INFLUENCE OF MATERNAL HIV-1 INFECTION ON THE VERTICAL TRANSFER OF EPSTEIN BARR VIRUS SPECIFIC IMMUNOGLOBULIN G

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for a degree or any other award.

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DEDICATION

I dedicate this thesis to my parents Hanna Koech and Luka Koech and my husband Mathew Kosgei in appreciation for their encouragement and overwhelming support.

ABSTRACT

More than 35% of infants residing in Chulaimbo regions are infected with Epstein–Barr virus (EBV) by six months of age, typically when maternal antibodies are expected to provide protection to the infants before they develop their own *denovo* antibodies. It has been reported that maternal immunoglobulin G (IgG) antibodies get transferred to the fetus from the second week of gestation and reach maximum levels by the third trimester. Transplacental transfer of these antibodies may be affected by several factors including: maternal infections such as human immunodeficiency virus (HIV-1). Inefficient transfer of EBV specific IgG during pregnancy may later predispose infants to early age of EBV infection. Therefore, this study determined the influence of maternal HIV-1 infection on the transfer of EBV specific antibodies to their neonates. The study specifically determined total IgG antibody levels in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates; determined levels of EBV specific IgG antibodies in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates and determined EBV specific IgG subclasses levels in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates. In this cross-sectional study, thirty-five HIV infected and thirty-five HIV uninfected pregnant women were randomly selected and recruited from Chulaimbo Sub-County Hospital and followed through pregnancy to delivery. The levels of total IgG, EBV specific IgG and IgG sub-classes in maternal venous blood and in cord blood was quantified using Enzyme-linked immunosorbent assay (ELISA). Total IgG antibody levels were compared between mothers and between neonates using Mann Whitney U test. Mann Whitney U test was also used to compare the median levels of EBV specific IgG antibodies and IgG subclasses between HIV infected and HIV uninfected mothers as well as between HIV exposed and HIV non-exposed neonates. Wilcoxon matched pairs test was used to compare EBV specific antibody levels in maternal and their corresponding neonates. The levels of total IgG were significantly higher in HIV infected mothers than HIV uninfected mothers and between HIV exposed neonates compared to HIV unexposed neonates (p=0.0001). HIV-1 exposed neonates had significantly lower levels of anti- EBV nuclear antigen (EBNA1) antibodies than in HIV-1 non-exposed neonates (p=0.0045). *In utero* exposure to HIV resulted in reduction in vertical transfer of; anti-EBNA1, anti-viral capsid antigen (VCA) IgG1 antibodies and anti-EBNA1 IgG1 antibodies (1.1%, 18.8% and 27.8% respectively). Levels of IgG1 and IgG4 against both VCA and EBNA1 were significantly higher in HIV infected mothers compared to HIV uninfected mothers (p=0.0357, 0.0072, 0.0458 and 0.0015 respectively). In addition, the levels of IgG3 against EBNA1 only were significantly high in HIV-infected mothers. These data suggest that, maternal HIV-1 infection contribute to impairment of transplacental transfer of EBV specific antibodies which may in turn lead to the early EBV infection in this high-risk population. These results are useful in development of EBV specific preventive and therapeutic measures tailored to children born to HIV infected mothers.

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LIST OF ABBREVIATIONS AND ACRONYMS

ART: – Antireviral therapy

BL: – Burkitt's lymphoma

CMR: – Cord to maternal ratio

EBNA: – Epstein Barr Virus Nuclear Antigen

eBL: – Endemic Burkitt's lymphoma

EBV: – Epstein Barr Virus

ELISA: – Enzyme linked immunosorbent assay

Fc: – Crystallizable fragment

Hb: – Hemoglobin G

FcRn: – IgG specific Fc receptor

HIV: – Human Immunodeficiency virus

HL: – Hodgkin's lymphoma

HRP: – Horse raddish peroxidase

IgG: – Immunoglobulin G

KEMRI: – Kenya medical Research institute

LCL's: – Lymphoblastoid cell lines

LMP: – Latent membrane protein

LP: – Leader Protein

NPC: – Nasopharyngeal carcinoma

OD: – Optical density

PBS: – Phosphate buffered saline

RPM: – Revolutions per minute

VCA: – Viral Capsid Antigen

DEFINITION OF TERMS

Cord to maternal ratio (CMR): the ratio of the level of specific antibody in cord blood to that in the maternal blood of the mother.

Syncytiotrophoblast: is the epithelial covering of the highly vascular embryonic

Placental villus which invades the wall of the uterus establishes nutrient circulation between the embryo and the mother.

Hyperimmunoglobulinemia: Abnormally high levels of immunoglobulin in serum/plasma

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CHAPTER ONE

INTRODUCTION

1.1. Background information

Burkitt lymphoma (eBL) is an aggressive B-cell malignancy with a high proliferative rate that may be fatal if not treated promptly (Evens *et al*., 2002). There are three types of Burkitt's lymphoma according to world health organization (WHO) classification ; Sporadic which is non-African, Immunodeficiency associated and Endemic Burkitt lymphoma(eBL) which affects mostly Africans and the most common form of childhood cancer in equatorial Africa (Mwanda *et al*., 2004). Its etiology has been linked to both early Epstein Barr virus (EBV) infection and repeated *Plasmodium falciparum* malaria infection during infancy (Rochford *et al*., 2005). However, the mechanism by which the two pathogens interact to cause malignant B cell clones is still under investigation.

The age of primary EBV infections varies by socio-demographics and by region with EBV infection being rare before the age of 5 among European and American children (Balfour *et al*., 2013; Svahn *et al*., 2006). In contrast, in developing countries, EBV infections manifest early during childhood (Jenson *et al*., 1999). A study done at Chulaimbo; a rural area of Kenya reported that 35% of infants in malaria holoendemic regions acquire primary EBV infection as early as 6 month of age (Piriou *et al*., 2012). Such early primary infections are unexpected since maternal antibodies acquired *in utero* protect infants against early infections, probably suggesting altered transfer of maternal antibodies to these infants.

The transfer of IgG across the placenta from mother to fetus is an active process that involves the neonatal Fc receptors (FcRn) at the syncytiotrophoblast that bind IgG molecules on the maternal side and transport them into foetal circulation (Moraes-Pinto *et al*., 1997). The neonate IgG levels typically correlate with maternal levels. However, previous studies have reported that placental transfer of total IgG is less efficient with increased maternal total IgG levels (Englund *et al*., 2007; Okoko *et al*., 2001). Maternal infections such as HIV-1 can potentially result in hypergammaglobulinemia (Moraes-Pinto *et al*., 1993). HIV-1 derived hyperimmunoglobunemia in maternal blood during the last trimester could compete for the Fc receptor (FcRn) thereby reducing active transport of total IgG as reported in the case of measles IgG antibodies (Farquhar *et al*., 2005). However, influence HIV-1 during pregnancy,

on the levels of total IgG in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding infants remains not fully elucidated.

Maternal antibodies that are passed through the placenta have been shown to protect the neonates against various infections before they develop *de novo* antibodies. A number of factors such as maternal infections, hypergammaglobulinemia, preterm birth and placental abnormities can affect the placental transfer of maternal antibodies to the neonates thus making them susceptible to infections during infancy (Palmeira *et al*., 2012). A recent study done at Chulaimbo Sub-County hospital, demonstrated that maternal malaria infection during pregnancy significantly altered only the transfer of anti-VCA and anti-EBNA1 EBV-specific antibodies to the neonates (Ogolla *et al.,* 2015). In addition, placental transfer of measles specific antibodies has been shown to be inhibited by high maternal HIV-1 viral loads (Farquhar *et al*., 2005). However, influence of maternal HIV infection on the levels of EBV-specific antibodies has not been investigated. Therefore, this study sought to determine the influence of maternal HIV-1 infection on the levels of VCA and EBNA1 in HIV-1 exposed and HIV-1 unexposed neonates.

Another factor that determines the level of antibodies transferred to the neonates is the antibody subclass. Indeed, a number of studies have reported the selective transfer of IgG subclasses across the placenta with IgG1 and IgG4 being transferred more efficiently than IgG2 and IgG3 (Costa-Carvalho *et al*., 1996). A previous study reported that the levels of VCA and EBNA1-specific IgG1 were significantly lower in cord blood of neonates whose mothers had malaria during pregnancy (Ogolla *et al*., 2015). Moreover, a recent study reported the transplacental passage of all the IgG subclasses being decreased in HIV positive mothers (Baroncelli *et al*., 2018). However, influence of HIV-1 infection on the levels of EBNA1 and VCA IgG subclass to the neonates has not been established.

1.2 Statement of the problem

Infants residing at Chulaimbo acquire EBV early in life by 6 months of age when maternal antibodies should typically protect them before they develop their own De novo antibodies. Moreover, HIV infected infants acquire EBV infection early compared to HIV uninfected infants thus the high prevalence of HIV infection among women residing in Chulaimbo region may have impacted negatively on placental transfer of antibodies thus leaving infants susceptible to early EBV infection.

This early age of EBV infection results in higher viral loads and poor control of the virus; a risk factor in the etiology of eBL. Endemic burkitt lymphoma is fatal if not diagnosed and treated early.

1.3 General objective

To evaluate the influence of maternal HIV-1 infection on the vertical transfer of EBV specific IgG antibodies.

1.3.1 Specific objectives

1. To determine the levels of total IgG antibody in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates.

2. To determine the levels of EBNA1 and VCA IgG antibodies in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates

3. To determine the levels of EBNA1 and VCA IgG antibody subclass in HIV-1 infected mothers, HIV-uninfected mothers and their corresponding neonates.

1. 3.2 Null Hypotheses

1. There is no difference in the levels of total IgG antibodies in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates.

2. There is no difference in the levels of EBNA1 and VCA IgG antibodies in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates.

3. There is no difference in EBNA1 and VCA IgG antibody subclass levels in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates.

