuse were excluded as these factors may cause adverse pregnancy outcomes (though none of the subjects admitted taking hard drugs). Maternal alcohol consumption has shown to suppress IFN regulatory factors which were associated with reduced expression of type I IFN. Maternal alcohol consumption may facilitate HIV infection and promote vertical transmission of HIV (Mastrogiannis et al, 2014). HIV uninfected non-pregnant women were excluded.

3.5 SAMPLE SIZE

Samples were randomly selected from a case controlled study and the sample size of 60 was determined using 3.1 % HIV sero-prevalence in Nigeria (UNAIDS, 2014). It was calculated using the following formula (Daniel, 1999):

$$\mathbf{n} = \frac{\mathbf{t^2 \times p(1-p)}}{\mathbf{m^2}}$$

Description:

n = required sample size

t = confidence level at 95% (standard value of 1.96)

p = HIV prevalence at 3.1 % = 0.031 (UNAIDS, 2014)

m = margin of error at 5% (standard value of 0.05)

$$\mathbf{n} = \frac{(1.96)^2 \times 0.031\,(1-0.031)}{(0.05)^2} = \mathbf{60}$$

\

Substituting the stated values in the formula above gives a sample size of 60. However, higher number of samples were collected to allow unforeseen circumstances.

3.6 Laboratory centers

Laboratory section of Mother of Christ Specialist Hospital, Ogui Enugu, Nigeria was used for the cell work and culture. The remaining analysis involved in the research was done in Prof. Lijun Rong Laboratory, Department of Microbiology and Immunology, University of Illinois at Chicago, U.S.A. The laboratory has fully approved "protocol for use of infectious agent/toxins in research" and "BSL-2 Laboratory" by the Office of Animal Care and Institutional Biosafety (OACIB) of the University-UIC, USA.

3.7 Specimen collection

About 10 ml blood sample was collected into heparin container from both test and control subjects. The whole blood was used for separation of peripheral blood mononuclear (PBMC) separation and B and T lymphocyte stimulation using mitogens while the remaining whole blood was separated and the plasma used for the analysis of Immunoglobulin (A, G, M). The culture supernatant from lymphocyte stimulation/transformation test was used for the analysis of cytokine assay. Separated plasma was stored frozen until ready for use.

3.8 Ethical consideration

Ethical clearance was obtained from the ethical committee of the Dept. of Medical Laboratory Science, Faculty of Health Science, Nnamdi Azikiwe University, Okofia (see **Appendix III**). Ethical approval letter was obtained from Mother of Christ Specialist, Hospital, Ogui Enugu (see **Appendix IV**).

3.9 METHODS OF INVESTIGATION AND PARAMETERS STUDIED

3.9.1 Lymphocyte Proliferation and Cytokine Detection Assay (Crowther, 1995)

A proliferation assay is a simple method of nonspecifically measuring lymphocyte activation and proliferation ability. The lymphocytes can be invitro stimulated using the following mitogens: Phytohemagglutinin (PHA) T-cell activation, Concanavalin (Con A) T-cell activation, Pokeweed mitogen (PWM) T-cell and B-cell activation.

Stages of assay procedure

1) Isolation of human peripheral blood mononuclear cell (PBMC) from whole blood:

PBMC isolation and lymphocyte (T cell and B cell) stimulation allow for a description of T cell responses without the influence of other whole blood components such as monocyte, erythrocyte, neutrophils and plasma. Cytokines are primarily produced by immune system but many other organs (liver, brain, endocrine gland) produce cytokines to influence immune response. After carefully checking the sample identification on the heparin blood tubes, 3 ml out of the 10 ml whole blood was collected into a 15 mL conical centrifuge tube. Equal volume of phosphate buffered saline (PBS) (Hank's balanced salt solution H9394-Sigma-Aldrich) was added into the same 15 ml conical centrifuge to bring the final volume to 6 ml. (phosphate buffered saline was `mixed with the whole blood to aid clear separation of different blood components). The whole blood-PBS solution was mixed with pipette. Five (5) ml 5 Histopaque (Sigma- Aldrich 1077 with density of 1.077g/ml) carefully layered into a second 15 mL conical centrifuge tube and was brought to room temperature. The 6 mL whole blood-Hank mixture in the first tube was collected with pasture pipette and carefully layered without disturbance unto the second tube tilting the second tube at angle 45^0 to avoid mixing. There was a formation of two distinct layers (blood-hank's mixture on top and histopaque at the bottom (see **Appendix V & VI**). The entire mixture was then centrifuged at room temperature for 30 minutes at 700×*g* (lower temperature such as 4° C may cause clumping and poor recovery of the cells). After centrifugation, four (4) distinct layers were formed namely (from top to bottom); plasma, PBMC, histopaque and red cell (see **Appendix VII**). Plasma (topmost) was removed and discarded to within 0.5 cm of the upper layer. Then the second layer which is the white cell interface/PBMCs was carefully removed and

transferred to a new 15 mL conical tube appropriately labeled. About 6 mL PBS (PBS should be about 3 times the volume of the PBMCs) was added unto the white cell, centrifuged for 10 min at room temperature at 200×*g* (this is called washing to remove any red cell, blood clot, plasma, histopaque trapped in the PBMC). After the wash, the supernatant was removed and discarded. The washing was done 3 times so that the white cell was properly washed. The washed white cell was then suspended in complete culture medium (enriched RPMI 1640 - R7388-Sigma Aldrich, Hank's isotonic PBS-H9394 can also be used for the suspension of the washed white cell).

2) Cell Viability Test by Trypan Blue Exclusion

Principle: This method is based on the principle that live (viable) cell do not take up certain dyes whereas dead (non- viable) cells do. Staining facilitates the visualization of cell morphology. TB has greater affinity for serum proteins than for cellular proteins. If the background is too dark, cells should be pelleted and re-suspended in protein free medium or salt solution prior to counting. If the cells are exposed to TB for a longer period of time, viable cells as well as non-viable cells may begin to take up dye.

3) Cytokine Assay (Crowther, 1995)

Principle:

IL-10 is captured by monoclonal antibody (mAb) to IL-10 adsrorbed in a a microtiter plate. Biotinylated secondary/detection antibody binds to the monoclonal antibody. The reaction mixture reacts with Streptavidin-PE conjugate, the reporter molecule and a substrate to produce a a color reaction. The intensity of the colour reaction is directly proportional to the amount of IL-10 bound. The concentration of IL-10 in the sample is determined by comparison with a standard curve of known protein concentrations. For the assay of IL2, TNF-α, IFN- γ and IL4, same principle and procedure is applied.

4. Immunoglobulin assay (Davies, 1994)

Principle of double antibody sandwich ELISA: The IgM present in samples/standard reacts with the anti-IgM antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti- IgM antibodies conjugated with

horseradish peroxidase (HRP) are added. These enzyme-labeled antibodies form complexes with the previously bound IgM. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3,5,5-tetramethylbenzidine (TMB). TMB substrate is used for color development at 450nm. The quantity of bound enzyme varies directly with the concentration of I IgM in the sample and directly proportional to color development, thus, the absorbance, at 450 nm, is a measure of the concentration of IgM in the test sample. A standard calibration curve is prepared in blocking buffer using dilutions of purified human IgM and is measured along with the test samples.

For the estimation of IgG and IgM, same principle and procedure as used in IgM assy is applied.

3.10 STATISTICAL ANALYSIS

Cytokine results were expressed as median and range values. Column analysis was done and Mann-Whitney test was used to calculate whether there is significant difference between medians of two (or more) groups in unpaired data. Immunoglobulin results were expressed as mean±(sd) values. Column analysis, one-way ANOVA and Bonferroni's Multiple Comparison Test were used for analysis and comparison of two or three groups. Statistical package used was Graph Pad Prism 5. Significant value was set at $P < 0.05$.

CHAPTER FOUR

RESULTS

Cytokine level of HIV infected HAART treated, HIV infected HAART naive and control pregnant women after T and B lymphocyte stimulation irrespective of trimester categorization is shown in table 4. 1. IFN-γ and IL-10 levels were significantly lower in pregnant women on HAART irrespective of stimulation when compared with the pregnant women who are HIV negative that served as control group. Tables 4. 2, 4. 3 and 4. 4 examined the cytokine levels of HIV infected HAART treated, HIV infected HAART naive and control pregnant women after stimulations of T and B lymphocyte during 1st, 2nd and 3rd trimesters using concanavalin A (con A), phytohemaglutinin (PHA) and pokeweed (PW). In the first trimester, IFN-γ stimulated by pokeweed had significantly lower value 1.0 pg/ml when compared with the control group 59.3 pg/ml. IFN-γ and IL-10 stimulated by phytohemaglutinin had significantly lower values than the control group (table 4. 2). Concanvalin A stimulation did not significantly affect cytokine levels between the pregnant women on HAART and the control group (table 4. 2).

Second trimester showed a rise in the level of IL-10 in the pregnant women on HAART irespective of stimulation as compared with the first trimester but pokeweed stimulation revealed a significant difference in the levels of IL-10 when pregnant women on HAART was compared with the control group (P = 0.038). In the third trimester, IL-10 values rose the more by pokeweed and phytohemaglutinin stimulations when compared with 1st and 2nd trimester (table 4. 4) but significantly lower than the values for control group when pokeweed stimulation was considered (P = 0.02). However, TNF-α, IL-2 and IL-4 secretions appear to be similar between HIV infected pregnant women on HAART and the control group.

Table 4.5 compared cytokine levels of HIV infected pregnant women on HAART after stimulation of B and T lymphocyte as the pregnancy progresses through 1st, 2nd and 3rd trimester using concanavalin A, pokeweed and phytohemaglutinin. Result showed a significantly (P = 0.02; 0.03) low secretion of TNF- α by phytohemaglutinin as pregnancy progressed to 3rd trimester.

Table 4. 1: Cytokine levels (in pg/ml, median and range) after B and T lymphocyte stimulation with pokeweed, concanavalin-A and phytohemaglutinin in the supernatant HIV infected HAART treated, HIV infected HAART naive and control pregnant mothers'peripheral mononuclear cell cultures.

	HIV uninfected control (n=72)	HIV infected HAART treated (n=122)	HIV infected HAART naïve (n = 13)	P value		
				control Vs treated	Control Vs naive	Treated Vs naive
IL-2 Con	3.5 (0- 13822)	0.0 (0-17056)	3.3 (0- 1232)	0.32	0.86	0.75
PW	3.3 (0- 1718)	0.0 (0-9060)	0.0 (0- 11.2)	0.28	0.10	0.42
PHA	0.2 (0-5064)	0.0 (0-105.5)	4.0 (0- 347)	0.58	0.39	0.22
TNF-α Con	257.4 (11-5707)	33.6 (0-13895)	38.0 (4.4-5444)	0.11	0.13	0.88
PW	169.8 (5.9-13633)	59.6 (2.3-17097)	57.0 (13.6- 2518)	0.30	0.59	0.69
PHA	149.6 (5.8-24325)	131.1 (0-12933)	19.6 (6.2- 2207)	0.54	0.15	0.31
IFN-γ Con	21.0 (0-4009)	3.3 (0- 14636)	12.4 (0- 573.6)	0.04*	0.38	0.44
PW	26.7 (15985)	5.3 (0- 17673)	22.4 (3.2- 11327)	0.01*	0.71	0.11
PHA	10.4 (0-17673)	3.7 (0- 8586)	4.2 (0.1- 8586)	0.01*	0.21	0.72
IL-4 Con	3.7 (0-18379)	0.0 (0-11070)	0.0 (0- 69.9)	0.03*	0.10	0.69
PW	0.0 (0-18379)	0.0 (0-16213)	0.0 (0- 7.9)	0.24	0.29	0.86
PHA	0.0 (0-16213)	0.0 (0-16213)	1.5 (0- 132.5)	0.37	0.92	0.51
IL-10 Con	14.4 (0-19577)	0.0 (0-12960)	2.7 (0- 817)	0.002*	0.31	0.29
PW	12.8 (0-19577)	0.0 (0-8486)	1.6 (0- 13945)	0.001*	0.10	0.27
PHA	8.2 (0-13945)	2.0 (0-1208)	3.7 (0- 15066)	0.02*	0.92	0.20

*Significant value set at ($P < 0.05$)

Table 4. 2: Cytokine levels (in pg/ml, median and range) of HIV infected pregnant women on HAART after stimulation of B and T lymphocyte during the 1st trimester using pokeweed, concanavalin-A and phytohemaglutinin.

Types of stimulation:					
Subjects:	IL-2	TNF- α	IFN-γ	IL-4	IL-10
Con A stimulation					
Pregnant women on HAART. n =122	0.0 (0-0.55)	18.5 (4- 6619)	0.2 (0- 4.7)	0.0 (0-0)	0.0 (0-0)
Control (n= 72)	2.4 (0- 60.9)	108.7 (11- 2035)	21.1 (0- 2920)	4.2 (0- 10756)	86.3 (1-17840)
P - value	0.09	0.28	0.11	------	--------
PW stimulation					
Pregnant women on HAART	0.0 (0- 282)	39.8 (2- 17097)	1.0 (0- 422)	0.0 (0- 737)	0.0 (0-0)
Control	3.7 (0- 616)	155.6 (6- 3203)	59.3 (3- 10150)	0.0 (0- 742)	72.1 (0-1445)
P - value	0.15	0.59	0.01*	0.33	-------
PHA stimulation					
Pregnant women on HAART	0.0 (0- 15.8)	867.7 (0- 10842)	0.0 (0- 162)	0.0 (0-0)	0.0 (0-1.9)
Control	0.0 (0- 13.4)	906.3 (8.4- 4225)	499.4 (4- 6017)	0.0 (0- 9876)	33.7 (0-13945)
P - value	0.83	0.79	0.03*	------	0.04*

Key: values asterisked showed ($P < 0.05$).

Table 4. 3: Cytokine levels (in pg/ml, median and range) of HIV infected pregnant women on HAART after stimulation of B and T lymphocyte during the 2nd trimester using pokeweed, concanavalin-A and phytohemaglutinin.

Types of stimulation:					
Subjects:	IL-2	TNF- α	IFN-γ	IL-4	IL-10
Con A stimulation					
Pregnant women on HAART. n =122	0.9 (0- 17056)	58.2 (0-13895)	7.3 (0- 5270)	0.0 (0- 6502)	1.1 (0-12960)
Control (n= 72)	4.6 (0-13822)	250.5 (17-2219)	30.0 (0- 4009)	1.4 (0- 18379)	19.7 (0-19577)
P - value	0.93	0.14	0.31	0.23	0.32
PW stimulation					
Pregnant women on HAART	3.1 (0-9060)	104.6 (3-11331)	5.5 (0- 17673)	0.0 (0- 4190)	0.5 (0-8067)
Control	1.1 (0- 85.4)	103.5 (7-13633)	70.6 (0-15985)	2.5 (0- 18379)	32.5 (0-3690)
P - value	0.81	0.57	0.19	0.45	0.03*
PHA stimulation					
Pregnant women on HAART	0.0 (0-105.5)	203.3 (4-12933)	3.8 (0- 8408)	0.3 (0- 97)	2.5 (0-937)
Control	0.9 (0- 34.8)	203.4 (6- 6475)	6.8 (0- 17673)	0.0 (0- 16213)	3.7 (0-581)
P - value	0.75	0.79	0.32	0.72	0.45

Key: values asterisked showed ($P < 0.05$).

Table 4. 4: Cytokine levels (in pg/ml, median and range) of HIV infected pregnant women on HAART after stimulation of B and T lymphocyte during the 3rd trimester using pokeweed, concanavalin-A and phytohemaglutinin.

Types of stimulation: Subjects:	IL-2	TNF- α	IFN-γ	IL-4	IL-10
Con A stimulation					
Pregnant women on HAART. n =122	0.5 (0- 8740)	33.6 (0-13495)	3.7 (0-4636)	0.3 (0-11070)	0.0 (0-4794)
Control (n= 72)	3.8 (0- 315)	386.0 (14.7-5707)	22.6 (0-2141)	5.3 (0-13000)	6.6 (0-6352)
P - value	0.64	0.68	0.28	0.39	0.11
PW stimulation					
Pregnant women on HAART	0.0 (0- 8438)	38.6 (9.1- 2965)	20.9 (0-17673)	0.0 (0- 16213)	1.1 (0-8486)
Control	3.5 (0- 1718)	202.2 (5.8- 2088)	26.6 (2.7-7806)	1.9 (0- 413)	10.4 (0-19577)
P - value	0.42	0.95	0.60	0.57	0.02*
PHA stimulation					
Pregnant women on HAART	2.4 (0- 57.1)	18.7 (0- 1995)	3.6 (0- 8586)	0.0 (0- 16213)	2.9 (0-1208)
Control	5.7 (0- 5064)	40.5 (5.8- 24325)	6.9 (0- 14243)	2.7 (0- 11785)	24.2 (0-376)
P - value	0.10	0.43	0.59	0.44	0.27

Key: values asterisked showed ($P < 0.05$).

