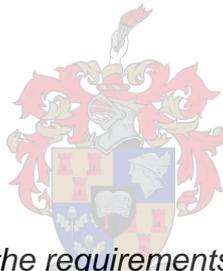


**LONGITUDINAL INVESTIGATION OF VACCINE SPECIFIC
ANTIBODY LEVELS AND CELLULAR MARKERS OF ADAPTIVE
IMMUNE RESPONSES IN HIV EXPOSED UNINFECTED (HEU)
AND UNEXPOSED (UE) INFANTS**

by

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in Medical Science at the University of Stellenbosch*

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DECLARATION

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SUMMARY

Background: In South Africa alone, 30% of women of child-bearing age are infected with HIV. With the increasing focus and success of prevention of mother-to-child transmission (PMTCT) programmes, an estimated 300 000 infants are born exposed to HIV every year. The underlying impact of *in utero* HIV exposure on infant immune health has not been extensively characterised. Clinical follow-up of these HIV-exposed uninfected (HEU) infants reveals increased infectious morbidity and mortality compared to their unexposed (UE) counterparts.

Objectives: (i) To evaluate and characterise adaptive immune properties by measuring vaccine-specific antibody levels in children from 2 weeks to 2 years of age in the presence and absence of maternal HIV infection. (ii) To investigate specific cellular markers of immune activation, immune regulation, apoptosis and B cell memory on T and B cell populations in HEU and UE children measured at 18 and 24 months of age.

Methods: This sub-investigation formed part of a collaborative pilot study between the universities of British Columbia (Vancouver, Canada) and Stellenbosch. A total of 95 HIV-positive and HIV-negative mothers were recruited after delivery at Tygerberg Hospital, and signed informed consent for their infants to be included in the study. Of these infants, only 27 HEU and 30 UE infants were eventually enrolled and followed up at various time points, starting at two weeks of age. Four of these infants were confirmed to be HIV-positive at 2 weeks and clinically followed up according to the protocol, but were excluded from statistical data analyses.

Blood was collected at 2, 6 and 12 weeks and again at 6, 12, 18 and 24 months of age. Quantitative IgG-specific antibodies to *Haemophilus influenzae B* (Hib), *Bordetella pertussis*, tetanus and pneumococcus were measured at each time point, using commercially available ELISA (Enzyme-Linked ImmunoSorbent) kits. Cellular markers of immune activation, immune regulation, apoptosis and memory were measured in various populations of T and B cells at 18 and 24 months only, by using four-colour flow cytometry and validated whole-blood staining methods. In addition, a functional assay was developed to evaluate cell susceptibility to apoptosis (spontaneously) by measuring the expression of Annexin V on both CD4⁺ T and CD20⁺ B cells after 16 and 24-hour incubation periods.

The statistical analysis of the antibody data was conducted by repeated-measures ANOVA (i.e. analysis of variance), using a mixed-model approach. Differences in the expression of the two groups' cellular markers were compared by employing one-way ANOVA. An F test p value (which assumes normality) was reported, while the non-parametric Mann-Whitney U test served as confirmatory tool. Repeated-measures ANOVA was used for the evaluation of the functional spontaneous apoptosis assay at three time points (*ex vivo*, 16 and 24 hours) on the 18-month samples, while one-way ANOVA was used for the 24-month samples.

Results: The HEU group (n = 23) displayed significantly lower levels of antibodies to pertussis (20.80 vs 28.01 Food and Drug Administration [FDA] U/ml; p = 0.0237), tetanus (0.08 vs 0.53 IU/ml; p < 0.001) and pneumococcus (31.67 vs 80.77 mg/l; p = 0.003) than the UE group (n = 23) at 2 weeks of age. No statistical differences were noted for Hib antibody levels between the two groups at this time point. At 6 weeks of age, HEU infants displayed lower mean levels of all antibodies measured; however, these differences did not reach statistical significance.

Following vaccination, compared to UE controls, the HEU group presented with statistically significantly higher antibody levels to pertussis at 6 months (155.49 vs 63.729 FDA U/ml; p = 0.0013), 12 months (26.54 vs 8.50 FDA U/ml; p < 0.001) and 18 months of age (1658.94

vs 793.03 FDA U/ml; $p = 0.0362$). A significant difference in tetanus antibody levels between the two groups was only evident at 24 months, with the HEU group displaying higher levels (3.28 vs 1.70 IU/ml; $p = 0.018$) than the UE group. No differences were observed between the two groups following vaccination for Hib.

At 18 and 24 months, the HEU group showed increased expression of cellular markers of immune activation (CD69 and CD40L) on CD4+ T cells compared to UE controls. The two groups showed similar expression of the cellular marker of activation CD38 on CD8+ T cells. The HEU group displayed significantly higher levels of CD127, the interleukin (IL) 7 receptor, on CD4+ T cells compared to UE controls at 18 months of age. The HEU group also showed increased expression of cellular markers of apoptosis on both CD4+ T and CD8+ T cells. No statistical significance was noted for the expression of Fas on CD4+ T cells at 18 and 24 months of age. However, at 24 months, the HEU group showed significantly increased expression of FasL on both CD4+ T and CD8+ T cells. During cell culture experiments, the HEU group displayed increased susceptibility to spontaneous apoptosis shown by increased Annexin V expression on CD4+ T cells after a 16-hour incubation period at both 18 and 24 months. At 18 and 24 months, no difference was noted in the two groups' immune regulation as measured by the expression of CTLA-4.

The HEU group displayed increased levels of the cellular markers of immune activation CD80 on CD20+ B cells at 18 and 24 months of age. The HEU group also showed significantly increased levels of CD69 on CD19+ B cells at 24 months. No statistical significance was reached for the expression of CD62L and CD10 at either 18 or 24 months. Although the HEU group displayed increased levels of apoptosis (Fas) on CD20+ B cells, no statistical significance was reached at 18 or 24 months of age. In addition, the HEU group showed no difference in the expression of programmed death 1 (PD-1) at 18 and 24 months. HEU and UE groups showed similar expression of Annexin V after 16 hours of incubation in the 18 and 24-month samples. The expression of the biomarker of B cell memory CD27 on CD20+ B and CD19+ B cells was comparable between the two groups at both time points.

Conclusion: At 2 and 6 weeks, lower mean antibody responses in HEU infants suggest poor placental transfer due to maternal HIV infection, while increased responses to specific antibodies may reflect an exaggerated immune response to immunisation. These robust responses may be due to the lack of competition with maternal antibodies, or may be ascribed to indirect stimulation of B cells via the activation of T cells.

A hyper-inflammatory state is an imminent danger, with increased expression of cellular markers of immune activation and apoptosis that may be consistent with early HIV exposure that persists following infancy. These observations may serve as contributing factors to the extensively documented increased susceptibility to infections in the HEU population. Although these findings are consistent with a primed immune system, larger studies are required to confirm these observations in relation to clinical outcomes and to assess further whether these differences persist in later years.

OPSOMMING

Agtergrond: In Suid-Afrika alleen het 30% van vroue van 'n vrugbare leeftyd MIV. Met die toenemende fokus en sukses van programme vir die voorkoming van moeder-na-kind-oordrag (sogenaamde PMTCT-programme) word ongeveer 300 000 babas jaarliks aan MIV blootgestel. Die onderliggende impak van intra-uteriene MIV-blootstelling op 'n baba se immuunstelsel is nog nie omvattend beskryf nie. Kliniese opvolgondersoeke van hierdie MIV-blootgestelde dog onbesmette babas (sogenaamde HEU's) dui op 'n hoër siekte- en sterftesyfer weens infeksies as hul nieblootgestelde eweknieë (sogenaamde UE's).

Doelstellings: (i) Om kinders met MIV-positiewe en MIV-negatiewe moeders se aangepaste (verworwe) immuun-eienskappe te beoordeel en te beskryf deur hulle vaksien-spesifieke teenliggaamvlakke vanaf die ouderdom van twee weke tot twee jaar te meet. (ii) Om ondersoek in te stel na bepaalde sellulêre merkers van immuunaktivering, immuunregulering, apoptose en B-selgeheue by die T- en B-selgroepe van sowel HEU's as UE's op die ouderdom van 18 en 24 maande.

Metodes: Hierdie subondersoek het deel uitgemaak van 'n samewerkende loodsondersoek tussen die universiteite van Brits-Columbië (Vancouver, Kanada) en Stellenbosch. Altesaam 95 MIV-positiewe en MIV-negatiewe moeders is gewerf nadat hulle by Tygerberghospitaal geboorte geskenk het, en het ingeligte toestemming verleen dat hul babas by die studie ingesluit kon word. Van dié babas is slegs 27 HEU's en 30 UE's uiteindelik in die studie opgeneem en in verskillende stadia vanaf die ouderdom van twee weke opgevolg. Vier van die babas is op twee weke as MIV-positief bevestig en volgens die protokol klinies opgevolg, maar is van die statistiese dataontleding uitgesluit.

Bloedmonsters is op twee, ses en 12 weke en weer op ses, 12, 18 en 24 maande geneem. Kwantitatiewe IgG-spesifieke teenliggame teen *Haemophilus influenzae B* (Hib), *Bordetella pertussis*, tetanus en pneumokokkus is telkens met behulp van kommersieel verkrygbare ELISA- ("Enzyme-Linked ImmunoSorbent"-)stelle bepaal. Sellulêre merkers van immuunaktivering, immuunregulering, apoptose en geheue is op slegs 18 en 24 maande by verskillende populasies T- en B-selle deur middel van 'n vierkleurvloei-sitometrie en geldig verklaarde volbloedkleuringsmetodes bepaal. Voorts is 'n funksionele toets ontwikkel om selvatbaarheid vir apoptose te bepaal deur die ekspressie van Annexin V op sowel CD4+ T- as CD20+ B-selle ná 16 en 24 uur van inkubasie te meet.

Die statistiese ontleding van die teenliggaamdata is met behulp van herhaaldemeterings-ANOVA (d.w.s. afwykingsontleding) volgens 'n gemengdemodel-benadering gedoen. Verskille in die twee groepe se sellulêre merkervlakke is deur middel van eenrigting-ANOVA vergelyk. 'n F-toets-p-waarde (wat normaliteit veronderstel) is bereken, terwyl die nieparametriese Mann-Whitney-U-toets as bevestigende instrument gedien het. Vir die 18 maande-monsters is herhaaldemeterings-ANOVA gebruik om die funksionele toets vir spontane apoptose in drie stadia (*ex vivo*, op 16 uur en op 24 uur) te beoordeel. Vir die 24 maande-monsters is eenrigting-ANOVA gebruik.

Resultate: Op die ouderdom van twee weke het die groep HEU's ($n = 23$) aansienlik laer teenliggaamvlakke teen kinkhoes (20.80 vs 28.01 Food and Drug Administration [FDA] U/ml; $p = 0.0237$), tetanus (0.08 vs 0.53 U/ml; $p < 0.001$) en pneumokokkus (31.67 vs 80.77 mg/l, $p = 0.003$) as die UE-groep ($n = 23$) getoon. In dié stadium is geen statistiese verskille in Hib-teenliggaamvlakke tussen die twee groepe opgemerk nie. Op ses weke het die groep HEU's laer gemiddelde vlakke van ál die betrokke teenliggame getoon, hoewel hierdie verskille nie statisties beduidend was nie.

In vergelyking met die UE-kontrolegroep het die groep HEU's ná inenting statisties beduidend hoër teenliggaamvlakke teen kinkhoes getoon op ses maande (155.49 vs 63.729 FDA U/ml; $p = 0.0013$), 12 maande (26.54 vs 8.50 FDA U/ml; $p < 0.001$) én 18 maande (1658.94 vs 793.03 FDA U/ml; $p = 0.0362$). 'n Beduidende verskil in die twee groepe se tetanus-teenliggaamvlakke het eers op 24 maande geblyk, met die groep HEU's s'n hoër (3.28 vs 1.70 IE/ml; $p = 0.018$) as die UE's s'n. Ná inenting teen Hib is geen verskille tussen die twee groepe waargeneem nie.

Op 18 en 24 maande het die HEU's verhoogde ekspressie van sellulêre merkers van immuunaktivering (CD69 en CD40L) op CD4+ T-selle getoon in vergelyking met die UE-kontrolegroep. Soortgelyke vlakke van die sellulêre merker van aktivering CD38 is ook op die CD8+ T-selle van die twee groepe opgemerk. Op 18 maande het die HEU-groep 'n beduidend verhoogde ekspressie van CD127, die IL-7-reseptor, op CD4+ T-selle getoon in vergelyking met die UE-kontrolegroep. Die HEU groep het ook verhoogde ekspressie van sellulêre merkers van apoptose op sowel CD4+ T- as CD8+ T-selle getoon. FAS-ekspressie op CD4+ T-selle op 18 en 24 maande was nie statisties beduidend nie, hoewel die HEU-groep op 24 maande beduidend verhoogde ekspressie van FasL op CD4+ T- sowel as CD8+ T-selle getoon het. In selkwekingseksperimente het die HEU-groep 'n verhoogde vatbaarheid vir apoptose getoon na aanleiding van die ekspressie van Annexin V op CD4+ T-selle ná 16 uur van inkubasie op sowel 18 as 24 maande. Op 18 en 24 maande was immuunregulering, aan die hand van die ekspressie van CTLA-4, bykans dieselfde by albei groepe.

Op sowel 18 as 24 maande toon die HEU's verhoogde ekspressie van die sellulêre merker van immuunaktivering CD80 op CD20+ B-selle. Op 24 maande het die HEU's ook aansienlik hoër vlakke van CD69 by CD19+ B selle getoon. Op nóg 18 nóg 24 maande was die ekspressie van CD62L en CD10 statisties beduidend. Hoewel verhoogde vlakke van apoptose (Fas) by CD20+ B-selle by die HEU-groep opgemerk is, was dit nie statisties beduidend op 18 óf 24 maande nie. Daarbenewens was daar ook geen verskil in die ekspressie van geprogrammeerde seldood 1 (PD-1) op 18 en 24 maande nie. Op 18 en 24 maande het die HEU's en UE's 'n soortgelyke ekspressie van Annexin V ná 16 uur van inkubasie getoon. Op sowel 18 as 24 maande was die twee groepe se ekspressie van die biomerker van B-selgeheue CD27 op CD20+ B- en CD19+ B-selle vergelykbaar.

Gevolgtrekking: Op twee en ses weke dui laer gemiddelde teenliggaamreaksies by HEU's op swak plasentale oordrag weens die moeder se MIV-infeksie, terwyl verhoogde reaksies op bepaalde teenliggame weer op oordrewe immuunreaksie op inenting dui. Hierdie robuuste reaksie kan toegeskryf word aan die gebrek aan mededinging met die moeder se teenliggame, of kan deur indirekte stimulasie van die B-selle via die aktivering van die T-selle veroorsaak word.

'n Hiperinflammatoriese toestand is 'n dreigende gevaar, met verhoogde ekspressie van sellulêre merkers van immuunaktivering en apoptose wat met vroeë MIV-blootstelling met 'n latere nawerking verbind kan word. Hierdie waarnemings kan bydraende faktore wees tot HEU's se goed gedokumenteerde verhoogde vatbaarheid vir infeksies. Hoewel hierdie bevindings met 'n geaktiveerde immuunstelsel strook, moet groter studies dit aan die hand van kliniese uitkomst bevestig en ook bepaal of hierdie verskille in later jare voortduur.

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“Do all you can with what you have, in the time you have, in the place you are...”

**Nkosi Johnson
(1989-2001)**

International AIDS Conference in Durban, South Africa, July 9, 2000

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of Variance
aP	Acellular pertussis
APCs	Antigen presenting cells
ART	Antiretroviral therapy
ARV	Antiretroviral
BCG	Bacillus Calmette-Guèrin
BCR	B cell receptor
BD	Beckton Dickinson
BSL3	Biosafety Level 3
CCMTS	Child Nutrition and Comprehensive Care
CCR5	CC-chemokine receptor 5
CD	Cluster of differentiation
CD40L	CD40 Ligand
cDNA	copy Deoxyribonucleic acid
CMV	Cytomeglovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte Antigen 4
CXCR4	CXC-chemokine receptor 4
DBS	Dry Blood Spot
DBSP	Dry Blood Spot Paper
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DOH	Department of Health
DRC	Democratic Republic of Congo

DTaP-IPV//Hib	Diphtheria-Tetanus-acellular Pertussis-Invactivated Polio Vaccine//Haemophilus influenzae b
DTP	Diphtheria-Tetanus-Pertussis
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme Linked ImmunoSorbent Assay
<i>env</i>	envelope protein
EPI	Expanded Programme on Immunisation
EPI-SA	Expanded Programme on Immunisation of South Africa
FasL	Fas Ligand
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
<i>gag</i>	Viral core protein
GERMSSA	Group for Enteric, Respiratory and Meningeal diseases Surveillance in South Africa
gp120	Glycoprotein 120
gp41	Glycoprotein 41
HAART	Highly Active Antiretroviral Therapy
Hb	Haemoglobin
Hep B	Hepatitis B
HEU	HIV exposed uninfected
Hib	<i>Haemophilus influenzae b</i>
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICH	Institute for Child Health
IFN	Interferon
IFN- γ	Interferon gamma
Ig	Immunoglobulin

IL	Interleukin
ICC	Intra-class correlation
IPD	Invasive pneumococcal disease
KIDCRU	Children's Infectious Disease Clinical Research Unit
LPS	Lipopolysaccharide
LS	Least Square
LTFU	Lost-to-follow-up
LUC	Large Unstained Cells
Mab	Maternal antibody
MDG	Millenium Development Goals
MHC	Major Histocompatibility complex
MRC	Medical Research Council
MtAb	Maternal antibodies
MTCT	Mother-to-Child Transmission
<i>nef</i>	Negative factor
NHLS	National Health Laboratory Service
NK cells	Natural killer cells
NVD	Normal Vaginal Delivery
NVP	Nevirapine
OD	Optical Density
OPA	Opsonophygcytic activity
OPV	Oral Polio Vaccine
OTS	Open tube sampler
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCP	Pneumococcal capsular polysaccharide
PCR	Polymerase Chain Reaction
PCV ₇	Pneumococcal Conjugate Vaccine 7

PD	Programmed death
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PMTCT	Prevention of mother-to-child Transmission
PRF	Poliomyelitis Research Foundation
PRP	Polyribosyl Ribitol Phosphate
PS	Phosphatidylserine
QC	Quality Control
<i>rev</i>	Regulator of virion protein
RNA	Ribonucleic acid
RCPA	Royal College of Pathologists of Australasia
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
RTHC	Road to Health Card/Chart
RV	Rotavirus
SANAS	South African National Accreditation System
SAVIC	South African Vaccine Immunisation Centre
SD	Standard Deviation
SIV	Simian immunodeficiency virus
SSC	Side Scatter
SST	Serum separating tubes
<i>tat</i>	Transactivator
TB	Tuberculosis
TCR	T cell receptor
Td Vaccine	Tetanus-Diphtheria Vaccine
Th cells	T helper cells
TMB	Tetramethylbenzidine

TNF	Tumour necrosis factor
TREC	T cell receptor circles
Treg	T regulatory cells
UE	Unexposed
UNAIDS	Joint United Nations programme on HIV/AIDS
UNICEF	United Nations Children's Fund
USA	United States of America
<i>vif</i>	Virion infectivity factor
<i>vpr</i>	Viral protein R
<i>vpu</i>	Viral protein U
WHO	World Health Organisation
wP	Whole-cell pertussis
ZVITAMBO	Zimbabwe Vitamin A for Mothers and Babies Project

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CHAPTER 1

INTRODUCTION

In 2010, approximately 21 000 children under the age of 5 years died each day. Sub-Saharan Africa currently experiences the highest rates of child mortality, where 1 in every 8 children dies before their fifth birthday. Here, compared to the developed world, childhood mortality is about 17 times greater (UNICEF, 2011).

In the year 2000, the top five causes of death in children under the age of 5 years featured HIV/AIDS in first place, followed by low birth weight, diarrhoeal diseases, lower respiratory tract infections and malnutrition (Bradshaw *et al.*, 2003). In 2009, approximately 5.6 million people were living with HIV/AIDS in South Africa which is believed to be more than in any other country (UNAIDS, 2010). About 1 in 3 women between the ages of 25 and 29 were at increased risk for infection in 2008 (Shisana *et al.*, 2009). UNAIDS (2010) reported that in East and Southern Africa about 68% of pregnant women living with HIV were receiving antiretroviral (ARV) treatment for the prevention of HIV transmission to their infants.

The risk of transmitting HIV from an infected mother to her infant is around 30% in the absence of PMTCT programmes. However, with comprehensive interventions, the rate of infection is decreased to less than 5% (De Cock *et al.*, 2000). With improved access to ARV therapy and PMTCT programmes, 300 000 infants are born annually HIV exposed but remain HIV uninfected (HEU) (Bobat *et al.*, 1996).

Despite their HIV negative status, documentation is accumulating on increased infectious morbidity and mortality in this group of infants, consequently classifying them as a vulnerable population (Marinda *et al.*, 2007, Filteau, 2009, Kuhn *et al.*, 2007, Thea *et al.*, 1993).

Compared to their unexposed (UE) counterparts, HEU infants show a greater mortality rate (Newell *et al.*, 2004). In addition, a number of studies have observed increased events of severe infections in HEU infants such as lower respiratory tract infections, meningitis, acute bronchiolitis, cytomegalovirus (CMV) as well as increased incidence of group B Streptococcal infection (Slogrove *et al.*, 2009, McNally *et al.*, 2007, Mussi-Pinhata *et al.*, 2010, Bates *et al.*, 2008, Epalza *et al.*, 2010). Furthermore, haematological abnormalities have been reported due to ARV exposure during foetal development as well as early infancy, therefore further highlighting the need for clinical follow-up during both pregnancy and early infancy (El Beitune and Duarte, 2006).

The reasons for the increased incidence of morbidity and mortality in HEU infants have not yet been defined clearly; however, they are believed to be multi-factorial. Contributing factors include low birth weight, reduced breastfeeding, ARV exposure, poor growth and nutrition, maternal disease severity, decreased acquisition of maternal antibodies and inadequate infant care (Filteau, 2009).

Several immune abnormalities have been described in infants and children born to HIV positive mothers. Differences in the proportions of T cell populations, as well as reduced CD4/CD8 ratios and decreased CD8+ naïve T cell percentages have been observed (Clerici *et al.*, 2000). Other studies have documented lower progenitor cells and decreased thymus outputs (Nielsen *et al.*, 2001).

During *in utero* development the growing foetus comes into contact with HIV viral particles. These particles serve as inducers of increased levels of immune activation, as well as apoptosis (Nielsen *et al.*, 2001, Miyamoto *et al.*, 2010). Increased levels of CD40L on

activated lymphocytes have been reported (Romano *et al.*, 2006), as well as increased B cell apoptosis (Miyamoto *et al.*, 2010).

Furthermore, the persistence of immune abnormalities due to HIV and ARV exposure beyond infancy and differences in even HEU adolescents have been documented (Miyamoto *et al.*, 2010, Clerici *et al.*, 2000). However, due to fact that HIV mainly uses CD4+ T lymphocytes as an entry point to invasion of the immune system, research has concentrated more on evaluating abnormalities of the T cell compartment and less on dysfunctions of B lymphocyte sub-populations (Clerici *et al.*, 2000).

A number of B cell abnormalities have been documented with HIV infection, some of which include hypergammaglobulinemia, polyclonal activation, as well as autoimmunity (De Milito, 2004). Abnormal levels of IgG that persist in HEU infants from birth until 24 months of age suggest an altered humoral immune response (Bunders *et al.*, 2010).

Vaccination is considered the most cost effective tool for preventing disease in the population at large; however, only a few studies have addressed the impact of HIV exposure on infant vaccination response (Abramczuk *et al.*, 2011, Jones *et al.*, 2011, Madhi *et al.*, 2005). Currently HIV exposed infants receive the same vaccine schedule as the unexposed. Considering that their immune system develops in a different antigenic environment due to maternal HIV infection, it merits further investigation to evaluate HEU vaccination responses.

The above studies have shown that HEU infants mount significantly different responses to certain vaccines. In comparison to UE controls, the response to vaccination is more robust and is believed to be a result of reduced passive specific maternal antibody levels at birth, thus allowing for less antibody interference (Madhi *et al.*, 2005, Jones *et al.*, 2011). Although these studies highlight significant differences and trends in vaccine specific responses in HEU infants, they have mainly evaluated responses to primary vaccination and not of follow-up immunisation through longitudinal studies. In addition, potential immunological differences, such as lymphocyte functionality as reasons for weak or heightened responses to certain vaccines need to be investigated (Abramczuk *et al.*, 2011).

In an attempt to address the immunological differences that exist within HEU and UE infants as potential contributing factors to the increased events of morbidity and mortality, we evaluated and described specific properties of the adaptive immune system. Vaccine specific IgG levels in HEU infants and UE controls were investigated as part of a longitudinal study from 2 weeks to 2 years of age. We hypothesised that infants born to HIV positive mothers respond differently to specific vaccinations of the scheduled Expanded Programme on Immunisation of South Africa (EPI-SA), due to increased antigenic exposure, decreased maternal antibody levels and differs according to the type of immunology required to mount responses to certain vaccines.

We further evaluated aspects of immune activation, regulation and apoptosis on both T and B cells by measuring the expression of various cellular markers of immune activation at 18 and 24 months of age at a time of observed decrease of vulnerability to infection (after the first 6 months). We also evaluated the expression of a cellular marker of B cell memory.

We hypothesised that HEU infants display increased levels of immune activation and apoptosis due to increased antigenic and ARV exposure on both T and B cell components of the adaptive immune system that may persist into late infancy potentially indicating long-term immunological differences. In addition, we hypothesised that HEU infants present with decreased levels of B cell memory as a result of increased apoptosis.