1.4 Significance of the study

Several studies have shown that maternal HIV-1 infection inhibit the placental transfer of maternal antibodies, thus results from this study enables a better understanding of how the levels of EBV specific IgG and IgG subclasses differs in neonates born to HIV-1 infected mothers against neonates born to HIV-1 uninfected mothers in an endemic area, thus raising

awareness and promote new approaches towards preventing acquisition of early EBV infections in HIV -1 exposed neonates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epstein Barr Virus

Epstein Barr virus (EBV) also known as Human herpesvirus4 (HHV-4), is a member of the herpes virus family. It primarily infects the human oropharynx epithelial cells and then replicates, EBV then establishes a growth transforming latent infection. This latent EBV persists in the individual's B cells for the rest of the individual's life (Amon, 2004). Despite EBV infection being a lifelong infection, its pathogenicity remains dormant unless the host immune system is suppressed. The age of primary EBV infections varies by socio-demographics and by region with EBV infection being rare before the age of 5 among European and American children (Balfour *et al*., 2013; Svahn *et al*., 2006). In contrast, in developing countries, EBV infections manifest early during childhood (Jenson *et al*., 1999). A study in rural areas of Kenya reported that 35% of infants in malaria holoendemic regions acquire primary EBV infection as early as 6 month of age (Piriou *et al*., 2012). HIV infected infants has also been demonstrated to acquire EBV earlier and more frequent than HIV uninfected infants (Slyker *et al*., 2013). In addition, acute EBV infection manifest clinically in HIV infected infants and distinctly from HIV uninfected infants (Slyker *et al*., 2013). The susceptibility of these infants to this early age of EBV infection remains unclear. Therefore, this study determined the influence of maternal HIV-1 infection on the vertical transfer of EBV specific antibodies.

2.1.1 Transmission of EBV

Epstein Barr Virus transmission involves maternal mastication of food which is common in some cultures, sharing of eating utensils and kissing (Alfieri *et al*., 1996). In addition, young children can acquire primary EBV from close contact that involves exchange of oral secretions via shared items such as toys and bottles. However, there is a possibility of spread through; genital transmission, blood transfusion and organ or bone marrow transplantation (Crawford *et al*., 2002; Ebell *et al*., 2004). EBV infection is usually contracted early in life, in low social background setting due to intra familial spread (Stephen *et al*., 2015). Thereafter, the infectious virus is shed in saliva at high concentration consistently for more than six

months following acute infection and intermittently at lower concentrations for life (Ling *et al*., 2003).

Primary EBV infection may manifest differently in HIV infected children and HIV uninfected children and cause substantial morbidity in HIV infected children (Slyker *et a*l., 2013). Moreover, in the same social economic status, HIV infected infants have been demonstrated to acquire EBV early in life as compared to HIV uninfected infants (Pedneault *et al*., 1996; Slyker *et al*., 2013). However, Jenson and colleagues study reported no difference in the manifestation of primary EBV infection between HIV infected and HIV uninfected infants (Jenson *et al*., 1999). Though contradictory, these studies suggests that, HIV-1 may impact primary EBV acquisition in infants but how maternal HIV-I infection influences placental transfer of EBV specific antibodies remains to be elucidated.

2.2 EBV and Burkitts lymphoma

Endemic Burkitt lymphoma (eBL) is an aggressive B-cell malignancy with a high proliferative rate that may be fatal if not treated promptly (Evens *et al*., 2002). It is the most common form of childhood cancer in equatorial Africa (Mwanda *et al*., 2004). There are three types of BL; endemic, sporadic and HIV associated. Despite various origins of sporadic and endemic BLs, a reproducible hallmark of all BLs is translocation and dysregulation of the c-*MYC* oncogene (Polack *et al*., 1996). A consistent expression of c-*MYC* in B cells of human infected with EBV may reproduce some phenotypic characteristics of BL-derived tumor cell lines(Polack *et al*., 1996). It is evidential that apart from an initiator of oncogenic events, c-*MYC* has pleiotropic influence since it acts as a universal amplifier of transcriptionally active genes (Lin *et al*., 2012).

The etiology of eBL has been linked to both early Epstein Barr virus (EBV) infection and repeated *Plasmodium falciparum* malaria co-infection during infancy (Rochford *et al*., 2005). An early study has shown that, the cycling memory B cells express EBV latent gene EBNA-1 which reflect the latent gene pattern in endemic BL since EBNA1 is the only latent protein that is expressed in Burkitt lymphoma tumors (Amoroso *et al*., 2010) along with various non-coding RNA species that are dispensable for transforming function such as leader protein (LP),latent membrane protein (LMP-1) (Amoroso *et al*., 2010). Throughout life cytotoxic T lymphocytes (CTL) plays a crucial role in the control of EBV infection since $CD8^+$ CTLs

specific for EBV lytic and latent proteins can be isolated and loss of EBV CTLs responses is associated with increase in development of EBV lymphomas (Khanna *et al*., 2000).

Epstein Barr Virus contributes to generation of aberrantly mutated cells by using their transforming potential to allow survival of mutated cells that can be scheduled to die by apoptosis in the absence of B cell receptor (BCR) as evidenced *in vitro*, where EBV can transform germinal centre B cells lacking immunoglobulin expression (Bechtel *et al*., 2005; Chaganti *et al*., 2005). This survival of mutated cells is enhanced by expression of latent membrane protein -2A (LMP2A) viral gene which can override BCR signal functions, since LMP2A contains an immune-receptor tyrosine based activation motif that can replace BCR signals (Brauninger *et al*., 2006). In addition, EBV can be directly involved in promoting genetic instability through the induction of DNA damage, induction of oxidative stress and telomere dysfunction (Gualandi *et al*., 2001; Kamranvar *et al*., 2013). However, the mechanism by which EBV contributes to carcinogenesis thus leading to eBL remains unclear.

2.3 Maternal total IgG

Infants are protected early in life by antibodies that are acquired from their mothers. It is therefore crucial for infants to acquire sufficient amounts of pathogen specific antibodies from their mothers since low levels can pre-dispose infants to early infections. The transport of antibodies across the placenta is an active process that involves the neonatal Fc receptors (FcRn) at the syncytiotrophoblast membrane that bind maternal IgG molecules, actively transported across the membrane and released into the fetal bloodstream(Moraes-Pinto *et al*., 1997). IgG transport from mother to fetus begins as early as 13 weeks and increases during the third trimester of pregnancy (Saji *et al*., 1999). The vertical transfer of maternal antibodies to the fetus can be affected by multiple infectious and non-infectious factors, placental abnormalities, maternal total and pathogen specific IgG levels and the gestational age (Palmeira *et al*., 2012). Moreover, maternal infections such as human immunodeficiency virus (HIV) and malaria during pregnancy can also affect the efficient vertical transfer of antibodies from mother to their neonates (Palmeira *et al*., 2012). Pinto and colleagues study, reported an increase in maternal total IgG titer in HIV infected mothers compared to HIV uninfected mothers (Moraes-Pinto *et al*., 1993). In addition, previous studies have reported that placental transfer of total IgG being less efficient with increased maternal total IgG

levels (Englund *et al*., 2007; Okoko *et al*., 2001). However, the influence of maternal HIV -1 infection on the total IgG levels in maternal and neonatal blood is not fully elucidated.

2.4 EBV specific IgG antibodies

During primary infection, antibodies against the viral capsid antigen IgG (VCA IgG) typically appear at the time of onset of the clinical symptoms of acute infection, and remain positive for life (Hess *et al*., 2004), whereas IgM antibodies (VCA IgM) usually appear at the same time as VCA IgG and disappear within a few weeks (Hess *et al*., 2004), their presence signifies an acute EBV infection (Dowd *et al*., 2003). Elevated levels of anti-VCA and anti-EBNA1 IgG signify evidence of past EBV infection (De Paschale *et al*., 2012). While presence of Early antigen diffuse (EAd) and ZEBRA(Zta) represence recent infections (De Paschale *et al*., 2012).

A number of factors have been demonstrated to affect the transplacenal transfer of EBV specific antibodies. Indeed, a recent study done at Chulaimbo Sub-County hospital, reported that maternal malaria infection resulted in altered transport of antibodies against anti-VCA and anti-EBNA1 only to the neonates (Ogolla *et al*., 2015). In addition, maternal immunosuppression and HIV-1 viremia has been reported early to influence infectious morbidity among infants by reducing passively acquired humoral immunity against important pathogens (Moraes-Pinto *et al*., 1998). A study by (Moraes-Pinto *et al*., 1996) reported that placental transfer of IgG against measles virus was reduced among HIV-1 infected than HIV-1 uninfected women. In the coastal region of Kenya, a reduction in cord blood levels of antibodies against tetanus in infants born to HIV positive mothers has been reported (Cumberland *et al*., 2007).

Farquhar and colleagues (Farquhar *et al*., 2005) demonstrated that, HIV-1 derived hyperimmunoglobulinemia in maternal blood during the last trimester (period when most of maternal antibodies are transferred from mother to infant) could compete for the IgG specific Fc receptor (FcRn) thereby reducing active transport of measles IgG antibodies from mother to infant. However, it remains to be determined whether this mechanism applies to VCA and EBNA1 levels in maternal venous blood and their corresponding cord blood.

2.5 Transfer of IgG subclasses

Maternal antibodies are transferred preferentially according to IgG subtype with some IgG being transferred efficiently than others. Antigens that elicit IgG1and IgG4 responses are transferred across the placenta more efficiently than either IgG2 or IgG3 (Costa-Carvalho *et al*., 1996). A previous study in a malaria holoendemic region of western Kenya, demonstrated that IgG1 was the dominant subclass of anti-VCA and anti-EBNA1 antibodies in cord blood of malaria exposed neonates (Ogolla *et al*., 2015). In addition, a recent study demonstrated that, the transplacental passage of all the IgG subclasses were decreased in HIV positive mothers and inversely correlated with high levels of maternal total IgG (Baroncelli *et al*., 2018). These previous studies did not determine the transfer of EBV-specific IgG subclasses. Subclass levels in maternal venous blood and cord blood in a prospective study of malaria positive mothers and their infants, showed that the levels of VCA and EBNA1-specific IgG1 were significantly lower in cord blood than in maternal venous blood with comparable levels of VCA specific IgG2, IgG3, and IgG4 in maternal venous blood and cord blood (Ogolla *et al*., 2015). In conclusion, these studies suggest that, IgG subtypes and maternal infections influence placental transfer of maternal antibodies. However, the levels of anti-VCA and anti-EBNA1 IgG subclasses in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates are yet to be determined.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was carried out at Chulaimbo Sub-County Hospital (**Figure 3.1)**. Chulaimbo Sub-County Hospital serves patients from predominantly rural area. It is located North West of Kisumu County of western Kenya. It was chosen because it is within the malaria holoendemic area with high prevalence of HIV infection especially in women and high incidence of EBV in children by six months of age as well as proximity to Academic Model Providing Access to Healthcare (AMPATH) which provides is an established study site where comprehensive services are offered to patients.