Table 4. 5: Cytokine levels (in pg/ml, median and range) of HIV infected pregnant women on HAART after stimulation of B and T lymphocyte during the 1st, 2nd and 3rd trimester using concanavalin A, pokeweed and phytohemaglutinin.

Concanavalin A stimulation:					
	IL-2	TNF- α	IFN-γ	IL-4	IL-10
Trimester:					
1st	0.0 (0-0.55)	18.5 (4-6619)	0.2 (0-4.7)	0.0 (0-0)	0.0 (0-0)
2nd	0.9 (0-17056)	58.2 (0-13895)	7.3 (0-5270)	0.0 (0-6502)	1.1 (0-12960)
3rd	0.5 (0-8740)	33.6 (0-13495)	3.7 (0-4636)	0.3 (0-11070)	0.0 (0-47944)
P- value					
1st vs 2nd	0.13	0.49	0.11	-------	--------
1st vs 3rd	0.16	0.62	0.36	-------	--------
2nd vs 3rd	0.54	0.76	0.75	0.52	0.58
Pokeweed stimulation					
1st	0.0 (0-282)	39.8 (2-17097)	1.0 (0-422)	0.0 (0-737)	0.0 (0-0)
2nd	3.1 (0-9060)	104.6 (3-11331)	5.5 (0-17673)	0.0 (0-4190)	0.5 (0-8067)
3rd	0.0 (0-8438)	38.6 (9.1-2965)	20.9 (0-17673)	0.0 (0-16213)	1.1 (0-8486)
P- value					
1st vs 2nd	0.13	0.71	0.25	0.23	-------
1st vs 3rd	0.32	0.92	0.07	0.27	-------
2nd vs 3rd	0.77	0.98	0.68	0.98	0.85
Phytohemaglutinin stimulation					
1st	0.0 (0-15.8)	867.7 (0-10842)	0.0 (0-162)	0.0 (0-0)	0.0 (0-1.9)
2nd	0.0 (0-105.5)	203.3 (4-12933)	3.8 (0-8408)	0.3 (0-97)	2.5 (0-937)
3rd	2.4 (0-57.1)	18.7 (0-1995)	3.6 (0-8586)	0.0 (0-16213)	2.9 (0-1208)
P - value					
1st vs 2nd	0.78	0.42	0.27	------	0.12
1st vs 3rd	0.59	0.02*	0.11	-------	0.16
2nd vs 3rd	0.65	0.03*	0.66	0.96	0.98

Key: values asterisked showed ($P < 0.05$).

Immunoglobulin results

Plasma total IgA (mg/dl) level in HIV infected HAART treated and HIV uninfected control at 1st, 2nd and 3rd trimester of gestation is shown in figure 4. 1. There was no significant (P>0.05) difference in plasma IgA through 1st, 2nd and 3rd trimester in HAART treated and control subjects. Furthermore, plasma IgA (mg/dl) level in HIV infected HARRT treated, HIV infected HARRT naive and HIV uninfected pregnant mothers also showed no significant (P>0.05) difference in plasma IgA level as shown in figure 4. 2.

Plasma total IgG (mg/dl) level in HIV infected HAART treated and HIV uninfected control at 1st, 2nd and 3rd trimester of gestation is shown in figure 4. 3. Plasma IgG was significantly higher (P<0.001) in 1st and 3rd trimester compared to 2nd trimester in HAART treated subjects. Plasma IgG level in control subjects was significantly lower (P<0.05) at 3rd trimester compared to 1st trimester as shown in figure 4. 3. On the other hand, plasma IgG (mg/dl) level in HIV infected HARRT treated, HIV infected HAART naive and HIV uninfected pregnant mothers (control) is shown in figure 4. 4. Plasma IgG levels in HIV infected HARRT naive and HIV infected HARRT treated pregnant mothers are significantly (P < 0.0001) higher compared to their control group. Plasma IgG level in HIV infected HAART naïve group is significantly higher than the HIV infected HAART treated pregnant women.

Plasma total IgM (mg/dl) level in HIV infected HAART treated and HIV uninfected control at 1st, 2nd and 3rd trimester of gestation is represented in figure 4. 5. Plasma IgM in HIV infected HAART treated pregnant women and control subjects showed no significant (P>0.05) difference throughout 1st, 2nd and 3rd trimester. However, the comparison of plasma IgM level in HIV infected HARRT treated, HIV infected HARRT naive and HIV uninfected pregnant mothers showed that plasma IgM level was significantly higher (P< 0.0001) in HAART naive and significantly lower (P< 0.0001) in HIV infected HARRT treated compared to control subjects as shown in figure 4. 6.

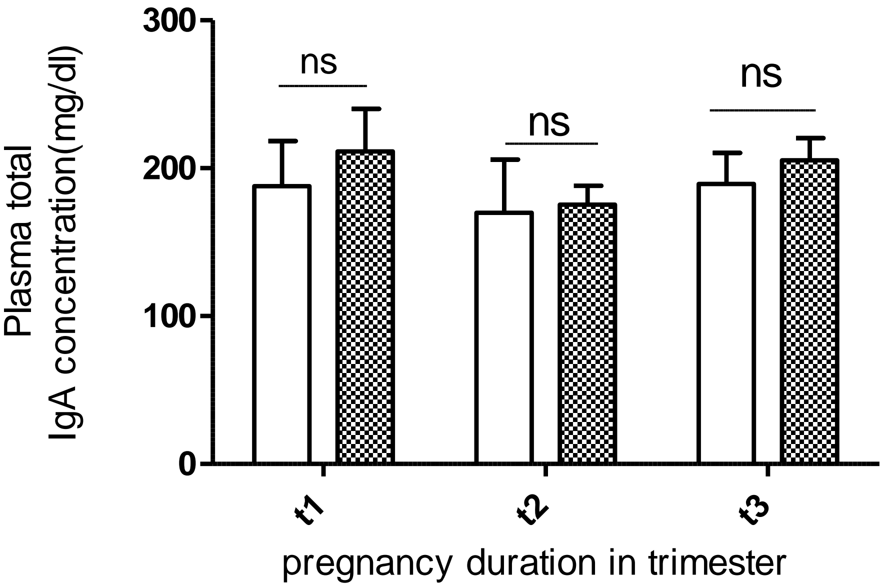


Figure 4.1: Plasma total IgA (mg/dl) levels in HIV infected HAART treated and HIV uninfected control at 1st, 2nd and 3rd trimester gestation.

ns+ - non significant between groups

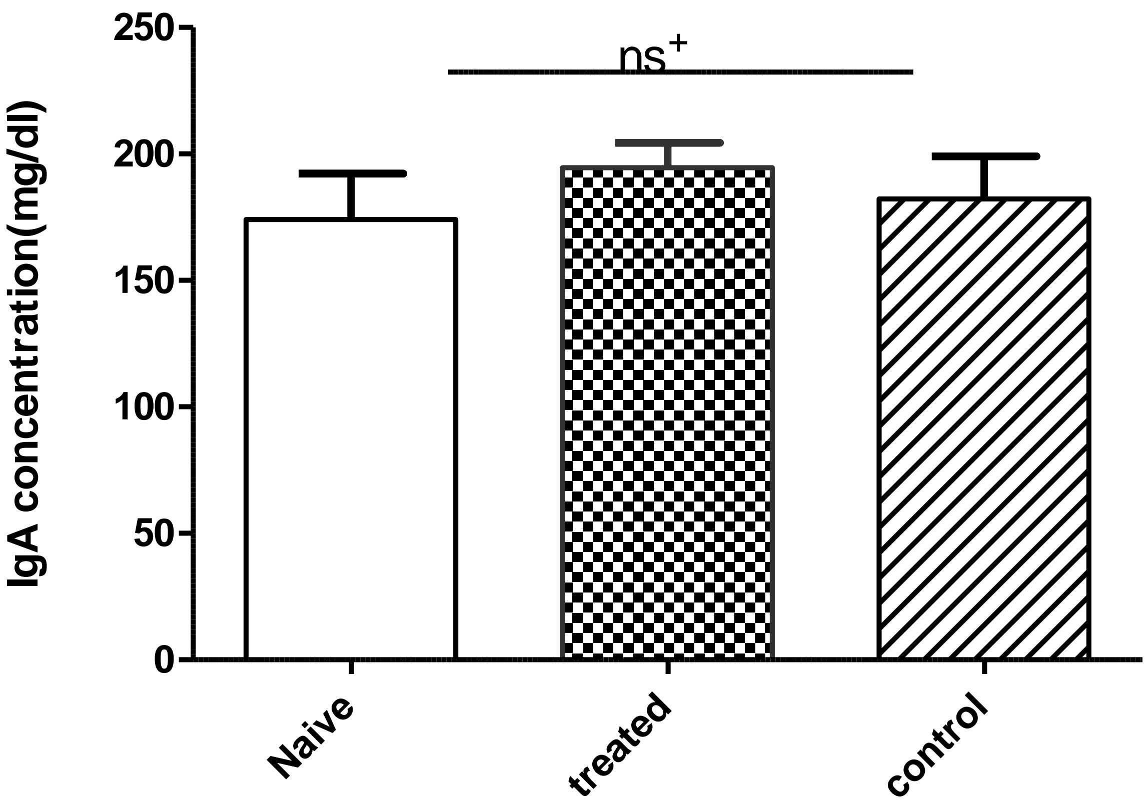


Figure 4. 2: Plasma IgA (mg/dl) level in HIV infected HARRT treated, HIV infected HARRT naive and HIV uninfected pregnant mothers.

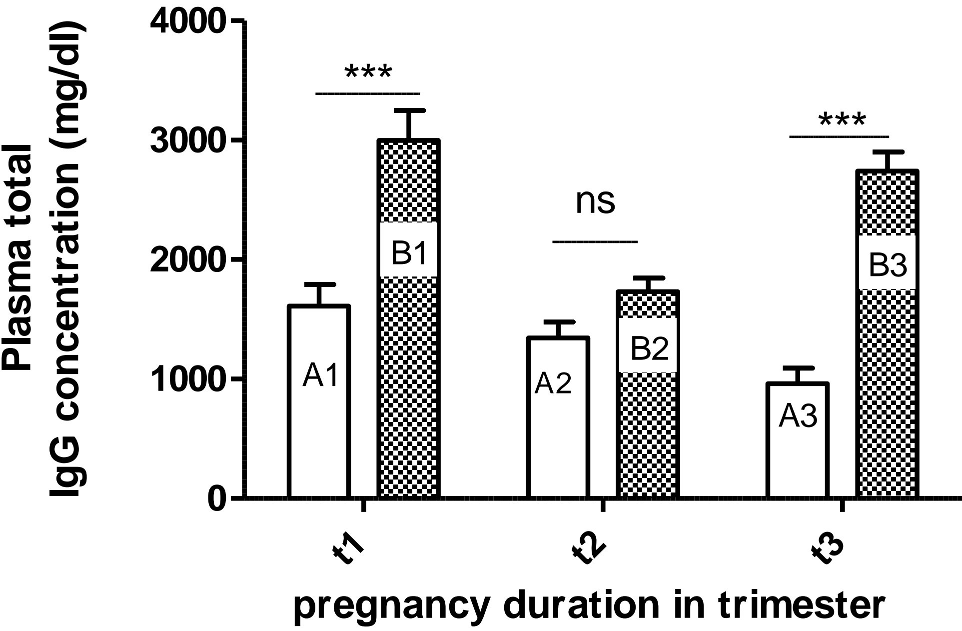


Figure 4. 3: Plasma total IgG (mg/dl) level in HIV infected HAART treated and HIV uninfected control at 1st trimester (t1), 2nd trimester (t2) and 3rd trimester (t3) gestation.

(***) - significant difference

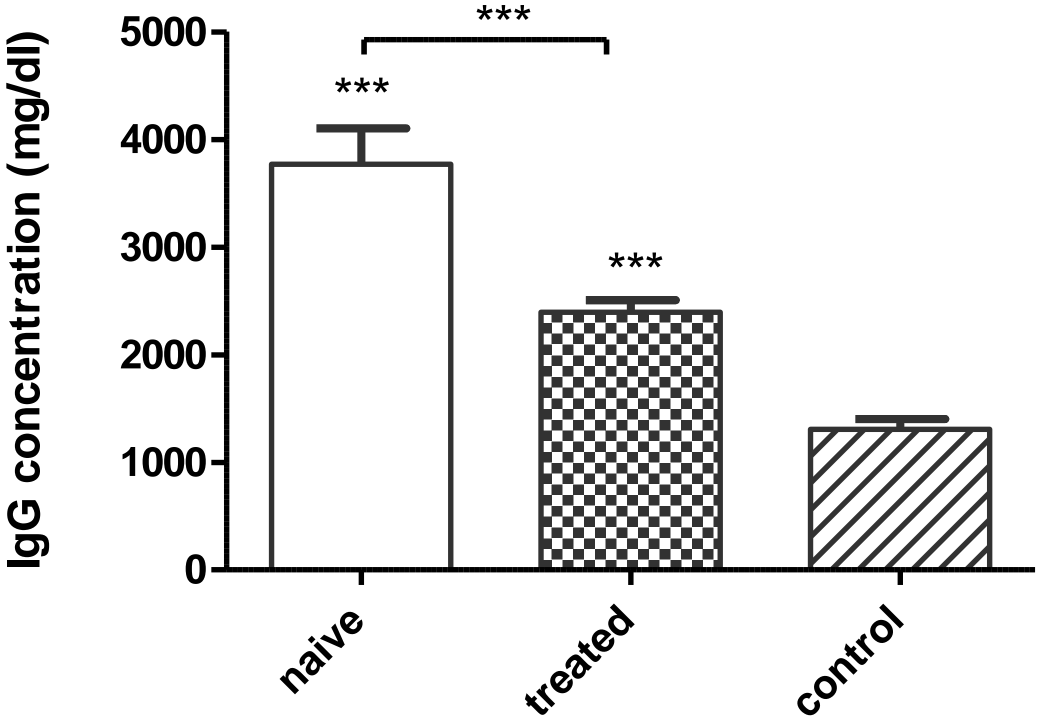


Figure 4. 4: Plasma IgG (mg/dl) level in HIV infected HARRT treated, HIV infected HAART naive and HIV uninfected pregnant mothers (control).

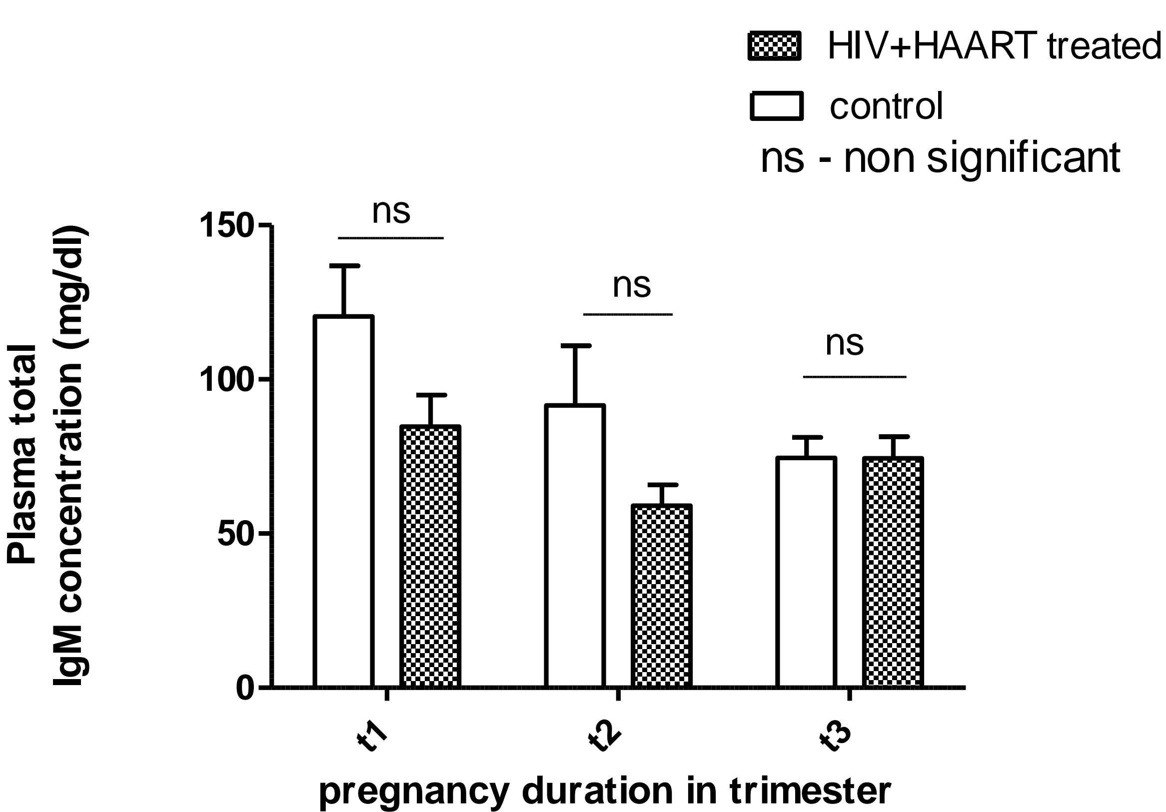


Figure 4. 5: Plasma total IgM (mg/dl) in HIV infected HAART treated and HIV uninfected control at 1st, 2nd and 3rd trimester gestation.