This study will therefore add valuable insight and baseline knowledge of the longitudinal vaccine specific antibody response of HEU infants that will serve as groundwork for larger

cohort studies. Furthermore, this study will address the long-term effects of HIV exposure on immune health and provide evidence that will coerce the need to enhance follow-up clinical evaluation of HEU infants.

A detailed understanding of mechanisms that account for the increased incidence of infectious morbidity and mortality in HEU infants is still missing. Therefore addressing potential immune mechanisms to structure appropriate strategies to improve the quality of life in the growing HEU population is relevant to the South African setting, a country with one of the most severe burdens related to the HIV pandemic.

CHAPTER 2

LITERATURE REVIEW

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2.1 OVERVIEW OF THE IMMUNE SYSTEM

Our immune systems are constantly challenged with infectious agents (pathogens) that may lead to disease and if not effectively controlled, may result in death. The ability of the human body to respond to infection and maintain resistance to infectious diseases is termed immunity. The synchronized reaction of cells, tissues and organs to the invasion of pathogenic elements is defined as the immune response. Therefore, the principle function of the immune system is to prevent and eliminate established infections, thereby preventing illness and fatality (Abbas and Lichtman, 2006-2007)

The defence mechanisms of the host comprises of two types of immunity: (1) Innate and (2) Adaptive. Innate immunity is the first protective mechanism against infections and the adaptive is described as a rather slow, but more specific and specialised response to infection (Abbas and Lichtman, 2006-2007). The specific properties of each of these immune compartments will be further described below with specific focus on the adaptive arm of the immune system.

2.1.1. Innate immunity

Innate immunity, also known as natural or native immunity, forms part of the early barriers of protection. Mechanisms of this type of protection detect and destroy pathogens rapidly and do not rely on clonal expansion of antigen-specific lymphocytes (adaptive immunity) (Murphy *et al.*, 2008).

The innate immune system serves well in warning the adaptive immune arm of the invasion of pathogens, thereby provoking the initiation of effective immune responses. Innate immune responses provide secondary signals for the activation of cells of the adaptive immune system, such as B and T lymphocytes (Murphy *et al.*, 2008).

Native immunity plays a key role in controlling infection whilst the adaptive immune arm prepares for defence. Most organisms have evolved to overcome the defences of early immunity, therefore prompting the need for specialised defences. The difference in adaptive compared to innate immunity lies in its specificity in antigen recognition.

2.1.2. Adaptive immunity

Adaptive immune responses develop over the life course of an individual in response to infections with specific pathogens. In contrast to innate immune responses, adaptive immunity is more specific and results in the generation of immunological long-term memory that offers protection against re-infection. Adaptive immune responses are classified as a

more specialised response to infection, thus making any human more vulnerable to disease if they have defects in this immune compartment (Alberts *et al.*, 2002).

Another specialised feature of adaptive immunity is its ability to avoid damaging responses against host molecules. In the event that this mechanisms fails, autoimmune diseases will occur which may lead to death (Alberts *et al.*, 2002).

The main properties that contribute to the specialisation of adaptive immunity is listed and described in Table 2-1.

Table 2- 1: Properties of adaptive immunity (Abbas and Lichtman, 2006-2007)

PROPERTY	SIGNIFICANCE FOR IMMUNITY TO MICROBES
Specificity	Capable of recognising and responding to various types of microorganisms
Memory	Ability to improve response to recurrent or persistent infections
Clonal Expansion	Possess the capacity to compete with rapidly proliferating microbes
Specialisation	Responses to distinct microbes are optimized for defence against these microbes
Non-reactivity to self	Prevents injurious immune responses against host cells and tissues

2.1.2.1 Cells involved in Adaptive immunity

Lymphocytes form the basis of adaptive immunity. They play a significant role in defining the adaptive immune response and occur in large numbers in the blood and lymph. A large portion of lymphocytes are found in lymphoid organs, such as the thymus, lymph nodes and spleen (Alberts *et al.*, 2002)

T and B lymphocytes are the two types of cells that contribute to defining the role of adaptive immunity in specificity and memory. T cells develop from the thymus and B cells from the bone marrow. However, both these cells are believed to develop from the same common lymphoid progenitor cells, which in turn are derived from haemopoietic stem cells that give rise to blood cells, such as red and white blood cell types (Alberts *et al.*, 2002).

In the presence of an antigen, T and B cells become activated, where after they proliferate and mature into effector cells. Effector B cells produce molecules called antibodies, which then mature into plasma cells. Effector T cells on the other hand, produce a diversity of cytokines, which serve as signalling proteins that act as cell mediators. T cells are divided into three main classes. The first is cytotoxic T lymphocytes (CTL), which are involved in the direct killing of infected host cells. The second class of T cells are T helper (Th) cells, which are involved in the activation of cells such as macrophages, dendritic cells (DCs) and B cells. Th cell activation occurs via the secretion of cytokines and the presentation of co-stimulatory proteins on their surface. The third T cell class are T regulatory (Tregs) or suppressor cells, which make use of similar strategies of Th cells and are involved in the control and regulation of the immune response (Alberts *et al.*, 2002).

Th cells are also known as CD4+ T cells. They interact with the major histocompatibility complex (MHC) II molecules on antigen presenting cells (APCs), such as DCs, macrophages and even B cells. There are two main types of Th cells. The first is Th1 cells, which are mainly involved in mediating inflammatory immune responses, and the second is Th2 cells, which play a key role in humoral immunity through the activation of B cells (Abbas and Lichtman, 2006-2007).

Cytotoxic T cells express CD8 molecules on their surfaces and are thus referred to as CD8+ T cells. These cell types bind to MHC type I molecules that present peptides from intracellular organisms, such as viruses, and results in the activation and killing action of

CD8⁺ T cells. The killing action by CTLs results in the release of various toxic molecules, such as perforin and granzymes, which induce the process of cell death or apoptosis. In addition, CTLs could also induce another pathway that occurs through the expression of Fas Ligand (FasL) on the surface of the CTLs, that interacts with Fas on the surface of the target cells, ultimately inducing apoptosis (Abbas and Lichtman, 2006-2007).

There are two forms of adaptive immunity; i.e. cell-mediated immunity and humoral immunity. Both forms are mediated by various cells and molecules and are intended to provide defence against intra- and extracellular pathogens respectively (Abbas and Lichtman, 2006-2007). Each of these responses will be described below.

2.1.2.2 Cell-mediated immunity

The role of cell-mediated immunity is to respond to intracellular microorganisms. This type of immunity is mediated by T lymphocytes.

Activation of T lymphocytes by recognition of MHC associated peptides

The T cell receptor (TCR) is comprised of an α and β chain, which are involved in antigen recognition. These receptors recognise displayed peptides and residues located around the peptide-binding cleft of MHC molecules located around the peptide-binding cleft. Antigens of protein nature from the extracellular environment are taken up into vesicles by APCs, which process them into peptides, which are then displayed by MHC class II complexes. Antigens that are present within the cytoplasm are processed and displayed by MHC class I molecules (Abbas and Lichtman, 2006-2007).

Each mature MHC restricted T cell expresses either CD4 or CD8 molecules called co-receptors, as they function with the TCR to bind to MHC molecules. During this time, the TCR recognises the MHC complexes that are bound to peptides. CD4⁺ T cells recognise extracellular microbial antigens displayed on MHC class II and CD8⁺ T cells recognise peptides from intracellular microbes displayed by MHC class I molecules (Abbas and Lichtman, 2006-2007).

T cell activation through co-stimulation

Well-defined co-stimulators of T cells are proteins known as CD80 (B7-1) and CD86 (B7-2) which are primarily expressed on professional APCs and whose expression is increased during the encounters of APC molecules with microbes. These proteins are recognised by a receptor expressed on T cells, called CD28. Signals that are provided by CD28, allow the binding of B7 proteins on APCs to T cells. This binding provides signals through the binding of T cells and co-receptors to MHC complexes on APCs. The CD28-mediated signals are key signals for initiating responses of naïve T cells. In addition, another set of molecules that provide increased co-stimulatory signals for T cells are CD40 Ligand (CD40L) that bind to CD40 on APCs. This binding stimulates or activates the APCs to express more B7 co-stimulation and secrete cytokines, such as IL-12, which further enhance T cell differentiation (Abbas and Lichtman, 2006-2007).

On the other hand, CD8⁺ T cells recognise antigen peptides from cytoplasmic proteins, such as viral proteins, in any nucleated cell. CD8⁺ cytotoxic lymphocytes in certain viral infections require the activation of CD4⁺ helper cells. During these types of infection, infected cells are taken up by specific APCs, called DCs, after which viral antigens are then “cross-presented”. The same APCs may present viral antigens from the cytosol in MHC I classes. In addition, CD4⁺ T cells may produce cytokines that assist in activating CD8⁺ T cells that lead to their clonal expansion and differentiation into effector and memory CTLs. This type of event may explain the defective CTL responses in individuals infected with HIV that result in the

destruction of CD4+ T cells and not necessarily the CD8+ T cells. However, other viral infections do not seem to require CD4+ T cell assistance (Abbas and Lichtman, 2006-2007).

The role of cytokines in T cell mediated immunity

The secretion of various cytokines with diverse functions is mainly produced by CD4+ T cells that occur in response to antigens and co-stimulation. These molecules are a large set of proteins that function as mediators of immunity and inflammation. Following activation of CD4+ T cells, the first cytokine to be produced is IL-2. This cytokine upregulates the ability of T cells to increase in numbers and respond to IL-2 by regulating the expression of the IL-2 receptor. In addition, the key action of IL-2 is to stimulate the proliferation of T cells; therefore it is often termed the T cell growth factor (Abbas and Lichtman, 2006-2007).

However, in comparison to CD4+ T cells, CD8+ T lymphocytes do not produce IL-2 in response to antigen stimulation. It is believed that antigen recognition drives the proliferation of CD8+ T cells without the requirement of IL-2 (Abbas and Lichtman, 2006-2007).

Th1 and Th2 cells

CD4+ helper T cells differentiate into subsets of effector cells that produce distinct sets of cytokines that possess various functions. These subsets are divided into Th1 and Th2.

Th1 cells are mainly involved in mediating inflammatory immune responses via the activation of macrophages. The most important cytokine produced by this subset is IFN γ , which serves as a potent stimulator of macrophages. In addition, it is also involved in the production of antibody isotypes that promote phagocytosis of microbes, as these antibodies bind to Fc receptors of phagocytes and activate complement. Furthermore, interferon gamma (IFN γ) also stimulates the expression of MHC II molecules and B7 co-stimulators on APCs, thus leading to the amplification of T cell responses. Another important cytokine produced by Th1 cells is the tumour necrosis factor alpha (TNF α) (Abbas and Lichtman, 2006-2007).

Th2 cells are primarily involved in humoral immunity through the activation of B lymphocytes. Cytokines such as IL-10 produced by Th2 cells are involved in the inhibition of macrophages. In addition, cytokines such as IL-4 and IL-10 are involved in the regulation/suppression of Th1 cell types (Abbas and Lichtman, 2006-2007).

2.1.2.3 Humoral immunity

Humoral immunity is mediated by antibodies and function in the elimination of extracellular microbes. Humoral immunity is important in defending the host against bacterial capsules rich in polysaccharides and lipids (Murphy *et al.*, 2008).

Introduction to Antibodies

Antibodies are produced by B lymphocytes and serve as the main mediators of humoral immunity through neutralisation and destruction of extracellular microbes (Murphy *et al.*, 2008).

The antibody molecule is Y-shaped and found in the components of blood or plasma, as well as extracellular fluid. It is composed of two regions: the constant region that holds one of five biochemically distinguishable forms, and the variable region that is composed of an unlimited variety of different amino acid sequences that vary in structure and allow for antibodies to bind to a wide range of antigens (Murphy *et al.*, 2008).

The binding specificity of the antibody is determined by the variable regions. In an antibody molecule there are two identical variable regions that has two identical antigen binding sites (Fab region). The constant region contributes to determining the effector function of the

antibody molecule that defines how the antibody will destroy the antigen once bound (Fc region). In addition, the antibody molecule is composed of two identical heavy and light chains, which both contribute to the variable and constant region, thus allowing the molecule to have a twofold axis of symmetry. The heavy and light chains of the variable regions combine to form the antigen-binding specificity of the antibody molecule (Murphy *et al.*, 2008).

Antibody function

Antibodies can participate in host defence in three main ways: neutralisation, opsonisation and complement activation. In addition to the above listed properties, antibodies also serve a fourth function which is its participation in the process of dependent cellular cytotoxicity where NK cells bind Fc receptors resulting in cell lysis and eventual phagocytosis.

Antibodies are produced after the stimulation of B lymphocytes by antigens in the peripheral lymphocyte organs. B lymphocytes stimulated by antigen then differentiate into antibody secreting cells to produce antibodies of different heavy chain classes or isotypes. Isotypes then enter the blood and progress towards the sites of infection, as well as the mucosal sections, to prevent infection by microbes that enter through the epithelia. This function of antibodies allows them to fight infection throughout the body. Some antigen-stimulated B lymphocytes differentiate into memory cells that do not secrete antibodies but wait for antigen. When encountering these antigens, memory cells subsequently differentiate into antibody producing cells, thus providing a large release of antibodies that are more effective in fighting infection. These properties of antibodies are utilised during the development of vaccines and aims to stimulate the development of long-lived antibody secreting cells and memory (Abbas and Lichtman, 2006-2007).

Immunoglobulin isotopes

There are a total of five known classes of antibodies, immunoglobulin (Ig) A, IgD, IgE, IgG and IgM, each of them possessing their own class of heavy chains (α , δ , ϵ , γ and μ) respectively. IgG and IgA isotypes have various subclasses e.g. IgG1-4 that differ in heavy chains. The differing heavy chains give a distinctive conformation to the hinge and tail regions of the antibodies so that each class and subclass is unique (Alberts *et al.*, 2002).

IgG is the main class of immunoglobulin in the blood. It is classified as a four chain monomer and is produced in large quantities during secondary antibody responses. The tail of the IgG molecules also binds to specific receptors expressed on macrophages and neutrophils by means of Fc receptors and in addition, is involved in the activation of the complement system (Alberts *et al.*, 2002).

Antibody responses to the first encounter with the antigen are termed the primary immune response. The second response to the same antigen is called the secondary immune response. Primary and secondary immune responses differ quantitatively and qualitatively. The antibody concentration during the primary response is much smaller than after repeated exposure (e.g. immunisation). During secondary responses with protein antigens, an increase in heavy chain class switching along with affinity maturation is achieved. This is due to the increase in T lymphocyte help. The secondary exposure to antigens results in improved capacity of antibodies to bind and neutralise microbial elements (Abbas and Lichtman, 2006-2007).

The course of an antibody response is depicted in Figure 2-1 below:

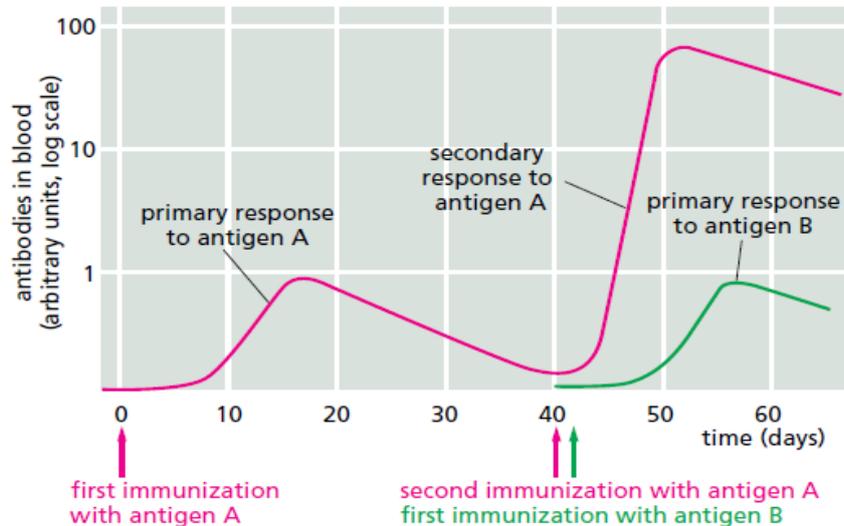


Figure 2- 1: Graphical representation of primary and secondary antibody responses. The secondary response caused by exposure to the same antigen is specific, thus highlighting the specificity of the adaptive immune system. The response to the second exposure is much faster and more efficient at eliminating the pathogen (Alberts *et al.*, 2002).

Antibody response to T-independent antigens

Antigens, such as polysaccharides, lipids and other non protein antigens, cannot bind to MHC molecules and therefore cannot be seen by T cells. These types of antigens elicit immune responses without assistance by T cells (Abbas and Lichtman, 2006-2007).

Defence against bacteria that contain polysaccharide-rich capsules is mainly mediated by antibodies through the process of phagocytosis. Polysaccharide and lipid antigens are believed to contain an arrangement of the same epitopes, which are able to cross-link many antigen receptors on a specific B cell (Abbas and Lichtman, 2006-2007).

The activation of B cells occurs through the clustering of the B cell receptor (BCR) and their related signalling molecules. Ig receptor-mediated signal transduction cross-linking occurs when two or more antigen molecules accumulate or when repeating epitopes of one antigen molecule bind to adjacent Ig molecules in the membrane of a B cell. This often occurs in the case of polysaccharides, lipids and other non protein antigens that contain similar epitopes that can bind to many Ig receptors at once. Receptors are non-covalently attached to proteins, called $Ig\alpha$ and $Ig\beta$, which form the BCR complex. Cross-linking of receptors induces B cell signalling and activation of transcription factors that result in the proliferation and differentiation of B cells (Abbas and Lichtman, 2006-2007).

Antibody responses to T-dependent antigens

Conversely, antigens, such as proteins, are recognised by Th cells, which play a key role in driving B cell activation, as well as heavy chain class switching and affinity maturation. Without T cell help, protein antigens produce weak antibody responses (Abbas and Lichtman, 2006-2007).

This process starts with the activation of T cells via antigen recognition and co-stimulation of APCs, preferentially by protein antigens that are administered with adjuvants. Antigens that stimulate $CD4^+$ Th cells are produced from extracellular microorganisms that are displayed on MHC II molecules on APCs. This process occurs in the cell rich zones of peripheral lymphoid tissues. $CD4$ T cells that recognise these antigens then differentiate into effector

cells that produce cytokines derived from Th1 and Th2 subsets. During this time, some T cells move toward the lymphoid follicles and the antigen-stimulated B and T cells move toward each other. Subsequent to B and T cells encountering each other at the edges of lymphoid follicles, B lymphocytes with bound protein antigen endocytose these antigens in endosomal vesicles and display MHC class II-associated peptides, which are recognised by CD4+ T cells (Abbas and Lichtman, 2006-2007).

CD40L on activated Th cells binds to CD40 on B cells and this result in signals that encourage the B cells to proliferate and secrete antibodies. In addition, Th cells also produce cytokines that bind to cytokine receptors on B lymphocytes and stimulate Ig production.

T cells also assist with signals that are required to stimulate heavy chain class switching and affinity maturation (Abbas and Lichtman, 2006-2007).

The adaptive immune system is designed to fight off infection and allow for the maintenance of good health. However, the immune system is often challenged by pathogens that have developed a number of mechanisms to overcome the protective mechanisms of immunity; one such pathogen is HIV.

2.2 THE HIV PANDEMIC

2.2.1 The global status of the HIV/AIDS pandemic

Nearly four decades after the first encounters with HIV, UNAIDS reports a reverse in the increase in HIV trends since 1999, which was thought to be the pinnacle years of the HIV epidemic. The number of new HIV infections per year has stabilised and deaths due to AIDS related diseases have decreased mainly due to increased access to antiretroviral (ARV) therapy (ART). An estimated 2.6 million new infections was documented in 2009, which has decreased by 19% since 1999 were 3.1 million new infections occurred. Despite these positive advancements towards limiting the spread of HIV, the number of individuals living with the disease is still relatively high and at the end of 2009 the number of infections stood at 33.3 million globally. In this same year, a total of 1.8 million deaths was reported (UNAIDS, 2010).

2.2.2 HIV pandemic in Sub-Saharan Africa

Sub-Saharan Africa continues to bear the greatest burden of the HIV/AIDS global pandemic. Currently it is host to 68% of the global total of infections of which 22.5 million people were living with HIV in 2009. In this region, countries such as South Africa, Ethiopia, Nigeria, Zambia and Zimbabwe are mainly affected by the pandemic. Southern Africa alone accounted for 34% of all HIV infection cases where an estimated total of 11 million people were living with the virus (UNAIDS, 2010).

2.2.3 HIV pandemic in South Africa

In South Africa an estimated 5.6 million people were living with HIV in 2009, classifying this country as the largest host of the HIV/AIDS pandemic. More recent statistics show an increase in the number of people living with HIV from 4.21 million in 2001 to 5.38 million in 2011. South Africa has an HIV prevalence of 10.5% of which 16.6% of the adult population between the ages of 15-49 are infected (StatsSA, 2011).

Furthermore, UNAIDS reports South Africa as being one of the few countries where an increase rise in mother and child mortality has been observed since the 1990s. AIDS is largely responsible for maternal mortality in South Africa and it also accounts for 35% of all deaths in children under the age of 5 years. Despite a decline in the prevalence of HIV, the

number of children orphaned by AIDS has risen and South Africa is currently as one of the 6 countries that has collectively contributed to 9 million HIV/AIDS orphans (UNAIDS, 2010).

2.2.4 HIV infection in women and children

HIV continues to contribute to the increased maternal and child mortality figures. In Sub-Saharan Africa, females between the ages of 15-24 are eight times more likely than men to be HIV positive (UNAIDS, 2010).

In 2009, 370 000 children contracted HIV during the perinatal and breastfeeding period and 260 000 children younger than 15 years died from HIV related diseases (UNAIDS, 2010). Mid-year population statistics for South Africa in 2011 reported 63 600 new HIV infections among children between the ages of 0-14 years of age (StatsSA, 2011).

In 2009, the Department of Health (DOH) of South Africa reported a 29.4% prevalence rate in women attending antenatal clinics after conducting a study across the nine provinces. They reported the highest prevalence in Kwazulu-Natal (39.5%) followed by Mpumalanga (34.7%) with the Northern Cape (17.2%) and Western Cape (16.9%) having the lowest number. In 2009, women between the ages of 30-34 presented the highest prevalence at antenatal clinics in South Africa (DOH, 2010b)

Mother-to-child transmission accounts for a large portion of HIV infection in children. With increased access to ART and the implementation of vigorous programmes to curb the transmission of HIV from mother to child, a decrease in transmission has been reported by UNAIDS. The total number of children born infected with HIV has decreased from 500 000 to 370 000 from 2001 to 2009. This equates to a 24% global decrease in mother-to-child transmission in the last 5 years (UNAIDS, 2010).

2.3 HIV PATHOGENESIS AND IMMUNOLOGICAL ABERRATIONS

HIV infection leads to immune dysfunction and subsequent depletion. In the past years there has been great progress in trying to understand the immunopathogenesis of HIV.

2.3.1 Structure of HIV

HIV-1 is a member of the lentivirus (from the Latin *lentus*, meaning “slow”) subgroup of retroviruses and is a family member of the HIV-2 and simian immunodeficiency virus (SIV). HIV-2 has been described and predominantly found in West Africa and causes less severe disease compared to HIV-1 (Murphy *et al.*, 2008).

The structure of the HIV-1 particle consists of two identical positive strand ribonucleic acid (RNA) copies of the viral genome, which codes for 9 viral genes and is encapsulated by a narrowed capsid, consisting of about 2000 copies of the viral protein, p24. The HIV particle is essentially spherical and is about 120 nm in diameter. A milieu of viral proteins surrounds the capsid, thus maintaining the integrity of the viral particle. A viral envelope surrounds the capsid and is composed of layers of phospholipids (Murphy *et al.*, 2008).

Various viral proteins, such as *gag* (viral core protein containing p24) and *env* (envelope proteins) consists of a cap comprised of three molecules called glycoprotein (gp) 120, as well as a stem consisting of three molecules of gp41 which are embedded in the capsid and play a role in anchoring of the viral envelope to the host cell. The HIV-1 genome contains additional genes, such as *tat* (transactivator), *rev* (the regulator of the virion protein) and *nef* (negative factor), which play a role in virus replication. Infectivity of the virus is further promoted by various accessory genes, such as *Vif* (virion infectivity factor), *Vpr* (viral protein R) and *Vpu* (Viral protein U) (Murphy *et al.*, 2008).

2.3.2 Viral entry and replication

Cellular tropism is essentially defined as the ability of HIV to enter particular cell types and is determined by the expression of specific receptors on the surfaces of those particular cells. HIV is known to bind mainly to cells, such as DCs, CD4⁺ T cells and macrophages. The entry of HIV into specific cell types involves a complex of two viral glycoproteins, gp120 and gp41, contained on the viral envelope. The portion of the gp120 glycoprotein binds with increased affinity to the cell-surface of the CD4 molecules. Prior to the synthesis and entry of the virus into the host cell, gp120 must also bind to a co-receptor on the membrane of the host cell. There are a number of chemokine receptors for HIV viral entry; the well described chemokine co-receptors CC-chemokine receptor 5 (CCR5), which is mainly expressed on DCs and macrophages, or the CXC-chemokine receptor 4 (CXCR4) chemokine, which is expressed on activated T cells. Binding of gp120 to gp41 subsequently results in the integration of the viral envelope with the cell plasma membrane, thus allowing the viral genome including associated proteins to enter the cell cytoplasm (Murphy *et al.*, 2008).

During the infection of a host cell, the RNA genome of the virus is reverse transcribed into copy deoxyribonucleic acid (DNA) (cDNA) by viral encoded reverse transcriptase. Viral integrase allows for viral cDNA to be integrated into the host's genome. The copy of cDNA is then referred to as the provirus. HIV can establish a latent infection during which the provirus remains dormant, which have been observed to occur in memory CD⁺ T cells and macrophages, thus serving as important reservoirs of infection (Murphy *et al.*, 2008).