MAP OF CHULAIMBO THE STUDY AREA

Figure 3.1 : Map of Chulaimbo Sub-County hospital

3.2 Study Design

This was a cross sectional study that randomly collected maternal and neonatal data at delivery. This was done at Chulaimbo Sub-County hospital.

3.3 Study population

This study was carried out among HIV-1 positive pregnant women attending the antenatal clinic and delivery ward at Chulaimbo Sub-County hospital. Pregnant women with no serologic evidence of HIV-1 infection from the same study area were also included.

3.4 Sample size determination

Formula below adapted from (Lamorte *et al*., 2016) was used to determine sample. This is a formula for two independent groups with continuous outcome.

$$n = 2\left(\frac{z\delta}{E}\right)^{2q}$$

Where;

n = desired sample size

z = value from standard normal distribution (1.96)

E = desired margin of error (5%)

δ = the standard deviation of the previous study [SD =10.12 (Ogolla *et al*., 2015)]

$$n = 2\left(\frac{1.96 \times 10.12}{5}\right)^{2}$$

n = 31.47

Formula for drop outs (Ball *et al*., 2002)

$$N'' = \frac{N}{1-q}$$

Where;

N" = the final sample size

N= the calculated sample size

q= Percentage of expected dropouts

$$N'' = \frac{31.47}{0.9}$$

N" =34.9

Sample size = 35 in each group

Total sample size = **70**

3.4.1 Inclusion criteria

Pregnant women of less than 30 weeks' gestation period, both HIV positive and negative, EBV positive, residence within 10 km from Chulaimbo Sub-County hospital and willingness to come back to the hospital for delivery and laboratory testing.

3.4.2 Exclusion criteria

Malaria positive women and those who did not sign an informed consent.

3.5 Methods of data collection

3.5.1 Collection of demographic data

A questionnaire was administered to the pregnant women who consented to the study **(Appendix 4).** The information obtained included: age, gravity, number of children they have, history of ARV use residence and intermittent preventative treatment in pregnancy (IPTp). Gestational age was calculated based on date of last menstrual period provided by the mothers.

3.5 .2 Sample collection

Immediately after delivery, the whole length of the cord was wiped using 70% alcohol to remove any blood clots. At least 5 ml of Cord blood was collected from large vein on the fetal side of the placenta with the needle pointing to the uterus into a heparinised tube. About 2ml maternal venous blood was also collected within 6 hours of delivery from a peripheral vein of the mother into a heparinized tube samples were then transported to SUNY/KEMRI

laboratory at the Centre for Global Health Research located in (CGHR) in Kisumu to be processed the same day.

3.5.3 Sample processing and preservation

Plasma fraction was separated from both peripheral blood and cord blood by spinning at 1200 revolutions per minute (RPM) for 5 minutes in a centrifuge (Jouan). Plasma was separated from whole blood using a serological pipet connected to pippetor and placed into a 2ml

Sarstedt tube (Sarstedt) then stored at -80°c ultra-low freezer for later batch analysis by enzyme linked immunosorbent assay (ELISA).

3.5.4 HIV detection

HIV status of all pregnant women was obtained from hospital records, since it is mandatory according to Kenya Ministry of Health National Guidelines that; all pregnant women be tested for HIV as part of control of mother-to-child-transmission.

3.5.5 Total IgG ELISA

Total IgG in maternal venous blood at delivery and cord plasma was determined by ELISA using human IgG total ELISA kit from eBioscience as per manufuctures instructions. High protein binding ELISA (Thermoscientific) plates were coated with 100µl/well of pre-titrated, purified anti-human IgG monoclonal antibody diluted in 1xPBS, pH 7.4 and incubated overnight at 4°C. Plates were then washed twice with 400µl/well wash buffer (PBS-Tween20) and blocked by adding 250µl/well of blocking buffer (1:10 dilution of Assay Buffer A concentrate (20x) in deionized water) then incubated for 2 hours at room temperature. The plates were then washed twice with 400µl/well wash buffer. Standards and prediluted samples were then added to the appropriate wells, sealed and incubated for 2 hours at room temperature. Plates were then washed four times with wash buffer and 100µl/well of pre-titrated, purified HRP-conjugated anti-human total IgG monoclonal antibody was added

then sealed and incubated for 1 hour at room temperature. Plate was then washed four times with PBS-Tween and 100µl/well of 5, 5, 3, 3, tetramethylbenzidine (TMB) substrate solution added. After 15 minutes developing time, 2N sulphuric acid was added to stop the reaction then plate read at 450nm on Imark microplate reader (Bio-Rad). Concentration in terms of optical densities (ODs) of total IgG in plasma was extrapolated from standard curves.

3.5.6 Determination of the levels of EBV specific IgG in Maternal venous and Cord blood

Plasma samples were analyzed for the presence of antibodies against EBV VCA and EBNA1 using an in-house ELISA Immulon 96-well plates (Immulon 4HBX, thermolabs) were coated with the VCA and EBNA1 peptides and incubated at 4°c overnight. Plates were then blocked using blocking buffer (PBS +3 %BSA) and then incubated at 37°C for one hour. 100ul/well

of samples and controls were then added to the plates and incubated for 1 hour at 37°C. The plates were then washed with wash buffer (PBS +0.05%Tween 20 (PBST)) and 100ul/well of rabbit anti-human IgG -Horseradish peroxidase (HRP) diluted 1:4000 in sample diluent was added and incubated for 1 hour at 37°C. The plates were then washed with wash buffer and developed using 5, 5, 3,3, tetramethylbenzidine (TMB) solution. The reaction was stopped using 1M sulphuric acid after 20 minutes incubation and the plates read using on Imark microplate reader (Bio-Rad) at 450nm.

3.5.7 Determination of the levels of EBV specific IgG subclasses in Maternal venous and Cord blood

The presence of IgG subclass antibodies to VCA and EBNA 1 was determined using an in-house ELISA. Immulon 96-well plates (Immulon 4HBX, thermolabs) were coated with 50µl per well of 1ug/ml of either VCA or EBNA1 peptide and incubated at 4°C overnight. After washing plates twice with 1XPBS, Plates were blocked using blocking buffer (PBS +3 %BSA) and then incubated at 37°C for one hour. Plates were then washed using wash buffer (PBS +0.05%Tween 20(PBST)) then 100µl/well of samples and controls diluted 1:100 in sample diluents was added to the plates and incubated for 1 hour at 37°c. the plates were then washed with wash buffer and 100µl/well of horseradish peroxidase (HRP) conjugated antibodies specific to detect different human IgG subclasses to each of the human IgG subclasses goat anti-human IgG1 Fc-HRP, goat anti-human IgG2 Fc-HRP conjugate, mouse anti-human IgG3-HRP and goat anti-humanIgG4 Fc-HRP) was added and incubated for 1 hour at 37°c. The plates were then washed with wash buffer and developed using 5,5,3,3, tetramethylbenzidine (TMB) substrate. The reaction was stopped using 1M sulphuric acid and plates read at 490 nm using Imark microplate reader (Bio-Rad) after 20 minutes.

3.6 Statistical Analysis

The levels of total IgG were compared between HIV positive mothers and HIV negative mothers as well as HIV exposed and HIV unexposed neonates using Mann Whitney U test.VCA and EBNA1 IgG antibodies levels were compared between HIV positive and HIV negative women as well as HIV exposed and HIV unexposed neonates using Mann Whitney U test. Wilcoxon matched pairs test was used to compare levels of EBV specific IgG antibodies between maternal venous blood and their corresponding cord blood

Levels of VCA and EBNA1 IgG subclasses were compared between HIV positive and HIV negative women as well as HIV exposed and HIV unexposed neonates using Mann Whitney U test. In addition, VCA and EBNA1 IgG subclasses levels were compared between maternal venous blood and their corresponding cord blood using Wilcoxon matched pairs test.

Cord to maternal ratio (CMR), the ratio of the level of specific antibody in cord blood to that in the maternal venous blood was used as a measure of placental transfer of antibodies. Comparison was made between HIV exposed and unexposed neonates.

3.7 Ethical considerations

Ethical approval was obtained from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (ERC) and Ethical Review Board of State University of New York, U.S.A **(Appendix 2).** Proposal approval for this study was obtained from Maseno University School of graduate studies **(Appendix 1).** In addition, written informed consent was obtained from all the study participants before any sample collection **(Appendix 3).**

Health information was kept confidential by keeping the consent forms and the sample collection forms in a locked cabinet. All study participants were assigned a number that appeared on the samples and no name appeared on all the samples collected. Study data was secured in password protected computers or lockable cabinets which were only accessible to the researcher.

CHAPTER FOUR

RESULTS

4.1 Characteristics of the study participants

The general, clinical and demographic characteristics of this study population are represented in **(Table 4.1).** Seventy pregnant women were enrolled during their first antenatal clinic visit and followed up until they delivered at the hospital. Of these, 50% (35) tested positive for HIV and 50% (35) tested negative for HIV as confirmed by HIV rapid test kit. All the mothers in this study were EBV seropositive as determined by VCA ELISA, **(Table 4.1).**

Underage mothers (below 18 years) in HIV positive and HIV negative were 6% (2) and 20% (7) respectively; Sixty percent (21) of the HIV positive mothers were on ARVs while 40% (14) were ARVs naive. The mean hemoglobin level (Hb) at enrollment in HIV positive mothers and HIV negative mothers was 9.7g/dl and 10.95g/dl respectively. Seventy one percent (25) of infants born to HIV positive mothers were term babies while 74% (26) of infants born to HIV negative mothers were term babies.