(*) - significant difference
(***) - significant difference

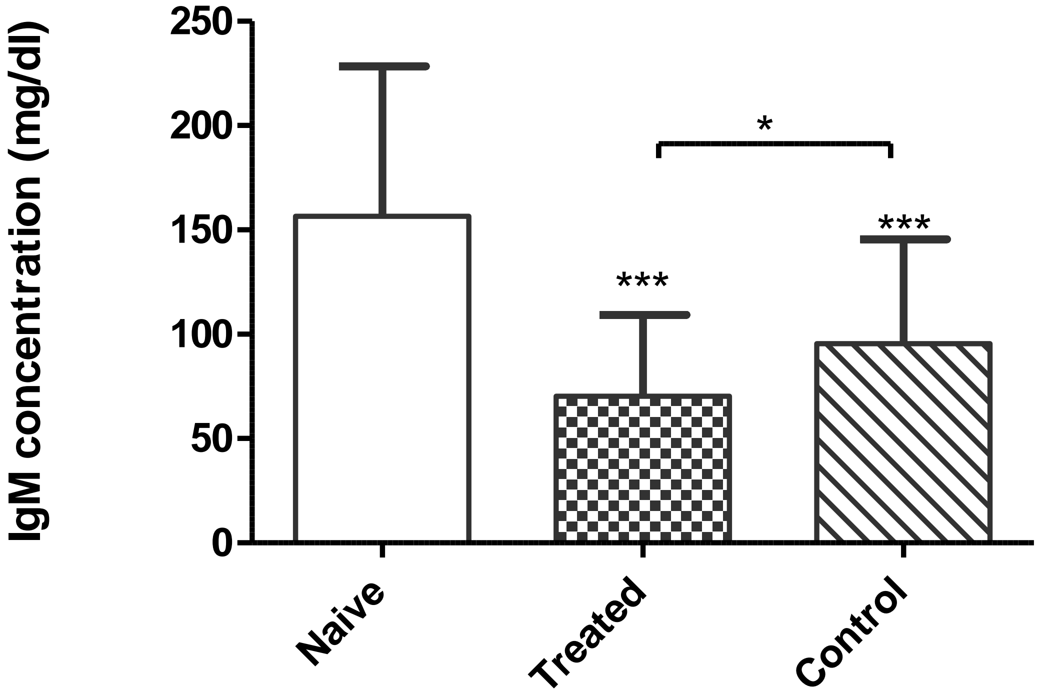


Figure 4. 6: Plasma IgM level in HIV infected HARRT treated, HIV infected HAART naive and HIV uninfected pregnant mothers.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

The present study considered the maternal immunoglobulin A, G M, and cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-10) in HIV infected HAART treated and uninfected pregnant women. The outcome of every pregnancy is determined by the immune strength of a pregnant mother. We assessed the lymphocyte function as an immune index. Lymphocyte stimulation assay specifically assess lymphocyte function as regards cytokine secretion. In the course of this study, IFN-γ, TNF-α and IL-2 were analysed to assess T-helper cell 1 cytokines (TH1-proinflammatory cytokine) while IL-4 and IL-10 assessed T-helper cell 2 cytokines (TH2– anti-inflammatory cytokine). The subjects were grouped into test subjects and control subjects. HIV infected HAART treated pregnant women who had normal blood pressure, non smokers, alcohol and drug abuse users and non diabetic were recruited as the test subjects. HIV uninfected pregnant mothers who had normal blood pressure, non smokers, alcohol and drug abuse users and non diabetic were recruited as the control subjects.

Results (irrespective of trimester categorization) showed that HIV infected HAART treated pregnant women had significant low levels of IFN-γ, IL-10 by concanavalin A (con-A), pokeweed (PW) and phytohemaglutinin (PHA) stimulations; and low level of IL-4 by con A stimulation. These mitogens were used because some individuals react differently in response to lymphocyte stimulation by mitogens. PHA and con A stimulate T lymphocyte while PWM stimulate B lymphocyte. Low levels of IFN-γ and IL-10 observed simoutenously in the three mitogens is a strong confirmation of low IFN-γ and IL-10 secretions by HIV infected HAART treated pregnant mothers unlike IL-4 which showed significant low secretion by concanavalin A stimulation only. Furthermore, the level of cytokine secretion was assessed according to trimester (1st, 2nd and 3rd), to find out if different stages of pregnancy can affect the level of cytokine secretion in HIV infected HAART treated and HIV uninfected pregnant mothers. Trimester categorization showed that HIV infected HAART treated pregnant women had significantly low IFN-γ and IL-10 secretions at 1st trimester. IL-10 was found to be consistently low throughout the trimester unlike IL-4 and and IFN-γ. The low production of IL-10 observed in HIV-infected HAART treated mothers in this study is synonymous to its regulatory function.

Low or aberrant expression of IL-10 enhances clearance of pathogens during an acute infection, but also exaggerate inflammatory response, resulting in exacerbated immunopathology and tissue damage. Though high IL-10 and IL-4 are preferred in normal pregnancy in other to curtail the adverse effect of pro-inflamatory cytokines such as TNF-α, IL-2, IFN-γ. (Mosser & Zhang, 2008; Iyer & Cheng, 2012). In this study, the low IL-10 and IL-4 is ideal because the pregnant women are infected with HIV and so need regulated clearance of the virus and opportunistic infection that may adversely affect the fetus. IL-10 is a TH2 cytokine which plays a crucial role in the establishment and maintenance of pregnancy, prevents fetus rejection and miscarriage as well as protecting the foetus from infections. IL-10 mediates the activation and maintenance of the humoral, or antibody-mediated, immune response (Iyer & Cheng, 2012).

The reason for continuous significant low secretion of IL-10 could be attributed to the HIV infection. IL-10 is a potent immune regulator and its low level in HAART treated pregnant women helps in pathogen clearance. IL-10 also maintains normal tissue homeostasis and avoid tissue damage caused by excessive inflammation by TH1 cytokines (Mosser & Zhang, 2008; Iyer & Cheng, 2012). Immune response to pathogens involves the rapid activation of pro-inflammatory cytokines that serve to initiate host defense against microbial invasion. However, excess inflammation can give rise to systemic metabolic and hemodynamic disturbances harmful to the host (Simona et al, 2006). In agreement with this finding, Taissa et al (2013) showed that IL-10 production was decreased in HIV infected HAART treated pregnant mothers compared to their control counterparts.

The low secretion of IFN-γ in HIV infected HAART treated pregnant women is preferred at pregnancy as it reduces pro-inflammatory effects. A successful pregnancy is characterized by an increase in TH2 cytokines and suppression of TH1 cytokine production (Fiore et al 2006; Cardoso et al, 2013). It is true that pregnancy is said to be successful when the maternal immune system does not reject the fetus and this is characterized by an increase in TH2 cytokines (IL-4, IL-10) and suppression of TH1 cytokine (TNF-α, IFN-γ, IL-2) production. The interplay between TH1 and TH2 cytokines is very important in maintenance of pregnancy, successful child birth and prevention from mother to child transmission (Lee et al 1997; Reinhard et al, 1998; Fiore et al 2006; Cardoso et al, 2013).

When the level each cytokine was compared through 1st, 2nd and 3rd trimester, TNF- α showed significantly low level by PHA stimulation as pregnancy progressed to 3rd trimester. The down regulation of maternal TNF- α by HAART as the pregnancy progressed to term in HIV infected HAART treated pregnant women helps to maintain the pregnancy and reduces possible adverse effects of unregulated inflammatory effect of TNF- α which could cause miscarriage, spontaneous abortion and infection of the fetus. HAART use in pregnancy is the standard care for HIV infected pregnant women and has resulted in the decline of mother to child transmission (MTCT) rate to below 1–2% (European Collaborative Study (a), 2005; and European Collaborative Study (b), 2005; Faye et al, 2007; Kfutwah et al, 2009)).

Some studies have shown varied cytokine levels at different stages of pregnancy. The study of Eliane Borges-Almeida et al, (2011) showed Lower IL-4 but higher TNFα among HIV infected HAART treated pregnant mothers at 9th month. Pornprasert et al., (2009) observed that HIV-1-infected and uninfected women did not show any difference in the expression of placental cytokine secretion except for a trend toward lower TNF-α in HIV infected women receiving HAART. While the study of Pornprasert et al., (2009) showed lower TNF-α secretion, ours showed that TNF-α secretion in HIV infected HAART treated pregnant mothers was similar to that in uninfected pregnant mothers. Fiore et al (2006) demonstrated the counter productiveness and immunomodulation induced by HAART in HIV infected HAART treated pregnant women who showed increased IL-2 and decreased IL-10. The finding of Fiore et al (2006) agrees with our finding that HIV infected HAART treated pregnant women had low IL-10. However, their finding on increasesd IL-2 production contradicts ours which showed similar IL-2 secretion amongst HIV infected HAART treated pregnant women and their uninfected control preganant women. The reason for differences in IL-2 secretions could result from the drug combinations used in HAART.

The role of specific humoral immunity in vertical transmission is still unclear. The association of prematurity with infant infection suggests a potentially protective effect of maternal humoral immunity, as active transport of antibody occurs late in pregnancy and antibodies have the potential to decrease the viral load to which the infant is exposed. There is paucity of information

elucidating the possible role of plasma Immunoglobulin in mother to child transmission. This study evaluated the level of total plasma immunoglobulin A, G and M in HIV infected HAART treated pregnant mothers and their healthy control. The pattern of changes of these Immunoglobulins as is relates to 1st, 2nd and 3rd trimester was also considered.

Plasma IgG and IgM levels were significantly higher in HIV infected HARRT naïve pregnant women compared with HIV HAART treated pregnant women and control pregnant women. HAART treated pregnant women showed significantly higher IgG but significantly lower IgM levels compared to control pregnant women. The high level of IgG and IgM among the HIV infected HAART naïve pregnant women could be attributed to the virus. The B-cell response to HIV infection and associated opportunistic infection might have contributed to higher total IgG and IgM in the HIV infected HAART naïve pregnant women. Total IgG and IgM were significantly lower in HIV infected HAART treated pregnant women compared to HIV infected HAART naïve pregnant women. This showed that HAART was able to reduce the spread and multiplication of HIV vrus and associated opportunistic infection. HIV infection has been associated with a wide range of B-cell defects, including decreased B-cell survival, enhanced expression of markers of B-cell activation, reduction of subsets of memory B-cells, polyclonal hypergammaglobulinemia, and impaired antibody responses to immunizations. Some studies have also shown that increased levels of immunoglobulins in HIV-infected untreated patients are caused by direct and indirect effects of the virus (Lane et al 1983; Chong et al 2004; De Milito et al 2004). Aylin et al (2014) has shown that HIV infected untreated non pregnant individual showed significantly higher concentratin of IgM and IgG compared to those on treatment.

The study of Ifeanyichukwu et al (2009) also showed increased level of IgG in symptomatic HIV infection compared with asymptomatic HIV infection. Their findings agree with the present study that HIV infected HAART naïve pregnant women showed significantly higher concentrations of IgG and IgM. This is because B cells are clonally expanded in the peripheral blood of HIV-1 infected untreated patients. Furthermore, Titanji et al (2000) showed that plasma IgG in HIV infected patients were significantly higher than in healthy pregnant women.

The pattern of Immunoglobulin levels through 1st, 2nd and 3rd trimester showed that there is no difference in IgA and IgM levels throught the period of pregnancy in HIV infected HAART

treated and control pregnant women. However, IgG was found to be significantly lower during second trimester in HAART treated pregnant women and significantly lower during 3rd trimester in control pregnant women. The different levels of mmunoglobulin at different trimester as seen in the healthy control pregnant women is synonymous with the pattern of IgG in normal pregnancy. Maternal antibodies of the IgG class are passively transferred to the fetus during pregnancy; protecting the neonate from new infections during the first weeks or months of life. At normal pregnancy during the first trimester, very little IgG is transferred from the mother to the fetus (Malek et al., 1996) and that is why plasma IgG level at 1st trimester in our control pregnant women was significantly higher compared to 3rd trimester. However, during 2nd trimester, the intraplacental transfer continues to increase reaching its peak at 3rd trimester thus making IgG level in 3rd trimester to be significantly lower. The reason for non-significant difference in the pattern of plasma IgA and IgM through 1st, 2nd and 3rd trimester in both HAART treated and control pregnant women is unclear; partly because IgA and IgM do not undergo placental transfer to the fetus.

One may ask the relevance of IgA in this study. The data in the present study showed that there was no significant difference in the concentration of plasma IgA in HAART treated, HAART naïve and their healthy control. Trimester categorization also showed no significant difference in IgA level through 1st, 2nd and 3rd trimester in the HAART treated and control group. Non significance in plasma IgA level could suggest nonexistence of mucosal infection. Markham et al, (1994) & Moodley et al (1997) found that IgA antibody concentration did not differ between HIV infected mothers who delivered infected children and HIV infected mothers who delivered uninfected children. Moodley et al (1997) concluded that their result failed to show association between vertical transmission and maternal antibody level, therefore implicating a non-protective role of this subclass of antibodies. Contrary to Moodley et al (1997) conclusion, Re et al (1992) found that the majority of HIV-1-seropositive mothers who delivered uninfected children showed IgA antibody to low-molecular-weight HIV-1 polypeptides during pregnancy. Among those who delivered infected babies, only one showed a weak IgA reactivity to HIV-1 during pregnancy, suggesting that the study of IgA may be a diagnostic adjunct to predict the risk of mother-to-child HIV-1 transmission. However, the studies of Moodley et al (1997) and Re et al (1992) did not include HIV infected HAART treated and HIV uninfected pregnant women.

5.2 Conclusion:

The findings in this study showed that there were low secretions of IL-10, IL-4 and IFN-γ in HIV infected HAART treated pregnant mothers irrespective of trimester categorisation.

However, when their values were considered based on trimester of the pregnancy, IFN-γ was the least value at 1st trimester while IL-10 maintained low levels throughout the trimesters.

TNF- α showed significantly low level by PHA stimulation as pregnancy progressed to 3rd trimester.

Irrespective of trimester categorization, plasma IgG and IgM levels were significantly higher in HIV infected HARRT naïve pregnant women. HAART treated pregnant women showed significantly higher IgG but significantly lower IgM levels. Plasma Immunoglobulin A showed no significant difference in all the groups.

Trimester categorization showed that plasma IgG was significantly lower at 2nd trimester but higher in 3rd trimester in HIV infected HAART treated subjects.

5.3 Recommendations

From the findings in this study, the following recommendations are drawn.

HAART treatment is recommended for use in HIV infected pregnant women as it has shown to upregulate cytokine secretions to similar level with the HIV uninfected control as the pregnancy progressed to term. HAART demonstarted effective immune response to protect the pregnancy.

Further study is required to assess the immune response on infants born to these HAART treated mothers. This is because some studies argued that HAART treatment can stop adverse pregnancy outcome and prevent mother to child transmission but adversely cause abnormal immune response on the uninfected exposed infants.

5.4 Limitations

The non-availability of HIV infected HAART naïve pregnant women posed a big limitation to this study.

5.5 Contribution to knowledge:

IL-10 maintained low level throughout 1st, 2nd and 3rd trimester among HIV infected HAART treated pregnant women while there was downregulation TNF- α by HAART as pregnancy progressed to term.

List of References

Abel, J., Conklin, J., Hunter, S., Empey, R., Tyler, E., Christensen, A., Talcott, K., Ballas, Z., Santillan, M., Santillan, D. (2013). Defining normal IgG changes throughout pregnancy. *Proceedings in Obstetrics and Gynecology*. **3** (2), 8 [2 p.].

Aluvihare, V. R., Kallikourdis, M., Betz, A. G. (2004). Regulatory T cells mediate maternal tolerance to the fetus. *Nature Immunology*., **5**, 266–271.

American Heritage Science Dictionary Copyright © 2005 by Houghton Mifflin Company. Published by Houghton Mifflin Company.

Amino, N., Tanizawa, O., Miyai, K., Tanaka, F., Hayashi, C., Kawashima, M., Ichihara, K. (1978). Changes of serum immunoglobulins IgG, IgA, IgM, and IgE during pregnancy. *Obstetrics and Gynecology*, **52** (4), 415-420.