2.3.3 The immune response following HIV infection

Upon HIV infection, both innate and adaptive immune systems respond by controlling HIV viral replication; nonetheless, a number of immune dysfunctions result in HIV-infected individuals. A number of viral strategies for overcoming the hosts immune control mechanism have been identified. Immune responses, such as phagocytosis, are simply not efficient; however, both cell-mediated and humoral immunity play central roles in controlling infection. In untreated individuals, viral replication is not completely controlled, thus leading to increased viraemia, disease progression and ultimately the development of AIDS (Murphy *et al.*, 2008).

HIV infection generates an immune response that controls the virus, but is unable to eradicate the disease. During the asymptomatic phase there are very low concentrations of HIV in the peripheral blood due to the persistent replication of virus in the lymphoid tissues. CD4⁺ T cell counts gradually decline and CTLs remain relatively high, however eventually become exhausted and lose their function resulting in increased concentrations of HIV in the peripheral blood. The host develops antibodies to the envelop protein (*env*) and the core protein (p24) (humoral immune response), which assist in trying to control and eliminate infection (Murphy *et al.*, 2008).

During the acute phase of viral infection, both adults and children present with increased levels of viraemia within 2 months of infection. The acute phases however, differ in adults and children with regards to the initial responses. Where adults experience a "brief burst" of high plasma viraemia, which is controlled by both cellular and humoral immunity, the levels of plasma viraemia in children lasts for much longer. The number of CD4⁺ T cells generated increases drastically to compensate for lost cells. These cells however, become new targets for viral replication and destruction. The lack of CTL functionality in infants, may explain the increased viraemia during the first stages of infection (Leal *et al.*, 2007).

2.3.4 Immune activation of T cells and chronic infection

It has been documented that HIV infection not only contributes to immunodeficiency, but also results in dramatic immune activation, which serves as one of the underlying mechanisms of

immune dysfunction. The exact mechanisms for T cell activation is still unclear, but is believed to be a major contributor to HIV pathogenesis. HIV pathogenesis is achieved when the virus depletes all CD4+ T cell populations, which is believed to occur within the first few weeks of infection (Hunt, 2007, Cohen *et al.*, 2008)

The review by Hunt (2007) highlights the potential mechanisms that may outline the reasons for T cell activation other than via the recognition of HIV antigens, which is believed to account for a relatively small portion of the T cell repertoire. Suggestions by evaluation of *in vitro* studies, implicate the binding of gp120 to CD4 or co-receptors of the molecule, thus inducing activation without direct infection. In addition, bystander T cells may be become activated by infected macrophages through the *nef* accessory protein. In addition, the regulatory T cells that play a key role in controlling T cell activation may be affected during HIV infection, rendering it unable to serve its function. T cell activation may also be triggered by constant activation of the innate immune system that produces inflammatory cytokines, which in turn triggers the constant stimulation of T cells (Hunt, 2007).

An overtly active immune system represents a prime feature of chronic immune activation. Studies have hallmarked the relationship between CD4+ T cell activation and subsequent depletion, which contributes to disease progression and continuous viral replication.

Activation of both CD4+ and CD8+ T cells express a number of surface activation markers, such as CD38 and human leukocyte antigen (HLA)-DR. The expression of CD38 on CD8+ T cells is classified as the most well described surrogate marker of immune activation and disease progression in HIV. It is even considered a better predictor than CD4+ T cell counts and HIV RNA levels (Giorgi *et al.*, 1999, Giorgi *et al.*, 2002).

CD69 is described as an early marker of immune activation of T cells. The expression of this protein molecule acts as a co-stimulatory molecule for T cell activation and proliferation. A study by Nielsen *et al* (2008) showed a correlation between lymphocyte proliferation and expression of CD69 in a group of HIV positive individuals (Nielsen *et al.*, 1998). Other studies have indicated impairment in the expression of CD69 in patients with advanced stages of HIV. These patients do not respond with an increase expression of CD69 upon stimulation with an antigen such as Staphylococcus enterotoxin B (Pitsios *et al.*, 2008).

Continuous activation of T cells causes the exhaustion and loss of both naïve and memory T cell pools, thus resulting in T cell homeostasis that leads to T cell depletion (Hazenberg *et al.*, 2003). A commonly described reason for T cell depletion during immune activation events is apoptosis.

2.3.5 Increased apoptosis during HIV infection

Introduction to apoptosis

Apoptosis or cell death is described as a normal physiological process that contributes to the maturation and maintenance of tissue homeostasis. The process is characterised by a number of morphological and biochemical features, such as loss of cell structure, chromatin condensation and breakdown of cell DNA. Apoptosis plays important roles during other physiological processes, such as the development of embryos and vascular systems, as well as during the modification of cells and tissues (Wyllie *et al.*, 1984).

Apoptosis also plays a crucial role in adaptive immune responses, such as the development of lymphocytes. Through the process of apoptosis, autoimmunity is prevented by negatively selecting those cells that do not bind correctly to appropriate structures (e.g. if a T cell binds too strongly to an MHC molecule). Apoptosis is responsible for the termination of an immune

response by deleting self-reactive T cell clones and by the elimination of malignant and virus infected cells (Sloand *et al.*, 1997).

During apoptotic events, a number of changes occur in the cell membranes of apoptotic cells. The loss of phospholipid asymmetry allows for the exposure of phosphatidylserine (PS), which then translocates to the outer cell membrane during the process of apoptosis. Annexin V is believed to be an early marker of apoptosis, which binds to PS with high affinity due to its calcium dependent phospholipid-binding nature and remains bound throughout the process of apoptosis. Therefore, by the use of flow cytometry and the conjugation of the Annexin V protein to a fluorescent dye, cells that are undergoing apoptosis can be detected (Fadok *et al.*, 1992, Vermes *et al.*, 1995, Zhang *et al.*, 1997).

Apoptosis during HIV infection

The depletion of T lymphocytes in HIV infection is believed to be due to the processes of apoptosis or induced cell death. Studies have described an increased level of spontaneous apoptosis of HIV infected and uninfected lymphocytes as a result of increased immune activation (Sloand *et al.*, 1997).

A number of studies have described T cell loss during HIV infection as either a direct destruction by the virus or through the defective depletion of T cells during HIV progression phases (Stan *et al.*, 2008, Yue *et al.*, 2005). It has also been documented that a large number of cells that undergo apoptosis are uninfected (Vassena *et al.*, 2007). Furthermore, by use of appropriate scientific models, researchers were able to study the influence of apoptosis on the dynamics of HIV infection, which was observed to play a key role in immunological failure (Mhaweji *et al.*, 2009).

Banda *et al.* (1992) has proposed that the initiation of apoptosis occurs when gp120 cross-links with the CD4 receptor (Banda *et al.*, 1992). Other studies have proposed that upon this cross-linking reaction, the upregulation of Fas occurs due to increased expression of IFN γ and TNF α (Oyaizu *et al.*, 1994). In addition, other viral proteins, such as *tat*, have been documented to increase the levels of cell apoptosis (Alimonti *et al.*, 2003). Some studies have even proposed that infected APCs, such as macrophages, are involved in directing the apoptosis of CD4⁺T cells (Badley *et al.*, 1996).

During HIV infection it is the Fas/FasL system which mediates T cell apoptosis. Fas or CD95 is a cell receptor that belongs to the TNF family. The Fas protein is expressed on activated mature lymphocytes, but do not occur on resting naïve T cells. Fas contain so-called death domains in its cytoplasmic tail, which are responsible for the activation of caspases involved in initiating the apoptotic process (Murphy *et al.*, 2008, Sloand *et al.*, 1997).

Elevated levels of Fas have been reported in HIV infected individuals and have been observed to play a role in increased disease progression (Poonia *et al.*, 2009)

2.3.6 Other T cell abnormalities associated with HIV infection

In addition to the loss of CD4⁺ T cells along with the loss of CTLs, HIV infection is also associated with impaired thymic output, thus leading to impaired naïve T cell pools (Gaulton, 1998). IL-7 is a pleiotropic cytokine that plays a key role in controlling T cell homeostasis, enhancing CTL function and increasing T cell survival. The IL-7 receptor complex (IL-7R), consists of two subunits; one of them being the IL-7R α chain (CD127) which is expressed on a number of cells including naïve and memory T cells and the other a common- γ chain receptor that is shared on a variety of cytokines. Disruption of the IL-7 signalling pathway thus results in impaired immunity (Fry *et al.*, 2001, Fry and Mackall, 2002, Hofmeister *et al.*, 1999). Studies have documented a decrease in CD127 expression on CD8⁺ T and CD4⁺ T cells, thus playing a role in disease progression amongst HIV positive individuals (Young

and Angel, 2011). The same study found that HIV infection modifies the pathway of IL-7 signalling, thus leading to a blockage in thymopoiesis and the “arrest” of T cell development. The study further concluded that the blocking mechanisms play no role in the expression of CD127. In contrast, other studies have found decreased expression of CD127 on CD8 T cells, which resulted in increased apoptosis of the same cell type (CD8 T cells), thus leading to chronic HIV infection. It was also found during *in vitro* studies, that with addition of IL-7, the expression of CD127 was restored and a decrease in apoptosis was noted. This study further highlighted the use of IL-7 as immunotherapy in HIV infected patients (Zhang *et al.*, 2009).

T cell responses are modulated through the balance of both co-stimulatory, as well as co-inhibitory signals that result in signalling molecules that play key roles in the regulation of T cell activation and tolerance. For example, pathways such as the B7:CD28 family that produce signals to activate and halt T cell responses, thus serve important roles in creating immune tolerance and preventing autoimmunity (Kaufmann and Walker, 2009). CD28 is the co-stimulatory molecule, whereas CTLA-4 and PD-1 serve as inhibitory molecules/T cell regulation. CD28 and CTLA-4 are known to share the same ligands, i.e. B7-1 (CD80) and B7-2 (CD86) and PD-1 interacts with B7-H1 (PD-L1) and B7-DC (PD-L2). During the recognition of MHC peptides by TCR, which serves as signal 1, and CD80 and CD86 by CD28, which serves as signal 2, activation, proliferation, differentiation and the production of cytokines occur. Following the TCR signal, PD-1 and CTLA-4 expression occurs. The binding of the TCR to one of these receptors leads to the arrest of the cell cycle, thereby terminating the T cell activation pathways (Kaufmann and Walker, 2009).

In HIV infection, the increased expression of PD-1 on HIV specific CTLs had been associated with CTL dysfunction. Cells with increased expression of PD-1 displayed impaired proliferating responses to the related antigen during *in vitro* studies. PD-1 expression was also associated with increased viral loads and disease progression, thus upon the receipt of ART resulted in a decrease in PD-1 expression (Petrovas *et al.*, 2006, Day *et al.*, 2006, Trautmann *et al.*, 2006). Studies have also shown a direct relationship between the expression of PD-1 and increased apoptosis. CTLs that expressed PD-1 were observed to be more vulnerable to undergoing both spontaneous, as well as Fas mediated apoptosis (Petrovas *et al.*, 2006).

The expression of CTLA-4 on the cell surface is induced after TCR signalling processes and consecutively presented on CD4⁺ FOXP3⁺ Treg cells. Naturally occurring FOXP3⁺ regulates Treg expression of CTLA-4 and dominantly controls T cell self tolerance. Blockage of CTLA-4 thus results in the abortion of Treg activity during both *in vitro* and *in vivo* studies. In addition, CTLA-4 absence such as lymphoproliferative diseases can be controlled by providing CTLA-4 sufficient T cells thus suggesting functional defects in Tregs in the absence of CTLA-4 (Sojka *et al.*, 2009, Jain *et al.*, 2010)

Various studies have conferred varying roles of CTLA-4 expression on T cells during HIV infection. Some studies showed that during progressive disease, overtly increased expression of CTLA-4 on CD4 T cells was noted that indirectly correlated with CD4 count (Leng *et al.*, 2002). Other studies have shown the expression of CTLA-4 during the acute HIV infection stage (Zaunders *et al.*, 2006). In addition, a study by Kaufmann *et al.* (2007) showed that CTLA-4 expression was shown to occur during various stages of HIV infection, except in individuals who had the ability to maintain virological control (without ART) (Kaufmann *et al.*, 2007).

CD40 ligand is mostly expressed on activated CD4⁺ T cells and plays a role in B-cell proliferation and Ig class switching. The interaction of CD40 with CD40L is also responsible for the ignition of the immune response (Romano *et al.*, 2006). Soluble CD40L levels have been found to be increased in HIV-infected patients that have undergone successful ART

(Sipsas *et al.*, 2002) and other studies have verified a long-lasting effect of ART on the maintenance of total lymphocyte counts (Bunders *et al.*, 2005a). The positive features of ARV drugs are not only to reduce viral replication, but also to promote viral immunity. Other studies have found similar findings in both HIV and non-HIV cases where other anti-virals were administered (Sipsas *et al.*, 2002). In addition, treatment with nucleoside analogues was also found to increase the expression of CD40L (Bergamini *et al.*, 2002).

The binding of CD40L on activated T cells to the CD40 receptor on B cells leads to the increased expression of costimulator molecules, such as CD80 on other APCs (O’Gorman *et al.*, 2001). Abnormal expression of CD40 ligand can lead to abnormalities in both the T and B cell compartments of the immune system. O’Gorman *et al.* (2001) showed that the levels of CD40L were aberrantly lower in a group of HIV infected children when compared to negative children born to HIV infected mothers. It is hypothesised that this could be due to the loss of a subpopulation of Th cells, which produce CD40L upon stimulation.

2.3.7 B cell abnormalities during HIV infection

Despite CD4+ T cells being the primary target for HIV infection, the virus ultimately affects all lymphocyte populations (De Milito, 2004). In fact, B cells were the first dysfunctional lymphocytes to be described in AIDS patients (Lane *et al.*, 1983). These patients were characterised by hypergammaglobulinaemia, polyclonal B cell hyperactivation and poor stimulation of B cells *in vitro*.

Aberrant hyperactivity of B cells during HIV infection may serve to be of benefit to the virus in order to avoid effective anti-viral B cell responses. Although a number of studies have extensively addressed the parameters of HIV pathogenesis, the underlying cause of B cell activation during infection remains unknown and has contributed to a number of debates in research (Haas *et al.*, 2011).

B cell dysregulation in HIV disease may be a result of irregular immune activation. Features of B cell hyperactivation in HIV disease include increased levels of Ig and autoantibodies, expansion in B cell areas of lymphoid tissue, increased expression of activation, proliferation and terminal differentiation markers on circulating B cells. In addition, activation of B cells during HIV infection has been documented to be the cause of B cell lymphomas (Moir and Fauci, 2008).

Whether HIV directly infects B cells are unknown. However, studies have detected viral products displayed on the surface of blood and lymph node isolations from infected individuals (Moir *et al.*, 2000). The described reason for this observation is linked to the interaction of complement-opsonised HIV virions to CD21 receptors expressed on B cells. This argument was further supported by evidence from Stoiber *et al.* who delineated the role of CD21 receptors in the capture of HIV virions opsonised with complement factors (Stoiber *et al.*, 2003).

The direct effects of HIV on B cells may not be the only phenomenon linked to B cell aberration during infection. Other effects may include the indirect stimulation by CD4+ T cells (Grossman *et al.*, 2006) or the binding of HIV through a superantigen effect (Berberian *et al.*, 1993).

In addition, Treg abnormalities during HIV infection may result in immune activation as a consequence of the development of abnormal T cell subpopulations (Eggena *et al.*, 2005). These Treg abnormalities may cause direct B cell dysfunction. Studies by Lim *et al.* (2005) explored the effect of Tregs on B cell Ig responses. Tregs may potentially migrate to B cell areas of the secondary lymphoid tissues and result in the suppression of T cell-dependent B cell IgG response. Researchers reported the presence of FOXP3+ Tregs in T-B areas of the

germinal centers which can cause direct suppression of B cell Ig responses. The suppression of B cells Ig production by Treg is accompanied by inhibition of Ig class switch recombination (Lim *et al.*, 2005). Thus during HIV infection, abnormal functioning of Treg cells may result in uncontrolled B cell proliferation.

A number of changes in B cell subpopulations have been observed during HIV infection. CD27 is a commonly defined marker of B cell memory, which has been shown to be depleted (De Milito *et al.*, 2001, Nagase *et al.*, 2001). Furthermore, there has also been evidence that indicate loss of memory B cells during early ART (Titanji *et al.*, 2005). Other subpopulations of B cells, such as CD10 (immature/transitional B cells), are increased with increased levels of immune activation (Malaspina *et al.*, 2006).

As noted with T cell activation, apoptosis occurs along with B cell activation and cell death during HIV infection. There are two major pathways described for apoptosis, which include (1) the intrinsic pathway triggered by mitochondrial apoptosis, and (2) the extrinsic pathway activated by the death receptor (Fas). Immature B cells are more susceptible to following the intrinsic pathway for the early deletion of defective B cells, as a result of the low expression of Bcl-2, which is often associated with cell survival. Mature or activated B cells are more likely to express Fas, which makes them more susceptible to apoptosis via interaction of FasL; therefore it makes use of the extrinsic pathway. Fas is considered as the most over-expressed death receptor on B cells in HIV patients with high viral loads (Moir *et al.*, 2004). During HIV infection, both mature and immature B cells are present in high quantities, thus making them more susceptible to cell death via either apoptotic pathways (Moir and Fauci, 2008).

A major contradiction observed with B cell aberration during HIV infection is the increased observation of activation *in vivo*; however, individuals respond poorly to *ex vivo* B cell stimulation, such as immunisation. This observation therefore highlights the role of viral replication as the driving force behind B cell hyperactivation and dysfunctions (De Milito, 2004). This idea was further supported by studies that have shown the link between HIV viraemia and the expansion of terminally differentiated B cells with high levels of Ig that do not respond to stimuli and are more susceptible to apoptosis (Moir *et al.*, 2001, Moir *et al.*, 2004).

The functionality of B cells can be assessed by evaluating the interaction or co-stimulatory effects between CD4+ T cells and B cells. Upon activation by antigen, B cells display antigen proteins to CD4+ T cells. As described in previous sections, this occurs via the interactions between CD80/CD86 receptors, which are upregulated after B cell activation and interaction with CD28 on CD4+ T cells. Studies have shown the inability of activated B cells to provide stimulatory signals to CD4+ T cells in HIV infection and in addition, are also associated with an impaired interaction between CD40L on T cells and CD40 on B cells (Malaspina *et al.*, 2003, Moir *et al.*, 2003).

As previously alluded to, ART does not restore memory B cell pools. Thus, HIV infected individuals on ARV therapy may not generate antigen specific B cells after immunisation (Malaspina *et al.*, 2005, Titanji *et al.*, 2006). In addition, memory B cell responses that require CD4+ T cell assistance may be impaired, thus reducing the generation of antigen specific B cells. Hart *et al* (2007) has demonstrated defective responses to T-cell independent vaccines, such as pneumococcal polysaccharides and measles, thus increasing susceptibility to pneumococcal disease (Hart *et al.*, 2007). In the paediatric population, poor antibody responses have been documented in both T-independent and T-dependent instances, which explains the increases susceptibility to bacterial infections in this population (Bliss *et al.*, 2008, Obaro, 2004, Dankner *et al.*, 2001, Titanji *et al.*, 2006).

2.4 EFFECTIVE INTRODUCTION OF PREVENTION OF MOTHER-TO-CHILD TRANSMISSION (PMTCT) PROGRAMMES

During pregnancy, HIV infected women have a 15-45% chance of transmitting the virus in the absence of prophylactic treatment. The UNAIDS (2010) reports that without preventative measures, HIV infected women have about a 1 out of 3 chance of transmitting the virus to her offspring. Almost half of the infants infected with HIV from their mothers will die before the age of 2 years without effective treatment.

In order to decrease the rate of transmission from an infected mother to her child, vigorous implementation of PMTCT programmes along with increased access to ART has resulted. Therefore, with the administration of ART during pregnancy and delivery to both mother and infant, the risk of transmitting the virus is decreased to less than 5% (DOH, 2009).

South Africa, along with Botswana, Namibia and Swaziland, has thus far achieved above 80% coverage of ART to prevent transmission. Coverage in South Africa and Zambia was reported to be around 95%, whereas Chad and the Democratic Republic of Congo (DRC) was reported to have coverage of only 6% and 9% respectively (UNAIDS, 2010).

The national PMTCT programme in South Africa was established and initiated in 2001, which was then followed by the initiation of the ART schedule in 2004. The original PMTCT programme involved using a single dose nevirapine (NVP) regime, which was then amended in 2008 to the use combination/dual therapy and included both AZT and NVP, which was recommended by the WHO (DOH, 2009). These initiatives were further enhanced and modified to address the increased mortality observed amongst women and children.

During the course of April 2010, revisions of the programme in keeping with the guidelines of the WHO, stipulated that all women with a CD4 cell count less than 350 cells/ μ l were considered eligible for ART. Pregnant women, who tested HIV positive, would receive treatment at 14 weeks gestation rather than in the final trimester. Previously, a CD4 count of 200 cells/ μ l served as the guideline for initiating therapy in pregnant mothers (DOH, 2010a).

These changes highlight constant improvements to decrease the spread of HIV from infected mothers to infants. In addition, it has been reported that along with a functioning health system, PMTCT programmes and interventions may be able to eliminate HIV infection in children (Sprague *et al.*, 2010).

2.5 THE HIV EXPOSED UNINFECTED (HEU) INFANT PROBLEM

2.5.1 The increasing number of HEU infants

The number of HIV positive pregnant women continues to grow annually around the globe. This growth is attributed to increased access to ART, as well as improved and expanded PMTCT services as highlighted in preceding sections, especially in low- and middle-income countries combined with revisions of the WHO guidelines (Heidari *et al.*, 2011). As these programs are increasingly accessed by pregnant women, the number of children born with HIV is decreasing. As a result, the number of infants born exposed to HIV during foetal development will increase annually around the world. Out of the 1 million babies born in South Africa annually, about 300 000 of these infants are born exposed to HIV (DOH, 2009). HEU infants represent 30% of all children born in some areas of South Africa (Shapiro and Lockman, 2010).

Although these HEU children remain HIV negative, there has been constant documentation supporting increased infectious morbidity and mortality that highlight the need for extensive clinical follow-up (Filteau, 2009, Slogrove *et al.*, 2009). These observations classify HEU infants as an increasingly important vulnerable group.

The reason for increased morbidity and mortality in the HEU population is yet to be delineated; however, it is believed to be multi-factorial. Factors such as low-birth weight, poor growth, exposure to ART, inadequate clinical follow-up, reduced breastfeeding, as well as immune abnormalities, have all been suggested as contributing risk factors (Figure 2-2) (Filteau, 2009). In addition, a review by Filteau (2009) also highlights the limited knowledge available on the health of HEU infants from low-socioeconomic backgrounds. Most of the current knowledge available on the HEU population is derived from developed countries, where issues such as health care, diet, exposure to environmental pathogens and poverty, differ drastically to third world countries.

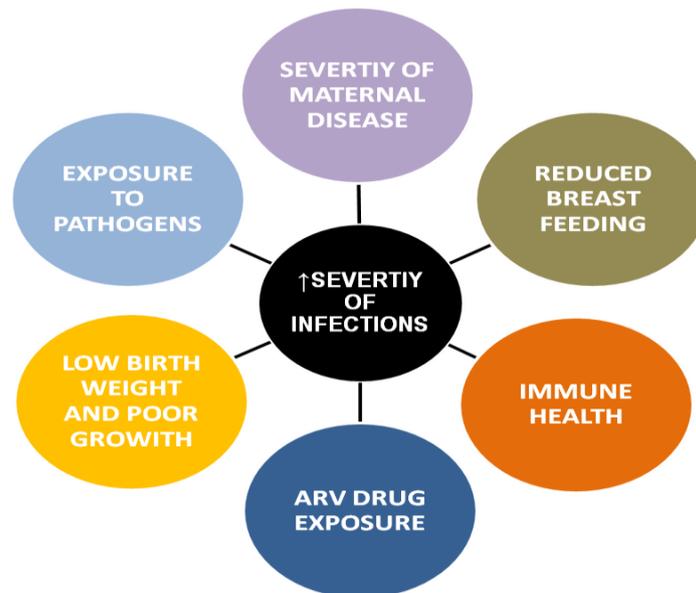


Figure 2- 2: Contributing factors that may account for the increased morbidity and mortality within the HEU population (Filteau, 2009).

2.5.2 Increased mortality in HEU infants

An increase in mortality has been well-documented in HEU cohorts from both developed and developing worlds.

A pooled analysis of seven MTCT trials of HIV infected women was conducted by Newell *et al* (2004), who observed the mortality rates of 3 468 children that were followed over a 15½ month period. The study found strong correlations of infant mortality with maternal death and severe maternal immunodeficiency (Newell *et al.*, 2004).

Studies conducted in the United States of America (USA) show increased mortality in both HEU and HIV infected infants in early infancy. They showed that infants born to HIV infected mothers have increased risk of death during the first two years of life (Paul *et al.*, 2005). Research conducted in Zimbabwe and Malawi showed similar mortality rates for the same infant age and further highlighted the impact of HIV exposure on long-term infant health (Marinda *et al.*, 2007, Crampin *et al.*, 2003). The latter study also observed that uninfected infants born to HIV infected mothers have at least twice the mortality risk than infants born to HIV negative mothers. They also showed correlations with HEU mortality and low birth weight, low maternal CD4 counts, maternal death and social morbidities, such as low household income. Death associated with birth weight was also shown in studies conducted in Tanzania (Wei *et al.*, 2004).