An infant was considered exposed to HIV, when the mother tested positive for HIV while an infant was considered HIV unexposed if the mother tested HIV negative. Using this criterion, 35 (50%) infants were HIV exposed while 35 (50%) infants were HIV non- exposed, the average Apgar score at 10 minutes for HIV exposed and HIV unexposed was 9.9 and 9.8 respectively **(Table 4.1).**

Table 4.1: Clinical, demographic and laboratory characteristic of study participants

Maternal characteristic

	HIV positive	HIV negative	Total
Number of participants, n,	35, (50)	35, (50)	70, (100)
Age, n,			
Age <18	2, (6)	7, (20)	9, (13)
Age >18	33, (94)	28, (80)	61, (87)
Gravida, n,			
Primigravida	4, (11)	14, (40)	18, (26)
Secundigravida	8, (23)	14, (40)	22, (31)
Multigravida	23, (66)	7, (20)	30, (43)
ARV use			
Experienced	21, (60)		21, (60)
Naïve	14, (40)		14, (40)
Mean Hb at enrolment(g/dl)	9.7	10.95	
Gestational Age <37wk	10, (29)	9, (26)	19, (27)
Gestational Age >37wk	25, (71)	26(74)	51, (73)
EBV seroprevalence	35, (100)	35, (100)	70, (100)

Neonatal characteristics

	HIV exposed	HIV unexposed
Number of Neonates	35, (50)	35, (50)
Mean Birth weight(g)	3120	3000
Mean Agpar score@ 10 min	9.9	9.8

Note: Values represent n (%) unless otherwise stated.

4.2 Levels of total IgG in maternal venous blood and neonatal cord blood

This study first determined the levels of total IgG in maternal venous blood at delivery as well as in cord blood of their infants. As demonstrated in **(Figure 4.1),** the median level of total IgG was significantly higher in HIV positive mothers compared to HIV negative mothers, 35.89ng/ml (interquartile range 34.45-39.44) versus 26.34ng/ml (interquartile range 21.17-36.14) respectively, $p<0.0001$ **[Figure 4.1(a)].** Similarly, the median concentration of total IgG was significantly higher in HIV exposed neonates 35.63ng/ml (interquartile range 34.88-39.33) than in HIV unexposed neonates 26.39ng/ml (interquartile range23.11-31.99) $p<0.001$, **[Figure 4.1(b)].**

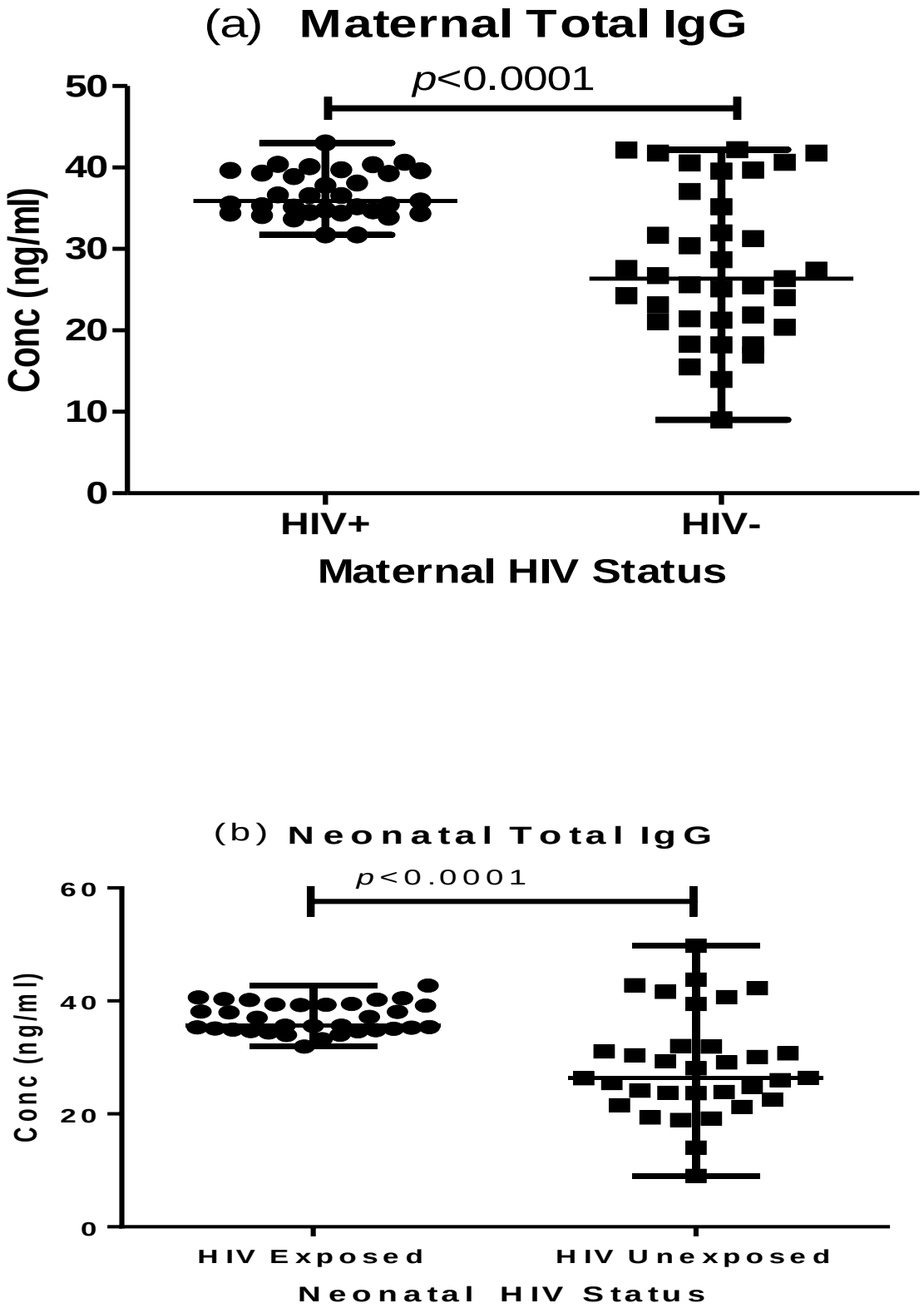

Figure 4.1: Total IgG in maternal venous blood **(a)** and neonatal blood **(b)**

Significant *p* values as determined by Mann Whitney U test are indicated in the figure

4.3 Levels of total IgG in maternal and their corresponding neonates

This study then determined the levels of total IgG in maternal venous blood at delivery and their respective neonates. As illustrated in **(Figure 4.2),** the median total IgG levels was significantly higher in HIV positive mothers compared to their HIV exposed infants, 35.89ng/ml (interquartile range 33.45-39.44) versus 36.63ng/ml (interquartile range 21.17-36.14), *p*=0.0034. However, there was no significant difference in the levels of total IgG between HIV negative mothers and their HIV unexposed infants, 26.34ng/ml (interquartile range 21.17-36.14) versus 26.36ng/ml (interquartile range 22.82-31.73), respectively *p*=0.9765.

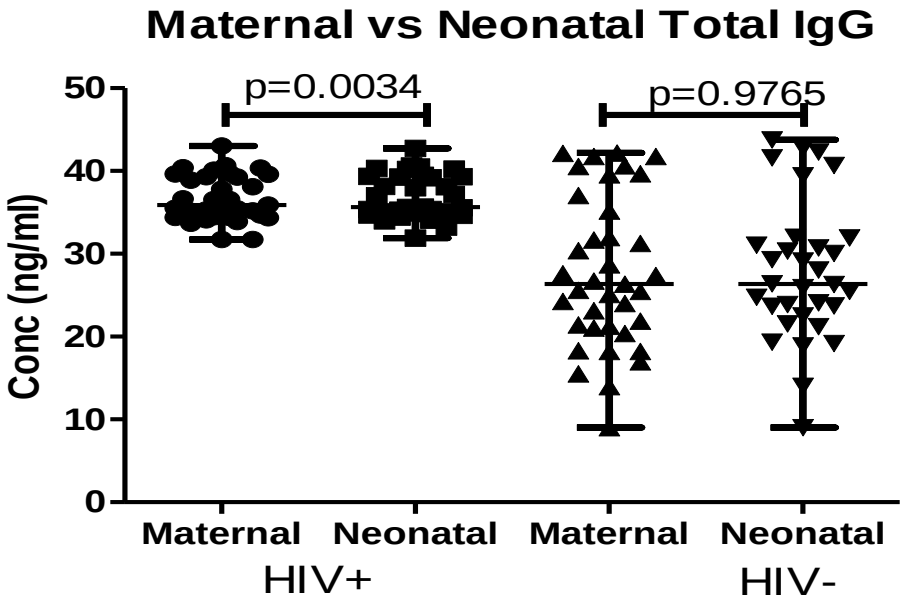

Figure 4.2: Levels of total IgG measured in maternal venous blood and their corresponding neonates**.** *P* values indicated on the figure are as determined by Wilcoxon paired test.

4.4 Levels of EBV specific antibodies in Maternal and their corresponding neonates

Next the study determined the levels of anti-VCA and anti- EBNA-I antibodies in maternal venous blood and their corresponding cord blood using ELISA. These levels were then compared between HIV positive mothers and HIV negative mothers and between the HIV exposed neonates and HIV non-exposed neonates.