Andrea Miyahira (2014). Types of immune cells present in human PBMC; http://technical.sanguinebio.com/types-of-immune-cells-present-in-human-pbmc/ accessed on 19.

Ann, N. Y. (2000). Cesarean section delivery to prevent vertical transmission of human immunodeficiency virus type 1. Associated risks and other considerations. *Academy of Science*, **918**, 115-121.

Anneke Rijnierse, Alfons B.A. Kroese, Frank A. Redegeld, Bart R.J. Blokhuis, Maurice W. van der Heijden, Andries S. Koster, Jean-Pierre Timmermans, Frans P. Nijkamp, Aletta D. Kraneveld. (2009). Immunoglobulin-free light chains mediate antigen-specific responses of murine dorsal root ganglion neurons. *Journal of Neuroimmunology,* **208** (1), 80-86.

Annelies Van Rie, Shabir A. Madhi, Jayvant R. Heera, Stephen Meddows-Taylor, Aaron M. Wendelboe, Fiona Anthony, Avy Violari, and Caroline T. Tiemessen. Gamma. (2006). Interferon Production in Response to Mycobacterium bovis BCG and Mycobacterium Tuberculosis Antigens in Infants Born to Human Immunodeficiency Virus-Infected Mothers. *Clinical and Vaccine Immunology*, **13** (2), 246-252.

Bahl Rajiv, (2005). "Infant feeding patterns and risks of death and hospitalization in the first half of infancy: multicentre cohort study", Bulletin of the World Health Organization **83** (6).

Barbara Lohman-Payne, Jennifer A. Slyker, Stephen Moore, Elizabeth Maleche-Obimbo, Dalton C. Wamalwa, Barbra A. Richardson, Sarah Rowland-Jones, Dorothy Mbori-Ngacha, Carey Farquhar, Julie Overbaugh, Grace John-Stewart. (2012). Breast milk cellular HIV-specific interferon γ responses are associated with protection from peripartum HIV transmission. *AIDS* **26**(16), 2007-2016.

Bartee, E. & McFadden, G. (2013). Cytokine synergy: an underappreciated contributor to innate anti-viral immunity. *Cytokine,* **63** (3), 237-240.

Benko, D. M., Schwartz, S., Pavlakis, G. N., Felber, B. K. (1990). "A novel human immunodeficiency virus type 1 protein, tev, shares sequences with tat, env, and rev proteins." *Journal of virology,* **64** (6), 2505–2518.

Berrington, J. E.; Barge, D., Fenton, A. C., Cant, A. J., Spickett, G. P. (2005). "Lymphocyte subsets in term and significantly preterm UK infants in the first year of life analysed by single platform flow cytometry". *Clinical and Experimental Immunology*, **140** (2), 289–292.

Biggar, R. J., Miotti, P. G., Taha, (1996). Perinatal intervention trial in Africa: effect of birth canal cleansing intervention to prevent HIV transmission. *Lancet,* **347**, 1647-1650.

Bloland, P. B., Wirima, J. J., Sketekee, R. W. (1995). Maternal HIV infection and infant mortality in Malawi: evidence for increased mortality due to placental malaria infection. *AIDS*, **9**, 721-726.

Boes, M. (2000). Role of natural and immune IgM antibodies in immune responses. *Molecular Immunoloy*, **37**, 1141–1149.

Borges-Almeida, E., Milanez, H. M., Vilela, M. M., Cunha, F. G., Abramczuk, B. M., Reis-Alves, S. C., Metze, K., Lorand-Metze, I. (2011). The impact of maternal HIV infection on cord blood lymphocyte subsets and cytokine profile in exposed non-infected newborns. *BMC Infectious Diseases*, **3**, 11-38.

Boswell, N. R, Carr, J. K., Salminen, M. (1995). The distribution of HIV-1 serotypes in Africa. IX International Conference on AIDS and STD in Africa, Kampala, Abstract MoA041.

Boyer, P. J., Dillon, M., Navale, M. (1994). Factors predictive of maternal-fetal transmission of HIV-1. *Journal of American Medical Association*, **217**, 1925-1930.

Braber S., Thio M., Blokhuis B.R. (2012) An association between neutrophils and immunoglobulin free light chains in the pathogenesis of chronic obstructive pulmonary disease. *American Journal of Respiratory Critical Care Medicine*, **185**(8):817–824

Bryson, Y. (1996). Perinatal HIV-1 transmission: recent advances and therapeutic interventions. *AIDS*, **10**(3), S33-S42.

Bulterys, M., Goedert, J. J. (1995). From biology to sexual behaviour - towards the prevention of mother to child transmission of HIV. *AIDS*, **10**, 1287-1289.

Bunders, M., Pembrey, L., Kuijpers, T., Newell, M. L. (2010). Evidence of impact of maternal HIV infection on immunoglobulin levels in HIV-exposed uninfected children. *AIDS,* **26**(9), 967-975.

Bunders, M., Thorne, C., Newell, M. L., European Collaborative Study. (2005). Maternal and infant factors and lymphocyte, CD4 and CD8 cell counts in uninfected children of HIV-1-infected mothers. *AIDS*, **19**, 1071-1079.

Burger, H., Kovacs, A., Weiser, B. (1997). Maternal serum vitamin A levels are not associated with mother-to-child transmission in the United States. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology*, **14**(4), 321-326.

Burke, D. S. (1997). Recombination in HIV: An Important Viral Evolutionary Strategy. *Emerging Infectious Diseases*., **3** (3), 253-259.

Burns, D. N., Landesman, S., Muenz, L. R., Nugent, R. P., Goedert, J. J, Minkoff, H., Walsh, J. H., Mendez, H., Rubinstein, A., Willoughby, A. (1994). Cigarette smoking, premature rupture of membranes, and vertical transmission of HIV-1 among women with low CD4+ levels. *Journal of Acquired Immune Deficiency Syndrome,* 7(7), 718-726.

Burton, G. J., O'Shea, S., Rostron, T. (1996). Physical breaks in the placental trophoblastic surface: significance in vertical transmission of HIV. *AIDS,* **10**(11), 1294-1295.

Cardoso, E. C., Pereira, N. Z., Mitsunari, G. E., Oliveira, L. M., Ruocco, R. M., Francisco, R. P., Zugaib, M., da Silva Duarte, A. J., Sato, M. N. (2013). TLR7/TLR8 Activation Restores Defective Cytokine Secretion by Myeloid Dendritic Cells but Not by Plasmacytoid Dendritic Cells in HIV-Infected Pregnant Women and Newborns. *PLoS One,* **27**, 8(6), e67036.

Carlos Del Rio and James W. Curran. (2010). Epidemiology and Prevention of Acquired Immunodeficiency Syndrome and Human Immunodeficiency Virus Infection. In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases: Expert Consult Premium Edition. Chapter 118 p 1635.

Carniel, E. F., Morcillo, A. M., Blotta, M. H., Da Silva, M. T. N., Mazzola, T. N., Antonio, M. A., Zanolli, M. L., Netto, A. A., Higashi, H. G., Raw, I., Vilela, M. M. (2008). Immunogenicity and safety of combined intradermal recombinant hepatitis B with BCG vaccines at birth. *Vaccine*, **26**, 647-652.

Casssatella, M. A. (2003.) The Neutrophil: An Emerging Regulator of Inflammatory and Immune Response, Karger Publishers, p. 66.

Cavacini, L. A., Kuhrt, D., Duval, M., Mayer, K., Posner, M. (2003). Binding and neutralization activity of IgG1 and IgG3 from serum of HIV infected individuals. *AIDS Research and Human Retroviruses*, **19**, 785–792.

Cavaco-Silva, P., Taveira, N. C., Rosado, L. (1998). Virological and molecular demonstration of human immunodeficiency virus type 2 vertical transmission. *Journal of Virology*, **72**(4), 3418-3422.

Chang, T.W., Wu, P.C., Hsu, C.L., Hung, A.F. (2007). Anti-IgE antibodies for the treatment of IgE-mediated allergic diseases. *Advance Immunology*, **93**, 63–119.

Charles Onyenekwe, Prince Ele, Nkiruka Ukibe, Michael Ezeani, Clement Ezechukwu, Grace Amilo, Pauline Umeanaeto, Martin Ifeanyichukwu. (2010). Assessment of White Blood Cell Count, Packed Cell Volume, Phagocytic Functions, Serum Albumin and Plasma Iron in HIV Infected Subjects. *Clinical Immunology*, **135**, S110.

Charles Shey Wiysonge, Muki Shey, Eugene J. Kongnyuy, Jonathan A. C Sterne, Peter Brocklehurst. (2011). Vitamin A supplementation for reducing the risk of mother-to-child transmission of HIV infection. Cochrane Database of Systematic Reviews, (1), CD003648

Chasela, C. S., Hudgens, M. G., Jamieson, D. J. (2010). Maternal or infant antiretroviral drugs to reduce HIV-1 transmission. *New England Journal of Medicine*, **362**, 2271–2281.

Chong, Y., Ikematsu, H., Kikuchi, K., Yamamoto, M., Murata, M., Nishimura, M., Nabeshima, S., Kashiwagi, S., Hayashi, J. (2004). Selective CD27+ (memory) B cell reduction and characteristic B cell alteration in drug-naive and HAART-treated HIV type 1-infected subjects. *AIDS Research and Human Retroviruses*, **20**(2), 219-226.

Clerici Mario, Sison Antonio, V., Berzofsky Jay, A., Rakusan Tamara, A., Brandt Carl, D., Ellaurie Maadhava, Villa MariaLuisa, Colie Christine, Venzon David, J., Sever John, L., Shearer Gene, M. (1993). Cellular immune factors associated with mother-to-infant transmission of HIV. *AIDS*, **7**(11), 1427-1428.

Clerici, M., Seminari, E., Suter, F., Castelli, F., Pan, A., Biasin, M., Colombo, F., Trabattoni, D., Maggiolo, F., Carosi, G., Maserati R. (2000). Different immunologic profiles characterize HIV infection in highly active antiretroviral therapy-treated and antiretroviral-naive subjects with undetectable viremia. *AIDS*, **14**(2), 109–116.

Collaborative Group. (1997). The effect of maternal viral load on the risk of perinatal transmission of HIV-1. *AIDS,* **11**, 437-444.

Colognesi, C., Halapi, E., Jansson, M. (1997). The role of virologic and immunologic factors in Conference, Geneva, Abstract 23268.

Connor, E. M., Sperling, R. S., Gelber, R. D., Kiselev, P., Scott, G. B., O'Sullivan, M. J., Van Dyke, R., Bey, M., Shearer, W. T., Jacobson, R. L., Jimenez, E., O'Neill, E., Bazin, B., Delfraissy, J. F., Culnane, M., Coombs, R. W., Elkins, M. M., Moye, J. J., Stratton, P., Balsey, J. (1994). Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. *New England Journal of Medicine*, **331**, 1173-1180.

Coovadia, H. M., Brown, E. R., Fowler, M. G. (2012). Efficacy and safety of an extended nevirapine regimen in infant children of breastfeeding mothers with HIV-1 infection for prevention of postnatal HIV-1 transmission (HPTN 046): a randomised, double-blind, placebo-controlled trial. *Lancet*, **379**, 221–228.

Coovadia, H. M., Rollins, N. C., Bland, R. M. (2007). Mother-to-child transmission of HIV-1 infection during exclusive breastfeeding in the first 6 months of life: an intervention cohort study. *Lancet*, **369**, 1107–1116.

Corthesy, B. (2007). Roundtrip ticket for secretory IgA: role in mucosal homeostasis? *Journal of Immunology*, **178**, 27–32.

Crowther, J. R. 1995. ELISA. Theory and Practice. Methods Mol. Biol. 42:1-223

Davies, C. 1994. Principles. In The Immunoassay Handbook. D. Wild, ed. Stockton Press, New York, p. 3-47.

D'Orsogna, L. J., Krueger, R. G., McKinnon, E. J., French, M. A. (2007). Circulating memory B-cell subpopulations are affected differently by HIV infection and antiretroviral therapy. *AIDS*, **14**, 1747–1752.

Datta, P., Embree, J. E., Kreiss, J. K. (1994). Mother-to child transmission of Human Immunodeficiency Virus Type 1: Report from the Nairobi study. *Journal of Infectious Disease*, **170**, 1134-1140.

De Rossi, A., Ometto, L., Masiero, S. (1997). Viral phenotype in mother-to-child HIV-1 transmission and disease progression of vertically acquired HIV-1 infection. *Acta Pediatrica*, **421**, 22-28.

Denise J. Jamieson, Charles S. Chasela, Michael G. Hudgens, Caroline C. King, Athena P. Kourtis, Dumbani Kayira, Mina C. Hosseinipour, Deborah D. Kamwendo, Sascha R. Ellington, Jeffrey B. Wiener, Susan A. Fiscus, Gerald Tegha, Innocent A. Mofolo, Dorothy S. Sichali, Linda S. Adair, Rodney J. Knight, Francis Martinson, Zebrone Kacheche, Alice Soko, Irving Hoffman, Charles van der Horst, for the BAN study team. (2012). Maternal and infant antiretroviral regimens to prevent postnatal HIV-1 transmission: 48-week follow-up of the BAN randomised controlled trial. *Lancet*, **379**(9835), 2449 – 2458.

Douglas, G. C., King, B. F. (1992). Maternal-fetal transmission of human immunodeficiency virus: a review of possible routes and cellular mechanisms of infection. *Clinical Infectious Diseases*, **15**, 678-691.

Duke University Medical Center (2014). Breast milk protein may be key to protecting babies from HIV. Assessed on 29 October 2014; http://www.sciencedaily.com/releases/2013/10/131021153200.htm.

Duliege, A. M, Amos, C. I., Felton, S. (1995). Birth order, delivery route and concordance in the transmission of human immunodeficiency virus type 1 from mothers to twins. *Journal of Pediatrics*, **126**, 625-632.

European Collaborative Study (1994). Caesarean section and the risk of vertical transmission of HIV-1 infection. *Lancet*, **343**, 1464-1467.

European Collaborative Study (a). (2005). Mother-to-child transmission of HIV-1 in the HAART era. *Clinical Infectious Diseases*, **40**, 458–465.

European Collaborative Study (b). (2005). Does exposure to antiretroviral therapy affect growth in the first 18 months of life in uninfected children born to HIV infected women? *Journal of. Acquired. Immune Deficiency Syndrome*, **40**, 364–370.

European Collaborative Study. (1992). Risk factors for mother-to-child transmission of HIV-1. *Lancet*, **339**, 1007-1012.

European Collaborative Study. (1996). Vertical transmission of HIV-1: maternal immune status and obstetric factors. *AIDS,* **10**, 1675-1681.

European Collaborative Study: Swiss HIV Pregnancy Cohort. (1997). Immunological markers in HIV-infected pregnant women. *AIDS*, **11** (15), 1859–1865.

Fang, G., Burger, H., Grimson, R. (1995). Maternal plasma human immunodeficiency virus type 1 RNA level: a determinant and projected threshold for mother-to-child transmission. *Proceedings of the National Academy of Science*, **92**, 12100-12104.

Faye, A., Pornprasert, S ., Mary, J-Y., Dolcini, G., Derrien, M ., Barré-Sinoussi, F., Chaouat, G ., Menu, E. for the ANRS 1267 study team and the HIV-1 PMTCT-PlaNet. (2007). Characterization of the main placental cytokine profiles from HIV-1-infected pregnant women treated with anti-retroviral drugs in France. *Clinical Experimental Immunology*., **149**(3), 430–439.

Federal Ministry of Health [Nigeria] (2013). National HIV & AIDS and Reproductive Health Survey, 2012 (NARHS Plus). Federal Ministry of Health Abuja, Nigeria.

Fenyo, E. M., Albert, J., McKeating, J. (1996). The role of the humoral immune response in HIV infection. *AIDS*, **10**(A), S97–106.

Filteau, S. (2009). The HIV-exposed, uninfected African child. *Trop Med Int Health,* **14**(3), 276-287.

Frank A. Redegeld, Maurice W. van der Heijden, Mirjam Kool, Bianca M. Heijdra, Johan Garssen, Aletta D. Kraneveld, Henk Van Loveren, Paul Roholl, Takashi Saito, Sjef J. Verbeek, Jill Claassens, Andries S. Koster, Frans P. Nijkamp. (2002). Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses**.** *Natur*e *Medicine* **8**, 694 – 701.

Friedman, J., Alam, S. M., Shen, X., Xia, S. M., Stewart, S., Anasti, K. (2012) Isolation of HIV-1-Neutralizing Mucosal Monoclonal Antibodies from Human Colostrum. PLoS ONE **7**

(5): e37648.

Garty, B. Z., Ludomirsky, A., Danon, Y. L., Peter, J. B., Douglas, S. D. (1994). Placental transfer of immunoglobulin G subclasses. *Clinical and Diagnostic Laboratory Immunology*., **1**, 667-669.