Similar studies by Zaba *et al* showed a relationship between maternal death and child death in a pooled analysis of three African countries (Tanzania, Uganda and Malawi) (Zaba *et al.*, 2005).

In a study conducted in a Ugandan cohort, maternal HIV status along with maternal survival seemed to be a common dominator for the outcomes of child mortality (Nakiyingi *et al.*, 2003). A Zambian study observed that increased mortality amongst HEU infants was associated with maternal death occurring within four months of delivery. Interestingly, the study observed a reduction in infant mortality with every 100 cell/ μ l of CD4+ T cell increase in the mother after adjusting for haemoglobin levels, birth weight and maternal death factors. The risk of death in infants whose mother died within 4 months of delivery was just under 48% (Kuhn *et al.*, 2005).

A recent study conducted by Kuhn *et al.*, observed a cumulative mortality rate of just under 14 % amongst 749 HEU infants who were followed up from 1-24 months of age. They further reported early weaning as a high risk for infant mortality in comparison to weaning beyond 18 months of age. In addition, they noted a greater infant mortality within the first year of life; however, the risk associated with weaning had progressed over time. Furthermore, researchers found that additional risk factors for child death were related to maternal death, limited access to food, as well as households with other children under the age of 5 years of age (Kuhn *et al.*, 2010). Shapiro *et al* also found an association of early weaning and mortality in HEU infants compared to UE counterparts (Shapiro and Lockman, 2010).

Although there have been a number of studies that have documented increased mortality amongst HEU infants, other studies have reported no significant differences between HEU and UE infants in Zambia and Malawi (Taha *et al.*, 1999, Sutcliffe *et al.*, 2008).

In general, the mortality of HEU infants is higher than that of an UE child. The reason behind the significant mortality is unclear but seems to be combination of factors that include maternal death, weaning, severity of maternal disease as well as low birth weight. Observations of many studies prompt the need for extensive clinical follow-up of both mother and infant pairs.

2.5.3 Increased morbidity in HEU children

A large Zimbabwean study consisting of 14 110 mother-infant pairs enrolled as part of the Zimbabwe Vitamin A for mothers and babies (ZVITAMBO) trial cohort reported a significantly higher morbidity amongst HEU infants compared to UE counterparts. They reported significantly increased levels of lower respiratory tract infections during the first half of infancy, which seemed to be transient after the first 6 months. A large proportion of HEU infants presented with oral thrush during infancy which seemed to be about 2 to 3 times greater compared to UE counterparts. Overall, increased morbidity was more profound in HEU infants whose mothers presented with more severe HIV disease. A significantly higher morbidity rate was noted in infants whose mothers had a CD4 cell count below 800 cells/ μ l (Koyanagi *et al.*, 2011).

A study conducted in Latin America and the Caribbean Countries found common infections related to skin or mucous membranes and upper and lower respiratory tract infections. A total of 17.5% of infants in this study were hospitalised with infection mostly related to lower respiratory tract diseases. In addition, this study also noted an association with maternal disease severity and HEU morbidity (Mussi-Pinhata *et al.*, 2007).

Kuhn *et al* also observed increased morbidity in a cohort of Zambian HEU infants also associated with severity of maternal HIV disease. This study observed increased hospitalisations related to pneumonia and/or sepsis, diarrhoea, as well as malaria.

Hospitalisations were also associated with low maternal CD4 count, as well as the lack of breastfeeding (Kuhn *et al.*, 2005)

A number of studies have documented increased cases of pneumonia amongst children born to HIV positive mothers. A South African study documented increased disease severity in 8 HEU infants, most notably to infections such as *Pneumocystis jiroveci* pneumonia, *Pseudomonas sepsis* and CMV colitis (Slogrove *et al.*, 2009). Similarly other studies have also documented increased events of the opportunistic infection, *Pneumocystis jiroveci* pneumonia in HEU infants (Heresi *et al.*, 1997, McNally *et al.*, 2007).

Earlier studies have documented an increased incidence of diarrhoeal disease in HEU children, which was observed to be twice as high when compared to UE infants (Thea *et al.*, 1993). Mussi-Pinhata *et al.* had documented an increase in lower respiratory tract infections amongst HEU infants. Acute bronchiolitis in the first 6 months was reported as a common infection amongst this group. In addition, this study also found an association between low maternal and infant CD4 counts as a risk factor for lower respiratory tract infections (Mussi-Pinhata *et al.*, 2010).

HEU infants in addition are also at increased risk of exposure to infections related to parental illness, such as tuberculosis. This may often be the case in high prevalence settings (Cotton *et al.*, 2008). They may also be exposed to other viral infections during intrauterine development, such as CMV, which is a common infection in immunodeficient patients (Bates *et al.*, 2008). In addition, due to the lack of breastfeeding, these infants may also be at greater susceptibility to dietary pathogens thus leading to enteric diseases.

Due to low birth weight and increased morbidity amongst HEU infants, abnormal growth parameters have been documented. Growth is characterised as a potential indicator of overall infant and child health. During the first half of infancy, HEU infants of the DRC have shown similar weights, but different lengths. However, no association between maternal HIV infection and growth was found (Bailey *et al.*, 1999). In contrast, a Zambian study observed both lower weight and length (after adjusting for gestational age) in HEU infants in the first 6 weeks of life (Makasa *et al.*, 2007).

The European Collaborative study (2003) however, showed increased weight for body mass index in HEU children, which may be a result of being formula fed. However, it seems as though HEU children from African descent tend to show a less rapid catch up growth compared to European cohorts (European-Collaborative-Study, 2003). The consequences of poor growth in these infants is yet to be delineated and whether there may be an association with poor health, obesity and chronic ailments (Filteau, 2009).

2.5.4 Effects of exposure to ART

There have been a number of documented effects of ART exposure during foetal and infant development. Mitochondrial dysfunction has been reported as a common side effect of ART in HEU infants (Noguera *et al.*, 2004). In contrast, the European Collaborative study (2005), found an association of ART and anaemia in the early life of HEU infants; but found no verification of ART associated mitochondrial abnormalities (European-Collaborative-Study, 2005).

Other studies have found relationships of ART exposure with abnormal T lymphocyte counts, as well as CTL counts in HEU children that persisted from infancy until 8 years of life (Bunders *et al.*, 2005b). ART has also been linked with abnormal neutrophil and red blood cell counts in the HEU populations (Pacheco *et al.*, 2006, Feiterna-Sperling *et al.*, 2007). Other effects of include decreased birth weights as noted in Thai infants; which seemed to decrease over time (Briand *et al.*, 2006)

Although the effects of ART should be carefully examined, the benefits of therapy far outweigh its side-effects, which seem to be transient. Severe clinical outcomes have been documented in HEU infants who have not been exposed to ART, thus indicating that its effects are not the only “alarm” for the poor health of HEU cohorts.

2.6 IMMUNE ABNORMALITIES IN HEU INFANTS

Despite an HIV negative status, HEU infants display a number of immune abnormalities that may represent the effects of early HIV exposure. These immune abnormalities may serve as contributing factors for the increased morbidity and mortality observed in these infants and children.

As previously outlined in this review, a number of multifaceted patterns in the changes of the immune system are associated with HIV infection. These changes include alterations in T and B cell populations, increases in CTLs, changes in cytokine profiles and major B lymphocyte defects.

During foetal development, the growing foetus of an HIV positive mother comes into contact with HIV particles and proteins. HIV-specific cellular immune responses have been reported in more than one-third of HEU infants evaluated by Kuhn *et al* (Kuhn *et al.*, 2002). The earliest of studies have even documented HIV specific lymphocyte proliferation following stimulation with HIV proteins (Borkowsky *et al.*, 1990) and other studies have shown CTL responses to HIV epitopes (Rowland-Jones *et al.*, 1993, Aldhous *et al.*, 1994). In addition, investigations have reported memory Th cell responses to HIV peptides at birth (Kuhn *et al.*, 2001b, Clerici *et al.*, 1993, Wasik *et al.*, 1999).

A study by Vázquez Pérez *et al* (2006) observed HIV-1 DNA in peripheral blood mononuclear cells (PBMC) from HIV exposed non-breastfed children at 3 months of age. The evidence of this study has prompted researchers to evaluate whether these findings may be associated with increased protection against HIV, which may explain HIV specific immune responses, but no infection (Vázquez Pérez *et al.*, 2006).

Evidence for immune activation and inflammation has also been documented. Studies by Khun *et al* (2001a) investigated the relationship between Th1 and Th2 cytokine production in cord blood leukocytes in both HIV infected and uninfected mothers. Infant cytokine levels were measured at birth and at 6 months of age. They showed that exposure to HIV *in utero* was associated with a greater increase of Th1 cytokines (IFN γ) than Th2 (IL-10) in cord blood cells of HEU infants than UE infants thus reflecting exposure to an inflamed environment (Kuhn *et al.*, 2001a). Similarly, more recent studies have shown decreased production in the levels of IL-4 and IL-7 in cord blood mononuclear cells of HEU newborns, but increased levels of IL-10 and IFN γ . Furthermore, they found that the expression of IFN γ and TNF α were higher in the supernatant of HIV positive mothers (Borges-Almeida *et al.*, 2011).

A study by Schramm *et al* (2006), evaluated a host of plasma markers, such as neopterin, β 2-microglobulin and soluble L-selectin in order to evaluate the level of immune activation in HEU infants in the presence and absence of a single-dose NVP administered to the mother at the onset of labour. They found that increased immune activation that did not result in HIV infection, was associated with exposure to HIV during foetal development, regardless of NVP exposure. In addition, they found that infants who were exposed to NVP displayed even higher levels of immune activation in cord blood samples. The outcome of this study suggested that NVP may synergise with HIV-1, thus resulting in an environment that endorses HIV-1 replication (Schramm *et al.*, 2006).

In addition to showing increased levels of pro-inflammatory cytokine and plasma markers, a number of changes in the peripheral blood counts of CD4+ and CD8+ T lymphocytes have been documented in HEU individuals (Blanche *et al.*, 1999, Clerici *et al.*, 2000, Pacheco *et al.*, 2006). Nielsen *et al.* reported a reduction in the number of naïve CD4+ T cells, which they then concluded could have been a result of fewer progenitor cells and decreased thymus output (Nielsen *et al.*, 2001). Other researchers have also highlighted changes in T cell populations that may result in abnormal responses to vaccines (Clerici *et al.*, 2000, Bunders *et al.*, 2005a, Ono *et al.*, 2008). A large cohort study conducted in Kenya found significant differences in CD8+ T cells in HEU infants that persisted until 1 year of age (Embree *et al.*, 2001). In addition, an Italian study found that these abnormalities continued in HEU children until 8 years of age (Clerici *et al.*, 2000).

A study by Economides *et al.* (1998) was the first to measure and compare the levels of apoptosis in CD4+ and CD8+ T cells of HIV infected and HEU newborns. They found that HEU newborns showed significantly more apoptosis after one day of *in vitro* culture than those obtained from UE newborns (Economides *et al.*, 1998). Studies have suggested that apoptosis may be as a result of immune activation (Hosaka *et al.*, 2000). In the HIV population, a strong correlation was observed between apoptosis and inflammation, rather than severity of disease. Studies of HIV infected adults and children show an impact of apoptosis on bystander cells not infected with HIV (Finkel *et al.*, 1995). In addition other studies have found increased apoptosis despite immune activation (Miyamoto *et al.*, 2010). Although very few studies have documented increased apoptosis in the HEU population, it may be worth evaluating considering increasing documentation of early immune activation.

HIV mainly uses CD4+ T lymphocytes as an entry point to invade the immune system. Studies have been concentrating on evaluating abnormalities of the T cell compartment with less emphasis on dysfunctions on the B lymphocyte abnormalities (Clerici *et al.*, 2000). A study by Bunders *et al.* (2010) showed persistent hypergammaglobulinaemia in children born to HIV positive mothers. They further showed that abnormal levels of IgG persisted in HEU children until 24 months of age, thus suggesting an altered humoral immune response (Bunders *et al.*, 2010). Furthermore, studies have documented exaggerated responses to primary vaccination with bacterial pathogens (Jones *et al.*, 2011). A recent study by Borges-Almeida *et al.* (2011) found increased levels of B cells (CD19/CD5+) in cord blood of HEU newborns (Borges-Almeida *et al.*, 2011). These studies therefore define the increasing evidence of B cell abnormalities that is associated with *in utero* HIV exposure; these studies further prompt the need to further evaluation.

The causes for the documented immune abnormalities found in the HEU cohort is yet to be delineated, however many of these abnormalities seem to be linked to HIV exposure, as they represent responses to HIV or exposure to an immunologically inflamed milieu. However, other maternal factors associated with HIV may also serve as mediators of immune dysfunction in HEU infants.

2.7 EARLY PREVENTION OF INFECTION (MATERNAL ANTIBODIES)

2.7.1 Role of maternal antibodies

During the first phases of growth and development, the neonatal immune system is relatively incompetent in fighting disease. This implies that innate mechanisms inherited during foetal development, play a key role in protecting the neonate against potential pathogens. This protection is offered by maternal antibodies, which are transferred from the mother to her infant. The protective role of maternal antibodies was first defined in 1846, by observing the protective capacity of antibodies in infants born to mothers who survived measles infection during an outbreak in Faroe Islands (Englund, 2007).

Placental transfer generally occurs during the first trimester and between 28-32 weeks gestation, where IgG levels in the foetus rises to about 50% of that of the mother (Malek, 2003). By 40 weeks, foetal IgG concentrations are higher than that of the mother (Englund, 2007). A healthy, full term neonate produces about 5% of adult levels of IgG including 20% of IgM and IgA at birth (de Moraes-Pinto *et al.*, 1998).

Some researchers consider the acquisition of maternal immunological memory as one of the fundamental elements required for the survival of newborns (Zinkernagel, 2001). The importance of maternal antibodies in protecting neonates is highlighted in infants diagnosed with agammaglobulinaemia, which is characterised by the inability to produce Ig. These infants are protected against infection in the first 3 to 12 months of life. Subsequent to the decay in maternal antibodies, infants are considered vulnerable to infectious diseases.

It is therefore essential that infants receive adequate levels that will confer protection against specific pathogens during their vulnerable period before receiving prescribed vaccinations (Mackay, 2006). The amount of antibodies transferred from the mother to the infant determines the length of its protection, as well as the response to vaccination, which will be described and discussed in subsequent sections (de Moraes-Pinto *et al.*, 1998, Farquhar and John-Stewart, 2003).

2.7.2 Mechanism of transfer of maternal antibodies

The transport of antibodies across the placenta is an active, selective and intracellular process. Only certain IgG subclasses (preferentially IgG1 and IgG3) are passed from mother to foetus through the placenta and transfer begins at around 17 weeks. This occurs through the contact of maternal blood with cells of the placenta that contain Fc receptors. These Fc receptors bind IgG molecules circulating through the blood which then serve as direct passages to the foetus. Antibody molecules that are attached to the receptors are taken up by placental cells through a process called receptor-mediated endocytosis. Following this, they are subsequently transported across the cell in vesicles, which are then released into the foetal blood by exocytosis. Other antibody subclasses or isotypes are unable to be transferred due to the structural inability to bind specific Fc receptors. IgG is also secreted into breast milk, which also offers protection against mucosal pathogens (Alberts *et al.*, 2002, Englund, 2007).

There are a number of factors that influence the effective transfer of maternal antibodies, some of which include placental abnormalities, total IgG concentration in maternal blood, vaccine-antigen type, gestational age of infant, as well as time duration of maternal vaccination and delivery (van den Berg *et al.*, 2010, Englund, 2007).

2.7.3 Influence of maternal antibodies on infant immune responses

Many infants still have relatively high levels of maternal antibodies at the initiation of primary immunisation. Studies have alluded to the interference of pre-existing maternal antibodies on vaccination responses. The extent of this effect varies with the type of vaccine antigen (Glezen, 2003).

A number of studies have observed lower than expected responses for vaccines in the presence of high maternal antibodies. Research into the effects of maternal antibodies on the infant's immune response showed a decrease of 25-56% in antibody levels to whole cell pertussis due to the presence of pre-existing antibodies. However, the same observation was not consistent with the administration of an acellular pertussis vaccine (Englund *et al.*, 1995a).

Other earlier studies have also documented lower than expected responses to both inactivated and oral live polio vaccines given at birth. The response was further decreased in

infants who were breastfed (Warren *et al.*, 1964, Simoes *et al.*, 1985). However, adequate responses were noted after immunisation at 2 months of age.

A study by Bjorkholm *et al.* (1995) evaluated infant responses to diphtheria toxoid after maternal vaccination given during pregnancy. Vaccination was given at 3, 5 and 12 months of age and serum was collected at 3, 7 and 18 months. They found that infants with pre-existing antibody levels greater than 0.1 IU/ml had decreased responses to primary vaccination. However, after the second dose given at 12 months of age, similar antibody responses were observed between infants with low and high pre-existing antibodies (Bjorkholm *et al.*, 1995). In another study that evaluated infant response to combination vaccines with Hib and tetanus after maternal immunisation with tetanus toxoid during pregnancy, found that Hib levels were unaffected however, tetanus antibody responses were reduced. Subsequently, the response to secondary vaccine doses was adequate (Nohynek *et al.*, 1999). These studies therefore imply that passively acquired antibodies play a role in suppressing the antibody response to immunisation during early infancy, but do not influence the response to booster vaccination.

Although antibody transfer occurs from mother to infant, there is no evidence that supports the transfer of cellular immunity. The priming of neonatal T lymphocytes when evaluating vaccination with tetanus toxoids or influenzae vaccines (T cell dependent response) is not observed (Englund, 2007).

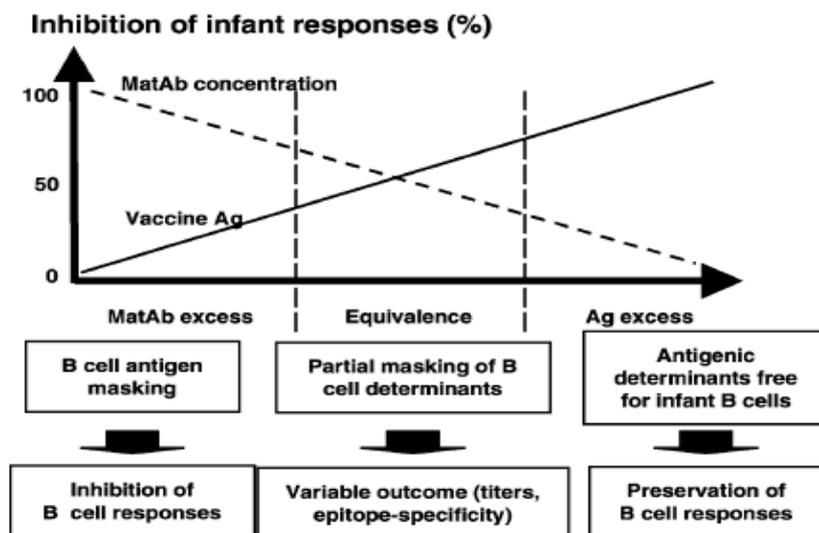


Figure 2- 3: Diagrammatic representation of the expected influence of maternal antibodies on vaccination responses in infants (Siegrist, 2003)

The mechanism where maternal antibodies interfere with the infants' immune response to vaccination is still unclear. However, it is believed to be epitope specific, mainly affecting B cell responses. It may be related to the maternal antibody to vaccine antigen ratio during the time of immunisation, which may result in epitope masking. Epitope masking refers to the concept whereby maternal antibodies disguise vaccine antigenic epitopes, therefore preventing the binding of infant B cells to these epitopes. In addition, it is suggested that a potential means of avoiding such immunological conditions would be to increase the dose of vaccine antigens, thereby increasing the abundance of antigenic epitopes that will allow sufficient binding of infant B cells (Figure 2-3) (Siegrist, 2003).

A diagrammatic representation depicting maternal antibody interference is shown in Figure 2-4.

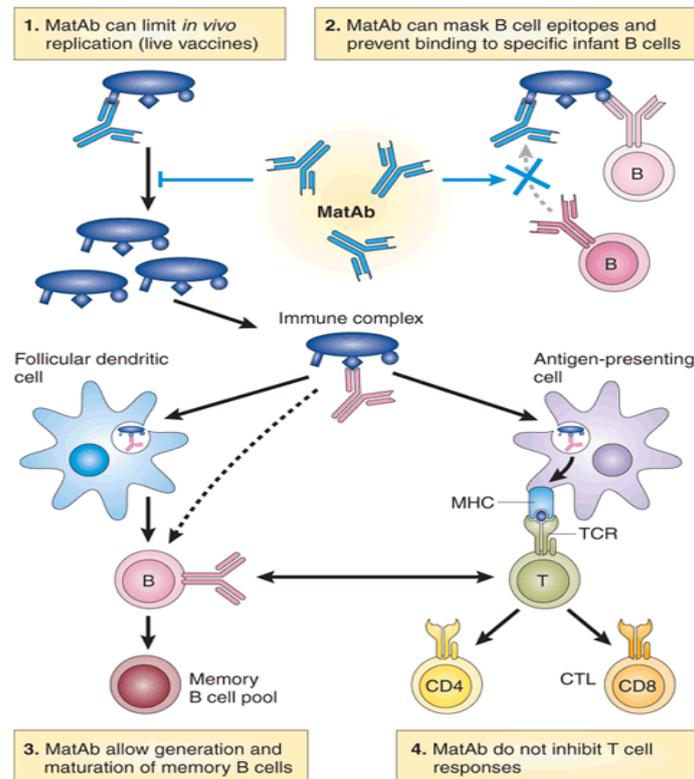


Figure 2- 4: Diagrammatic representation of the dual effect of maternal antibodies on infant responses to vaccines. The presence of maternal antibodies inhibits B cell responses to masked epitopes, but do not affect the generation of memory B cells. Maternal antibodies also play a role in increasing T cell responses by enhancing antigen capture and presentation (Lambert *et al.*, 2005).

2.7.4 Maternal antibody levels in HEU infants

In previous sections of this literature review, we have alluded to the increased morbidity and mortality that exist within the HEU population. Although the reasons for this increased vulnerability is considered multifactorial, maternal antibodies or the deficiency thereof, may serve as contributing factors.

It has been speculated that HIV impairs the transport of IgG from an infected mother to her foetus (de Moraes-Pinto *et al.*, 1998, Farquhar and John-Stewart, 2003). Therefore, an infant born to an HIV positive mother may be deprived from early protection against disease. In addition, the effective transfer of maternal antibodies is influenced by the integrity of the placenta. Diseases, such as malaria and HIV, have negative impacts on the transfer. In the case of malaria, studies have shown impairment in the Fc receptors (Englund, 2007). Increased concentrations of HIV specific antibodies present in maternal blood during the final trimester may also obstruct Fc receptors, thus inhibiting the active transport antibodies (Farquhar *et al.*, 2005).

Jones *et al* (2011) observed significantly decreased levels of maternal antibodies to pertussis, tetanus, Hib and pneumococcus in HEU compared to UE infants at birth. In addition, they found that compared to HIV negative women, HIV positive women had significantly lower levels of antibodies to Hib and pneumococcus. However, the levels to tetanus and pertussis were similar between both maternal groups (Jones *et al.*, 2011).

Similarly, a Malawian study investigating the influence of maternal HIV infection on the transfer of specific IgG antibodies to *Streptococcus pneumoniae*, showed a low transplacental antibody transfer (a reduction of 86%) in HEU infants. The presence of IgG antibodies to the tetanus toxoid and measles, however, was not reduced (de Moraes-Pinto *et al.*, 1998, Farquhar and John-Stewart, 2003).

Similar findings have also been related to viral antibodies. A Zambian study showed that infants born to HIV positive women had lower antibody levels to measles than infants born to HIV negative women. Levels were even lower in infants infected with HIV. This suggests that prior to vaccination, HEU and HIV infected infants are at a larger risk for contracting measles (Scott *et al.*, 2005). The findings of this study was also supported by other investigations in Kenya and Brazil, that showed a decrease of measles antibodies in cord blood samples of infants from HIV positive women (Scott *et al.*, 2005, de Moraes-Pinto *et al.*, 1993). In addition, another study by Scott *et al* (2007) showed that infants born to HIV-1 infected women were less likely to have maternally derived antibodies that would be functional in neutralising the measles virus. Due to this increased risk, they suggested that children receive measles vaccination before the scheduled time of 9 months (Scott *et al.*, 2007).

A Kenyan showed that maternal HIV infection was associated with a 52% reduction of tetanus antibodies in infants after compensating for maternal vaccination. They also found that 5.3% of the mothers and 7.8% of the newborns were seronegative to tetanus. The study concluded that HIV interferes with the transfer of maternal antibodies, thus resulting in the insufficient transfer of maternal antibodies (Cumberland *et al.*, 2007).

It is unclear whether HIV viral load may interfere with the mechanism of transfer. Although Jones *et al* (2011) found a 23% reduction in the placental transfer for Hib, 40% for pertussis and 27% for tetanus, there was no association between maternal CD4 count or HIV viral load and placental transfer. This goes in contrast to a study by Farquhar *et al* (Farquhar *et al.*, 2005), who showed a positive correlation with maternal viral load and reduced transfer of maternal antibodies.

Other studies have shown that mothers with a higher total IgG concentration transferred lower levels of antibodies to their infants (Hartter *et al.*, 2000). This was confirmed in a Malawian study that showed that maternal HIV infection and hypergammaglobulinaemia were associated with a reduction in the transfer of IgG antibodies to *S. pneumoniae* (de Moraes-Pinto *et al.*, 1996). Hypergammaglobulinaemia is often associated with HIV infection and Hartter *et al* (2000) has proposed that during hypergammaglobulinaemia, IgG antibodies compete for available receptors. They also observed that despite German mothers having only half the IgG concentration of that of Nigerian women, they still transferred a higher amount of measles antibody to their foetuses. The increased total IgG observed in Nigerian women may be indicative of constant antigenic stimulation thus resulting in limited passive transfer of antibodies to their infants.