There was no significant difference in the median levels of VCA specific IgG between mothers and between neonates **(Figure 4.3).** There were comparable levels in the median of EBNA-1 specific IgG antibodies between mothers in the two groups, median levels of EBNA-1 specific IgG in HIV non-exposed group were significantly higher than HIV exposed group p=0.0045 **(Figure 4.3).**

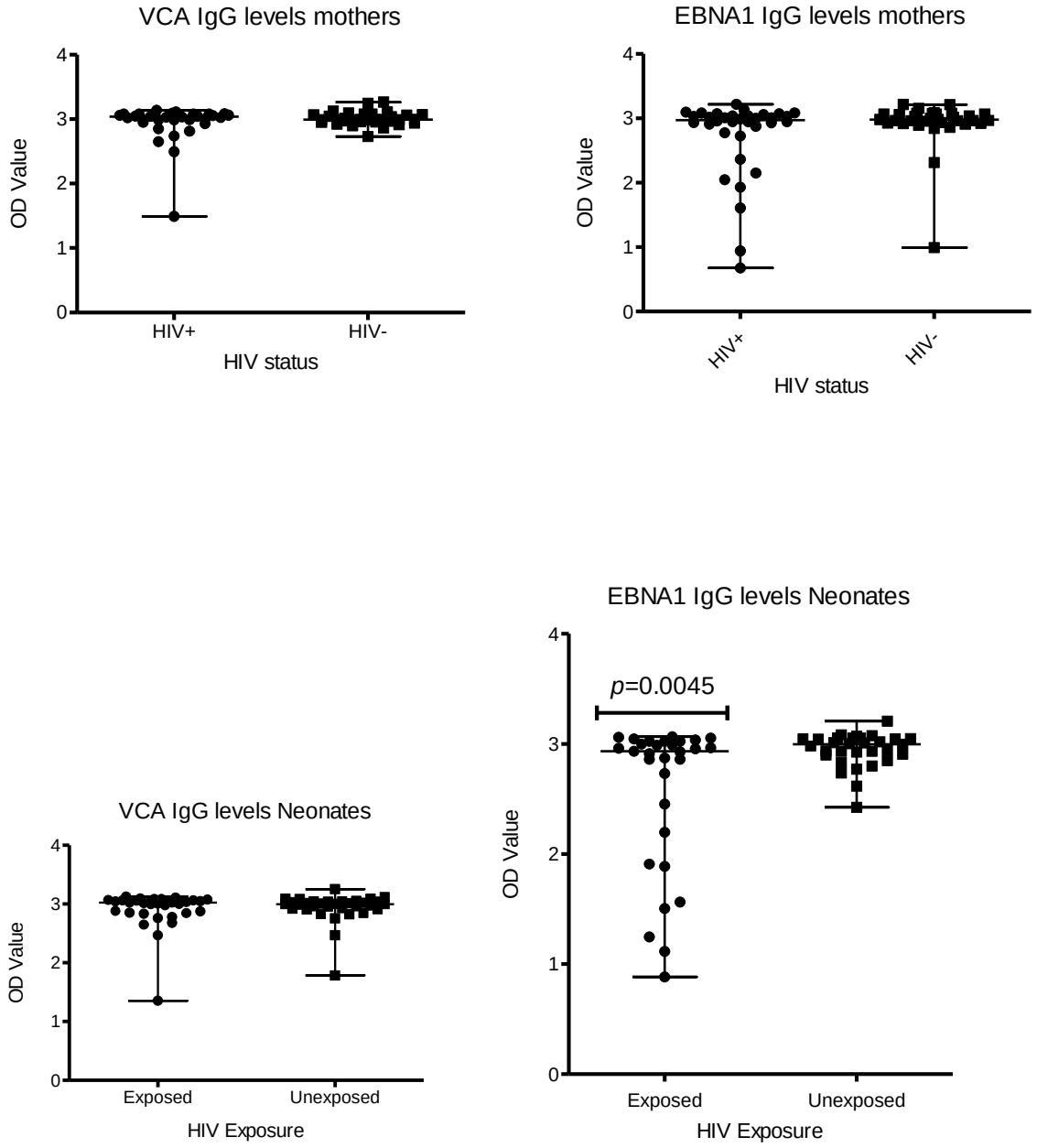

Figure 4.3: EBV specific IgG antibodies levels in maternal venous blood and their corresponding neonates. The *p* values as determined by Mann Whitney u test.

Comparison was then made between mothers and their corresponding neonates in the two groups; median levels of VCA specific IgG in HIV positive mothers was significantly higher than in their HIV exposed neonates (p=0.0370), **(Figure 4.4).** However, HIV negative mothers had lower median levels of VCA specific IgG compared to their HIV non-exposed **(Figure 4.4).** This was statistically significant (p=0.0197). Median levels of EBNA1 in HIV positive mothers were significantly higher than their HIV exposed neonates 2.973 versus 2.758 (p=0.0051) **(Figure 4.4).** However, there was no significant difference in the median levels of EBNA-1 specific IgG in HIV negative mothers compared to their HIV unexposed neonates **(Figure 4.4).**

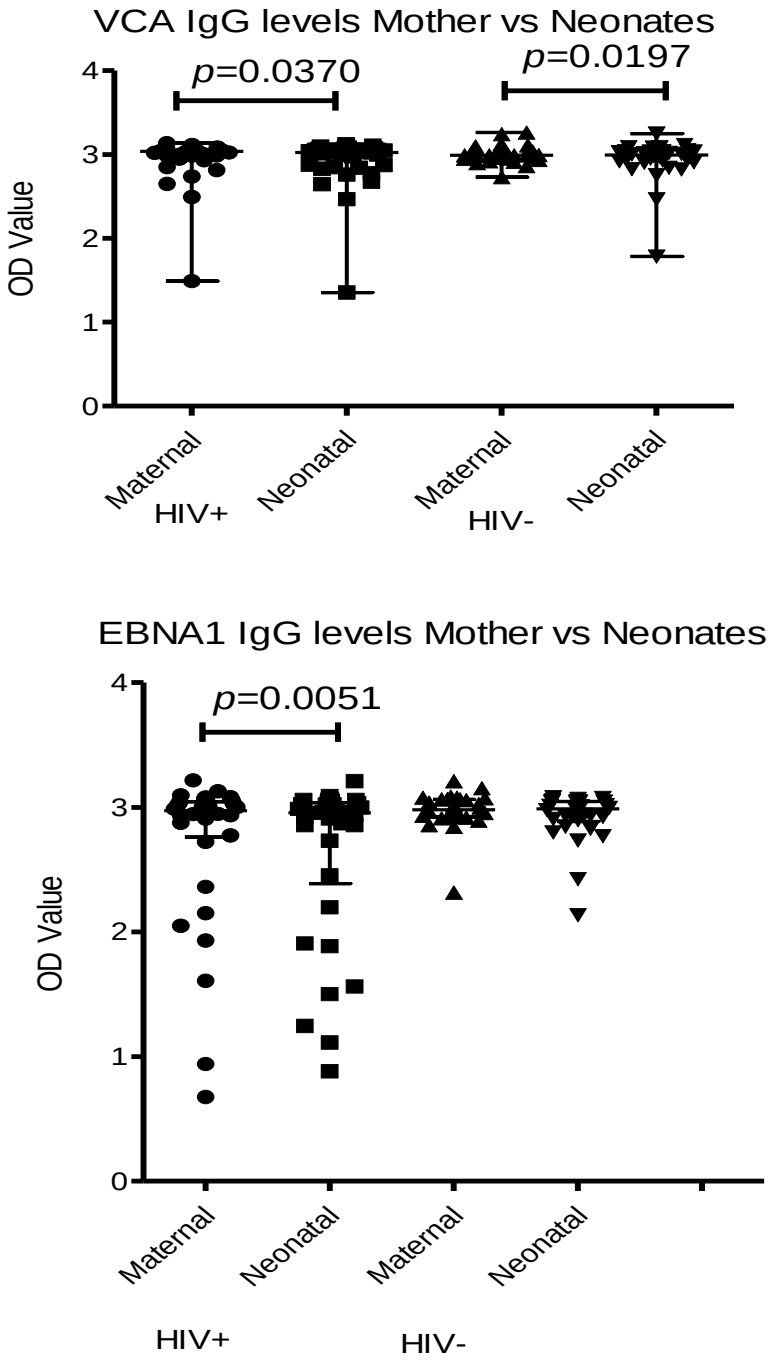

Figure 4.4 EBV specific IgG antibodies levels in maternal venous blood and their corresponding neonates. The *p* values as determined by Wilcoxon matched pairs test

4.5 Placental transfer of VCA and EBNA1 specific IgG antibodies from mother to their neonates

This study then determined the placental transfer of EBV specific antibodies from mothers to their neonates. The ratio of the levels of specific IgG antibodies in cord blood to that in the maternal venous blood at delivery CMR (cord blood/maternal ratio) was used as a measure of placental transfer.

The transfer of anti-EBNA-1 antibodies from HIV positive mothers to their HIV exposed was significantly reduced by 1.1% **(Table 4.2).** However, there was no significant reduction in the transfer of anti-VCA from HIV negative mothers to their HIV non-exposed neonates in the transfer of anti-VCA **(Table 4.2).**

Table 4.2: Placental transfer of EBV specific antibodies in HIV exposure

	Exposed	Non-Exposed	% reduction	*p* value
VCA	0.995 (0.894 - 0.997)	0.993 (0.979 - 1.00)	-0.2	0.935
EBNA1	0.983 (0.873 - 0.997)	0.994 (0.976 - 1.00)	1.1	**0.048**

Transplacental transfer of VCA and EBNA1 specific IgG antibodies. Values are median CMR (cord blood/maternal ratio). CMR was used to determine placental transfer of antibodies. Percentage reduction due to HIV infection was determined as follows: [(CMR of non-exposed neonates-CMR of exposed neonates) ×100]. Significant difference in CMR between the exposed and unexposed group was determined by Mann Whitney U test where $p<0.05$ was considered significant represented in bold.

4.6 EBV specific IgG subclass levels in maternal venous blood at delivery

Prior to determining the transfer of EBV specific antibody subclasses from mother to their infants, the levels of anti-VCA and anti-EBNA1 IgG subclasses in maternal venous blood at delivery were evaluated, as illustrated in **(Table 4.3).** HIV positive mothers had significantly higher levels of anti-VCA IgG1, IgG4 and anti-EBNA1 IgG1, IgG3 and IgG4 compared to HIV negative mothers ($p=0.0072$, $p=0.0458$, $p=0.0357$, $p=0.0096$ and $p=0.0015$), respectively. On the other hand, HIV positive mothers had significantly lower levels of anti VCA IgG2, IgG3 and EBNA1 IgG2 compared to HIV negative mothers, $p=0.0072$, $p=0.0114$ and $p=0.0016$ respectively.