Geisberger, R., Lamers, M., Achatz, G. (2006). The riddle of the dual expression of IgM and IgD. *Immunology*, **118**, 429–437.

Gereda, J. E., Leung, D. Y. M., Thatayatikom, A., Streib, J. E., Price, M. R., Klinnert, M. D. (2000). Relationship between house dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *Lancet,* **355**, 1680–1683.

Ghys, P. D., Fransen, K., Diallo, M. O. (1997). The associations between cervicovaginal HIV Shedding, sexually transmitted diseases and immunosuppression in female sex workers in Abidjan, Cote d'Ivoire. *AIDS*, **11**(12), F85-F93

Goedert, J. J. (1997). Vertical transmission of human immunodeficiency virus type 1: insights from studies of multiple pregnancies. *Acta Pediatrica*, **421**, 56-59.

Gougeon, M. L., Lecoeur, H., Sasaki, Y. (1999). Apoptosis and the CD95 system in HIV disease: impact of highly active anti-retroviral therapy (HAART). *Immunology Letters.*, **66**, 97-103.

Gray, G. (2000). The PETRA study: early and late efficacy of the three short ZDV/3TC combination regimens to prevent mother-to-child transmission of HIV-1. XIII International AIDS Conference, Durban. South Africa, 9–14 July 2000: 17.

Greenberg, B. L, Semba, R. D., Vink, P. E. (1997). Vitamin A deficiency and maternal-infant transmission of HIV in two metropolitan areas in the United States. *AIDS*, **11**, 325-332.

Hague, R. A., Yap, P. L., Mok, J. Y. Q., Eden, O. B., Coutts, N. A., Watson, J. G., Hargreaves, F. D., Whitelaw, J. M. (1989). Intravenous immunoglobulin in HIV infection: evidence for the efficacy of treatment. *Archives of disease in childhood*, **64**, 1146-1150.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B. (1993). Naturally occurring antibodies devoid of light chains. *Nature,* **363**, 446–448.

Harry W. Schroeder and Lisa Cavacini. (2010). Structure and function of immunoglobulins. *Journal of Allergy and Clinical Immunology*, **125**(2), S41–S52.

Hart, M., Steel, A., Clark, S. A., Moyle, G., Nelson, M., Henderson, D. C., Wilson, R., Gotch, F., Gazzard, B., Kelleher, P. (2007). Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease. *Journal of Immunology*, **14**, 8212–8220.

Henin, Y., Mandelbrot, L., Henrion, R. (1993). Virus excretion in the cervicovaginal secretions of pregnant and nonpregnant HIV-infected women. *Journal of Acqired Immune Deficiency Syndrome*, **6**, 72-75.

Holmes, C. B., Losina, E., Walensky, R. P., Yazdanpanah, Y., Freedberg, K. A. (2003). "Review of human immunodeficiency virus type 1-related opportunistic infections in sub-Saharan Africa". *Clinical Infectious Diseases,* **36**(5), 656–662.

Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A., Murphy, K. M. (1993). "Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages". *Science,* **260**(5107), 547–549.

Husson, R. N., Lan, Y., Kojima, E. (1995). Vertical transmission of human immunodeficiency virus type 1: autologous neutralizing antibody, virus load and virus phenotype. *Journal of Pediatrics*, **126**, 865-871.

Ifeanyichukwu, M., Onyenekwe, C. C., Ele, P. U., Ukibe, N. K, Meludu, S. C., Ezeani, M. C., Ezechukwu, C. C. (2009). Evaluation of immunoglobulin classes (IgA, IgG and IgM) levels and complement fixation activity in HIV infected subjects. *International Journal of Biological and Chemical Sciences,* **3**(6), 1504-1508.

International Perinatal HIV Group. (1999). The mode of Delivery and the Risk of Vertical Transmission of Human Immunodeficiency Virus Type 1 — A Meta-Analysis of 15 Prospective Cohort Studies. *New England Journal of Medicine*, **340**, 977-987.

Isabelle Bekeredjian-Ding, Sandra Foermer, Carsten J. Kirschning, Marijo Parcina, Klaus Heeg. (2012). Poke weed mitogen requires Toll-Like receptor ligand for proliferative activity in human and murine B lymphocytes. *Plos one,* **7**(1): e29806.

Italian Register for HIV Infection in Children. (2004). Combined antiretroviral therapy reduces hyperimmunoglobulinemia in HIV-1 infected children. *AIDS*, **18**, 1423–1428.

Janeway Charles, Paul Travers, Mark Walport, Mark Shlomchik (2001). Immunobiology, 5th edition. New York and London: Garland Science. ISBN 0-8153-4101-6.

John, G. C., Kreiss, J. (1996). Mother-to child transmission of human immunodeficiency virus type 1. *Epidemiologic Review,* **18**(2), 149-157.

John, G. C., Nduati, R. W., Mbori-Ngacha, D. (1997). Genital shedding of human immunodeficiency virus type-1 DNA during pregnancy: association with immunosuppression, abnormal cervical and vaginal discharge and severe vitamin A deficiency. *Journal of Infectious Diseases*, **175**(1), 57-62.

Kafulafula, G., Hoover, D. R., Taha, T. E. (2010). Frequency of gastroenteritis and gastroenteritis-associated mortality with early weaning in HIV-1-uninfected children born to HIV-infected women in Malawi. *Journal of Acquired Immune Deficiency Syndrome*, **53**, 6–13.

Katzmann, J. A., Clark, R. J., Abraham, R. S., Bryant, S., Lymp, J. F., Bradwell, A. R., Kyle, R.A. (2001). "Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains". *Clinical Chemistry*, **48**(9), 1437–1444.

Kelso, A. (1993.) "The enigma of cytokine redundancy", *Immunology and Cell Biology*, **72**, 97-101.

Kilewo, C., Karlsson, K., Ngarina, M. (2009). Prevention of mother-to-child transmission of HIV-1 through breastfeeding by treating mothers with triple antiretroviral therapy in Dar es Salaam, Tanzania: The Mitra Plus study. *Journal of Acquired Immune Deficiency Syndrome*, **52**, 406–416.

King Steven R. (1994) HIV: Virology and Mechanisms of disease. *Annals of Emergency Medicine,* **24**, 443-449.

Kolte, L. (2013). Thymic function in HIV-infection. *Danish Medical Journal*, **60**(4), B4622.

Kongnyuy, E. J., Wiysonge, C. S., Shey, M. S. (2009). A systematic review of randomized controlled trials of prenatal and postnatal vitamin A supplementation of HIV-infected women.
International Journal of Gynecology & Obstetrics, **104** (1), 5–8.

Koup, R., Yunzhen, C., Ho, D. (1996). Lack of maternal viral threshold for vertical transmission of HIV-1. Third International Conference on Retroviruses and Opportunistic Infections, Washington DC, Abstract LB2.

Kuhn, L., Sinkala, M., Kankasa, C. (2007). High uptake of exclusive breastfeeding and reduced early post-natal HIV transmission. *PLoS One*, **2**, e1363.

Kuhn, L., Sinkala, M., Semrau, K. (2010). Elevations in mortality due to weaning persist into the second year of life among uninfected children born to HIV-infected mothers. *Clinical Infectious Disease*, **54**, 437–444.

Kuhn, L., Coutsoudis, A., Moodley, D., Mngqundaniso, N., Trabattoni, D., Shearer, G. M., Clerici, M., Coovadia, H. M. (2001a). Interferon-gamma and interleukin-10 production among HIV-1-infected and uninfected infants of HIV-1-infected mothers. *Pediatric Research*. **50**(3), 412-416.

Kuhn, L., Meddows-Taylor, S., Gray, G., Trabattoni, D., Clerici, M., Shearer, G. M., Tiemessen, C. (2001b). Reduced HIV-stimulated T-helper cell reactivity in cord blood with short-course antiretroviral treatment for prevention of maternal-infant transmission. *Clinical and Experimental Immunology*, **123**(3), 443-450.

Kumwenda, N. I ., Hoover, D. R., Mofenson, L. M. (2008). Extended antiretroviral prophylaxis to reduce breast-milk HIV-1 transmission. *New England Journal of Medicine*, **359**, 119–129.

Lallemant, M, Le Coeur, S., Samba, L. (1994). Mother to child transmission of HIV-1 in Congo, *Lancet*, **339**, 1007-1012.

Lane, H.C., Masur, H., Edgar, L. C., Whalen, G., Rook, A. H., Fauci, A. S. (1983). Abnormalities of B-cell activation and immunoregulation in subjects with the acquired immunodeficiency syndrome. *New England Journal of Medicine*, **309**, 453–458.

Lee, B. N., Ordonez, N., Popek, E. J., Lu, J. G., Helfgott, A., Eriksen, N ., Hammill, H ., Kozinetz, C., Doyle, M ., Kline, M ., Langston, C., Shearer, W. T., Reuben, J. M. (1997). Inflammatory cytokine expression is correlated with the level of human immunodeficiency virus (HIV) transcripts in HIV-infected placental trophoblastic cells. *Virology,* **71**(5), 3628–3635.

Lindsay J. Whilton and Micheal B. A. Oldstone. (1996). Immune Response to Viruses. In: Fields Virology, Fiels BN, Knipe DM, Howley PM (eds), Third Edition. Lippincott-Raven Publishers, Philadelphia, 346-369.

Loussert-Ajaka, I., Mandelbrot, L., Delmas, M. (1997). HIV-1 detection in cervicovaginal secretions during pregnancy. *AIDS,* **11**(13), 1575-1581.

Lugada, E. S., Mermin, J., Asjo, B., Kaharuza, F., Downing, R., langeland, N., Ormaasen, V., Bruu, J., Awor, A. C., Ulvestad, E. (2004). Immunoglobulin Levels Amongst Persons with and without Human Immunodeficiency Virus Type 1 Infection in Uganda and Norway Scandinavian. *Journal of Immunology*, **59**(2), 203–208.

Luscher, M. A., Choy, G., Embree, J. E. (1998). Anti-HLA alloantibody is found in children but does not correlate with a lack of HIV type 1 transmission from infected mothers. *AIDS Research and Human Retroviruses*, **4**(2), 99-107.

MacDonald, K. S., Embree, J., Njenga, S. (1998). Mother-child class I HLA concordance increases perinatal human immunodeficiency virus type 1 transmission. *Journal of Infectious Diseases*, **177**(3), 551-556.

Madeleine Bunders, Lucy Pembrey, Taco Kuijpers, Marie-Louise Newell. (2009). Research and Human Retroviruses. *AIDS,* **26**(9), 967-975.

Malek, A., Sager, R., Kuhn, P., Nicolaides, K. H., Schneider, H. (1996) Evolution of maternofetal transport of immunoglobulins during human pregnancy. *American Journal of Reproductive Immunology*, **36**, 248-255.

Mandelbrot, L., Landreau-Mascaro, A., Rekacewicz, C., Berrebi, A., Bénifla, J. L., Burgard, M., Lachassine, E., Barret, B., Chaix, M. L., Bongain, A., Ciraru-Vigneron, N., Crenn-Hébert, C., Delfraissy, J. F., Rouzioux, C., Mayaux, M. J., Blanche, S. (2001). Agence Nationale de Recherches sur le SIDA (ANRS) 075 Study Group: Lamivudine-Zidovudine combination for prevention of maternal-infant transmission of HIV-1. *Journal of American Medical Association*, **285**, 2083-2093.

Mandelbrot, L., Le Chenadec, J., Berrebi, A., Bongain, A., Benifla, J. L., Delfraissy, J. F., Blanche, S., Mayaux, M. J. (1998). Perinatal HIV-1 transmission: interaction between zidovudine prophylaxis and mode of delivery in the French Perinatal Cohort. *Journal of American Medical Association*, **280**(1), 55-60.

Mandelbrot, L., Mayaux, M. J., Bongain, A. (1996). Obstetric factors and mother-to-child transmission of human immunodeficiency virus type 1: the French perinatal cohorts. *American Journal of Obstetrics and Gynecology*., **175**, 661-667.

Mangano, A., Prada, F., Roldan, A. (1998). Distribution of the CCR-5 delta32 allele in Argentinian children at risk of HIV-1 infection: its role on vertical transmission. *AIDS*, **12**(1), 109-110.

Markham, R. B. 1., Coberly, J., Ruff, A. J., Hoover, D., Gomez, J., Holt, E., Desormeaux, J., Boulos, R., Quinn, T. C., Halsey, N. A. (1994). Maternal IgG1 and IgA antibody to V3 loop consensus sequence and maternal-infant HIV-1 transmission. *Lancet*, **343**(8894), 390-391.

Martin Ifeanyichukwu, Sam Meludu, Prince Ele, Nkiru Ukibe, Charles Onyenekwe, Michael Ezeani, Clement Ezechukwu, Grace Amilo, Pauline Umeanaeto. (2010). Evaluation of some cellular immune index in HIV infected participants. *International Journal of Biological and chemical Sciences,* **5**(3), 1310-1315.

Martine Braibant, Francis Barin. (2013). The role of neutralizing antibodies in prevention of HIV-1 infection: what can we learn from the mother-to-child transmission context? *Retrovirology*, **10**, 103.

Matheson, P. B., Schoenbaum, E., Greenberg, B., Pliner, V. and the New York City Perinatal HIV transmission Collaborative Study. (1997). Association of maternal drug use during pregnancy with mother-to-child transmission. *AIDS,* **11**(7), 941-942.

Matheson, P. B., Thomas, P. A., Abrams, E. J. (1996). Heterosexual behavior during pregnancy and perinatal transmission of HIV-1. *AIDS,* **10**, 1249-1256.

Mayaux, M. J., Blanche, S., Rouzioux, C., Le Chenadec, J., Chambrin, V., Firtion, G., Allemon, M. C., Vilmer, E., Vigneron, N. C., Tricoire, J. (1995). Maternal factors associated with perinatal HIV-1 transmission: the French Cohort Study: 7 years of follow-up observation. The French Pediatric HIV Infection Study Group. *Journal of Acquired Immune Deficiency Syndrome and Human Retrovirology*, **8**(2), 188-194.

Mayaux, M. J., Dussaix, E., Ispoet, J. (1997). Maternal viral load during pregnancy and mother-to child transmission of human immunodeficiency virus type 1: The French Perinatal Cohort Studies. *Journal of Infectious Diseases*, **175**, 172-175.

Meena A. khirwadkar, Jayant R. kher. (1991). Short communication study of serum immunoglobulins in normal pregnancy. *Indian Journal of Physiology and Pharmacology*, **35**(1), 69-70

Merriam-Webster Dictionary. Retrieved 28 October 2011.

Michael H. Chung, James N. Kiarie, Barbra A. Richardson, Dara A. Lehman, Julie Overbaugh, John Kinuthia, Francis Njiri, Grace C. John-Stewart. (2008). Highly active antiretroviral therapy versus zidovudine/nevirapine effects on early breast milk HIV type-1 Rna: a phase II randomized clinical trial. *Antiviral Therapy*, **13**(6), 799-807.

Miedema, F., Petit, A. J., Terpstra, F. G. (1988). Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. HIV affects the immune system before $CD4^{+}$ T helper cell depletion occurs. *Journal of Clinical Investigation*, **82**, 1908–1914.

Miles, D. J, Gadama, L., Gumbi, A., Nyalo, F., Makanani, B., Heyderman, R. S. (2010). Human immunodeficiency virus (HIV) infection during pregnancy induces CD4 T-cell differentiation and modulates responses to Bacille Calmette-Guérin (BCG) vaccine in HIV-uninfected infants. *Immunology,* **129**(3), 446-454.

Miller, E. C., Abel, W. (1984). Changes in the immunoglobulins IgG, IgA and IgM in pregnancy and the puerperium. *Zentralblatt fur Gynakol*ogie, **106**(15), 1084-1091.

Miller-Keane (2003). Encyclopedia and Dictionary of Medicine, Nursing, and Allied Health, Seventh Edition. Saunders, an imprint of Elsevier, Inc

Misrahi, M., Teglas, J. P., N'Go, N. (1998). CCR5 chemokine receptor variant in HIV-1 mother-to-child transmission and disease progression in children. French Pediatric HIV Infection Study Group. *Journal of American Medical Association*, **279**(4), 277-280.

Mocroft, A., Ledergerber, B., Katlama, C., Kirk, O., Reiss, P., d'Arminio Monforte, A., Knysz, B., Dietrich, M., Phillips, A. N., Lundgren, J. D. (2003). EuroSIDA study group. Decline in the AIDS and death rates in the EuroSIDA study: an observational study. *Lancet*, **362**, 22–29.

Mofenson, L. M. (1997). Interaction between the timing of perinatal human immunodeficiency virus infection and the design of preventive and therapeutic interventions. *Acta Pediatrica*, **421**, 1-9.