2.8 VACCINATION IN PREVENTING DISEASE

2.8.1 History of vaccination

The period before vaccination led to a number of feared epidemics around the world. Millions of people died from diseases of which held very little understanding (Baker, 2010). The genesis of vaccination, and more importantly the field of immunology, developed along with the emergence and discovery of smallpox, which was suspected to emerge after 10 000 BC and claimed the lives of millions of people around the world. The origin of smallpox is unknown, however is believed to have been an animal poxvirus that had undergone mutations for transmission to humans (Parrino and Graham, 2006).

The mortality rate of pathogenic smallpox was around 30%. Following exposure to this pathogen, the incubation period lasted for about 10-12 days after which time individuals would show no symptoms of infection. The incubation phase was then followed by a pre-eruptive phase that resulted in fever, headache, nausea and vomiting. Individuals would thereafter develop characteristic rashes that progress into vesicles and pustules within a week, which was then followed by the formation of a scab. Those individuals who developed scars after surviving smallpox were observed to be immune to re-infection. The concept of variolation was then explored and attempts were made to expose individuals to smallpox matter by means of inoculating them with smallpox pus or scabs. Through the process of developing milder forms of the disease, protection against smallpox infection was demonstrated (Parrino and Graham, 2006).

The start of the vaccine era began with Edward Jenner in 1796 when he developed a vaccine against smallpox by inoculating a young boy with material from a cowpox lesion from the hand of a milkmaid. Upon second exposure to the pathogen, the boy showed protection against infection. Jenner's work was widely regarded as the foundation of immunology despite the origins of variolation starting in India and China (Baker, 2010, Riedel, 2005).

It was around 1979 that the World Health Organisation (WHO) sanctioned the eradication of smallpox. Since this milestone, the aim was to eradicate poliomyelitis and measles infections, thus significantly contributing to the reduction in various disease burdens caused by infectious diseases. These objectives have led to the successful implementation of immunisation programmes, as well as a number of public health interventions (Baker, 2010).

2.8.2 Importance of vaccination in preventing disease

In 2008, approximately 8 million children died before the age of 5 years around the world. Of these, 6 million (68%) were the result of infectious diseases and about 41% of these deaths occurred in neonates (Black *et al.*, 2010). In 2002, the WHO reported that an estimated 14% (1.4 million) of deaths could have been prevented by the administration of routine vaccinations (WHO, 2002).

The introduction of vaccination has had great impact on global health. Some of the successes of vaccination include a decrease in polio cases (300 000 cases in 1980 to only 2 000 cases in 2002), the eradication of neonatal tetanus and a decrease in measles deaths since 1974 (Bloom *et al.*, 2005).

Vaccination has a major impact on limiting mortality and is often referred to as the most cost-effective and the most powerful, high impact public health tool that contributes to saving millions of lives each year. The eradication of a number of diseases has emphasised the global action against fighting disease (Jamison *et al.*, 2006).

The importance of vaccination therefore lies in its ability to prevent and eradicate diseases, thus decreasing the number of unwanted deaths and contributing to improving the life of many individuals.

2.8.3 The Expanded Programme on Immunisation (EPI)

The WHO created the EPI in 1974, which aimed to make immunisation available to every child by 1990. At this time, less than 5% of children in developing countries were receiving immunisations. Children under the age of 1 year were being vaccinated against 6 killer diseases, which included tuberculosis, diphtheria, tetanus, pertussis, polio and measles. By 1988, yellow fever was added to the immunisation programme in countries with high prevalence and the introduction of hepatitis B (Hep b) followed in 1992. By 1990, about 80%

of children under the age of 1 year were vaccinated, which resulted in a the prevention of 3 million deaths each year (Jamison *et al.*, 2006).

2.8.4. EPI-SA

In 1995, the EPI-SA was introduced and primarily covered the six killer diseases listed above. Since then, a number of advancements have been made to the schedule, which had a major impact on the disease burden of South Africa. The introduction of combination vaccines have allowed for better administration and success of implementing larger coverage. A number of milestones have been reached in the history of the EPI-SA vaccine programme. The introduction of Hep B and Hib vaccines in 1995 and 1999 were one of the first important highlights of the programme. The year 2002 marked the elimination of neonatal tetanus in 2002 and in 2006, a declaration of being polio-free was put forward. More recent advancements include the introduction of the conjugated pneumococcal and rotavirus vaccines in 2009 as well as the change from whole cell to acellular pertussis and the implementation of a Hib booster at 18 months of age (Baker, 2010).

The EPI-SA is viewed as the leader of introducing new vaccines into the programme. In 2003, South Africa individually financed the implementation of Hib into the schedule (Ngcobo, 2008).

The mission of the EPI-SA is ultimately “to reach and protect all targeted children in South Africa with safe high quality vaccines that are delivered to the recipient with recent technology whilst promoting and developing local capacity and skills” (Ngcobo, 2008).

The current vaccine schedule for South African infants and children is listed in Table 2-2.

Table 2- 2: EPI-SA Revised Childhood Immunisation Schedule from April 2009

(<http://www.doh.gov.za/docs/epi-f.html>).

Age of Child	Vaccines Required
At Birth	OPV (0) Oral Polio Vaccine BCG Bacillus Calmette Guerin Anti-tuberculosis Vaccine
6 Weeks	OPV (1) Oral Polio Vaccine RV (1) Rotavirus Vaccine DTaP-IPV//Hib (1) Diphtheria, Tetanus, Acellular Pertussis, Inactivated Polio Vaccine, <i>Haemophilus influenzae</i> type B Combined Hep B (1) Hepatitis B Vaccine PCV ₇ (1) Pneumococcal Conjugated Vaccine
10 Weeks	DTaP-IPV//Hib (2) Diphtheria, Tetanus, Acellular Pertussis, Inactivated Polio Vaccine, <i>Haemophilus influenzae</i> type B Combined Hep B (2) Hepatitis B Vaccine
14 Weeks	RV (2) Rotavirus Vaccine* DTaP-IPV//Hib (3) Diphtheria, Tetanus, Acellular Pertussis, Inactivated Polio Vaccine, <i>Haemophilus influenzae</i> type B Combined Hep B (3) Hepatitis B Vaccine PCV ₇ (2) Pneumococcal Conjugated Vaccine
9 Months	Measles Vaccine (1) PCV ₇ (3) Pneumococcal Conjugated Vaccine
18 Months	DTaP-IPV//Hib (4) Diphtheria, Tetanus, Acellular Pertussis, Inactivated Polio Vaccine, <i>Haemophilus influenzae</i> type B Combined Measles Vaccine (2)
6 Years	Td Vaccine Tetanus and reduced strength of Diphtheria vaccine
12 Years	Td Vaccine Tetanus and reduced strength of Diphtheria vaccine

* Rotavirus Vaccine should NOT be administered after 24 months

Although a number of positive outcomes have been experienced with eradicating diseases and preventing a large proportion of deaths, child mortality is still relatively high. One of the Millennium Development Goals (MDGs) is to reduce child mortality by two thirds by 2015 (Duclos *et al.*, 2009). A number of challenges still stand in the way of achieving this goal. The challenges that face South Africa highlighted by Ngcobo (2008), state that there is a great need to maintain the achievements made in the control of disease through vaccination. The second challenge highlights the need for the incorporation of EPI with other child survival strategies, such as Infant and Child Nutrition and Comprehensive Care, Management and Treatment Services (CCMTS) for children infected with HIV. Lastly, attention on incorporating the support of nations and provincial districts, as well as health professionals, is required to further improve and strategise programmes.

2.9 IMMUNOLOGY OF VACCINATION

The immunology following vaccination is defined by the process of stimulating a protective adaptive immune response against infection of microorganisms by exposing an individual to a weakened form or component of the pathogen (Abbas and Lichtman, 2006-2007).

2.9.1 Requirements for an effective vaccine

Vaccine efficacy is defined by the generation of a qualitative antibody response. In addition, the vaccine should offer long-term protection against re-infection by the pathogen and result in the generation of immunological memory. These factors together serve as critical parameters of long-term vaccine efficacy (Siegrist, 2008).

Both T and B cells play essential roles in the developing effective immune responses to vaccines. Although B cells are commonly thought to dominate the role in vaccine efficacy, T cells are critical for the development of high-affinity antibodies and immune memory. More importantly, the optimisation of immunisation strategies lies in the identification of immune correlates of protection (Siegrist, 2008, Bojak *et al.*, 2002, Igietseme *et al.*, 2004).

The type of infecting organism determines the efficiency of a successful vaccine. Extracellular organisms for example require antibodies to provide the important mode of defences in comparison to infection with intracellular organisms where an effective CD8 T-lymphocyte response is required (Murphy *et al.*, 2008).

2.9.2 Effectors of vaccine responses

The main effectors of vaccine immunity are antibodies or Ig, which are produced by B lymphocytes. Antibodies essentially bind to the pathogens and promote its destruction via various mechanisms, such as neutralisation, opsonophagocytosis or activation of the complement cascade. The other effectors involved in vaccine induced immunity include those of cytotoxic CD8⁺ T lymphocytes that are mainly involved in killing intracellular pathogens. These effector cells work either by direct killing action through the release of perforin or granzymes, or through the release of antimicrobial cell-communicators, cytokines. As illustrated in Figure 2-5 below, both these cell type responses are generated and maintained by signals of CD4⁺ T helper cells, which are divided into Th1 and Th2 cells. Th1 cells produce cytokines, such as IFN γ , TNF α and β and IL-2, which support the activation of B cells, macrophages and cytotoxic T cells. On the other hand, Th2 cells produce cytokines, such as IL-4, IL-5 and IL-10 that promote the activation and differentiation of B cells only, thereby promoting the generation of humoral immune responses (Murphy *et al.*, 2008).

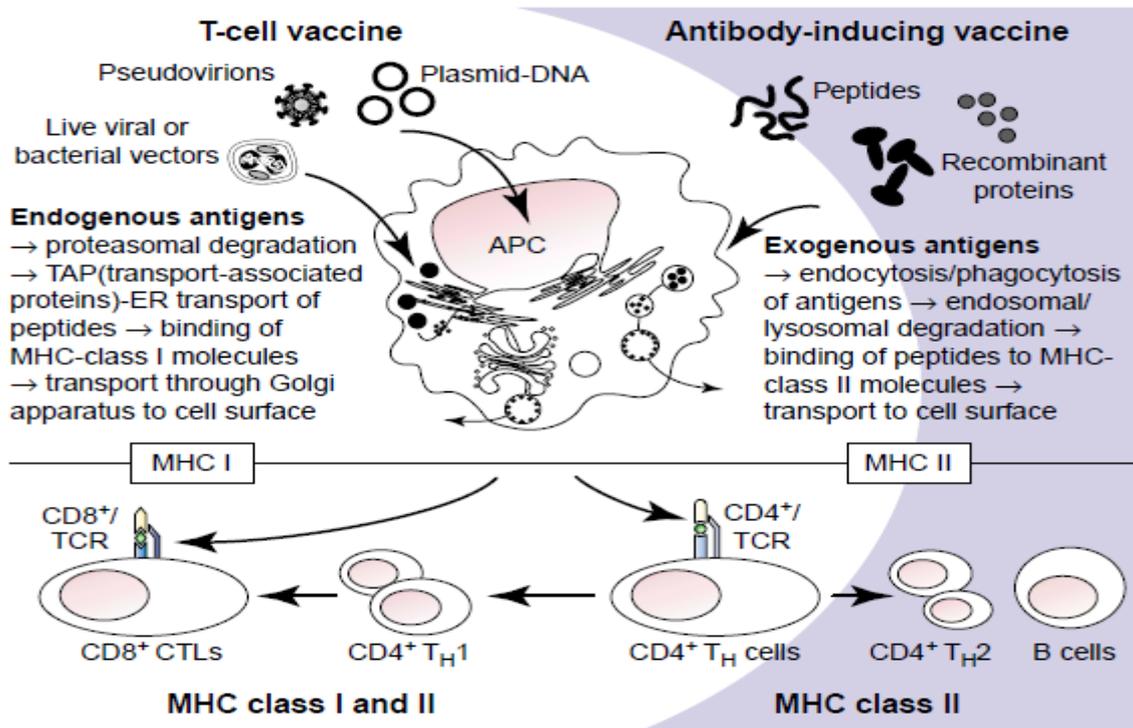


Figure 2- 5: Illustration of the immune response after introduction of vaccination as proposed by (Bojak *et al.*, 2002). T cell vaccines, such as live viral vectors, induce MHC class I processing and T-independent vaccines, such as subunit vaccines, induce MHC class II responses.

The type of vaccine administered determines the type of immune response that ultimately determines the level of protection offered by the specific vaccines. For example, *S. pneumoniae* and *H. influenzae* unconjugated polysaccharide vaccines evoke B cell responses without T cell assistance (T independent responses), although emerging research promotes the role of CD4 T cell help (Malley *et al.*, 2005). T cell dependent immune responses are achieved by toxoid, protein and live attenuated viral vaccine types, which tend to induce both high affinity antibodies, as well as immune memory (Siegrist, 2008). Most of the current vaccines mediate protective immunity through the induction of vaccine specific antibodies (Hanekom, 2005).

Polysaccharide vaccines are classified as long chains of sugar molecules that comprise of the surface capsule of specific bacteria. The immune response to a pure polysaccharide vaccine such as pneumococcus and Hib (no longer available) are characterised as T cell independent. These types of vaccines are not consistently immunogenic in children under the age of 2 years which may be related to their immaturity of their immune system. Unlike inactivated protein vaccines that result in the increase of antibody levels after each booster dose, polysaccharide vaccines do not cause increase in antibody levels. In addition, the antibodies induced by polysaccharide vaccines have decreased functionality due to the majority of antibodies produced are IgM and very little IgG (Siegrist, 2008 and Siegrist, 2003).

In order to increase the immunogenicity of polysaccharide vaccines, vaccines are combined to protein molecules in during a chemical process termed conjugation. Conjugated vaccines therefore alter the immune response from T-cell independent to T-cell dependent thus invoking a more immunogenic response upon boosting. Conjugated vaccines such as pneumococcus and Hib are further described below (Siegrist, 2008 and Siegrist, 2003).

The immunological process for the production of antibodies via both T-independent and T-dependent mechanisms have been explained in preceding sections of this review.

2.9.3 The role of adjuvants

Adjuvants play an important role in vaccine design with the aim of eliciting an adequate and protective immune response. This role of adjuvants is highlighted by the importance of co-stimulation required for T cell activation. Antigens of protein nature produce T-independent immune responses. However, improved immunity by evoking T cell dependent responses, antigens can be administered along with adjuvants that allow for the activation of macrophages, as well as other APCs. These substances aid the expression of co-stimulators on APCs, which are also stimulated to produce a host of cytokines that function in activating T cells. In most instances¹, adjuvants represent products of microbial components or alternatively, substances that imitate microbial elements. Essentially, adjuvants imitate pathogens by altering inactive protein antigens (Abbas and Lichtman, 2006-2007). Current adjuvants include a variety of formulations including cell wall components, alum and carbomers (Garlapati *et al.*, 2009).

2.9.4 Generation of immunological memory

The most enviable aim of any vaccination is the generation of long-lasting protection through immunological memory. Following the primary immune response, lymphocytes proliferate and undergo phenotypic changes. Memory cells are both quantitatively and qualitatively different from cells that have not yet encountered antigen and thus contain extended replicates of lymphocytes with differing functionality (Beverley and Maini, 2000).

The differentiation of memory B cells mainly occurs on the outside of the lymphoid follicles. Affinity maturation and heavy chain class switching occur in the germinal centres. After exiting the germinal centres, B cells differentiate into memory cells that migrate through the blood towards the extrafollicular areas of the spleen and nodes. It is here that they rest and await exposure to their specific antigens. After secondary exposure to antigens, these memory B cells proliferate and further differentiate into plasma cells, thus secreting large quantities of high affinity antibodies. These antibodies are then detected in serum/plasma within a few days (Siegrist, 2008).

2.9.5 Correlates of protection

Correlates of protection in vaccine-induced immunity is specifically important not only for basic research, but for linking immunity with protection. These factors provide insight into the reliability of vaccine production, susceptibility to infection within individuals and communities and in addition serve well in the certification of efficacy trials. A correlate defined by Plotkin is “an immune response that is responsible for a statistically interrelated protection” (Plotkin, 2010).

The important characteristic of determining correlates of protection is to firstly define protection. It is also important to note that the mechanism of protection offered by vaccination may vary by the mechanism required to respond to infection. In addition, the ideal way of evaluating correlates of protection involve the assessment of longitudinal studies with the evaluation of the outcome being those who get the disease (Plotkin, 2010).

Correlates may differ both in quantity and quality and in addition, may depend on the objective related to the type of infection it is to prevent. Most vaccines are mediated by antibodies of which correlates of protection may relate to functionality and specific levels. In addition, immune memory is also defined as a critical correlate of protection (Plotkin, 2010).

The correlates of protection for various vaccines will be discussed in subsequent sections of this review.

2.10 SELECTED VACCINES OF THE EPI

This section of the review will evaluate and describe four important vaccine elements that form part of the EPI-SA schedule: pertussis, tetanus, Hib and PCV₇. These vaccine antigens also formed the basis of this investigation.

Three of the four vaccine antigens listed above are currently routinely administered as part of a 5-in-1 combination vaccine, Pentaxim™ (Sanofi Pasteur) that provides immunisation against diphtheria, tetanus, pertussis, poliomyelitis and Hib. The vaccine is administered at 6, 10 and 14 weeks and a booster dose is given at 18 months of age. Pentaxim™ was first licensed in Sweden during 1997 and is at present in use in over 100 countries (Plotkin *et al.*, 2011). The vaccine is currently given to infants as part of the South African vaccine schedule.

The components and relative quantity of the Pentaxim™ vaccine is listed in Table 2-3 below (Plotkin *et al.*, 2011)

Table 2- 3: Components and relative quantity of Pentaxim™

Component per 0.5ml dose	Quantity
Diphtheria toxoid	30 IU ^a
Tetanus toxoid	40 IU ^a
<u>Bordetella pertussis antigens:</u>	
Toxoid	25 µg
Filamentous haemagglutinin	25 µg
Inactivated poliomyelitis virus type 1	40 DU ^b
Inactivated poliomyelitis virus type 2	8.0 DU ^b
Inactivated poliomyelitis virus type 3	32 DU ^b
Haemophilus influenzae type b polysaccharide conjugated with tetanus protein (PRP-T)	10 µg
Aluminum hydroxide (adjuvant)	0.3 mg

^aInternational Units ^bD-antigen levels

A number of studies have evaluated the efficacy, immunogenicity and safety of the Pentaxim™ vaccine (Plotkin *et al.*, 2011). Several clinical trials have verified immunogenicity and seroconversion after administration of the primary vaccine dosages. In addition, studies have shown protective levels one month following primary vaccination to all vaccine antigens. Seroprotective levels ranged from 92.3-100% for diphtheria, tetanus and polyribosyl ribitol phosphate (PRP) antibodies and for pertussis, seroprotective levels ranged from 83.9-100%. Furthermore, these levels were comparable to antibody responses produce upon separately administered vaccines (Plotkin *et al.*, 2011).

A recent study conducted in South Africa assessed these aspects after the administration of the primary vaccine dose as part of the EPI-SA vaccine schedule at 6, 10 and 14 weeks of age. The study showed increased immunogenicity for all vaccine antigens measured. Following the last dosage about 95% of participants showed an anti-PRP level greater than or equal to 0.15 µg/ml with a mean level of 2.0 µg/ml. The response to pertussis antigens were 97.5%, which represented a 4 fold increase in antibody concentration and a 100%

seroconversion rate for tetanus (≥ 0.01 IU/ml) was also noted. In addition, other vaccine types, diphtheria and polio also showed high immunogenicity. The latter study was comparable with a reference study conducted in France using a vaccine regimen of dosages administered at 2, 3 and 4 or 2, 4 and 6 months (Madhi *et al.*, 2011).

The four pathogens alluded earlier, namely, pertussis, tetanus, Hib and pneumococcus will be further discussed in this section.

2.10.1 Pertussis

Pertussis is considered one of the 10 leading causes of childhood deaths globally. Each year an estimated 290 000 deaths occur primarily in non-vaccinated children (WHO, 2002, Pereira *et al.*, 2010). For four decades there has been a gradual decline in the number of pertussis cases globally. However, there has been concern regarding the increase in pertussis incidence, particularly in the adolescent and adult populations. While the trend has not been documented in South Africa, these groups of individuals in the developed world serve as potential hosts that may contribute to the spread of disease, particularly to the unimmunised community (Madide, 2006, Pereira *et al.*, 2010).

Bordetella pertussis is a Gram-negative coccobacillus that contains many virulent factors that contribute to infecting its host by means of attachment to ciliated respiratory epithelium and use of filamentous haemagglutinin. After attachment it then produces and releases toxins (tracheal cytotoxin). The pertussis toxins play a crucial role in the dysfunction of epithelial cells by preventing clearance of debris through altering the movement of cilia. This organism is classified as an acute, extremely contagious respiratory disease. Subsequent to the incubation period which ranges from about 3 to 12 days, patients present with clinically defined symptoms such as a cough lasting for about 2 weeks, vomiting and whooping. Diagnosis is made by laboratory confirmed cell culture, PCR or positive paired serology (Madide, 2006).

In South Africa the efficacy of both whole-cell and acellular pertussis vaccines have been shown to be in the ranges of 83-98%. However, it is mentioned that the extent of the immune response to pertussis is proportionate to the number of doses administered (SAVIC, 2009b).

A study by Tejiokem *et al* (2009) showed that after 3 doses of a whole-cell pertussis vaccine only 19.2% of HIV positive children and 48% of HEU children possessed detectable antibodies. The initial antibody response was much lower in HIV-infected infants than HEU infants; however, it is unclear whether HIV-infected children may be at risk of severe or more frequent pertussis disease relative to HEU infants (Tejiokem *et al.*, 2009).

Defining correlates of protection to pertussis is relatively complex, as in addition to the pertussis toxin, the vaccine contains one or more attachment factors which may confer additional protection. Thus, correct correlates of protection would need to be determined by antibody levels against the pertussis toxin, as well as haemagglutinins. The exact antibody levels to each of these are arguable (Plotkin, 2010). Furthermore, it has been suggested that protection induced against pertussis may depend on a number of immunological factors relating to the humoral and cell-mediated response. Bearing in mind the decay of maternal antibodies, a definitive vaccine response represents a 4-fold increase in antibody concentration between the pre- and post-vaccination samples (Godfroid *et al.*, 2004). Similarly, Capeding, *et al* (2008) described pertussis seroconversion as a 4-fold increase in antibody titres that occurred in 95.3% of their study population (Capeding *et al.*, 2008).

2.10.2 Tetanus

Clostridium tetani is a Gram-positive, rod shaped, anaerobic bacterium, which is generally present in soil. Tetanus infection occurs by the bacterial release of exotoxins

(tetanospasmin), which then results in painful muscular spasms due to its effect on the central nervous system. The disease is not contagious, but is acquired when the individual, who may have open wounds or cuts, comes into contact with infectious substances (released spores). Several complications result from tetanus infection, some of which include respiratory failure, cardiovascular instability, as well as renal failure (Poudel *et al.*, 2009).

In 2010, there were an estimated 9 683 reported tetanus cases globally and in 2008, 61 000 estimated deaths occurred in children under the age of 5 years (WHO, 2002). Although tetanus is considered a relatively rare disease in developed countries, incidence in developing worlds contribute to a large number of deaths. In 1998, the WHO estimated 410 000 deaths due to tetanus, of which 409 000 occurred in developing countries, where immunisation is less widespread (Aristegui *et al.*, 2003, WHO, 2002, Poudel *et al.*, 2009). The occurrence of tetanus in neonates is most often a result of contamination of the umbilical cord or in the case where mothers did not have sufficient antibodies to passively transfer in order to confer protection in the infant.

Correlates of protection against tetanus have been well defined. A level of 0.01ug/ml confers sufficient protection and a level greater than 0.1 µg/ml is associated with complete protection against tetanus. Tetanus diseases have been reported to occur despite having sufficient antibody concentrations, which is believed to be a result of insufficient diffusion into sites of toxin production (Plotkin, 2010).

2.10.3 Hib

Haemophilus influenzae b is a Gram negative encapsulated coccobacillus that is believed to be the leading cause of bacterial pneumoniae and meningitis in children under the age of 5 years. Disease usually occurs in unvaccinated individuals and includes other clinical outcomes, such as septicaemia, cellulitis and epiglottitis (Mangtani *et al.*, 2010).

About 8.1 million serious events and 371 000 deaths occur due to Hib disease every year. In addition, 8 100 deaths occur in HIV infected children (Watt *et al.*, 2009).

In 1994, Hussey *et al* observed that 65.5% of invasive Hib disease occurred in children less than 5 years of age and that 1 in every 250 children contracted Hib within the first year of life (Hussey *et al.*, 1994). It was this epidemiological study that prompted the introduction of the Hib vaccine into the EPI, making South Africa the first African country to introduce Hib into the immunisation schedule of infants. Following the implementation of this vaccine, the number of Hib related cases decreased (Baker, 2010).

In 2010, a total of 82 cases of Hib were reported amongst children less than 5 years of age. According to the Group for Enteric Respiratory and Meningeal disease Surveillance in South Africa (GERMS-SA) audit (GERMS-SA, 2010), a total of 91 Hib disease cases were reported in South Africa, of which the Western Cape represented the third highest (21 cases). The highest incidence was in the Eastern Cape, followed by Gauteng.