TABLE 4.3: **Maternal levels of EBV specific IgG subclasses**

	IgG Subclass	HIV Positive	HIV Negative	*p* value
	IgG1	0.0950(0.0450-0.1940)	0.0555(0.0345-0.1335)	**0.0072**
	IgG2	0.0205(0.0085-0.1105)	0.0380(0.0005-0.2525)	**0.0072**
	IgG3	0.0290(0.0025-0.0785)	0.0750(0.0308-0.1163)	**0.0114**
VCA	IgG4	0.0260(0.0145-0.1150)	0.0200(0.0120-0.0285)	**0.0458**
	IgG1	0.1075(0.0360-0.2120)	0.0737(0.0376-0.0102)	**0.0357**
	IgG2	0.0280(0.0115-0.0510)	0.0510(0.0318-0.0988)	**0.0016**
	IgG3	0.1355(0.0530-0.1810)	0.0550(0.0313-0.1028)	**0.0096**
EBNA1	IgG4	0.0360(0.0195-0.0850)	0.0225(0.0143-0.0285)	**0.0015**

Values are median levels of EBV specific IgG subclass in maternal blood (in parenthesis are the 25^{th} and 75^{th} percentiles). *P* values were determined by Mann Whitney U test as indicated in the table. Significant *P* values are indicated in bold.

4.7 EBV specific IgG Subclass levels in cord blood

The levels of anti-VCA and anti-EBNA1 IgG subclasses in cord blood were also evaluated in this study. The median levels of VCA specific IgG4 and EBNA1-specific IgG1, IgG3 and IGg4 was significantly higher in HIV exposed neonates compared to HIV unexposed neonates p=0.0012, p=0.0194, and p=0.0001, respectively **(Table 4.4).** The median levels of VCA specific IgG2, IgG3 and EBNA1-specific IgG2 were significantly higher in HIV unexposed neonates compared to HIV exposed p=0.0003 p=0.0375 and p=0.0017, respectively **(Table 4.4).** However, there were comparable levels in the median levels of VCA IgG1 between HIV exposed and HIV unexposed neonates.

TABLE 4.4: **EBV specific IgG subclasses levels in neonates**

	IgG Subclass	**HIV Exposed**	HIV Unexposed	***p* value**
	IgG1	0.0665 (0.0490-0.2175)	0.0595 (0.0420-0.1760)	0.3526
	IgG2	0.0185 (0.0115-0.0325)	0.0460 (0.0250-0.0590)	**0.0003**
	IgG3	0.0340 (0.0145-0.0650)	0.0495 (0.0308-0.1225)	**0.0375**
VCA	IgG4	0.0220 (0.0155-0.0515)	0.0150 (0.1225-0.0220)	**0.0129**
	IgG1	0.0785 (0.0455-0.1705)	0.0445 (0.0235-0.0778)	**0.0194**
	IgG2	0.0280 (0.0100-0.0490)	0.0490 (0.0313-0.0713)	**0.0017**
	IgG3	0.1080 (0.0480-0.1615)	0.0285 (0.0183-0.0685)	**0.0001**
EBNA1	IgG4	0.0360 (0.0275-0.0515)	0.0200 (0.0130-0.0358)	**0.0012**

Values are; EBV specific IgG subclass levels in neonatal blood represented as median ODs in parenthesis are the 25th and 75th percentiles. *p* values indicated on the table as determined by Mann Whitney u test. Significant *P* values are indicated in bold.

4.8 EBV specific IgG Subclass levels in maternal and their corresponding neonates

This study then compared the levels of EBV specific IgG subclasses in maternal venous blood and their corresponding neonates (cord blood) in the two groups; HIV positive and HIV negative. The median level of VCA IgG1 in HIV positive Mothers was 0.0975 while in their corresponding neonates was 0.0665. This difference was statistically significant *p*=0.0276, **(Table 4.5).** HIV exposed neonates had high levels of EBNA IgG1 compared to their HIV positive mothers, 0.2120 versus 0.1075 p=0.0056, **(Table 4.5).** However, there were comparable levels in VCA IgG2, IgG3, IgG4 and EBNA1 IgG2, IgG3, IgG4 subclasses between HIV positive mothers and their HIV exposed neonates.

In the HIV negative group, mothers had significantly higher levels of EBNA IgG2, IgG3 compared to their infants 0.0510 (IQ 0.0318-0.098) versus 0.0490 (IQ 0.0313 - 0.0713) *p*=0.0095, 0.0530 (IQ 0.0309 - 0.0958) versus 0.0283(0.0181- 0.0660) *p*=0.0017, respectively and comparable levels of EBNA1 IgG1, IgG4 and VCA IgG subclasses, **(Table 4.5).**

TABLE 4.5: **VCA and EBNA-1 IgG Subclass levels in maternal and their corresponding Neonates**

		HIV Positive		HIV Negative			
	IgG Subclass	**Maternal**	**Neonatal**	**Maternal**	**Neonatal**	***p* value a**	***p* value b**
	IgG1	0.0975(0.0450-0.1940)	0.0665(0.0490-0.02175)	0.0600(0.0380-0.1505)	0.0575(0.0395-0.1705)	**0.0276**	0.3974
	IgG2	0.0205(0.0085-0.0310)	0.0185(0.0115-0.0325)	0.0380(0.0180-0.0868)	0.0460(0.0250-0.0590)	0.08314	0.2418
	IgG3	0.0290(0.0180-0.0785)	0.0340(0.0145-0.0650)	0.0750(0.0308-0.1163)	0.0495(0.0308-0.1225)	0.4081	0.1496
VCA	IgG4	0.0260(0.0145-0.1150)	0.0220(0.0155-0.0515)	0.0200(0.0128-0.1175)	0.0150(0.0123-0.0223)	0.2455	0.1073
	IgG1	0.1075(0.0360-0.2120)	0.2120(0.0455-0.1705)	0.0615(0.0318-0.0988)	0.0445(0.0235-0.0778)	**0.0056**	0.1202
	IgG2	0.0280(0.0115-0.0510)	0.0280(0.0100-0.0490)	0.0510(0.0318-0.0988)	0.0490(0.0313-0.0713)	0.6816	**0.0095**
	IgG3	0.1355(0.0530-0.1810)	0.1615(0.0480-0.1615)	0.0530(0.0309-0.0958)	0.0283(0.0181-0.0660)	0.1565	**0.0017**
EBNA1	IgG4	0.0360(0.0195-0.0850)	0.0360(0.0275-0.0515)	0.0225(0.0143-0.0285)	0.0200(0.0130-0.0358)	0.7124	0.3943

Values are: EBV specific IgG subclass levels in maternal blood and their corresponding neonates. P values *a* compares HIV positive mothers and their HIV exposed neonates. *b* compares HIV negative mothers and their HIV unexposed neonates as determined by Mann Whitney U test. Significant *P* values are indicated in bold.

4.9 Transplacental Transfer of EBV specific IgG subclass antibodies from mother to their neonates

This study then determined the transplacental transfer of EBV specific IgG subclass. Placental transfer was measured as ratio of the level of antibodies in cord blood to that of maternal venous blood at delivery, [cord blood to maternal blood ratio (CMR)].

Transplacental transfer of anti-VCA IgG1, IgG3 and anti-EBNA1 IgG1, IgG4 from HIV-1 positive mothers to their HIV-1 exposed neonates were significantly reduced by 18.86**%**, 25.11**%**, 27.78**%** and 22.04**%** respectively **(Table 4.6).** However, there was no significant reduction in the transfer of anti-VCA IgG2, IgG4 and anti-EBNA1 IgG2, IgG3 (p=0.352, p=0.395, p=0.978 and p=0.283 respectively) **(Table 4.6).**

TABLE4.6: **Transplacental transfer of anti-VCA and anti-EBNA1 specific IgG subclass antibodies**

	IgG Subclass	Exposed	Non-Exposed	% reduction	*p* value
	IgG1	0.780(0.633 - 1.057)	0.968 (0.698 - 1.306)	18.8	**0.046**
	IgG2	0.887 (0.487 - 1.594)	0.894 (0.679 - 1.633)	0.72	0.352
	IgG3	0.749 (0.536 - 1.065)	1.00 (0.693 - 1.356)	25.11	**0.047**
VCA	IgG4	0.954 (0.615 - 1.125)	0.833 (0.509 - 1.207)	-12.15	0.395
	IgG1	0.671 (0.563 - 0.927)	0.949 (0.636 - 1.164)	27.78	**0.028**
	IgG2	1.000 (0.466 - 1.202)	0.899 (0.684 - 1.184)	-10.09	0.978
	IgG3	0.841 (0.658 - 1.087)	0.746 (0.372 - 1.096)	-9.43	0.283
EBNA1	IgG4	0.930 (0.572 - 1.144)	1.151 (0.806 - 1.571)	22.04	**0.024**

Values are median CMR (cord blood/maternal ratio). Significant p values on the table as determined by Mann Whitney u test.
CMR was used to determine placental transfer levels of antibodies. Percentage reduction due to HIV infection was determined as follows:(CMR of non -exposed neonate-CMR of exposed neonates) ×100. Significant P values are indicated in bold.

CHAPTER FIVE

DISCUSSION

5.1 Total IgG antibody levels in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates

Infants living in malaria holoendemic regions of Kenya are infected by EBV early in life when maternal antibodies should protect them against infection. This early age of EBV infection results in higher viral loads and poor control of the virus; a risk factor for eBL. This raises the question whether maternal HIV infection affects the transfer of maternal antibodies to the infants, thus rendering them vulnerable to early EBV infection.

This study first determined total IgG levels in maternal venous blood and cords blood plasma and found that; median concentration of total IgG was higher in HIV positive mothers compared to HIV negative mothers. These were similar to results from other studies (Babakhanyan *et al*., 2016; Moraes-Pinto *et al*., 1993; Moraes-Pinto *et al*., 1998; Moraes-Pinto *et al*., 1996). This similarity may be due to HIV existence since it may cause unspecific B cell hyperactivation leading to high secretion of IgG.

The higher levels of total IgG in HIV exposed neonates compared to HIV unexposed neonates is consistent with (Babakhayan *et al*.,2016) study which demonstrated high total IgG levels in HIV exposed compared to HIV unexposed infants. This observation may be attributed to higher levels of maternal total IgG demonstrated in their mothers. A previous study had earlier demonstrated that neonatal IgG antibody levels is linearly correlated with their maternal antibody levels (Moraes-Pinto *et al*., 1993).