Moir, S., Malaspina, A., Pickeral, O. K., Donoghue, E. T., Vasquez, J., Miller, N.J., Krishnan, S. R., Planta, M. A., Turney, J. F., Justement, J. S., Kottilil, S., Dybul, M., Mican, J. M., Kovacs, C., Chun, T. W., Birse, C. E., Fauci, A. S. (2004). Decreased survival of B cells of HIV-viremic subjects mediated by altered expression of receptors of the TNF superfamily. *Journal of Experimental Medicine,* **14**, 587–599.

Montagnier Luc. (1999). Human Immunodeficiency Viruses (Retroviridae). Encyclopedia of Virology (2nd Ed.) 763-774.

Moodley, D., Coovadia, H. M., Bobat, R. A., Sullivan, J. L. (1997). HIV-1 specific immunoglobulin A antibody as an effective marker of perinatal infection in developing countries. *Journal of Tropical Pediatr*ics, **43**(2), 80-83.

Mor, G., Straszewski-Chavez, S. L., Abrahams, V. M. (2006). Macrophage–trophoblast interactions. *Methods in Molecular Medicine,* **122**, 149–163.

Morris, L., Binley, J. M., Clas, B. A. (1998). HIV-1 antigen-specific and -nonspecific B cell responses are sensitive to combination antiretroviral therapy. *Journal of Experimental Medicine*, **188**, 233–245.

Mosser DM, Zhang X. (2008). Interleukin-10: new perspectives on an old cytokine. *Immunology Review,* **226**, 205–218.

Müller, F., Tjonnfjord, G. E., Nordoy, I., Kvale, D., Mellbye, O. J., Aukrust, R., Froland, S. S. (2002). Immunophenotypic analyses of CD34+ cell subsets in bone marrow from HIV-infected subjects during highly-active antiretroviral therapy. *European Journal of Clinical Investigation*, **32**, 535–540.

Mushahwar Isa K. (2007) Human Immunodeficiency Viruses: Molecular Virology, pathogenesis, diagnosis and treatment. *Perspectives in Medical Virology,* **1**, 75-87.

Nahlen, B., Steketee, R., Ayisi, A. (1998). Association between placental malarial infection and increased risk of mother to infant transmission of HIV-1 in western Kenya. 12th World AIDS Conference, Geneva, Abstract 23268.

Nduati, R. W., John, G. C., Richardson, B. A. (1995). Human immunodeficiency virus type 1-infected cells in breast milk: association with immunosuppression and vitamin A deficiency. *Journal of Infectious Diseases*, **172**(6), 1461-1468.

Newell, M. L., Huang, S., Fiore, S., Thorne, C., Mandelbrot, L., Sullivan, J. L., Maupin, R., Delke, I., Watts, D. H., Gelber, R. D., Cunningham, C. K. (2007). PACTG 316 Study Team. Characteristics and management of HIV-1-infected pregnant women enrolled in a randomised trial: differences between Europe and the USA. *BMC Infectious Diseases*. **7**, 60.

Nicola, N. A. (1994.) "Cytokine pleiotropy and redundancy: a view from the receptor", *Stem Cells*, **12**(1), 3-12.

Nielsen, S. D., Jeppesen, D. L., Kolte, L., Clark, D. R., Sørensen, T. U., Dreves, A. M., Ersbøll, A. K., Ryder, L. P., Valerius, N. H., Nielsen, J. O. (2001). Impaired progenitor cell function in HIV-negative infants of HIV-positive mothers' results in decreased thymic output and low CD4 counts. *Blood*, **98**, 398-404.

Notermans, D. W., De Jong, J. J., Goudsmit, J. (2001). Potent antiretroviral therapy initiates normalization of hypergammaglobulinemia and a decline in HIV type 1-specific antibody responses. *AIDS Research and Human Retroviruses*, **17**, 1003–1008.

Olivier Garraud, Gwenoline Borhis, Gamal Badr, Severine Degrelle, Bruno Pozzetto, Fabrice Cognasse, Yolande Richard. (2012). Revisiting the B- cell compartment in mouse and human: more than one B-cell subset exists in the marginal zone and beyond. *BMC Immunology* **13**, 63.

Oluwaseun O. Akinpelu, Yetunde A. Aken'Ova, Ganiyu O. Arinola. (2012). Levels of Immunoglobulin Classes Are Not Associated with Severity of HIV Infection in Nigerian Subjects. *World Journal of AIDS*, **2**, 232-236.

Omer, S. B. (2011). Six Week Extended-Dose Nevirapine Study Team Twelve-month follow-up of Six Week Extended Dose Nevirapine randomized controlled trials: differential impact of extended-dose nevirapine on mother-to-child transmission and infant death by maternal CD4 cell count *AIDS*, **25**, 767–776.

Ono, E., Nunes dos Santos, A. M., de Menezes Succi, R. C., Machado, V. D., de Angelis, S. A., Salomão, R., Kallás, E. G., de Moraes-Pinto, M. I. (2008). Imbalance of naive and memory T lymphocytes with sustained high cellular activation during the first year of life from uninfected children born to HIV-1-infected mothers on HAART. *Brazilian Journal of Medical and Biological Research*, **41**(8), 700-708.

Pacheco, S E., McIntosh, K., Ming, L., Mofenson, L. M., Diaz, C., Foca, M., Frederick, M., Handelsman, E., Hayani, K., Shearer, W. T. (2006). Women and Infants Transmission Study: Effect of perinatal antiretroviral drug exposure on hematologic values in HIV-uninfected children: an analysis of the women and infant's transmission study. *Journal of Infectious Diseases,***194**, 1089–1097.

Palombi, L., Marazzi, M. C., Voetberg, A., Magid, N. A. (2007). Treatment acceleration program and the experience of the DREAM program in prevention of mother-to-child transmission of HIV. *AIDS*, **21** (4), S65–S71.

Peeters, M., Courgnaud, V. (2002). "Overview of Primate Lentiviruses and their Evolution in Non-human Primates in Africa". In Kuiken, C., Foley, B., Freed, E., Hahn, B., Korber, B., Marx, P. A., McCutchan, F. E., Mellors, J. W., Wolinsky, S. HIV sequence compendium. Los Alamos, NM: Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, 2–23.

Peeters, M., Courgnaud, V., Abela, B. (2001). "Genetic Diversity of Lentiviruses in Non-Human Primates". *AIDS Reviews* **3**, 3–10.

Peltier, C. A., Ndayisaba, G. F., Lepage, P. (2009). Breastfeeding with maternal antiretroviral therapy or formula feeding to prevent HIV postnatal mother-to-child transmission in Rwanda. *AIDS*, **23**, 2415–2423.

Peter J. Delves, Seamus J. Martin, Dennis R. Burton, Ivan M. Roitt. (2011). Roitt's Essential Immunology, 11th Ed. ISBN 978-1-4051-3603-7. John Wiley & Sons.

Pitt, J., Brambilla, D., Reichelderfer, P. (1997). Maternal immunologic and virologic risk factors for infant human immunodeficiency virus type 1 infection: findings from the Women and Infants Transmission Study. *Journal of Infectious Diseases*, **175**, 567-575.

Plaeger, S., Bermudez, S., Mikyas, Y., Harawa, N., Dickover, R., Mark, D., Dillon, M., Bryson, Y. J., Boyer, P.J., Sinsheimer, J. S. (1999). Decreased CD8 cell-mediated viral suppression and other immunologic characteristics of women who transmit human immunodeficiency virus to their infants. *Journal of Infectious Diseases*, **179**(6), 1388-1394.

Plantier, J. C., Leoz, M., Dickerson, J. E., De Oliveira, F., Cordonnier, F., Lemée, V., Damond, F., Robertson, D. L., Simon, F. (2009). A new human immunodeficiency virus derived from gorillas. *Nature Medicine*., **15**(8), 871-872.

Pongsakdi Chaisilwattana, Kulkanya Chokephaibulkit, Amphan Chalermchockcharoenkit, Nirun Vanprapar, Korakot Sirimai, Sanay Chearskul, Ruengpung Sutthent, Nisarat Opartkiattikul. (2002). Short-Course Therapy with Zidovudine Plus Lamivudine for Prevention of Mother-to-Child Transmission of Human Immunodeficiency Virus Type 1 in Thailand. *Clinical Infectious Dis*eases, **35**(11), 1405-1413.

Pornprasert, S., Mary, J. Y., Faye, A., Leechanachai, P., Limtrakul, A., Rugpao, S., Sirivatanapa, P., Gomuthbutra, V., Matanasaravoot, W., Le Coeur, S., Lallemant, M., Barré-Sinoussi, F., Menu, E., Ngo-Giang-Huong, N., ANRS 1267 study team and HIV-1 PMTCT-PlaNet. (2009). Higher placental anti-inflammatory IL-10 cytokine expression in HIV-1 infected women receiving longer zidovudine prophylaxis associated with nevirapine. *Current HIV Res*earch, **7**(2), 211-217.

Porter, K., Babiker, A., Bhaskaran, K., Darbyshire, J., Pezzotti, P., Walker, A. S. for the CASCADE Collaboration. (2003). Determinants of survival following HIV-1 seroconversion after the introduction of HAART. *Lancet,* **362**, 1267–1274.

Re, M. C., Furlini, G., Vignoli, M., Ricchi, E., Ramazzotti, E., Bianchi, S., Guerra, B., Costigliola, P., La Placa, M. (1992). Vertical transmission of human immunodeficiency virus type 1. Prognostic value of IgA antibody to HIV-1 polypeptides during pregnancy. *Diagnostic Microbiology and Infectious Disease*., **15**(6), 553-536.

Reggy, A., Simonds, R.J., Rogers, M. (1997). Preventing perinatal HIV transmission. *AIDS,* **11**(A), S61-S66.

Reinhard, G., Noll, A., Schlebusch, H., Mallmann, P., Ruecker, A. V. (1998). Shifts in the TH1/TH2 balance during human pregnancy correlate with apoptotic changes. *Biochemical and Biophysical Research Communications*, **245**(3), 933-938.

Reitter, A., Stücker, A., Linde, R., Königs, C., Knecht, G., Herrmann, E., Schlößer, R., Louwen, F., Haberl, A. (2014). Pregnancy complications in HIV-positive women: 11-year data from the Frankfurt HIV Cohort. *HIV Medicine*, **15**(9), 525-536.

Riesbeck, K., Nordstrom T. (2006). Structure and immunological action of the human pathogen *Moraxella catarrhalis* IgD-binding protein. *Critical Reviews in Immunology*, **26**, 353–376.

Romagnani, S. (2000). T-cell subsets (Th1 versus Th2): Ann Allergy Asthma *Immunology*, **85**(1), 9-18.

Roux, K.H., Greenberg, A.S., Greene, L., Strelets, L., Avila, D., McKinney, E.C. (1998). Structural analysis of the nurse shark (new) antigen receptor (NAR): molecular convergence of NAR and unusual mammalian immunoglobulins. *Proceedings of National Academy of Sciences*, **95**, 11804–11809.

Sachdeva, N., Oshima, K., Cotter, A., Ashman, M., Davila, L., Okazaki, T., Inaba, N., Asthana, D. (2008). Analysis of immunological markers associated with pregnancy and HIV-1 infection: relevance in perinatal transmission in HIV-1-infected pregnant women with low plasma viral load. *American Journal of Reproductive Immunol*ogy, **60**, 264–273.

Schüpbach, J., Wunderli, W., Kind, C. (1989). Frequent detection of HIV-and IgG-specific IgM and IgA antibodies in HIV-positive cord-blood sera: fine analysis by western blot. *AIDS*, **3**, 583–589.

Semba, R. D., Miotti, P. G., Chiphangwi, J., Hoover, D. R. (1994). Maternal vitamin A deficiency and mother to child transmission of HIV-1. *Lancet*, **343**, 1593-1597.

Sepkowitz, K. A. (2001). "AIDS—the first 20 years". *New England Journal of Medicine*, **344** (23), 1764–1772.

Shankar Subramanian Iyer, Genhong Cheng (2012). Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease. *Critical Review in Immunology,* **32**(1): 23–63

Shapiro, L., Hughes, M. D., Ogwu, A. (2010). Antiretroviral regimens in pregnancy and breast-feeding in Botswana. *New Engandl Journal of Medicine*, **362**, 2282–2294.

Shirai, A., Cosentino, M., Leitman-Klinman, S. F., Klinman, D. M. (1992). Human immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation. *Journal of Clinical Investigation*, **89**, 561–566.

Simona Fiore, Marie-Louise Newell, Daria Trabattoni, Claire Thorne, Linsay Gray, Valeria Savasi, Cecilia Tibaldi, Enrico Ferrazzi, Mario Clerici. (2006). Antiretroviral therapy-associated modulation of Th1 and Th2 immune responses in HIV-infected pregnant women. *Journal of Reproductive Immunology*, **70**(1-2), 143–150.

Smith, K.A., Nelson, P.N., Warren, P., Astley, S.J., Murray, P.G., Greenman, J. (2004). Demystified recombinant antibodies. *Journal of Clinical Pathology*, **57**, 912–917.

Steihm, E. R. (1996). Newborn factors in maternal-infant transmission of pediatric HIV infection. *Journal of Nutrition*, **126**, 2632S-2636S.

Stubbe, H., Berdoz, J., Kraehenbuhl, J.P., Corthesy, B. (2000). Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing *Clostridium difficile* toxin A damaging of T84 monolayers. *Journal of Immunology*, **164**, 1952–1960.

SWEN Study Team. (2008). Extended-dose nevirapine to 6 weeks of age for infants to prevent HIV transmission via breastfeeding in Ethiopia, India, and Uganda: an analysis of three randomised controlled trials. *Lancet*, **372**, 300–313.

Taha, T. E., Hoover, D. R., Chen, S. (2011). Effects of cessation of breastfeeding in HIV-1-exposed, uninfected children in Malawi. *Clinical Infectious Diseases*, **53**, 388–395.

Taissa M. Kasahara, Joana Hygino, Bernardo Blanco, Luciana Xavier, Carlos Fernando Araújo-Lima, Landi V.C. Guillermo, Vera Carolina B. Bittencourt, Vander Guimarães, Arnaldo F. B. Andrade, Cleonice A. M. Bento. (2013). The impact of maternal anti-retroviral therapy on cytokine profile in the uninfected neonates. *Human Immunology*, **74**(9), 1051–1056.

Temmerman, M., Nyong'O, A. O., Bwayo, J. (1995). Risk factors for mother-to-child transmission of human immunodeficiency virus-1 infection. *American Journal of Obstetrics and Gynecology*, **172**, 700-705.

Thea, D. M., Steketee, R. W., Pliner, V. and New York City Perinatal HIV Transmission Collaborative Group. (1997). The effect of maternal viral load on the risk of perinatal transmission of HIV-1. *AIDS,* **11**, 437-444.

Thior, I., Lockman, S., Smeaton, L. M. (2006). Breastfeeding plus infant zidovudine prophylaxis for 6 months' vs formula feeding plus infant zidovudine for 1 month to reduce mother-to-child HIV transmission in Botswana: a randomized trial: The Mashi Study. *Journal of American Medical Association*, **296**, 794–805.

Thomas, T. K., Masaba, R., Borkowf, C. B. (2011). Triple-antiretroviral prophylaxis to prevent mother-to-child HIV transmission through breastfeeding—the Kisumu Breastfeeding Study, Kenya: a clinical trial *PLoS Medicine*, **8**, e1001015.

Thorne, C., Semenenko, I., Pilipenko, T., Malyuta, R. (2009). Ukraine European Collaborative Study Group. Progress in prevention of mother-to-child transmission of HIV infection in Ukraine: results from a birth cohort study. *BMC Infectious Diseases*., **9**, 40.

Titanji, K., Chiodi, F., Bellocco, R., Schepis, D., Osorio, L., Tassandin, C., Tambussi, G., Grutzmeier, S., Lopalco, L., De Milito, A. (2005). Primary HIV-1 infection sets the stage for important B lymphocyte dysfunctions. *AIDS*, **19**(17), 1947-55.

Torres, R.M., Imboden, J., Schroeder, H.W. (2008). Antigen receptor genes, gene products, and co-receptors. In: Rich, R.R., Fleisher, T.A., Shearer, W.T., Schroeder, H.W., Frew, A.J., Weyand, C.M. (Eds.), Clinical immunology: principles and practice (3rd ed), Mosby Elsevier, London, 53–77.

Turner, B. J., Hauck, W. W., Fanning, T. R., Markson, L. E. (1997). Cigarette smoking and maternal-child HIV transmission. *Journal of Acquied Immune Deficiency Syndrome and Human Retrovirology*, **14**(4), 327-337.

Ugen, K. E., Srikantan, V., Goedert, J. J (1997). Vertical transmission of Human immunodeficiency virus type 1: seroreactivity by maternal antibodies to the carboxy region of the gp41 envelope glycoprotein. *Journal of Infectious Diseases*, **175**, 63-69.