Vaccine efficacy rates for the Hib conjugate vaccine in South Africa ranges from 90-100% and immunogenicity is said to differ according to the type of carrier proteins they contain. Hib conjugated vaccines are however documented to be effective from 6 weeks of age (SAVIC, 2009a). Hib conjugate vaccines have also shown to be greatly effective in preventing about 25% of radiographically confirmed pneumonia (Trotter *et al.*, 2008). Following the implantation of the vaccine in 1999, the number of Hib cases declined by an estimated 65% in children younger than 1 year of age (von Gottberg *et al.*, 2006)

HIV-infected individuals have a 6-8 fold increased risk of invasive Hib disease. A study by Jeena (2008) found that the initial immunological response to vaccination in HIV-infected individuals was 54%, compared to 90% in uninfected infants. They further found a

sequential loss of memory by 15 months of age (Jeena, 2008). Madhi *et al* (2005) investigated both the quantity and quality (bactericidal activity and avidity) of Hib conjugate vaccine in both HIV-infected and uninfected infants. The study concluded that only a few HIV positive infants were unlikely to have antibody concentrations above the indicated protective levels, when compared to uninfected infants. In addition, a limited proportion of HIV infected infants had antibody levels considered to be functional. The concentration of antibody required for 50% killing of Hib bacteria, through bactericidal activity, was much higher in HIV infected infants. The risk for vaccine failure was much higher for HIV infected infants than HIV uninfected infants (Madhi *et al.*, 2005). Mangtani *et al* (2010) therefore recommended booster doses in HIV infected infants after 2 years of age (Mangtani *et al.*, 2010).

Correlates of protection for anti-capsular antibodies are best defined by the opsonophagocytic (OPA) or bactericidal activity of antibodies. However, the relative binding of antibodies serve as useful surrogates and are routinely used to identify protection. Plotkin (2010) suggests that an antibody level of 0.15 µg/ml is most likely to offer protection against bacteraemia (Plotkin, 2010).

2.10.4 PCV

Streptococcus pneumoniae or pneumococcus is a Gram-positive, catalase negative coccus that is characterised into more than 90 serotypes and are responsible for a large portion of bacterial meningitis, bacteraemia and otitis media (Zar *et al.*, 2001). Pneumococcal diseases account for an estimated 700 000 to 1 million child deaths each year globally. During 2000, an estimated 14.4 million severe pneumococcal disease events were reported. In the same year, an estimated total of 826 000 deaths in children between 1 and 59 months of age occurred. Of this estimated total, 91 000 were HIV positive and 735 000 were HIV negative. About 61% of all HIV negative child deaths occurred in ten African and Asian countries (O'Brien *et al.*, 2009). Furthermore, the highest mortality rates occurred in sub-Saharan Africa and south Asia. In African HIV-infected children, the risk of dealing with invasive pneumococcal disease (IPD) is 40 fold greater (Madhi *et al.*, 2000). These estimates not only highlight the significant burden of pneumococcal disease in children and the HIV infected population, but illustrate the need for effective vaccination to prevent morbidity and mortality.

Pneumococcal conjugate vaccines (PCV) have been shown to be effective in both HIV infected and uninfected children. Trials conducted in South Africa and the United States of America (USA) showed an overall reduction of 83-94% of vaccine serotype specific IPD in uninfected children. Although lower than in HIV uninfected children, PCV was shown to have a 54% efficacy in preventing serotype specific IPD in HIV infected children not receiving ART (Klugman *et al.*, 2003, Black *et al.*, 2000). Two randomised placebo control trials documented the evaluation of PCV9-CRM in South Africa and Gambia. The South African trial conducted from 1998 to 2001 demonstrated the efficacy against pneumococcal disease in both HIV infected (65% efficacy) and uninfected children (83% efficacy) (Klugman *et al.*, 2003).

PCV₇ (Pevnar[®], Pfizer, previously Wyeth Pharmaceuticals) was registered in South Africa during 2005 and was initially only available through the private health-sector. The vaccine was made available to the public sector during 2008 and formed part of the routine infant immunisation schedule in one health district (Ukhahlamba district) in the Eastern Cape. The latter locality served as a region for piloting the implementation of the vaccine through conducting fridge space audits, training of nurses and evaluating the availability of cold chain accessibility. Pevnar[®] was available nationally in April 2009 and the date of execution differed between geographical areas. Children born after 15 Feb 2009 that were 6 weeks of age on 1 April 2009 could receive Pevnar[®]. There were no catch-up programmes during 2008 and 2009. Pevnar[®] includes 7 serotypes (4, 6B, 9B, 9V, 18C, 19F and 23F) that cause

70% of all childhood IPD in South Africa (von Gottberg *et al.*, 2007, von Gottberg, 2010). Prevnar13[®] (Pfizer) has already been rolled-out during early 2011 and includes 13 serotypes namely, 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23 F) which are documented to contain 83% of the serotypes known to cause IPD in children (von Gottberg, 2010).

PCV₇ consists of a solution of the capsular antigens of *S. pneumoniae* serotypes 4 (2 µg), 6B (4 µg), 9V (2 µg), 18C (2 µg), 19F (2 µg) and 23F (2 µg). Each serotype is individually conjugated to the diphtheria protein CRM₁₉₇ (non-toxic variant of diphtheria toxin) (20 µg), which is a nontoxic variant of diphtheria toxin isolated from cultures of *Corynebacterium diphtheria* strain C7 (β197). The formulation also consists of 0.125 mg of aluminum per 0.6 ml dose as aluminum phosphate adjuvant (information obtained from package insert).

In South Africa, PCV₇ was introduced in the routine infant immunisation programme as a 2-dose primary series at 6 and 14 weeks of age and a booster dose at 9 months of age. Generally, children younger than 2 years of age do not respond relatively well to vaccines of capsular polysaccharide nature. These polysaccharides are therefore conjugated to carrier proteins that thus lead to T-cell dependent immune activation which results in the induction of high-quality antibodies and subsequent immunological memory (Trotter *et al.*, 2008). In addition, conjugate vaccines are important for inducing mucosal immunity which is defined as their ability to prevent new acquisition of nasopharyngeal serotypes (von Gottberg, 2010).

The WHO has proposed a protective concentration of IgG anticapsular polysaccharide antibodies of ≥ 0.35 µg/ml measured by ELISA one month after primary immunisation. Further studies have indicated that this level is suitable for the level of protection conferred in a larger population rather than individual immunity (Siber *et al.*, 2007).

2.13 RESEARCH HYPOTHESIS

Primary Hypothesis

HEU infants demonstrate an altered adaptive immune response in early infancy which persists into childhood. Aberrations of this type of immunity are related to *in utero* HIV exposure and result in increased morbidity and mortality.

Secondary Hypotheses

- Baseline antibody levels prior to specific EPI vaccination in HEU and UE infants at 2 and 6 weeks of age show significant differences between the two groups reflecting differences in passively acquired maternal antibodies. These represent an inability of the HIV positive mother to transfer sufficient antibodies to her infant which results in increased infectious morbidity during infancy.
- Specific antibody levels after routine scheduled vaccination of HEU compared to UE infants differ significantly and these differences may be related to HIV exposure and the consequence of impaired transfer of maternal antibodies.
- Due to increased antigenic exposure during foetal development and early infancy, HEU infants' upregulate the expression of various cellular markers of immune activation, regulation and cell death (apoptosis). The elevated expression of these cellular markers is present on both T and B cells of the adaptive immune system measured at 18 and 24 months of age.

- HEU infants display decreased expression of the cellular marker of B cell memory as a result of early HIV exposure. The deficient memory B cell responses are linked to variations in antibody levels to vaccination.

CHAPTER 3

AIMS AND OBJECTIVES

3.1 PRIMARY AIM OF STUDY

The primary aim of this study was to evaluate specific characteristics of the adaptive arm of the immune system in HEU infants in comparison to an UE control group

3.2 OBJECTIVES OF STUDY

3.2.1 Primary objectives

- a) To assess the IgG levels to specific vaccines (i.e. pertussis, tetanus, Hib and PCV₇) as administered according to the Expanded Programme on Immunisation of South Africa (EPI-SA) from 2 weeks to 2 years of age and document differences in the antibody profiles of infants born to HIV positive mothers to those born to HIV negative mothers.
- b) To evaluate the effects of HIV exposure on the upregulation of specific cellular markers of immune activation, regulation and apoptosis in HEU and UE groups at 18 and 24 months of age

3.2.2 Secondary objectives

- a) To investigate the possible role of *in utero* HIV exposure on acquisition of maternal antibody levels by observing the specific antibody levels of infants at 2 and 6 weeks of age before the receipt of their primary vaccination
- b) To investigate possible differences or trends that may exist between the HEU and UE control groups with regards to specified vaccine antibodies at 12 weeks and 6, 12, 18 and 24 months of age
- c) To evaluate the effects of HIV exposure on immune activation by investigating various cellular markers on both T and B lymphocytes in both HEU and UE control groups at 18 and 24 months of age subsequent to the vulnerable period (first 6 months of life) of susceptibility to infections
- d) To evaluate the effects of maternal HIV infection, antiretroviral exposure (mother and infant) and immune activation on the degree of cell death (apoptosis) in the HEU group compared to the UE group by investigating specific cellular markers on both T and B lymphocytes at 18 and 24 months of age after the vulnerable period of susceptibility to infections.
- e) To evaluate immune regulatory properties of both T and B lymphocytes by evaluating the expression of specific cellular markers, such as CTLA-4 and PD-1, at 18 and 24 months of age in both the HEU and UE control infant groups
- f) To assess B cell memory by evaluating the expression of memory marker CD27 at 18 and 24 months of age in both the HEU and UE control groups

- g) To address potential aberrant immunological mechanisms in the adaptive immune compartment of the HEU compared to UE control groups that may elucidate the potential reasons for the increased morbidity and mortality observed in the HEU population
- h) To generate hypotheses for a larger cohort study with the above data

CHAPTER 4

MATERIALS AND METHODS

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4.1 OVERVIEW OF STUDY OUTLINE

4.1.1 Study Design

This was a prospective comparative study aimed at observing specific characteristics of the adaptive immune response in infants born to HIV positive mothers. This investigation (Project Number N08/09/262) formed part of a larger pilot study (Project Number N08/10/289) of which probable innate abnormalities in HEU and unexposed (UE) controls were investigated. This multi-collaborative pilot study was also aimed at investigating a potential difference for a range of immunological, social, nutritional and clinical parameters between these infant groups from birth to 2 years of age.

In this sub-study we observed IgG antibody responses in the two infant groups (HEU and UE) to routine vaccinations as part of the EPI in South Africa at community clinics within the Western Cape region. Specific antibody levels at 2, 6, 12 weeks and 6, 12, 18 and 24 months of age were investigated in HEU and UE infant groups respectively. Specific antibody levels to pertussis, tetanus, Hib and pneumococcal capsular polysaccharide (PCP) were determined at the various time points. The UE group served to define the normal ranges within the specified age clusters to which the exposed group were compared and documented for any differences.

Antibody levels at 2 and 6 weeks in both infant groups represented the presumed transfer of maternal antibodies. Infants received routine vaccines as outlined in the EPI schedule (Table 2-2). At birth infants received a dose of the oral polio vaccine (OPV) (Sanofi Pasteur, Lyon, France), as well as the Bacillus Calmette Guérin (BCG; Danish strain 1331, Statens Serum Institute) vaccine. At 6, 10 and 14 weeks and 18 months of age, infants were given a vaccine combination consisting of diphtheria, tetanus toxoid, pertussis and Hib (DTP-Hib; Sanofi Pasteur), as well as Hepatitis (Hep) B (Heber Biotec, Havana, Cuba) and OPV. Changes to the EPI schedule occurred from July 2009 and included the replacement of DTP-Hib with diphtheria, tetanus toxoid and acellular pertussis (aP) vaccine combined with inactivated polio and Hib (DTaP-IPV/Hib; Sanofi Pasteur). Additions to the schedule included two new vaccines viz. the pneumococcal 7-valent (PCV₇) conjugate (Wyeth, Andover, Massachusetts) and the rotavirus (RV) (GlaxoSmithKline, Rixensart, Belgium) vaccine. The roll-out of PCV₇ had occurred after the commencement of this study resulting in only a few infants receiving the prescribed vaccination as outlined in the schedule. Although the IgG levels for the vaccine was determined for all infants at each visit, only the presumed maternal antibody levels at 2 and 6 weeks will be reported. As the mothers of the infants in this study did not receive the described vaccination, the maternally derived antibody levels in these infants will thus be due to natural exposure to *Streptococcus pneumoniae*.

Based on preliminary data generated from post-vaccination responses and haematological data at earlier time points (discussed in following chapters), we then investigated specific cellular markers of immune activation on CD4+ T, CD8+ T, CD19+ B and CD20+ B cells as well as cellular markers of apoptosis and regulation on the same cell types. A functional assay was designed and optimised to measure the expression levels of cellular marker of early apoptosis, Annexin V, on both CD4+ T and CD20+ B cells after observing minimal expression during *in vitro* staining of cells. Furthermore, we investigated the expression of the cellular marker of B cell memory (CD27+) on both CD19+ and CD20+ B cells. In addition, we assessed a cellular marker that plays a role in the function of homing of naive

T-cells to peripheral lymph nodes (CD62L+) on CD20+ B cells as well as the expression of CD127 (IL-7 receptor) on CD4+ T cells that serves to control pro-T cell growth and maturation (See Table 4-5 for list of marker functions). Data generated from the investigation of these various biomarkers was completed for the 18 and 24 month time point only.

4.1.2 Ethical Aspects

The study was conducted according to the Helsinki declaration, as well as the guidelines from the Medical Research Council (MRC) and the Institute of Child Health (ICH). Ethical approval for the broader pilot and sub-study was granted by the Committee for Human Research, University of Stellenbosch (Faculty of Health Sciences). Informed consent was obtained from mothers by a suitable study nurse in the participants' home language before they were included in the study and at any point in time, mothers could withdraw consent and exclude their infants from further participation. Blood was collected in a pleasant environment and in a manner that is least painful for the study participants. The area of venepuncture was treated with EMLA[®] local anaesthetic cream (lidocaine 2.5% and prilocaine 2.5%) 10-20 minutes before the procedure to minimise pain during blood collection. Mothers who withdrew their infants from the study were under no circumstances deprived of medical care and infants who underwent seroconversion due to failed PMTCT (an estimated 5% of enrolled participants) received full medical treatment and all mothers had access to HIV counselling.

All babies received regular health check-ups by a qualified doctor experienced in paediatric medicine and the mothers' travel expenses for study visits were reimbursed. The same total volume (5ml) of blood was collected from both the HEU and UE infants. Phlebotomy was performed by qualified staff at the KIDCRU (Children's Infectious Diseases Clinical Research Unit) clinical Facilities at Tygerberg Hospital.

All participant information was handled in a confidential manner at all times and reference to the infant and his/her clinical information was restricted to the use of a subject number.

4.1.3 Sample Size and Method of Sampling

4.1.3.1 Study population

The characteristics of the enrolled study participants are listed in Table 4-1 below. A total of 57 participants were enrolled which included 27 HEU infants and 30 UE control infants.

Table 4- 1: Characteristics of enrolled population from 2 week time point

Characteristics	HIV-Infected Women and Exposed Infants (HEU)	HIV-Uninfected Women and Unexposed (UE) Controls	P-value	n
General Maternal Characteristics				
Maternal age at delivery (median, range)	25.00 (18.0-41.0)	26.00 (18.0-41.0)	0.63 ^a	n=27 HEU n=30 UE
Maternal primigravidity (%)	7 (26%)	9 (31%)	0.40 ^b	n=27 HEU n=29 UE
Infants delivered by normal vaginal delivery (NVD) (%)	26 (96%) ^c	29 (100%) ^d	0.64 ^e	n=27 HEU n=29 UE
Social Characteristics				
Household lives in informal structure (%)	16 (59%)	5 (17%)	0.002^b	n=27 HEU n=30 UE
Households that have inside access to running water (%)	11 (41%)	26 (87%)	0.0001^b	n=27 HEU n=30 UE
People in same household as infant (median, range)	3.5 (1.00-10.00) SD (2.40)	5.5 (2.00-10.00) SD (2.13)	0.0075^a	n=26 HEU n=30 UE
People sleeping in the same room as infant (median, range)	2.00 (1.00-5.00) SD (1.12)	3.00 (1.00-6.00) SD (1.46)	0.0059^a	n=26 HEU n=29 UE
Maternal HIV Diagnosis				
During pregnancy (%)	14 (52%)	N/A	0.96 ^f	n= 27 HEU
Prior to pregnancy (%)	13 (48%)	N/A		
Gestational age when therapy started-PMTCT (median, range), weeks	30 (8-35) SD (8.15)	N/A	-	n= 13 HEU
Mothers on HAART (%)	4 (15.4%)	N/A	-	n=26 HEU
Mothers on Dual Therapy (%)	19 (73.1%)	N/A	-	
No PMTCT (%)	3 (11.5%)	N/A	-	
Maternal CD4 Count (extracted from patient files)				
2 weeks (median, range)	337 (131-673) SD (150.83)	N/A	-	n=23 HEU
6 months	394 (150-620)	N/A	-	n=21 HEU

Table 4-1 (cont.): Characteristics of enrolled population from 2 week time point

(median, range)	SD (122.63)			
12 months (median, range)	433 (200-757) SD (132.48)	N/A	-	n= 18 HEU
18 months (median, range)	444 (178-800) SD (161.97)	N/A	-	n=15 HEU
24 months (median, range)	460.50 (203-609) SD (142.09)	N/A	-	n= 14HEU
Infant Birth Characteristics				
Gestational age at birth (median, range), weeks	38 (30-41) SD (2.74)	38 (33-42) SD (2.40)	0.65 ^a	n=24 HEU n =29 UE
Birth Weight (median, range), kg	2.980 (1.90-3.82) SD (0.42)	3.12 (2.08-4.10) SD (0.51)	0.11 ^a	n=27 HEU n= 30 UE
Gender				
Females (%)	20 (74%)	15 (50%)	0.09 ^g	n=27 HEU n=30 UE
Males (%)	7 (26%)	25 (50%)		
Ethnic Grouping				
African (Xhosa) (%)	19 (70%)	8 (27%)	<0.001^g	n=27 HEU n= 30 UE
Coloured (%)	5 (19%)	21 (70%)		
African (Zimbabwean) (%)	1 (4%)	0		
African (Malawian) (%)	2 (7%)	0		
White (%)	0	1 (3%)		
Exclusive Breastfeeding				
Birth (%)	1 (4%)	28 (97%)	<0.001	n=26 HEU n=29 UE
2 weeks (%)	2 (7%)	24 (86%)	<0.001	n=27 HEU n=28 UE
6 weeks (%)	1 (4%)	17 (63%) ^h	<0.001	n=27 HEU n=27 UE
12 weeks (%)	1 (4%)	11 (44%)	<0.001	n= 27 HEU n=25 UE
Weight over time				
2 weeks (mean, range), kg	3.28 (2.10-4.37) SD (0.52)	3.34 (2.16-4.50) SD (0.50)	0.64 ^a	n=28 HEU n= 29 UE
6 weeks (mean, range), kg	4.38 (3.27-5.4) SD (0.60)	4.31 (2.90-5.30) SD (0.66)	0.66 ^a	n=28 HEU n= 27 UE
12 weeks (mean, range), kg	5.69 (4.30-7.06) SD (0.74)	5.40 (3.60-7.32) SD (0.86)	0.30 ^a	n=28 HEU n= 26 UE
6 months (mean, range), kg	7.72 (6.04-9.57) SD (1.29)	7.20 (5.60-10.20) SD (1.08)	0.09 ^a	n=25 HEU n= 22 UE
12 months (mean, range), kg	9.50 (7.65-11.70) SD (1.29)	8.33 (6.45-11.40) SD (1.242)	0.12 ^a	n=23 HEU n= 21 UE
18 months (mean, range), kg	11.19 (8.90-14.30) SD (1.69)	10.06 (7.25-12.90) SD (1.4068)	0.03^a	n=18 HEU n= 21 UE

Table 4- 1 (cont.): Characteristics of enrolled population from 2 week time point

24 months (mean, range), kg	12.46 (9.50-16.10) SD (2.06)	11.20 (9.00-13.80) SD (1.30)	0.03^a	n=17 HEU n= 20 UE
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^a Student's t-Test^b Pearson Chi-Square^c In the HEU group, one infant was born via C-section.^d Mode of delivery for one infant in the control group was not documented^e Fisher exact, one tailed test^f Two way ANOVA^g A Pearson Chi-Square analysis was followed using a cross-tabulation form of analysis^h One infant in the UE group were breast fed from two different women. Infant was thus not counted as part of exclusive breastfeeding criteria

4.1.3.2 PMTCT

During the study period, all HIV-positive women were assessed according to the WHO clinical staging guidelines as part of routine care. As soon as the women were confirmed to be HIV positive, CD4 counts were measured.

As part of the clinical guidelines for routine care, mothers with a CD4 count greater than 200 cells/ μ l followed the PMTCT regimen. This consisted of dual therapy for both mother and infant and involved the initiation of zidovudine at 28 weeks or more gestation, followed by zidovudine for 1 month to the infant, as well as a single dose of NVP to both infant and mother.

Mothers, who were in WHO clinical stage 4 with a CD4 count less than 200 cells/ μ l, were eligible for Highly Active Antiretroviral Therapy (HAART). Of the 27 HEU infants who were enrolled, 23 maternal CD4 counts were extracted from the patient files. Four of the CD4 counts were not listed. A total of 6 mothers had a CD4 count below 200 cells/ μ l. Of these, 3 mothers were initiated on HAART. One mother with a CD4 count of 293 cells/ μ l also received HAART during pregnancy.

HIV positive mothers were provided with free infant feeding formula for 6 months.

All information regarding PMTCT for both mother and infant, including maternal CD4 counts were extracted from the patient hospital records and documented if available.

4.1.3.3 Inclusion and Exclusion Criteria

Mothers who delivered clinically healthy babies of appropriate birth weight and gestational age with no congenital abnormalities were eligible for the study. Selected mothers were recruited at Tygerberg Hospital maternity ward postnatally and signed informed consent to enrol their infants in the study. Infants with acute illnesses and an HIV positive polymerase chain reaction (PCR) at 2 and 6 weeks (suggesting a failed PMTCT programme) were excluded from the statistical analysis, but remained part of the study and their health followed up at each visit.

Maternal exclusion criteria included:

- Mothers that had or were currently undergoing tuberculosis (TB) treatment
- Unstable or untreated hypertension during pregnancy (including pre-eclampsia)
- Proteinuria loss greater than 300mg/L/d]

- Perinatal pyrexia of more than 38°C during labour
- Current infection or undergoing treatment for syphilis
- Any form of malnutrition or immunodeficiency (other than being HIV positive)
- Anaemia (haemoglobin levels less than 8g/L)

4.1.3.4 Sample Collection and Storage

Enrolment of participants for the pilot study occurred over a period of 4 months (March-June 2009). Approximately 95 mothers were recruited for the study shortly after delivery at the Tygerberg Hospital Maternity Ward and signed informed consent for enrolment of their infants. Patients were referred to the KIDCRU unit and the infants were followed up at 2, 6 and 12 week and 6, 12 and 18 and 24 month time points. The final study group consisted of 27 HEU and 30 UE infants. At the 2 week follow-up 4 infants were confirmed HIV PCR positive.

Preparation of Heparinised Syringes for Blood Collection

Sodium heparin syringes were prepared 1 hour prior to blood collection in a Biosafety Level 3 (BLS3) laboratory at the Division of Medical Virology, University of Stellenbosch, Tygerberg. Five millilitre (5ml) sterile syringes (Neomedic, UK) were primed with sodium heparin (Pharmaceutical Partners of Canada) using sterile 23 gage needles (BD Biosciences, USA). Approximately 250µl of heparin (10 000 USP Units/ml) was transferred into the syringe, which was then capped (BD Biosciences, USA), sealed and left at room temperature until used (within 24 hours).

Blood Collection

At each time point, a maximum of 4.5 ml of blood was collected into pre-primed sodium heparin syringes and about 500µl was collected into paediatric EDTA tubes (BD Biosciences, USA). Blood was collected by venepuncture of a peripheral vein in the cubital or external jugular vein in the neck. Plasma for antibody studies was collected from sodium heparin blood by centrifugation at 3200 rpm for 5 minutes (with brake). About 5 aliquots of plasma (approximately 400µl in each tube) were collected into Eppendorf tubes and frozen at -80°C. Three hundred and fifty microlitres (350µl) of whole blood was allocated at the 18 and 24 months time point for flow cytometric biomarker analysis. For each flow panel, consisting of four fluorochromes, 50µl of whole blood was allocated. Innate immune analysis was completed at the 2 and 6 weeks, as well as at the 6, 12 and 24 months time points respectively.

Confirmation of immunisation

Infant immunisation status was documented on Road to Health Cards (RTHC) by appropriate clinic nurses after the administration of the vaccine. RTHC's were then collected, copied and certified at each time point. Only infants with confirmed vaccination dates formed part of the analysis.

Clinical Assessment

Mothers and their infants were referred to KIDCRU at each visit where a full clinical assessment was completed and the information documented on a data capture form. Infants were examined by a medical doctor experienced in paediatric medicine for infections and

abnormal health. Information concerning growth, feeding regimens, demographics, medication, hospitalisations and maternal health was documented at each time point.

4.1.4 Routine Laboratory Tests

4.1.4.1 HIV Polymerase Chain Reaction (PCR) Analysis

An HIV PCR was done for all infants on the study at 2, 6 and 12 weeks and 24 months of age. Approximately 75µl of EDTA whole blood was dispensed onto dried blood spot paper (DBSP), which was labelled with the patient number and date. The dried blood spots were sent to the Division of Medical Virology, NHLS Tygerberg for analysis.