5.2 Levels of EBV specific IgG antibodies in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates

This study reported significantly higher levels of anti-EBNA1 in HIV non -exposed neonates as compared to HIV exposed neonates. This is consistent with the data from Brazil that reported a reduction in the transfer of type 2 poliovirus antibody in HIV positive group as compared to HIV negative group (Moraes-Pinto *et al*., 1993). In addition, another study has also reported lower measles concentration in infants born to women with lower CD4 count (Farquhar *et al*., 2005). This similarity may be due to high maternal total IgG observed in

their HIV positive mothers which are usually associated with low placental transfer ratio of pathogen specific antibodies. High maternal total IgG has been reported before to reduce placental transfer in Malawian population (Moraes-Pinto *et al*., 1998).

In this study, anti-VCA IgG and anti-EBNA1 levels were higher in HIV positive mothers compared to the levels in their HIV exposed neonates; this can either be due to maternal HIV infection which can produce defective IgG in HIV infection thus impairing the binding of its Fc portion to the receptor in the trophoblast thus inhibiting transfer of maternal antibodies to their neonates. Though this study reports no significant difference in anti-VCA IgG levels between HIV exposed and HIV non exposed infants, an earlier study had reported high VCA –IgG titers in HIV-1 infected children compared to HIV-1 uninfected children (Jenson *et al*., 1999). The infant HIV infection status may have played a key role in the expression of anti-VCA IgG antibodies thus the contradicting results.

Measurement of placental transfer as ratio of the level of antibodies in cord blood to that of maternal venous blood at delivery; cord blood/maternal blood ratio (CMR), show that HIV positive mothers had significant reduction in the transfer of anti-EBNA1 antibodies to their HIV unexposed neonate. This is consistent with a study in Kenya that reported reduction in CMR and tetanus antibody levels among HIV-1 infected women (Cumberland *et al*., 2007). In addition, (Moraes-Pinto. *et al*., 1996) reported, placental transfer of IgG against measles virus being reduced among HIV-1 infected than HIV-1 uninfected women. Similarly, (Babakhayan *et al*., 2016) demonstrated a significant reduction in placental transfer of maternal antibodies among Cameroonian pregnant women. Moreover, Farguyah's study reported a reduction in maternal fetal antibody transfer in women with lower CD4 count (Farquhar *et al*., 2005) . The plausible explanation for this similarity may be that, HIV infection lead to decreased levels of Fc receptor. Another possible explanation may be that, HIV-1 derived hyperimmunoglobulinemia in maternal blood which could compete for the IgG specific Fc receptor (FcRn) thereby reducing active transport of IgG antibodies from mothers to their infants.

5.3 EBV specific IgG subclasses levels in HIV-1 infected mothers, HIV-1 uninfected mothers and their corresponding neonates.

Previous studies have demonstrated that, vertical transfer of IgG from mother to the neonate is subclass specific with some subsets being transported more than others (Costa-Carvalho *et*

al., 1996; Ogolla *et al*., 2015). While comparing the levels of EBV specific IgG subclasses in maternal venous blood and their neonatal cord blood; this study demonstrated that, the median anti-VCA IgG1 in HIV positive group was significantly higher in maternal than their corresponding neonates. This results are consistent with a previous study that reported higher levels of anti-VCA IgG1 and EBNA-1 IgG1 antibodies in maternal blood compared to that in their infants (Ogolla *et al*., 2015). In addition (Baroncelli *et al*., 2018) demonstrated lower IgG subclass levels in infants than their mothers. This observation may be attributed to maternal HIV-1 infection since viral infections predominantly elicit production of IgG1 subclass.

In the transfer of EBV antibodies, this study reports IgG1 as the most dominant subclass in both anti-VCA and anti-EBNA-1 than any other subclass. This is consistent with (Costa-Carvalho *et al*., 1996) who reported that, IgG1 was transferred more efficiently from mothers to their neonates. This results are also consistent with (Ogolla *et al*., 2015) results which reports IgG1 as the dominant subclass in both anti-VCA and anti-EBNA1. A possible explanation for this may lie in the competition of these subclasses to bind to the Fc receptors to initiate active transport. However (Baroncelli *et al*., 2018) contrasted with this study since they reported similar passage for all the four IgG subclasses.

CHAPTER SIX

SUMMARY OF THE FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Study Findings

This study compared total IgG levels between HIV-1 infected mothers and HIV-1 uninfected mothers and between HIV exposed and HIV unexposed neonates. Results indicated that HIV infected mothers had significantly high total IgG levels compared to HIV uninfected mothers. Similarly, total IgG levels were found to be significantly higher in HIV exposed compared to HIV-1exposed neonates. However, anti-EBNA1 levels were significantly reduced in HIV-1 exposed neonates than HIV-1 non-exposed neonates. EBNA1 transplacental transfer was significantly reduced due to maternal HIV-1 infection. Immunoglobulin G subclasses results demonstrate that, IgG1 and IgG4 levels against both VCA and EBNA1 were higher in HIV infected mothers compared to uninfected mothers. Overall this would suggest that, maternal HIV-1 infection is a risk factor for reduced levels of EBV specific IgG in neonates.

6.2 Conclusions

i. The increased levels of total IgG in HIV-1 infected pregnant women were as a result of HIV-1 infection.
ii. Maternal HIV-1 infection significantly reduced the levels of anti-EBNA1 IgG in HIV -1 exposed neonates.
iii. There was a significant reduction in the placental transfer of anti-VCA IgG1 and IgG 3 and anti-EBNA1 IgG1and IgG4 in HIV unexposed neonates.

6.3 Recommendations from this study

i. The high total IgG antibody levels in HIV-1 infected pregnant women do not mean protection against EBV acquisition
ii. Since the placental transfer of anti-EBNA-1 is inhibited by maternal HIV-1 infection, an EBNA1 specific therapeutic measure should be designed, tested and administered to HIV exposed neonates to protect them against early EBV infection.

iii. There is a reduction in the placental transfer of anti-VCA IgG1 and IgG3 and anti-EBNA1 IgG1, IgG4 in HIV infected mothers therefore, a therapeutic measure specific to VCA IgG1, IgG3 and EBNA1 IgG1, IgG4 therapeutic measures should be designed, tested and administered to HIV exposed neonates to protect them against early EBV infection.

6.4 Recommendations for future studies

i. Future studies should measure CD4 count in HIV-1 infected mothers to find out if low CD4 count contributes to elevation of total IgG levels in HIV-1 pregnant women.
ii. Future studies should investigate whether the reduction in placental transfer of EBV specific antibodies is associated with the risk of early EBV infection in HIV-exposed neonates.
iii. Further studies should investigate why the placental transfer of EBNA1 IgG1, IgG4 and VCA IgG2, IgG3 are inhibited in HIV-1 exposed neonates

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APPENDICES

Appendix 1: Proposal Approval

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: MSc/PH/00069/2013

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

Date: 29th JUNE, 2017

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR KOECH JEPKEMBOI EMMILY— MSc/PH/00069/2013

The above named is registered in the Master of Science degree programme of the School of public Health and Community Development, Maseno University. This is to confirm that his research proposal titled "***Influence of maternal HIV-1 Infection on the Vertical Transfer of Epstein virus Specific Immunoglobin G***" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

MASENO UNIVERSITY
30 JUN 2017
DEAN SCHOOL OF GRADUATE STUDIES

Prof: J. O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Maseno University *ISO 9001:2008 Certified*

Appendix 2: Ethical Approval

KENYA MEDICAL RESEARCH INSTITUTE

KENYA MEDICAL RESEARCH INSTITUTE
CENTRE FOR GLOBAL HEALTH RESEARCH
P.O. Box 1578 - KISUMU

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **January 14, 2011,**

TO: **DR. ROSEMARY ROCHFORD (PRINCIPAL INVESTIGATOR)**
DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY, SUNY,
UPSTATE MEDICAL UNIVERSITY, SYRACUSE, NEW YORK

THRO': **DR. JOHN VULULE,**
THE DIRECTOR, CGHR,
KISUMU

FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

RE: **SSC PROTOCOL NO. 1910 (*INITIAL SUBMISSION*): EFFECT OF MALARIA AND HIV INFECTION ON EBV PERSISTENCE IN INFANTS AND THEIR MOTHERS:**

Make reference to your letter dated December 15, 2010 received on January 6, 2011. Thank you for your response to the issues raised by the Committee. This is to inform you that the issues raised during the 184th meeting of the KEMRI/ERC meeting held on 23rd November 2010, have been adequately addressed.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **14th day of January 2011,** for a period of twelve (12) months.

Please note that authorization to conduct this study will automatically expire on **13th January 2012.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **15th September 2011.**

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

ROTKithinji

Caroline Kithinji,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE

Appendix 3: Consent form

SUNY Upstate Medical University Kenya Medical Research Institute Consent for Human Investigational Studies

Effects of malaria and HIV on EBV persistence in infants and their mothers

Study Participant Number: _____.

Background and Purpose:

Burkitt's lymphoma is a cancer that affects children and is thought to be caused by the presence of malaria and a virus called the Epstein Barr Virus (EBV). We know that children who live in malaria areas get EBV earlier in life than children that live in areas without malaria. These children are also likely to carry high levels of the virus. In this study we will look at the reasons why this is so.

Women who live in areas with high malaria such as Chulaimbo, when they become pregnant can get malaria parasites in their placenta. The presence of the parasite in the placenta has been known to cause many complications in babies after they are born. We think that mothers who suffer from malaria when they are pregnant may cause the higher levels of virus in their babies. The purpose of this study is to see if the presence of parasites in the placenta may result in babies getting high levels of EBV earlier in life and if this is also the reason that some children suffer from Burkitt 's lymphoma.

Dr. Rosemary Rochford from SUNY Upstate Medical University (SUNY Upstate) in Syracuse, USA and Dr Peter Odada Sumba at of the Kenya Medical Research Institute (KEMRI) are inviting you and your newborn child to participate in this research study.

We are approaching you at the antenatal clinic so that you may agree to participate in the study before your baby is born. If you agree to be in the study we will ask you to fill a form that will give us information about your pregnancy, where you live and when you are expected to deliver. When you come back to the hospital to deliver we will identify you as a study participant so that we may pay for your delivery costs.