UNAIDS (2014) 'Epidemiology Fact Sheet on HIV and AIDS in Nigeria' - See more at: http://www.avert.org/professionals/hiv-around-world/sub-saharan-africa/nigeria#footnote2_31532g5. Retrieved on July 14, 2015.

Van de Perre, P., Hitimana, D. G., Simenon, A. (1992). Postnatal transmission of HIV-1 associated with breast abscess. *Lancet,* **339**, 1490-1491.

Van Leeuwen, R., Katlama, C., Murphy, R. L. (2003). "A randomized trial to study first-line combination therapy with or without a protease inhibitor in HIV-1-infected subjects". *AIDS* **17** (7), 987–999.

Vincke, C., Loris, R., Saerens, D., Martinez-Rodriguez, S., Muyldermans, S., Conrath. K. (2009). General strategy to humanize a camelid single-domain antibody and identification of a universal humanized nanobody scaffold. *Journal of Biological Chemistry*, **284**, 3273–3284.

Vogel, M., Schwarze-Zander, C., Wasmuth, J. C., Spengler, U., Sauerbruch, T., Rockstroh, J. K. (2010). "The treatment of subjects with HIV". *Deutsches Ärzteblatt international*, **107**(28–29), 507–515.

Votteler, J. and Schubert, U. (2008) Human Immunodeficiency Viruses: Molecular Biology. Encyclopedia of Virology. (3rd ed.) p. 517-525.

WHO (2010). Antiretroviral drugs for treating pregnant women and preventing HIV infection in infants: recommendations for a public health approach World Health Organization, Geneva.

WHO (2013). 'Global update on HIV treatment: Results, impacts and oppurtunities available at http//www.avert.org/hiv-and-breastfeeding. Assessed on sept 16, 2014.

WHO. (2010). Guidelines on HIV and infant feeding: principles and recommendations for infant feeding in the context of HIV and summary of evidence. Accessed Jan 27, 2012.

Wicherek, L., Basta, P., Pitynski, K., Marianowski, P., Kijowski, J., Wiatr, J., Majka, M. (2009). The characterization of the subpopulation of suppressive B7H4(+) macrophages and the subpopulation of CD25(+) CD4(+) and FOXP3(+) regulatory T-cells in decidua during the secretory cycle phase, Arias Stella reaction, and spontaneous abortion – a preliminary report. *Am Journal of Reproductive Immunol*ogy, **61**, 303–312.

Williams, A.F., Barclay, A. N. (1988). The immunoglobulin superfamily—domains for cell surface recognition. *Annual Review of Immunology*, **6**, 381–405.

Wood, L. (1996). Perinatal Transmission of HIV-1 (letter). *Journal of American Medical Association*, **276**(16), 1300.

Woof, V. and Mestecky, J. (2005). Mucosal immunoglobulins. *Immunology Review*, **206**, 64–82.

World Health Organization (2011). The strategic use of antiretrovirals for treatment and prevention of HIV infection. Report of a WHO technical consultation, 14–16 November 2011, Geneva, Switzerland. accessed 15 May 2013. (https://extranet.who.int/iris/restricted/bitstream/10665/70912/5/9789241503808_eng.pdf ,

Yeh, S.W., Cavacini, L.A., Bhol, K.C., Lin, M.S., Kumar, M., Duval, M. (2006). Pathogenic human monoclonal antibody against desmoglein. *Journal of Immunology*, **120**, 68–75.

Zachar V, Fazio-Tirrozzo, G., Fink, T., Roberts, D. J., Broadhead, R. L., Brabin, B., Ebbesen, P. (2000). Lack of protection against vertical transmission of HIV-1 by interferons produced during pregnancy in a cohort from East African republic of Malawi. *Journal of Medical Virology*, **61**(2), 195-200.

Zenclussen, A. C. (2005). CD4(+) CD25 + T regulatory cells in murine pregnancy. *Journal of Reproductive Immunol*ogy, **65**, 101–110.

Appendix I: Standard questionnaire for test/control subjects

PATIENS' QUESTIONIRE

Mother's identification no: Enrolment Date:

Age: Gestational Age: Weight & Height of the mother:

When did you test positive for HIV: Parity: Blood pressure:

Most recent CD4: CD4 value before the treatment:

When last did you treat malaria: Phone number:

How often (how many times per year) do you treat malaria:

Are you diabetic: Any other infection:

Baby identification no: Baby's Date of Birth: Sex:

Baby's Date of Enrolment: Weight &Height of the baby:

Baby's head circumference: Baby's treatment at the time of sample collection:

Mode of baby's delivery: Any complication prior to delivery:

APPENDIX II: SUBJECT'S CONSENT FORM

I Mrs/Miss/Miss ---, having understood the benefits of the research entitled "Neutrophil phagocytic function, lymphocyte transformation and detection of viral antigen in mother to child transmission" as explained by the researcher Ewenighi Chinwe O. to me, my child and the society at large, and having known that I have the right to participate or withdraw at any time, I hereby give my consent.

Subject ID:

Signature:

Date:

Appendix III: Ethical approval from NAU

FACULTY OF HEALTH SCIENCES AND TECHNOLOGY
COLLEGE OF HEALTH SCIENCES
NNAMDI AZIKIWE UNIVERSITY, NNEWI CAMPUS
P.M.B. 5001 NNEWI ANAMBRA STATE, NIGERIA

OFFICE OF THE DEAN

Our Ref: ______ *Your Ref:* ______ *Date:* 12th August, 2013

Enwenighi Chinwe Obianuju (Reg. No.: 2011347005F)
c/o The Head
Department of Medical Laboratory Science
Faculty of Health Sciences and Technology
Nnamdi Azikiwe University
Nnewi Campus.

Re: Request for Ethical Approval to carry out Ph.D Research Project

I wish to inform you that the corrected copy of your research proposal titled "Neutrophil Phagocytic function, lymphocyte Transformation and Detection of viral antigen on HIV – Positive pregnant mother and exposed child" has been reviewed.

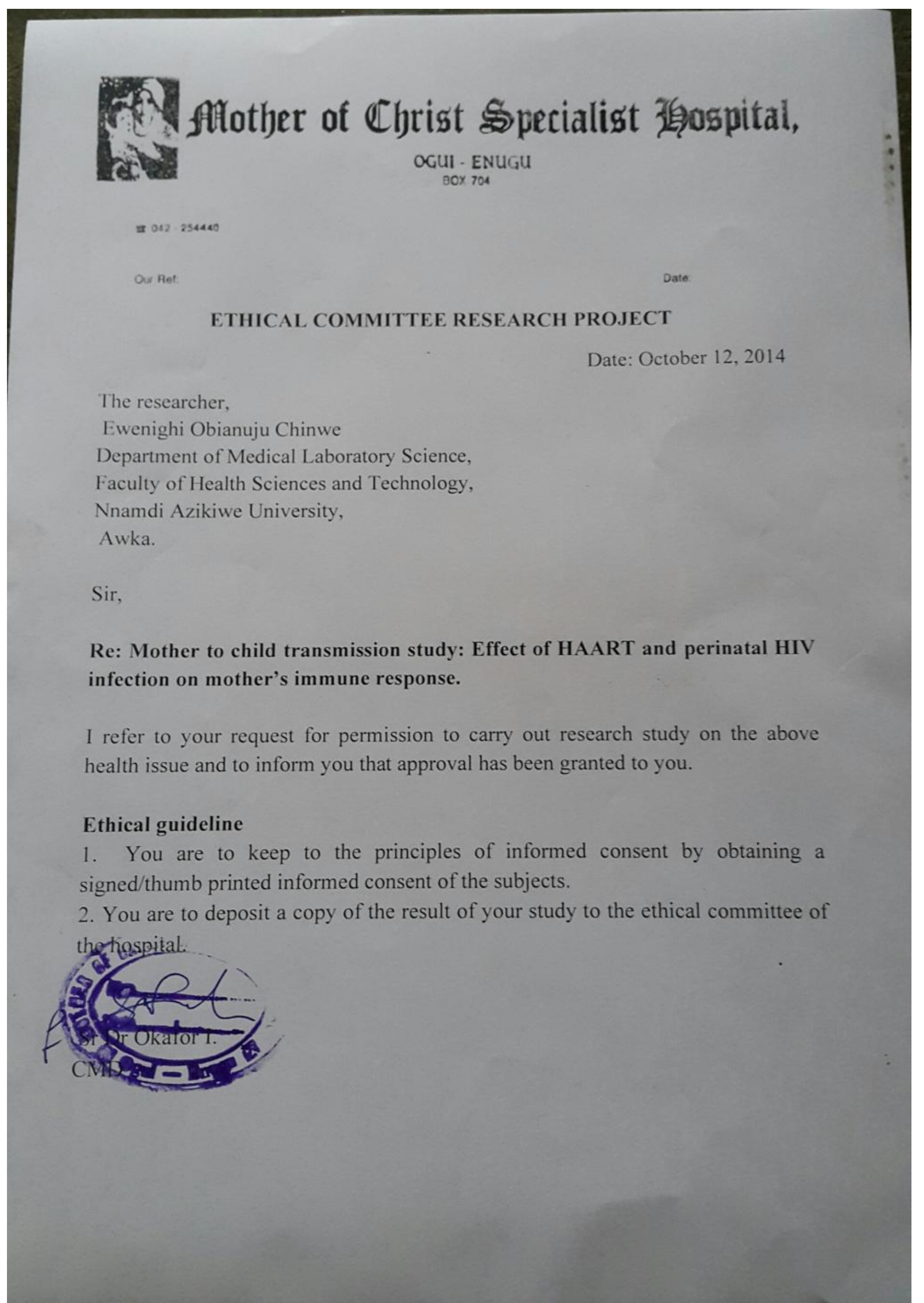
You are granted ethical approval to carry out the study.

Thank you.

Yours Sincerely,

Dr. Chiejina E.N.
Chairman
For FHST Ethical Committee.

Appendix IV: Ethical clearance letter from Mother of Christ Specialist, Hospital, Ogui Enugu



Mother of Christ Specialist Hospital,

OGUI - ENUGU

BOX 704

☎ 042 - 254440

Our Ref: Date:

ETHICAL COMMITTEE RESEARCH PROJECT

Date: October 12, 2014

The researcher,
Ewenighi Obianuju Chinwe
Department of Medical Laboratory Science,
Faculty of Health Sciences and Technology,
Nnamdi Azikiwe University,
Awka.

Sir,

Re: Mother to child transmission study: Effect of HAART and perinatal HIV infection on mother's immune response.

I refer to your request for permission to carry out research study on the above health issue and to inform you that approval has been granted to you.

Ethical guideline

1. You are to keep to the principles of informed consent by obtaining a signed/thumb printed informed consent of the subjects.
2. You are to deposit a copy of the result of your study to the ethical committee of the hospital.

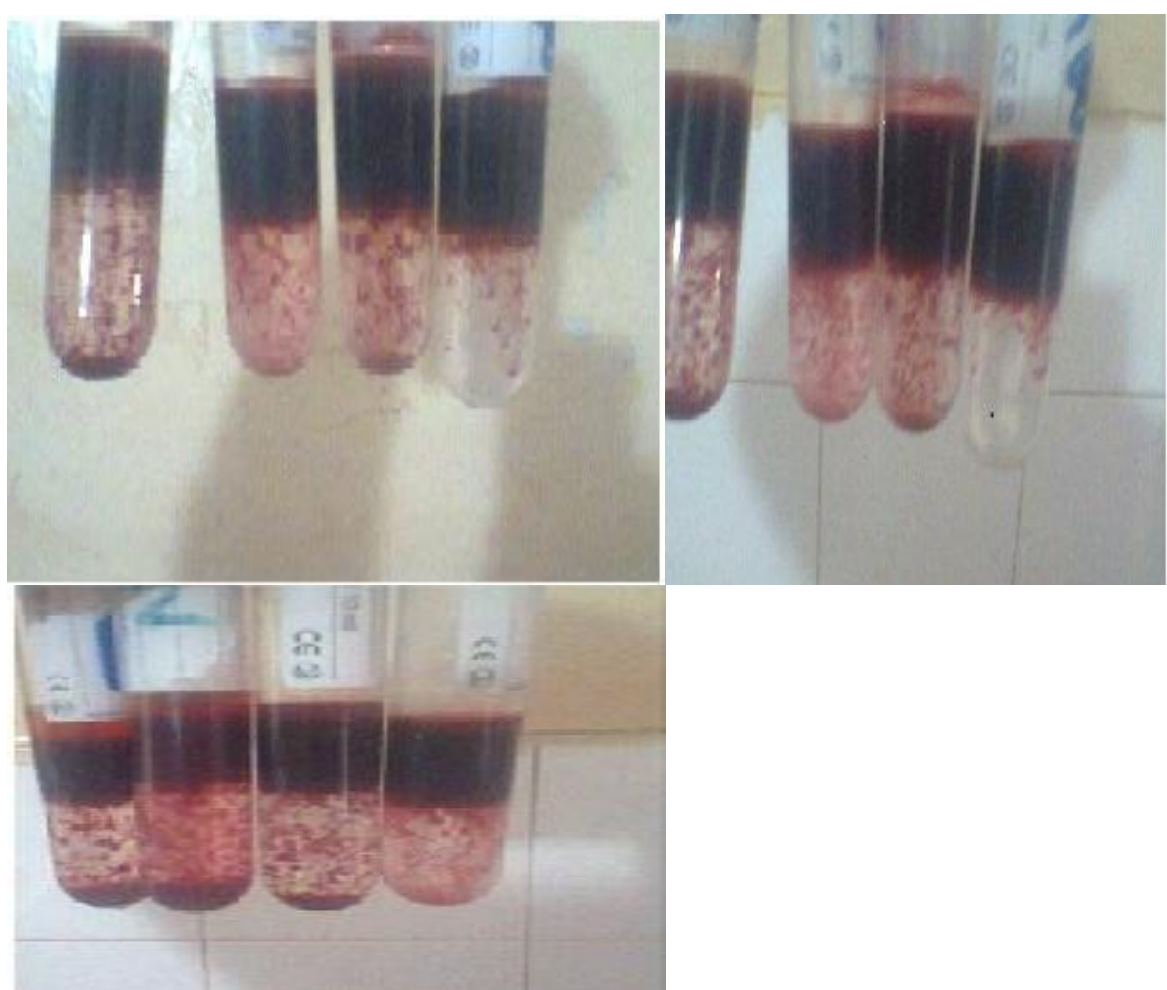
Sr Dr Okafor I.
CMD

Appendix V

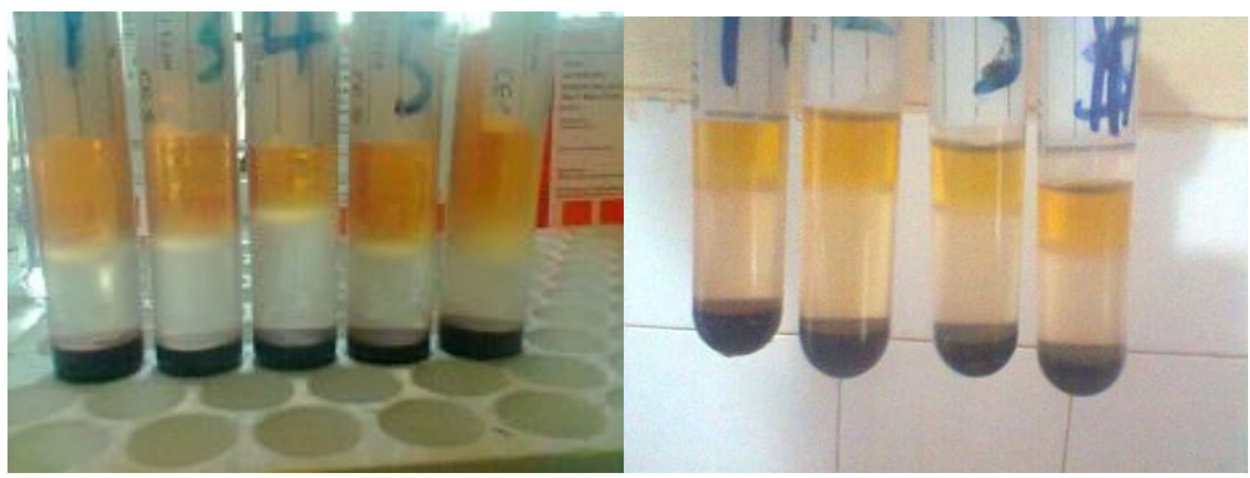
Separation of peripheral blood mononuclear cells (PBMC) using histopague/ficolpaque.

Appearance of the whole blood - phosphate buffered saline PBS (Hank's solution) mixture immediately after addition unto ficolpaque. The ficolpaque settles at the bottom of the tube while the whole blood is at the top.