A qualitative AMPLICOR HIV-1 DNA test, version 1.5 (Roche Molecular Systems Inc, Branchburg, New Jersey) was performed on the DBSs from infants at 2 and 6 weeks, after which the COBAS AmpliPrep/COBAS TaqMan HIV-1 Qualitative HIV-1 DNA test using AMPLILIMK Software, version 3.2 Series (Roche Molecular Systems Inc, Branchburg, New Jersey) was employed for the 12 week and 24 month time points. The latter method allowed for greater sensitivity (98.8%) and specificity (97.1%) with a positive predictive value of 90.9% and a negative predictive value of 99.6%.

AMPLICOR HIV-1 DNA test, version 1.5

This test was based on four key procedures: (1) Sample preparation, (2) PCR amplification or target DNA using HIV-1 specific complementary primers, (3) hybridisation of the amplified products to oligonucleotide probes specific to the target DNA and (4) the detection of the probe-bound amplified products by calorimetric determination.

An area of the DBS was punched after which 1ml of Amplicor BLD wash solution was added and rotated for 20 minutes. The supernatant was then discarded.

A working extraction mix was prepared and transferred to a 15ml conical tube. Eighty microlitres (80µl) of the HIV-1C reagent also added to the tube. The positive and negative HIV-1 controls (50µl of each) were prepared separately. Two hundred microlitres (200µl) of the working mix was added to each pellet including the control tubes. Samples were then incubated on a heating block at two intervals of 30 minutes at 60°C and 100°C.

After incubation, 50µl of each sample was added to the analogous working mastermix tubes after which amplification was done using the Perkin Elmer 9600 PCR machine. Amplicons were then denatured by the addition of 100µl of DN solution.

Amplicon detection involved the addition of 100µl of HIV-1 HYB to each well in the source plate. Twenty-five microlitres (25µl) of the denatured and control amplicon samples were added to the plate and mixed until a colour change from blue to yellow was noted. Subsequent to incubation (37°C for 1 hour), the plate was washed using AMPLICOR HIV-1 wash buffer. One hundred microlitres (100µl) of AV-HRP was added to each well and incubated for 15 minutes at 37°C. The wells were washed and 100µl of substrate, prepared by mixing 2.0ml of SUB A and 0.5ml of SUB B, was added to each well. A colour reaction was allowed to develop by incubating the plates in the dark at room temperature for 10 minutes. One hundred microlitres (100µl) of Stop solution was added. The absorbance was read at 450nm using the Anthos Reader (Biochrom Ltd, UK). Results were either negative or positive. Inconclusive and positive test results were repeated.

COBAS AmpliPrep/COBAS TaqMan HIV-1 Qualitative HIV-1 DNA test

This is mainly an automated procedure and involves 4 key processes: (1) Sample pre-extraction and incubation, (2) Sample preparation to isolate HIV-1 target nucleic acids, (3) Reverse transcription of the target RNA to generate complementary DNA and (4) Simultaneous amplification of target cDNA or proviral DNA by PCR and detection of cleaved dual-labelled oligonucleotide detection probe specific to the target.

The COBAS instrument was loaded with the necessary reagents for processing. Control standards, HIV-1 QUAL CS1 were placed on a specific reagent rack of which HIV-1 QUAL CS2, HIV-1 QUAL CS3 and HIV-1 QUAL CS4 was placed onto a separate reagent rack.

One thousand one hundred microlitres (1100µl) of SPEX was then added to each tube containing 12mm punched DBSs. Vials for the CTM negative and HIV-1L positive control were vortexed and 1000ul transferred to tubes placed at position 1 and 2.

Sample tubes were placed in an Eppendorf Thermomixer Comfort at 56°C with continuous shaking at 1000 rpm for 10 to 30 minutes in to lyse blood clots. After incubation samples were then loaded in corresponding positions and the machine instructed to process samples.

The analyser automatically determines the presence of HIV-1RNA or proviral DNA and HIV-1C RNA in the samples and controls. During this analysis, the cycle threshold value as well as fluorescence intensity is determined for either the HIV-1 RNA or proviral DNA and the HIV-1C RNA of both sample and controls. The analyser also determines if HIV-1L positive and CTM negative controls are valid. Should controls fall out of the predicted range, a FLAG is generated and samples were repeated.

Samples that are indicated as positive and have a cycle threshold value greater than 32 or an AFI value less than 5 were repeated for confirmation.

4.1.4.2 Lymphocyte Count (CD3/CD4/CD8)

Routine lymphocyte analyses were completed for all patients at each time point by the Division of Medical Virology, University of Stellenbosch at the Faculty of Health Sciences, Tygerberg. In summary, 50µl of whole EDTA blood was incubated with an aliquot of 20µl MultiTest™ (BD Bioscience, USA) monoclonal antibody mixture (CD3-FITC/CD4-APC/CD8-PE/CD45-PerCP) in a TruCount beads tube for 15 minutes at room temperature in the dark due to the light sensitive characteristic of the monoclonal antibodies. After the first incubation, 450µl of FACS Lysing solution (FACSLyse, BD Bioscience, San Jose, USA) was added and cells incubated at room temperature for a further 15 minutes. Samples were subsequently analysed on a flow cytometer (FACSCalibur, Becton Dickinson) using the automated MultiSet™ software. The CD3, CD4 and CD8 results as well as the CD4:CD8 ratio of each sample analysed were reported as percentages of each subset, as well as absolute values. These results were determined using the beads of known concentration in each tube specific to the batch number.

4.1.4.3 Full and Differential Blood Counts

At each time point, full and differential blood counts were performed on all infants enrolled into the study. Collected EDTA blood samples were sent to the Division of Haematology, NHLS Tygerberg Hospital for analysis.

The Advia 2120 Haematology Analyser® (Siemens Healthcare Diagnostics, USA) is a fully automated diagnostic instrument which employs the principles of flow cytometry and

involves cytochemical reactions. Cell classification, counts, size and haemoglobin levels are measured by the conversion of cytometric data into recognisable results.

Paediatric tube specimens were run via a manual open tube sampler (OTS). Two hundred microlitres (200µl) of the sample was passed through the system for measurement of various parameters. For the measurement of haemoglobin, modified cyanmethaemoglobin, a surfactant, lyses the red cells and releases haemoglobin. When the protein is denatured, haem combines with cyanide and the product is read in the colorimeter. Red blood cells were lysed, fixed and stained in the peroxidase chamber. A precipitate containing peroxidase and a chromogen is added together with hydrogen peroxide. In lymphocytes and large unstained cells (LUC), peroxidase is absent; these cells are identified when moving past a pair of detectors in a tungsten light-based optics chamber. The cell types described above are characterised in combination with their size (scatter) and peroxidase activity.

A manual differential count was performed on both study groups to further classify LUC that were often noted in automated counts, particularly the HEU group.

The optimal staining of cellular elements was done using a Romanowsky stain, which assists in the identification of abnormal cells, as well as mature and immature leukocytes, which may be the result of underlying disorders.

4.2 VACCINE SPECIFIC ANTIBODY RESPONSES

The quantitative determination of specific antibody levels to pertussis, tetanus, Hib and pneumococcus was performed by the use of standard commercial ELISA assays. Analysis was performed within a South African National Accreditation System (SANAS) accredited Immunology routine diagnostic laboratory, NHLS, Tygerberg Hospital, using validated methods as specified by the kit instructions for the determination of antibody levels. All ELISA procedures were done under the supervision of a senior Medical Technologist. The external quality assurance for the ELISA laboratory falls under the Royal College of Pathologists of Australasia (RCPA) programme.

4.2.1 Instrumentation and Reagents

The Bio-Rad PhD™ system (Bio-Rad Laboratories, Inc., Hercules, USA), a robotic liquid handling method was employed. Quantitative IgG plasma antibody levels were measured according to the manufacturers' instructions provided by commercial ELISA kits.

All lines of the ELISA Analyser (Bio-Rad PhD™) were decontaminated and primed with distilled water and appropriate wash buffer before use as part of the daily quality control. Specific IgG levels to pertussis and tetanus were evaluated using SERION ELISA *classic* kits (ESR120G and ESR108G Serion Immunodiagnostica GmbH, Würzburg, Germany) in accordance with the manufacturers' guidelines.

Hib capsular polysaccharide and PCP specific IgG were measured using VaccZyme™ Human Anti-Hib and Anti-PCP ELISA kits (MK016 and MK012; The Binding Site Ltd, Birmingham, England). Microwells in the pneumococcal assay were pre-coated with PCP antigens 1-5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

All kits were stored at 4°C and brought to room temperature before use. Controls and standards provided as part of each kit were included with the samples in all runs.

All samples were frozen and batched thawed on the day of analysis. Each thawed batch was processed immediately. Samples were not run in duplicate due to limited sample volume.

4.2.2 Quantitative IgG Measurement for Pertussis and Tetanus

Samples with positive and negative controls (controls provided with kits) were diluted 1:100 in dilution buffer (phosphate buffer with protein and Tween 20). One hundred microliters (100µl) were dispensed in pre-coated microtiter plates for pertussis and tetanus toxoid. The sample-antigen complex was then incubated for 60 minutes at 37°C to ensure optimal binding of antibodies to antigen (solid phase). Microwells were then washed four times with 300µl of *concentrated washing solution* (sodium chloride containing Tween 20 and 30mM of Tris) to ensure removal of all unbound antibodies and proteins. Thereafter, 100µl of the *APC-conjugate solution* (anti-human-IgG, IgA, IgM from polyclonal goat antibodies conjugated to alkaline phosphatase and stabilised with protein stabilisation solution) was added to the immune complex and incubated for 30 minutes at 37°C. A second washing step was done to ensure removal of all unbound conjugate. Addition of 100µl of *pNPP-substrate solution* (para-nitrophenylphosphate, solvent free buffer) resulted in a formation of a coloured product which was due to a reaction with the conjugate after 30 minutes of incubation at 37°C. A STOP reagent (1.2N sodium hydroxide) was added to halt the enzyme reaction which subsequently resulted in the formation of a yellow coloured product relative to the amount of specific antibody bound. The optical density (OD) was measured spectrophotometrically at 405nm within 60 minutes of adding the STOP solution against the substrate blank with a reference wavelength between 405nm and 620nm. SERION easy base 4PL-Software was used to calculate the antibody concentration in IU/ml from the OD obtained.

4.2.3 Validation and Interpretation of Test Results for Pertussis and Tetanus

Results were valid if the substrate blank had an OD of <0.25. The negative control should always represent a negative concentration for pertussis (calculated by the software programme) and for tetanus it should be less than 0.1IU/ml. The positive control value must be within the validity range specified on the LOT specific quality control (QC) certificate. Tetanus values below 0.1IU/ml were regarded as lower than the detection limit.

Tetanus antibody levels were classified as providing sufficient protection if more than 0.1U/ml.

The normal (protective) ranges for antibodies to pertussis, defined by the manufacturer is listed in Table 4-2 below.

Table 4- 2: References ranges for pertussis antibody levels defined by the manufacturer

Concentration	Evaluation of vaccination titres
>30 FDA U/ml	Normal Protector Ranges
20-30 FDA U/ml	Equivocal
<20 FDA U/ml	None

The WHO classifies tetanus antibody levels greater than 0.1 IU/ml providing sufficient protection (WHO, 2006)

The SERION ELISA kit further classifies the antibody levels to tetanus as described as in Table 4-3 below. The significance of these test results were evaluated against several parameters, including age, vaccination history and clinical history.

Table 4- 3: Reference ranges for tetanus toxoid antibody levels defined by the manufacturer

Concentration	Evaluation of vaccination titres
<0.01 IU/ml	no immunity, depending on vaccination history: booster shot or basic immunisation
0.01-0.1 IU/ml	no safe immune protection, booster shot recommended
0.11-0.5 IU/ml	sufficient immune protection
0.51-1.0 IU/ml	sufficient immune protection, booster shot not necessary
1.1-5.0 IU/ml	long term protection, titre control after 5-10 years
>5.0 IU/ml	long term protection, titre control after 10 years

4.2.4 Quantitative Antibody Levels to Hib and PCP

Patient samples and six calibrators provided in the kit were diluted 1:100 in dilution buffer specific for Hib and PCP and added to each of the pre-coated anti-Hib or anti-PCP microtitre wells (solid phase). The sample-antigen complex was then incubated for 30 minutes at room temperature to ensure optimal binding of antibodies contained in patient serum (mobile phase). Microwells were washed three times with 300µl of wash buffer to ensure all unbound antibodies and proteins within patient sera were removed. After washing, 100µl of conjugate solution (purified peroxidase labelled antibody to human IgG) was added to the immune-complexes and incubated for 30 minutes at room temperature. A second washing step was carried out to ensure all unbound conjugate were removed. Addition of 100ul of substrate solution (*3,3',5,5' tetramethylbenzidine (TMB)*) was added and allowed to react, which resulted in a blue coloured product. After the addition of the substrate solution, 100µl of STOP reagent (3M phosphoric acid) was added to each of the wells. This latter step ensured that the enzyme-substrate reaction was terminated, resulting in the formation of a yellow colour, which is relative to the amount of specific antibody bound.

The OD was measured spectrophotometrically at 450nm within 30 minutes of adding the STOP solution. A calibration curve was constructed for both anti-Hib IgG and anti-PCP antibody with concentration on the log scale against the OD on the linear scale for each calibrator. The accuracy of the curve is limited by the sensitivity below 0.33mg/L.

4.2.5 Validation and Interpretation of Test Results for Hib and PCP

The values for controls (high and low controls) were specified on the QC certificate of the respective kits. The standard curve was constructed using known concentrations indicated in the kit. The generated curve of the test run was compared to that of the QC certificate for further verification of a valid test run.

Anti-Hib antibody titres of more than 1.0mg/L were regarded as protective (Agbarakwe *et al.*, 1995).

There has been no defined level of protective immunity for the collective response to pneumococcal serotypes indicated for the PCV₇ vaccine.

4.2.6 Validation of Plasma Samples for the use of Hib and PCP kits

Virion Serion kits are validated for use of both plasma and serum. However, according to the manufacturing guidelines for the MK016 and MK012 kits (The Binding Site Ltd, Birmingham, England), plasma samples were not validated to be used with these kits. A subsequent in-house comparative validation assay was done to compare and correlate the results found with plasma and serum for Hib and PCP kits. For this validation, 10 adult samples were collected in sodium heparin (for plasma) and serum-separating tubes (SST) (for serum). Samples were centrifuged at 4500 rpm to separate the plasma and serum from the red blood cells and then analysed according to the methods described above under all specified laboratory conditions. Results showed no difference between the two sample types. Statistical methods were employed to compare the results. An intra-class correlation (ICC) analysis was used to determine if any differences between the two sample types exists. Results for the Hib kit showed an ICC value of 0.967 (SEM 0.697) and for the PCP kit an ICC value of 0.999 (SEM 3.165) was calculated. An ICC value close to one is indicative of the similarity in the methods used. Based on the statistical outcomes, we therefore concluded that there were no significant differences between the results obtained for serum and plasma.

4.3 CELLULAR MARKERS OF IMMUNE ACTIVATION, APOPTOSIS AND B CELL MEMORY

4.3.1 Experimental Design of Study

This part of the study was designed to investigate the levels of immune activation, apoptosis and regulation on adaptive immune cells (CD4+ T, CD8+ T, CD19+ B and CD20+ B cells) in HEU and UE infants at 18 and 24 months of age. In addition, a functional assay was developed and optimised to evaluate the concept of spontaneous apoptosis by measuring the expression of the early apoptotic marker, Annexin V, on CD4+ T and CD20+ B cells at 16 and 24 hours. At the 24 month time point, the expression of Annexin V was only measured at 16 hours.

In addition, this part of the study was also aimed at investigating whether adequate B-cell memory is generated by HEU infants, in comparison to UE infants. Therefore, the expression of cellular markers of memory (CD27) on CD20+ and CD19+ B cells was measured.

For comparing various cellular markers of immune activation, apoptosis and B cell memory, and each subject had four polystyrene round bottom non-pyrogenic falcon tubes (BD Bioscience, USA) labelled panel 1-7. Table 4-4 below describes the various panels and cellular markers with appropriate function. After sample processing and staining, acquisition was conducted using a four colour flow cytometer (BD™ FACSCalibur).

Table 4- 4: Summary of cellular markers investigated in HEU and UE infants at 18 and 24 months of age (on which markers were measured is highlighted in blue)

PANELS	FLOROCHROMES	MARKER	MEASURED FUNCTION
PANEL 1	PE	FasL	Expressed on activated T cells, signalling pathway for apoptosis
	FITC	Fas	Expressed on T cells, initiates a signalling cascade leading to cell death
	PERCP	CD69	Expressed on activated T cells involved in signalling
	APC	CD4	Class II MHC restricted T cell, signalling and adhesion
PANEL 2	PE	CD80	Expressed on activated B cells, co-stimulator for T cell activation
	FITC	Annexin V	Measure early apoptosis on B and T cells in this panel
	PERCP	CD20	Expressed on all B cells, Involved in B cell activation (general B cell marker)
	APC	CD4	Class II MHC restricted T cell, signalling and adhesion
PANEL 3	FITC	Fas	Expressed on B cells, initiated a signalling cascade leading to cell death
	PERCP	CD20	Expressed on all B cells, Involved in B cell activation (general B cell marker)
	APC	CD62L	Expressed on B-cells, homing of naive T cells to peripheral lymph nodes
PANEL 4	PE	CD10	Expressed on mature B cells
	PERCP	CD20	Expressed on all B cells, Involved in B cell activation (general B cell marker)
	APC	CD27	Memory B cell marker, mediates co stimulatory signals for T and B cell activation
PANEL 5	PE	CTLA-4	Expressed on cytotoxic T lymphocytes, involved in the regulation of the immune system by transmitting inhibitory signals to T cells
	FITC	CD127	Marker of late pro-T cell growth, proliferation and maturation, IL-7 receptor
	PERCP	CD4	Class II MHC restricted T cell, signalling and adhesion
	APC	CD40L	Expressed on activated T cells and involved in promoting B cell proliferation
PANEL 6	PE	PD-1	Involved in cell regulation during activation, extended member of CTLA-4 family of proteins
	FITC	CD69	B cell activation marker
	PERCP	CD19	B cell co-receptor subunit
	APC	CD27	Memory B cell marker, mediates co stimulatory signals for T and B cell activation
PANEL 7	PE	FASL	Expressed on activated T cells, signalling pathway for apoptosis
	PERCP	CD8	Expressed on cytotoxic T cells (MHC class I receptor)
	APC	CD38	Well described marker of immune activation in HIV, expressed on T cells

Spontaneous apoptosis was measured by analysing the expression of cellular apoptotic marker, Annexin V, using two polystyrene round bottom non-pyrogenic falcon tubes (BD Biosciences, USA) each, labelled 16 and 24 hours. After sample processing and staining acquisition was conducted using the Becton Dickinson BD™ FACSCalibur.

4.3.2 Study Subjects and Blood Collection

The subjects involved in this segment of the study included infants that formed part of the larger pilot study and arrived for the 18 and 24 month visit. Analysis for panels 1-7 listed above was completed for a total of 98 infants.

At 18 months, a total of 41 infants (18 HEU, 21 UE and 2 HIV positive) were analysed for cellular markers listed in panel 1-4. During later stages of the 18 months time point, additional panels (5-7) were added and analysed for 18 infants (9 HEU and 9 UE). The measurement of Annexin V as part of the spontaneous apoptosis assay (also initiated later in the time point), was completed for a total of 15 infant samples which included 6 HEU, 8 UEs and 1 HIV positive infant.

At the 24 month time points, a total of 39 infants were analysed (17 HEU, 20 UE and 2 HIV positives) for panels 1-7 including the measurement of Annexin V expression at 16 hours only.

Four hundred and fifty microlitres (450µl) of the total 5ml of collected blood (described above) was allocated for this part of the study.

4.3.3 Reagents and Buffers

Reagents

- a) **Monoclonal antibodies:** CD4-APC (*Clone: OKT4*-Biolegend, USA), Fas-FITC (*Clone: DX2*-BD Biosciences, USA), FasL-PE (*Clone: MFL3*-eBioscience, UK), CD69-PerCP (*Clone: FN50*-BD Biosciences, USA), Annexin V-FITC (BD Biosciences, USA), CD20-PerCP (*Clone: L27*-BD Biosciences, USA), CD80-PE (*Clone: L307.4*-BD Biosciences, USA), CD62L-APC (*Clone: Dreg56*-BD Biosciences, USA), CD10-PE (*Clone: HI10a*-BD Biosciences, USA), CD127-FITC (*Clone: HL-7R-M21*-BD Biosciences, USA), CD27-APC (*Clone: 0323*-eBioscience, UK) CD4-PerCP (*Clone: SK7*-BD Biosciences, USA), CTLA-4-PE (*Clone: BNI3*-BD Biosciences, USA), CD40L-APC (*Clone: TRAP1*-BD Biosciences, USA), CD19-PerCP (*Clone: HIB19*-Biolegend, USA), PD-1-PE (*Clone: MIH4*-BD Biosciences, USA), CD69-FITC (*Clone: FN50*-BD Biosciences, USA), CD8-PerCP (*Clone: SK1*-BD Biosciences, USA), CD38-APC (*Clone: HIT2*-eBioscience, UK) and FasL-PE (*Clone: MFL3*-eBioscience, UK)
- b) **Antibody cocktails:** prepared according to each panel listed in Table 4-4. For each panel an antibody cocktail was made using equal volumes of each monoclonal antibody. The volume of antibody cocktail aliquoted to each tube is described below. The optimisation (by use of titration and Fluorescence-minus-one experiments) of required volume was determined by ongoing projects within the laboratory.

Buffers and Solutions

- a) **10% FACS lysing solution:** working solution was prepared by diluting the 10X concentrate (BD Biosciences, USA) in MilliQ water. The solution was used to lyse red blood cells and suspend white blood cells. The solution was stored at 4°C and brought to room temperature before use.
- b) **Staining buffer:** this solution was prepared by adding 1.25ml of heat inactivated (56°C for 1 hour) Foetal Bovine Serum 10106 (FBS;

Gibco[®], Invitrogen, New Zealand) to 498.75ml of Dulbecco's Phosphate Buffered Saline 14190 (DPBS; Gibco[®] Invitrogen, New Zealand). This buffered salt solution was used during washing stages of cell preparation. Solution was stored at 4°C and brought to room temperature before use.

- c) **Cell culture media:** media for spontaneous apoptosis assay was prepared using sterile endotoxin-free RPMI 1640 (Lonza, Biowhittaker, USA) with 10% heat inactivated FBS. Cell culture media was stored at 4°C and was warmed up in a 37°C incubator an hour before use.

4.3.4 Cell Preparation and Staining

Daily maintenance and quality control of flow cytometer

The basic daily maintenance of the flow cytometer includes start-up and shut-down procedures. The start-up procedure includes switching on the flow cytometer, followed by the computer at least 15 seconds later to ensure connectivity. The sheath container is filled with FACSTFlow[®] (sheath fluid) and the waste container emptied and filled with 200ml of FACSClean[®]. The system is then pressurised using the vent valve. Air within the sheath filter is released by using the bleed tube. Two primes are performed to ensure that the flow chamber contains no bubbles. About 4ml of distilled water is run through the machine on Hi for 5 minutes to ensure effective cleaning of the system.

After cleaning procedures, calibration and colour compensation of the flow cytometer is followed.

Colour compensation is done using FACSCComp[®] beads. For four colour compensation and time delay calibration, two tubes were set up using one drop of unlabelled CaliBRITE[®] (BD Biosciences) beads and one drop of APC-labelled CaliBRITE[®] beads in 1ml of sheath fluid (FACSTFlow[®]). In the tube used for colour compensation, 1 drop of each unlabelled FITC, PE, PerCP and APC labelled CaliBRITE beads in 3ml of sheath fluid was added. By use of the FACSCComp[®] programme, the machine is monitored for its performance in automated time delay calibration and colour compensation.

As part of the daily shut-down procedure, FACSRinse[®] is run through the machine with the support arm in the un-engaged position. Once the machine aspirates about 2ml of FACSRinse[®], the support arm is placed in the engaged position and continued to run for 5minutes. The same procedure is followed for FACSClean[®] and distilled water. On completion of this process, the sheath container is de-pressurised by releasing the vent valve and selecting the standby mode.

Staining for markers of immune activation, regulation, apoptosis and B cell memory

The process for staining cells for the list of markers in Table 4-5 include pipetting 50µl of whole blood into four polystyrene round bottom non-pyrogenic falcon tubes labelled panel 1-7. Five microliters (5µl) of the pre-made antibody cocktail as described above was then added to each one of the four panels. Contents were gently mixed by small vortex pulses and incubated for 20 minutes in the dark at room temperature.

After incubation, 500µl of BD FACSLysing Solution™ (1X) was added to lyse the red blood cells and was thereafter vortexed. Further incubation for 20 minutes in the dark at room temperature followed. Subsequently, 500µl of staining buffer was added, vortexed and centrifuged for 5 minutes at 1300 rpm also at room temperature.

After centrifugation, 750µl of the supernatant was removed and the pellet gently re-suspended. Three hundred and fifty microlitres (350µl) of the staining buffer was added to the pellet, which was then stored in the dark at 4°C until measured on a flow cytometer.

Staining for Annexin V as cellular marker for spontaneous apoptosis

One hundred microlitres (100µl) of subject whole blood was added to two polystyrene round bottom non-pyrogenic falcon tubes and mixed with 100µl of RPMI 1640 + 10% foetal calf serum (FCS) (cell culture media). Samples were thereafter incubated at 37°C (5% CO₂) for 16 and 24 hours respectively. The time points (16 and 24 hours) were chosen based on optimisation protocols, were samples were previously incubated at 4 and 16 hours. It was concluded that 4 hours was insufficient time for the measurement of spontaneous apoptosis and 16 hours seemed to be optimal.

At 16 and/or 24 hours, samples were removed from incubation and centrifuged at 1300 rpm for 5 minutes at room temperature. One hundred and fifty microlitres (150µL) of the supernatant was removed in order to leave a volume of 50µl of whole blood.