We will ask for consent from you for your participation in the study. We will also ask you for consent for your newborn child to participate in this study.

Permission will be required from you for both you and your child. We are hoping to enroll 400 women and 400 infants into this study.

Study Procedures 1. Sample Collection: In order to understand how strongly your child's body responds to malaria and to EBV, we have to know if you had malaria during your pregnancy and if your placenta has malaria parasites in it. We also want to know when your child is first infected with malaria and how many infections he/she will get and when he/she is infected with EBV. We will collect a small sample of blood from your finger today to test for malaria and also for some studies that will be carried out in our laboratories in Kisian. Each time that you come to the antenatal clinic, we will collect a small sample of blood from you. We will collect your placenta at delivery and will look for malaria parasites in it. We are also requesting for a teaspoon of blood from you once you are rested after your baby's delivery, we will use a needle attached to a syringe to obtain the blood from your arm. We will also request a small blood sample from your child at delivery, which we will obtain from the umbilical cord.

After the birth of your baby, we will set up appointments for you to return to the clinic with your baby at 6, 10, 14, and 18 weeks of age. We will request to take blood samples from your baby at these appointments. A small amount of blood (a few drops), will be taken from your child's heel or finger. We will also schedule return visits to the clinic for when your baby is 6 months old, and at every 3-month interval to 24 months (2 yrs of age). Starting at the 6-month visit, we will request to take from your child a larger blood sample, equal to about 2 teaspoons. This sample will be drawn from a vein in your child's arm using a needle attached to a blood collection tube. It is important if you agree to be in this study that you will be around at least for the next year.

We will also collect from you breast milk and saliva samples to see if the EBV virus is in the milk and saliva and if this is how your baby gets infected. Breast milk and saliva will be collected at 6, 10, and 14-week visits. We will require about two teaspoons of breast milk and a teaspoon of saliva.

All samples will be transported to the SUNY Upstate/KEMRI laboratory in Kisumu. Tests done in the laboratory will tell us if you had malaria during pregnancy and when your child is infected with malaria and EBV and how your child's body protects him/her against malaria and against EBV. We will also carry out tests that will inform us of your child's immune

system. We will monitor your child's growth every time we visit by measuring his/her height and weight.

2. Genetic studies:

We also would like to use part of your child's blood at the SUNY Upstate/KEMRI laboratory to do genetic studies. Genes are composed of the genetic material called DNA. DNA is the part of the cell that is responsible for providing hereditary characteristics (such as eye color) and is used to build proteins. We would like to test his/her blood for genes including sickle cell trait, G6PD deficiency and HLA type that may protect against malaria. We may also test for other genes that are known to affect malaria and EBV. If we get any results from the lab studies that may affect your child's status with respect to malaria, we will inform you. We will also look at genes belonging to the malaria parasite and to EBV. This is important to see if your child has built defenses against different types of malaria parasites or EBV and similar viruses. Since the significance of the tests for changes in the malaria parasite or EBV is not known to you, we will not release the results of any genetic tests associated with the EBV and malaria parasite testing.

Do you accept for yours and your child's samples to be used for Genetic Studies? YES_____ NO_____

3. Sample Storage:

Samples collected will be stored in the SUNY-KEMRI laboratories in Kisumu for the full duration of the study and the period required to analyze results. We may save some of your samples for many years to further study Burkitt's lymphoma in the SUNY/KEMRI Labs in Kisian or in the labs of Dr Rochford at the SUNY Upstate Medical University. If at any time you wish to withdraw your agreement for us to save your and your child's samples, please contact Dr. Peter Odada Sumbab, the Research Project Manager, at KEMRI Kisumu Tel. 254-57-2022989 or 254-733-746854/254-720-766550 and we will destroy the samples.

Do you accept for yours and your child's samples to be stored and when necessary to be shipped to SUNY Upstate Medical University in the US for further investigations?

YES_____ NO_____

Risks:

There are few risks in having your blood taken from the placenta or your arm after delivery. There are also few risks from taking your breast milk and saliva. Minimal risks are associated with collecting your child's blood. The blood drawings include a little bleeding, pain, bruising and, rarely, infection. All of these are uncommon but may occur in very few people. We have never experienced these problems in our previous studies.

Answering questions during the antenatal enrolment interview will not cause any risk to you or your child.

Benefits:

We will be testing for malaria in your placenta, which is not normally done and is therefore a benefit. It is important to know if you had malaria in your placenta because it will tell us how your baby will fight off malaria and EBV later in life. Also, as a benefit to your child, we will be making a visit every month to your home to collect samples at which time if your child is sick we will treat your child for fever, malaria, diarrhea and anemia according to the Kenya Ministry of Health Guidelines. Also, the information that we get from this study will be important for prevention programs for Burkitt' s lymphoma.

Alternatives:

You do not need to participate in this study to receive medical care for you and your baby. If you choose not to participate, you may still obtain normal delivery care at the hospital and medical care as provided by the Kenya Ministry of Health, including free testing and treatment for malaria.

Voluntary Participation:

Your participation in this research study is entirely voluntary. Refusing to participate will not alter your usual health care or involve any penalty or loss of benefits. If you decide to join the study, you may withdraw you and or your child at any time and for any reason.

Costs/ Payments:

We will pay for your delivery costs if you participate in this study. We will meet both bed and delivery fees**.** There are no additonal costs to you or your child for participating in this study.

During your participation in the study, you and your child will receive free medical care and attention from the study assigned Clinical Officer for the duration of the study. If you withdraw from the study before you deliver, the study will not pay for your delivery costs. You will however be able to obtain normal delivery care at the hospital but the costs will be your responsibility. If you withdraw from the study after delivery, you will not receive the free medical care and medication for study participants but you can still attend the clinic and receive services provided by the Ministry of Health. If you withdraw at any OTHER time, after our 1st home visit, your child will continue to receive free medical care from the study appointed clinical officer for the duration of the study period.

Neither you nor your child will be paid for participating in this study.

Questions:

If you have any questions about this study you may speak to Dr. Peter Odada Sumba, the Research Program Manager, at the Center for Global Health Research, Kenya Medical Research Institute, PO Box 1578, Kisumu at 254-57-2022989 / 254-733-746854 / 254- 720-766550 or to The Director of CGHR, KEMRI in Kisumu at 254-57-2022924. Queries pertaining to research subjects' rights may be made to the KEMRI/National Ethical Review Committee (ERC), PO Box 54840, Nairobi at 254- 2-02722541 or The Director of KEMRI, PO Box 54840, Nairobi at 254- 2-02722541.

In Case of Injury:

In the event of illness or physical injury resulting from taking part in this research study, please contact The Director of the Center for Global Health and Research (CGHR) at KEMRI in Kisumu at PO Box 1578, Kisumu 40100. Tel: 254-57-2022924 or Dr. Peter Odada Sumba at 254-57-2022989/ 254-733746854/254-720-766550

SUNY Upstate Medical University has no plans to give you money if you are injured. You have not waived any of your legal rights by signing this form.

Confidentiality of Records and Authorization to use/share protected health information for research: If you agree to participate in the study and if you allow your child to participate in this research, yours and your child's health information will be kept confidential. We will assign a number to you and your child that will appear on all the

samples that we collect. Your name WILL NOT appear on any of these samples. This is being done to protect your and your child's medical information. There will be a few people that will have your name and numbers. These will be Dr. Rochford, Dr Odada Sumba and the study assistants. If for any reason your samples need to be shipped to the US for analysis, these samples will have only your study number and no names will be included. When we publish or present any of the findings from this study, your names will never be revealed. All information that we collect from you will be will be stored in locked cabinets at CGHR, KEMRI with only a few people in the Research Project, having access to these cabinets. This is also to ensure privacy and protection of your and your child's medical information.

Consent to participate in Research:

The nature and the purpose of the above research study have been explained to me. Signing below indicates that I have been informed about the research study in which I voluntarily agree to my participation and my child's participation. I have asked questions about the study and the information given to me has permitted me to make a fully informed and free decision about my and my child's participation in the study. By signing this consent form, I do not waive any legal rights, and the investigators are not relieved of any liability they may have. I can withdraw from this study at any time. A copy of this consent form will be provided to me.

Signature of Parent_____Date_____

Signature of Person Obtaining Consent/Authorization_____ Date _____

Signature of Witness_____Date_____

Appendix 4: Questionnaire

Maternal Prenatal (ANC) Visit Form

PARTICIPANT ID: ECH-__ | __ | __ | __ | - M

Completed By: _____ Date: ____/_____/_____

(dd/mmm/yyyy)

Visit Number: ANC1 ANC2 ANC3 ANC4

1. Estimated gestational age (in weeks): _____
2. Fundal Height (cm): _____

Symptoms No /Yes

3. Is the patient sick today?
4. Does the patient have a fever today?
5. Fever in the past two days?
6. Cough in the past two days?
7. Headache in the past two days?
8. Chills in the past two days?
9. Diarrhea in the past two days?
10. Stomach ache in the past two days?
11. Is a bed net being used?
12. Other symptoms in the past two days?

If yes, please describe: _____

Physical Exam

13. Weight (kg): _____(13)
14. Height (cm): _____(14)
15. Mid-Upper Arm Circumference [MUAC] (cm): _____(15)
16. Heartbeat Rate (beats per minute) _____(16)

17. Respiratory Rate (breaths per minute) _____(17)

18. Blood Pressure _____ / _____ (18)

19. Axillary Temperature (degrees C): _____(19)

Medications Given at Visit

Was the medication given Yes or No

20. Iron Tables (N=30)

21. Folic Acid (N=30)

22. Fansider Tables (Malaria prophylaxis) (N=3)

23. Tetnus booster given?

24. Deworming (Membendazole500gms)

25. ITN (INSECTS treated net)

26. ARV (prophylaxis) AZT+NVP

27. Other medications

If yes, please specify: _____

---

28. Clinical Diagnosis: _____

For Data Entry Use Only

Entered By:_____ Date (dd/mmm/yyyy):_____/_____/_____

Reviewed By:_____Date (dd/mmm/yyyy):_____/_____/_____