Appendix VI: Appearance of the whole blood- hank mixture few minutes after the addition unto ficolpaque. The red cell starts settling at the bottom of the tube



Appendix VII: Appearance of the whole blood- hank mixture after density gradient centrifugation at room temperature (30 minutes at 700×*g*). The mixture shows 4 distinct layers in this order, starting from the top to the bottom: **plasma, PBMC, histopaque and red cell**



APPENDIX VIII: Lay-out for cytokine assay

Lay-out for cytokine assay

Cytokine assay Second batch.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0 standard background	Standard # 4 80 pg/ml	QC-2 control	113T1B	133T3 B	136T2 A	121T1 C	130T3 C	C034 T2B	C016 T1C	C040 T3C	C013 T2A	
B	0 standard background	Standard # 4 80 pg/ml	QC-2 control	083T2B	100T3 B	107T2 A	105T1 C	131T3 C	C019 T2B	C014 T1C	C018 T3C	C010 T2A	
C	Standard # 1 10,000pg/ml	Standard # 5 16 pg/ml	124T3N B	120T2B	128T3 B	127T2 A	colourless 088T1 C	126 T3C	C028 T2B	C023 T1C	C030 T3C	C028 T2A	
D	Standard # 1 10,000 pg/ml	Standard # 5 16 pg/ml	132T2NB	136T2B	131T3 B insufficient	078T3 A	080T1C	099 T3C	C071 T2B	C032 T1C	C003 T3C	C067 T2A	
E	Standard # 2 2000 pg/ml	Standard # 6 3.2 pg/ml	096T3NA	138T2 B	121T1 A	099T3 A	135T2 C	C023 T1B	C030 T3B	C027 T2C	C016 T1A	C018 T3A	
F	Standard # 2 2000 pg/ml	Standard # 6 3.2 pg/ml	084T3NA	134T2 B	125T1 A	098T3 A	087T2 C	C062 T1B	C018T3 B	C043 T2C	C022 T1A	C020 T3A	
G	Standard # 3 400 pg/ml	QC-1 control	096T3N C	130T3B	117T1 A	131T3 A	118T2 C	C006 T1B	C020 T3B	C048 T2C	C023 T1A	C030 T3A	
H	Standard # 3	QC-1 control	132T2 NC	114T3 B	120T2 A	133T3 A	085T2 C	C016 T1B	C069 T3B	C047 T2C	C024 T1A	C004 T3A	

APPENDIX IX: Subjects' description

Characteristics	HIV-1 neg[1] (n = 72)			HIV-1 pos HAART. t[2] (n = 122)			HIV-1pos HAART.n[3] n = 13
	T_1[4] n = 12	T_2[5] n = 35	T_3[6] n = 25	T_1[4] n = 16	T_2[5] n = 52	T_3[6] n =54	
Age, years (median, IQR[7])	27.5(23-42)	29(19-36)	29(21-42)	27.5(24-34)	32(22-38)	30(23-38)	32.5(23-45)
Duration of HIV-1 infection(median,IQR[7])	-	-	-	3(1-6)	3(1-16)	3(1-10)	<1 (<1-6)
Duration of HAART treat. (median,QR[7])	-	-	-	3(1-6)	3(1-7)	2(1-8)	-
Gestational age, months (median, IQR[7])	3(2-3)	5(4-6)	8(6-9)	3(1-3)	5(4-6)	8(7-9)	-
Parity, (alive - dead)	17	31-1	30-1	20-2	75-5	52-6	7-1
Blood pressure, mean (mm/Hg)	109/66	107/69	103/63	103/80	107/73	111/72	104/69
Weight/Height (kg/cm)	78/169	78/168	79/166	67/ 154	74/165	90/165	71/166
BMI index(kg/m^2)	27.3	27.6	28.7	28.3	27.2	33.9	28.7

All HIV infected pregnant mothers were enrolled into HAART irrespective of their CD4 count. Only one out of 122 HIV infected pregnant women had miscarriage. All the HIV infected pregnant women had normal delivery but for 3 who had assisted delivery. Pregnant women with any other infection besides HIV-1, who smoked or used illegal substances, were not included in the study, as these factors may cause adverse pregnancy outcomes. All the pregnant women in this study were given anti-malaria drug as a routine preventive therapy because malaria parasite is endemic in Africa. Pregnant women with diabetes, pre-eclampsia and high blood pressure were excluded from this study.

- HIV-1 neg[1] –- Healthy HIV negative pregnant mothers. Total of 72 subjects were seen in this group.
- HIV-1 pos HAART. t[2] –- HIV infected pregnant mothers treated with HAART (highly active anti-retroviral therapy). Total of 122 subjects were seen in this group. Mothers in this category were either on:

Combivire/Nivarapine (Lamovidine + Zidovudine + Nivarapine) OR

Truvada/Efavirenz (Emtricitabine + Tenofovir + Efavirenz).

Lamovidine/Zidovudine = Nucleoside Reverse Transcriptase Inhibitors NRTIs.

Emtricitabine/Tenofovir = Nucleoside Reverse Transcriptase Inhibitors NRTIs.

Nivarapine/Efavirenz = Non- Nucleoside Reverse Transcriptase Inhibitors NNRTIs.

- HIV-1 pos HAART.n[3] – HIV positive HAART naïve. This group includes HIV-1 infected pregnant mothers yet to start HAART treatment. Total of 13 subjects were seen in this group.

- T_1[4] – First trimester pregnancy (1-3 months gestational age)
- T_2[5]– Second trimester pregnancy (4-6 months gestational age)
- T_3[5]– First trimester pregnancy (7-9 months gestational age)
- IQR – Interquartile range

APPENDIX X: **Cell Viability Test by Trypan Blue Exclusion**

The number of viable leukocytes was determined by trypan blue exclusion test. There is need to test the viability of the cells to make sure that viable cells are used. About 0.3 mL of PBS and 0.2 mL whole blood were mixed unto a test tube and 0.5 mL of 0.4% TB (w/v) added and mixed thoroughly. The entire mixture was allowed to stand for 5-15 minutes. A small amount of TB-cell suspension mixture is transferred unto hemocytometer chamber and covered with coverslip. Live/viable cells were seen unstained and transparent nucleus while very minute dead/non-viable cells were seen stained blue-nucleus. Viable and non-viable cells were counted. Cell viability was calculated as thus: Cell viability percentage (%) = total viable cell (unstained) ⁄ total cells (stained and unstained) X 100%. The cells were found to be above 98% viable.

APPENDIX XI: Lymphocyte Stimulation and Culture

The suspended PBMC cells containing lymphocytes and monocytes were dispensed into 96-well tissue culture plates (100 µl per well). Lymphocytes were stimulated with 100 µl enriched RPMI 1640 and 100 µl of ConA, 100 µl of PW, 100 µl of PHA per well. In the 12 X 8 well-plate format, Con A was added in 1 X 8 wells, so do PHA and PW per sample. Four (4) samples were used in each 96 well plate. Cultures were stimulated at 37°C in 5-10% CO_2 for 3 days for maximal proliferation. At the end of the 3rd day, culture was pulled from 8 wells each representing con A, PHA and PW, centrifuged at 5 minutes at $13{,}000 \times g$ and the cell free supernatants were harvested put in vials and stored frozen.

APPENDIX XII: Description and preparation of the individual reagents used for the culture

a) RPMI 1640 + 2 mM Glutamine (R11875-093 Gibco by life technologies-USA). RPMI as a basal media is supplemented/enriched with penicillin-streptomycin (10 ml/L), amphotericin B (2.5 mg/L; normal usage in maintenance of antifungal cell cultures is 2.5 mg/L with penicillin and streptomycin used in the medium) and 10% v/v FBS (fetal bovine serum). **N/B:** Penicillin-Streptomycin is to control bacterial contamination, Amphotericin B is to control fungal infection while FBS is a growth supplements for cell culture, prefers over human serum because of its high content of embryonic growth promoting factors, no antibodies and better for T-cell and B-cell stimulation.

b) Mitogens:

bi) Pokeweed mitogen - PWM (lectin from phytolacca Americana L9379; Sigma-Aldrich) at mitogenic activity of 0.3 µg/mL (range of mitogenic activity is 0.03-0.3µg/mL). Preparation of 0.3 µg/mL mitogenic activity. Stock is 10 mg/10 ml in 0.9% sodium chloride normal saline N/S): dissolve 10 mg PWM in 10 ml N/S to give 1mg/ml. Converting 0.3 µg/mL to mg gives 0.0003mg/ml. apply RV/O where R= required concentration (0.0003mg/ml), V = required volume (10 ml) and O = original concentration (1mg/ml).

Original volume = RV/O = 0.0003 x 10/1 = 0.003 ml of PWM stock. Pipette 0.003ml of PWM stock and make up to 10 ml with N/S to get the required working concentration of 0.3 µg/ml.

Bii) Phytohemaglutinin PHA (lectin from phaseolus vulgaris phytohemagglutinin P 8754; Sigma-Aldrich) at mitogenic activity of 5 µg/mL (range of mitogenic activity is <10 µg/mL).

Preparation of 5 µg/ml PHA: stock = 5mg/ml ie 5mg of PHA dissolved in 1ml of N/S (5mg/ml). Chosen mitogenic activity of PHA =5 µg/mL. Convert µg to mg: 1000 µg = 1 mg, therefore 5 µg is 5/1000 = 0.005mg/ml chosen concentration. Using RV/O

Required concentration = 0.005mg/ml, Original concentration = 5mg/ml, Required volume = 10ml. RV/O = 0.005 X 10/5 = 0.01ml of the stock PHA is made up to 10ml with N/S to get 5 µg/ml being the working concentration.

Biii) Concanavalin A - ConA (lectin from from canavalia Ensiformis type IV C5275; Sigma) at mitogenic activity of 70 µg/mL (range of mitogenic activity is <=75 µg/mL). Preparation of 70 µg/mL: stock = 5mg/ml ie 5mg of CONA dissolved in 1ml of N/S (5mg/ml). Chosen mitogenic activity of CONA = 70 µg/mL. Convert µg to mg: 1000 µg = 1 mg, therefore 70 µg is 7/1000 = 0.07mg/ml chosen concentration. Using RV/O

Required/chosen concentration = 0.07mg/ml, Original concentration = 5mg/ml, Required volume = 10ml. RV/O = 0.07 X 10/5 = 0.14ml of the stock CONA is made up to 10ml with N/S to get 70µg/ml being the working concentration.

APPENDIX XIII; shipment of frozen culture supernatant

The supernatant from lymphocyte stimulation culture was aliquoted in vals and stored frozen (-40^{0}C) at the freezer belonging to the laboratory section of Mother of Christ Hospital, Ogui, Enugu-Nigeria. Permit to transport infectious sample was requested by my supervisor Prof. Rong Ligun, University of Illinois at Chicago (UIC) to CDC (Center for Disease Control, USA). The permit was later obtained and scanned to me through email by my supervisor at UIC. With this permit, the frozen supernatant from lymphocyte stimulation culture was shipped to Prof. Rong Ligun laboratory UIC, USA through FedEX before my departure. The specimen was packed in a cooler and sufficient ice-gels were packed in between the specimen and the cooler was tightly closed. It took few days to get to UIC and immediately the specimen was stored frozen at -90^{0}C.

APPENDIX XIV: Cytokine Assay

Cytokine assays were performed using supernatants from lymphocyte stimulation cultures. Cytokines in the class of TH1 (IL-2, TNF-α, IFN-γ) and TH2 (IL-4, IL-10) were assayed using Milliplex MAP Kit for human cytokine/chemokine magnetic bead panel. Customized kit of 96 well plates was used because only 5 cytokines (IL-2, TNF-α, IFN-γ, L-4, and IL-10) were requested to be customized by the company. Catalog # HCYTOMAG-60K, HCYTOMAG-60K-PX29, HCYTOMAG-60K-PX30, HCYTOMAG-60K-PX38, HCYTOMAG-60K-PX41. Control catalog # MXH6060-2 Lot # HCY-108 and HCY-208. Cytokine reagent is from EMD Millipore Corporation, 290 Concord Road, Billerica MA 01821 USA, 636 441-8400, 636 441-8050 (fax). Milliplex is a registered trademark of Merck, Darmstadt, Germany. (see **Appendix VIII**) for pictures and lay-out of cytokine assay)**.**

Procedure- MILLIPLEX

Assay was performed in duplicate. 200 µL of Wash Buffer was added into each well of the 96-well plate, sealed and mixed on a plate shaker for 10 minutes at room temperature (25°C). The Wash Buffer was decanted and the residual wash buffer was removed from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. 25 µL of Assay Buffer was

added to the sample wells. 25 µL cell culture supernatant was added into the appropriate sample wells. The standard was prepared and used within 1-hour preparation. 25 µL of each diluted Standard was added into the appropriate wells for standard. 25 µL of control culture medium solution was added to the background/blank, standard and control wells (The assay buffer should be used for 0 pg/mL standard - background). The mixing bottle was Vortexd and 60 µL from each antibody bead vial were mixed in the mixing bottle, and brought to final volume of 3ml with bead diluent. 25 µL of the Mixed or Premixed Beads was added to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.) the plate was sealed with a plate sealer. The plate was wrapped with foil and incubated with agitation on a plate shaker overnight at 4°C (or 2 hrs at room temperature (25°C). (An overnight incubation (16-18 hrs) may improve assay sensitivity for some analytes). After the incubation, the plate was placed into hand-held magnetic plate for 60 seconds to allow magnetic settling of the beads, well content was removed by gently decanting the plate in waste receptacle. The plate was tapped gently on absorbent pads to remove residual liquid. The plate was then removed from handheld magnetic plate, and 200 µL wash buffer was added in each well The plate was shaken for 30 seconds, reattached to hand-held magnetic plate, letting beads settle for 60 seconds, inverted to remove contents as described earlier. The washing was repeated 2 times. 25 µL of detection antibodies was added into each well. (Note: Detection Antibodies were allowed to warm to room temperature prior to addition.)

The plate was sealed with cover with foil and incubated with agitation on a plate shaker for 1 hour at room temperature (20-25°C). About 5 µL Streptavidin-Phycoerythrin was added to each well containing the 25 µL of Detection Antibodies. The plate was sealed with plate cover and incubated with agitation on a plate shaker for 30 minutes at room temperature (25°C). The well

plate was washed 2 times following the same washing procedure described above. Afterwards, 150 µL of Sheath Fluid was added to all wells and the plate shaken for 5 minutes to re-suspend the beads. The plate was run on Milliplex HTS software. Median Fluorescent Intensity (MFI) data was calculated using spline curve-fitting method for calculating cytokine/chemokine concentrations in samples.

APPENDIX XV: Immunoglobulin assay

Estimation of Human IgA/G/M was done using ELISA kit from Research Innovative,Inc.USA. Catalog # of kits used were IHUIGMKT Lot # 17274, IHUIGGKT Lot # 415, IHUIGAKT Lot # 315.

Principle of double antibody sandwich ELISA: The IgM, IgA, IgG present in samples/standard reacts with the anti-IgM antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound proteins by washing, anti- IgM, IgA, IgG antibodies conjugated with horseradish peroxidase (HRP) are added. These enzyme-labeled antibodies form complexes with the previously bound IgM, IgA, IgG. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3,5,5-tetramethylbenzidine (TMB). TMB substrate is used for color development at 450nm. The quantity of bound enzyme varies directly with the concentration of IgM, IgA, IgG in the sample and directly proportional to color development, thus, the absorbance, at 450 nm, is a measure of the concentration of IgM, IgA, IgG in the test sample. A standard calibration curve is prepared in blocking buffer using dilutions of purified human IgG and is measured along with the test samples.

Procedure:

Assay was performed in duplicate. A serial sample dilution of 1:1000,000 of sample dilution was prepared with assay buffer. 100µl of diluted IgG std and sample were added into wells. The plate was shaken at 300 rpm for 30 mins at room temperature using a plate shaker. The plate was washed 3 times with 300 µl wash buffer. excess wash buffer was removed by tapping on kimwipe. 100 µl of reconstituted primary antibody was added to all wells. The mixture was

shaken at 300 rpm for 30 minutes. The plate was washed 3 times with wash buffer. 100 µl diluted streptavidine was added to all wells. The mixture was shaken at 300 rpm for 3 minutes. After incubation, the plate was washed 3 times with wash buffer. 100 µl of TMB substrate was added to all wells and the mixture shaken for for 5 minutes. The reaction was quenched by adding 50 µl stop solution (1N H_2SO_4). Plate was read at 450 nm in a microtiter plate spectrophotometer. Standard curve was plotted by plotting A450 against the amount of IgG in the standards. Straight line was fit through the linear points of the standard curve. The amount of IgM, IgA, IgG in the sample was determined from the curve. Then the IgM, IgA, IgG concentration obtained was multiplied by the dilution factor (1000,000).