The staining procedure for Annexin V then followed which included incubation in the dark at room temperature for 20 minutes with 5µl of pre-made antibody cocktail, which included monoclonal antibodies, Annexin V-FITC, CD4-APC and CD20-PerCP (BD Biosciences, The Scientific Group, South Africa).

After incubation, 500µl of BD FACSLysing Solution™ (1X) was added to lyse the red blood cells and vortexed thereafter. Further incubation for 20 minutes in the dark at room temperature followed. Subsequently, 500µl of staining buffer was added, vortexed and centrifuged for 5 minutes at 1300 rpm also at room temperature.

After centrifugation, 750µl of the supernatant was removed and the pellet was gently re-suspended. Three hundred and fifty microlitres (350µl) of the staining buffer was then added to the pellet which was then stored in the dark at 4°C until measured on the flow cytometer.

4.3.5 Flow Cytometric Analysis

Prepared samples were acquired on a flow cytometer (Becton Dickinson BD™ FACSCalibur). CellQuest™ software was used for the acquisition and the threshold was set on forward scatter (FSC) to exclude cell debris. A total of 20,000 events in gate 1 (as shown in Figure 4-1 below) were acquired. During both 18 and 24 month time points, a panel of unstained cells were acquired. The analysis of these unstained panels served as negative controls and assisted in the visualisation of the negative population as well as defining populations of interest.

Defining Lymphocyte Population (Gating strategy)

A dot plot of forward scatter (FSC), which is an indication of cell size and side scatter (SSC), an indication cell granularity, was constructed for each sample. Three cell populations were

noted with the dot plot. Neutrophils represent the highest population of cells with the highest FSC and SSC. Monocytes have similar FSC as neutrophils and intermediate SSC. Lymphocytes are located at the bottom of the plot and have the lowest SSC and FSC, implying that they are smaller in size and have the least complexity.

Figure 4-1 below indicates the distinct population groups that exist, as well as the gating strategy for lymphocytes.

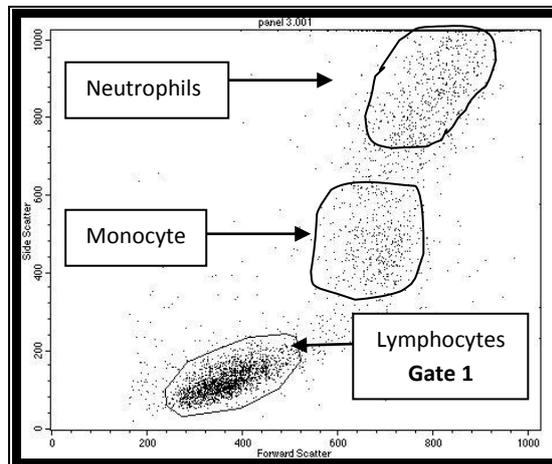


Figure 4- 1: Definition of lymphocyte and other cell types indicated by a dot plot. Lymphocytes, monocytes and neutrophils were identified and gated according to their different characteristics of size and granularity.

The lymphocyte gating strategy was employed during the acquisition of each of the seven panels.

A dot plot was constructed for each panel after which the quadrant statistics was used to determine the percentage of cells with marker expression on T and B subpopulations (See figures 4-2 to 4-8 below).

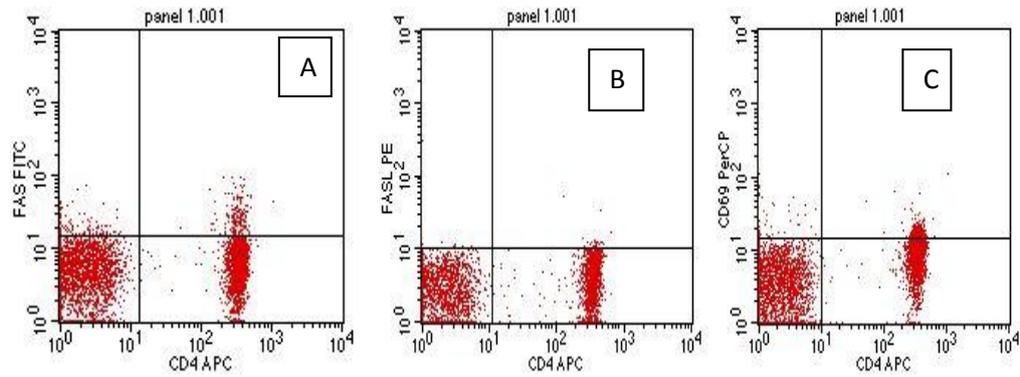


Figure 4- 2: For panel 1, dot plots visualised above were created to measure dual populations (top right quadrants) for each of the parameters indicated by A, B and C. (A) dot plot created to measure the expression of FAS-FITC on CD4-APC. (B) Dot plot created to measure expression of FASL-PE on CD4-APC. (C) Dot plot created to measure expression of CD69-PerCP on CD4-APC.

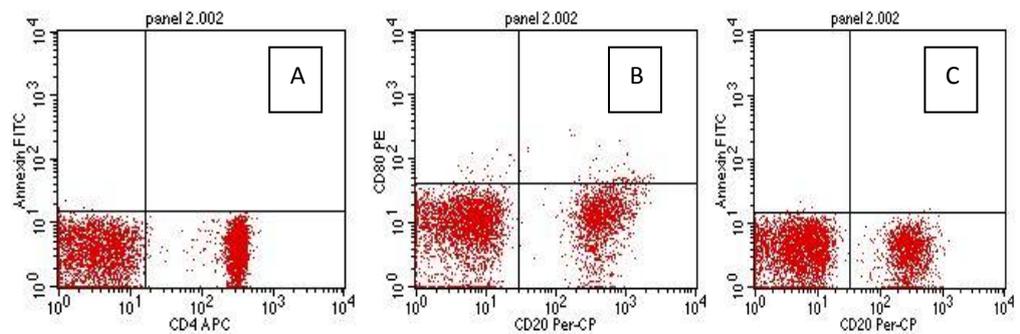


Figure 4- 3: For panel 2, dot plots visualised above were created to measure dual populations (top right quadrants) for each of the parameters indicated by A, B and C. (A) dot plot created to measure the expression of Annexin V-FITC on CD4-APC. (B) Dot plot created to measure expression of CD80-PE on CD20-PerCP. (C) Dot plot created to measure expression of Annexin V-FITC on CD20-PerCP.

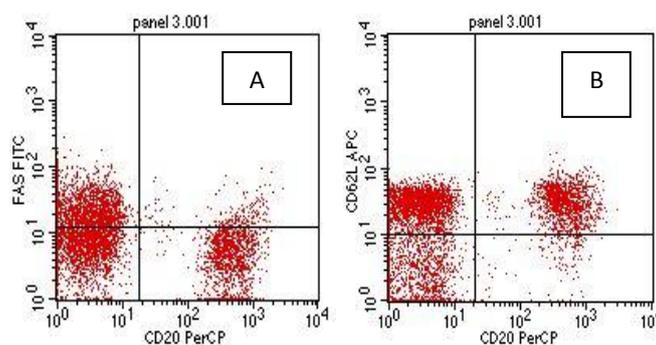


Figure 4- 4: For panel 3, dot plots visualised above were created to measure dual populations (top right quadrants) for each of the parameters indicated by A and B. (A) dot plot created to measure the expression of FAS-FITC on CD20-PerCP. (B) Dot plot created to measure expression of CD62L-APC on CD20-PerCP.

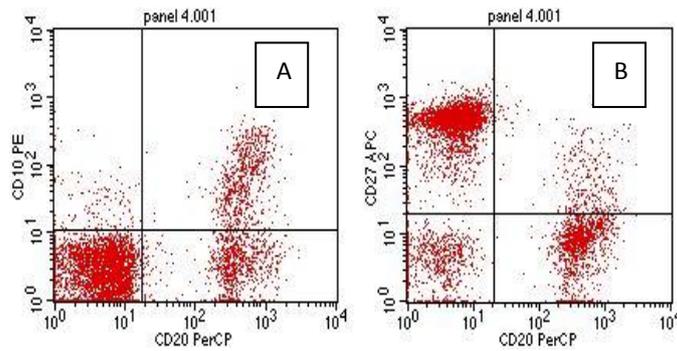


Figure 4- 5: For panel 4, dot plots visualised above were created to measure dual populations (top right quadrants) for each of the parameters indicated by A and B. (A) Dot plot created to measure expression of CD10-PE on CD20-PerCP. (B) Dot plot created to measure expression of CD27-APC on CD20-PerCP.

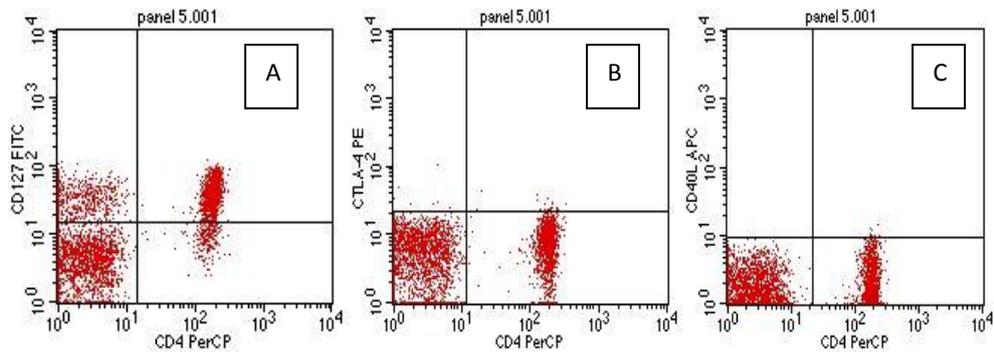


Figure 4- 6: For panel 5, dot plots visualised above were created to measure dual populations (top right quadrants) for each of the parameters indicated by A, B and C. (A) dot plot created to measure the expression of CD127-FITC on CD4-PerCP. (B) Dot plot created to measure expression of CTLA-4-PE on CD4-PerCP. (C) Dot plot created to measure expression of CD40L-APC on CD4-PerCP.

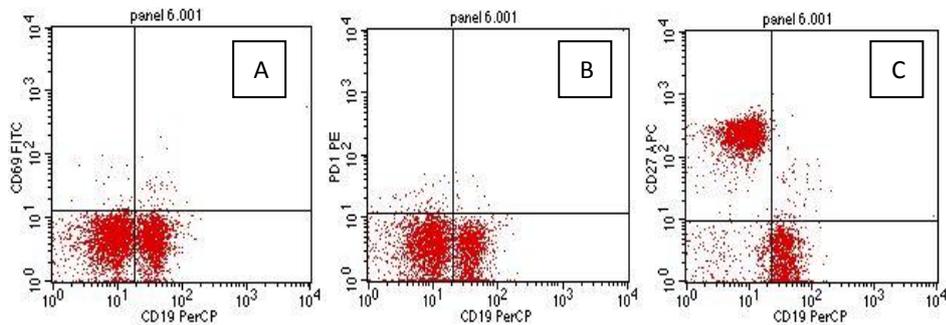


Figure 4- 7: For panel 6, dot plots visualised above were created to measure dual populations (top right quadrants) for each of the parameters indicated by A, B and C. (A) dot plot created to measure the expression of CD69-FITC on CD19-PerCP. (B) Dot plot created to measure expression of PD1-PE on CD19-PerCP. (C) Dot plot created to measure expression of CD27-APC on CD19-PerCP.

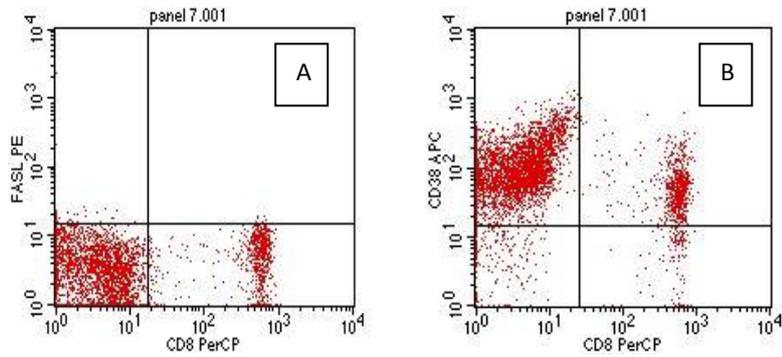


Figure 4- 8: For panel 7, dot plots visualised above were created to measure dual populations (top right quadrants) for each of the parameters indicated by A and B. (A) Dot plot created to measure expression of FasL-PE on CD8-PerCP. (B) Dot plot created to measure expression of CD38-APC on CD8-PerCP.

Measurement of Spontaneous Apoptosis

The determination of Annexin V binding was also performed on the FACSCalibur™ flow cytometer. A total of 20 000 events were acquired using CellQuest™ software. The gating of lymphocytes for this section was the same as described previously (Figure 4-1 above). The binding of Annexin V on CD4+ T cells and CD20+ B cells was determined by constructing a dot plot and defining quadrant statistics. Figures 4-9 to 4-11 below show dot plots of Annexin V binding for T and B cells before and after *ex vitro* stimulation. Stimulation occurred at 16 and/or 24 hour time points.

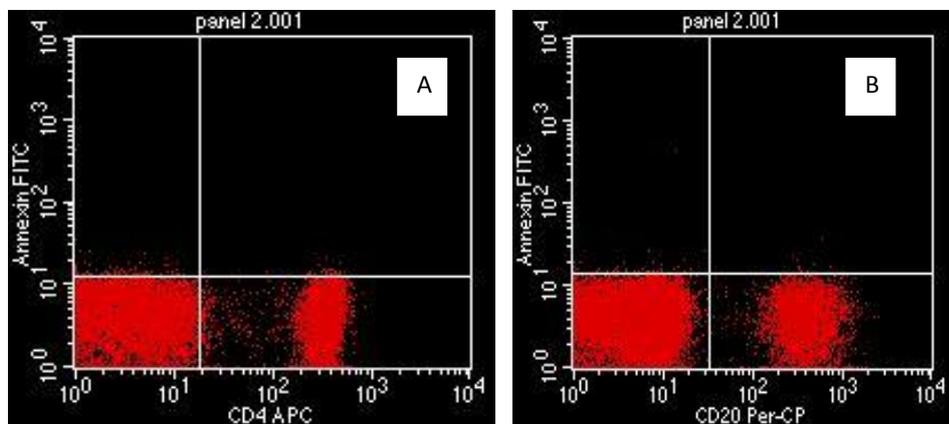


Figure 4- 9: Unstimulated expression of Annexin V on CD4-APC cells (A) and CD20-PerCP (B) cells. There is no indication of dual populations for both CD4-APC and CD20-PerCP.

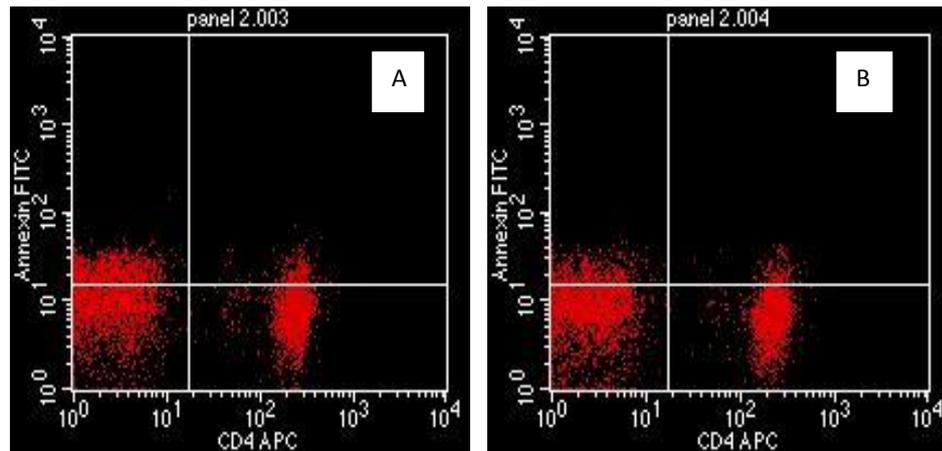


Figure 4- 10: Expression of Annexin V after 16 and 24 hour stimulations. (A) The generation of a dot plot indicates the expression of Annexin V-FITC on CD4-APC (dual population in the top right quadrant) after 16 hours. (B) The generation of a dot plot indicates the expression of Annexin V-FITC on CD4-APC (dual population in the top right quadrant) after 24 hours.

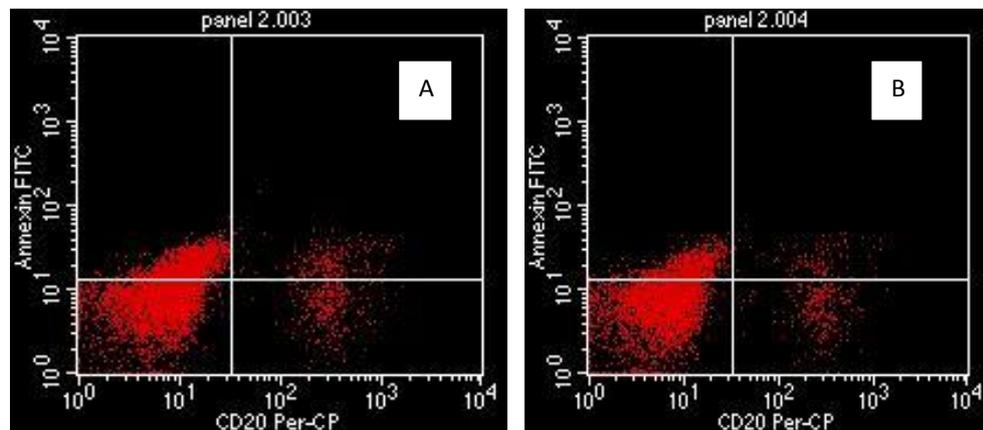


Figure 4- 11: Expression of Annexin V after 16 and 24 hour stimulations. (A) The generation of a dot plot indicates the expression of Annexin V-FITC on CD20-PerCP (dual population in the top right quadrant) after 16 hours. (B) The generation of a dot plot indicates the expression of Annexin V-FITC on CD20-PerCP (dual population in the top right quadrant) after 24 hours.

4.4 DATA COLLECTION AND PROCESSING

Data was collected using Microsoft Excel software (2007) spreadsheets. Basic descriptive statistics was done using the same programme. Further statistical analysis was performed by the Centre for Statistical Analysis, University of Stellenbosch using *Statistica version 10* software. All data sets were analysed for normality. Data that represented a non-normal distribution were log transformed and appropriate statistical methods employed for analysis. All comparisons were pre-defined except for the comparison of the 18 and 24 month cellular makers over time for the two groups, which was post-hoc. Missing data points were removed from all analysis.

Confidence intervals of 95% were used during analysis and p values less than 0.05 ($p < 0.05$) and 0.01 ($p < 0.01$) were considered as statistically significant and highly significant respectively. HIV positive infants ($n=4$) were excluded from all analysis.

4.4.1 Population Characteristics

The population characteristics were divided into categorical and numerical data.

Categorical data included mode of delivery, gender, racial group, housing structure, access to running and maternal HIV diagnosis (before or after pregnancy). Numerical data included gravidity, maternal age, number of people living in the same household as infant, number of people sleeping in the same bed as infant, gestational age, birth weight and weight over time.

Other categorical (PMTCT regime and breastfeeding) and numerical data (gestational age when PMTCT therapy started and maternal CD4 count) were listed but not statistically analysed due to these factors being related to only one group (HEU) or showed vast differences amongst the groups (e.g breastfeeding).

The Student's T test was employed for the analysis of all numerical data. All data represented normal distribution.

A non parametric Pearson Chi-Square test was employed for categorical data representing housing structure and access to running water and a Fisher's exact one tailed test p-value (for the comparison of proportion) was recorded for mode of delivery. Pearson Chi-Square cross tabulations was also used for the analysis of contingency tables presented for gender and racial groups. Maternal HIV diagnosis (only for the HEU group) was analysed using a two-way Analysis of Variance (ANOVA).

The minimum and maximum ranges were also displayed for each numerical data set.

4.4.2 Antibody data

The antibody levels to specific vaccines (pertussis, tetanus, Hib and pneumococcus) at each time point for both groups (HEU and UE) were compared by repeated measures ANOVA using a mixed model approach which compensated for infants who missed follow-up visits. Data was log transformed to compensate for non-normal distribution. Results were displayed graphically using Type III decomposition vertical bars with 95% confidence intervals. The p-values were calculated using by comparing the Least Square (LS) means of the HEU and UE groups at the specific time points.

HEU and UE subjects were categorised from the 12 week blood draw into groups according to vaccination doses given. These groups were stratified and analysed by use of log transformation and the employment of ANOVA analysis (report of the F-test p-value) to determine the effect of the administration of 1 or 2 doses on the IgG levels to pertussis, tetanus and Hib. Median days from vaccination to blood draw was calculated at 12 weeks, 6, 12 and 18 months of age. Infants who did not receive the required dose(s) by the time of the specific blood draw were removed from the analysis. Those infants who received "catch-up" vaccinations were included in subsequent analysis. The median days from vaccination to blood draw were then re-calculated accordingly.

Spearman correlations were employed to show a relationship between the specific vaccine IgG responses at various time points with maternal age, gravidity, housing structure, gender, birth weight, racial group, number of household members and access to running water.

4.4.3 Flow cytometry cellular markers

Differences in the expression of the various cellular markers between the two groups were compared by employing a one-way ANOVA analysis. An F-test (which assumes normality) p-value was reported of which the non parametric Mann-Whitney U test was used as a confirmatory tool to test the hypothesis that stipulates a difference between the two groups. In cases where the Mann-Whitney U and F-test showed differing outcomes, data was log-transformed and the F-test reported. The 18 and 24 month time points were compared separately and then over time by using a two-way ANOVA.

The evaluation of the functional “spontaneous apoptosis” assay at 18 months for the comparison of expression of Annexin V at three time points (*ex vivo*, 16 and 24 hours) were compared by using a repeated measures ANOVA analysis. Results were displayed graphically using Type III decomposition vertical bars with 95% confidence intervals. The p-values were calculated by comparing the LS means of the HEU and UE groups at the three time points.

At 24 months, the comparison of the expression of Annexin V at two time periods (*ex vivo* and 16 hours) was compared by using a one-way ANOVA analysis. Results were displayed graphically using Type III decomposition vertical bars with 95% confidence intervals. The p-values were calculated by comparing the LS means of the HEU and UE groups at the two time points.

CHAPTER 5

RESULTS

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5.1 STUDY POPULATION

5.1.1 Overview

In this study, children born to HIV positive and negative mothers were assessed longitudinally (2 weeks to 24 months) for vaccine-specific IgG responses to pertussis, tetanus, Hib and pneumococcus. The 2 and 6 week time points indicated the presumed maternally derived antibody levels and thereafter subsequent time points (12 weeks, 6, 12, 18 and 24 months) represented the post-vaccination response as scheduled according to the EPI-SA. Although the IgG levels for the pneumococcal vaccine were determined for all infants at each visit, only the presumed maternal antibody levels at 2 and 6 weeks will be reported.

In addition, various specific cellular markers of immune activation, apoptosis, regulation and B cell memory on CD4+ T, CD20+ B, CD19+ B and CD8+ T cells were assessed at 18 and 24 months. Furthermore, we evaluated cell susceptibility to undergoing spontaneous apoptosis at 18 and 24 months through the development of a functional *ex vivo* assay and subsequent staining procedures for Annexin V.

5.1.2 Characteristics of Study Population

In this longitudinal comparative pilot study, a total of 95 mothers were recruited for participation after delivery. Of these, 43 were HIV positive (45%) and 52 were HIV uninfected (55%). The study also included 3 sets of twin births, all in the HEU group.

At the 2 week time point, a total of 61 participants were followed up, which included 27 HEU (44%), 30 (49%) UE controls and 4 (7%) HIV positive (PCR confirmed at 2 weeks) infants. During the time of recruitment and the 2 weeks visit (first time point), 34 patients were lost-to-follow-up (LTFU) or excluded from the study. Of these 34 mothers, 22 were HIV negative and 12 were HIV positive. See Table 5-2 for reasons of exclusion. At the final time point (24 months), a total of 39 participants were followed up and included 17 HEU, 20 UE and 2 HIV positive babies (See Tables 5-1 and 5-2 for participant retention and attrition). HIV positive babies were excluded from statistical analysis; however, remained part of the study and were clinically followed up.

There were 2 infant deaths during the course of this study. One from the UE group who died before the 6 month visit (died of a disseminated adenovirus infection) and an HIV positive infant who died before the 12 week visit (was admitted to Red Cross Children's Hospital and died of pneumonia).

Table 5- 1: Summary of patient visits (retention) from 2 weeks to 24 months

Time point	Status	Number	Total and % Retention from last visit
2 weeks	HEU	27	61 (64% of recruited population retained for follow-up)
	UE	30	
	HIV POS	4	
6 weeks	HEU	27	59 (97%)
	UE	28	
	HIV POS	4	
12 weeks	HEU	27	57 (97%)
	UE	27	
	HIV POS	3	
6 months	HEU	25	50 (88%)
	UE	22	
	HIV POS	3	
12 months	HEU	23	47 (94%)
	UE	21	
	HIV POS	3	
18 months	HEU	18	41 (87%)
	UE	21	
	HIV POS	2	
24 months	HEU	17	39 (95%)
	UE	20	
	HIV POS	2	

According to the patient files from the HIV positive mothers (n=27), 52% were diagnosed during pregnancy. The majority of these (73.1%) received dual therapy, 15.4% were on (HAART) and 11.5% did not receive any form of PMTCT care during pregnancy. A total of 23 maternal CD4 counts were extracted from the patient records at 2 weeks, which produced a median value of 337 cells/ μ l (SD 150.83). All except one (4%) of HIV infected mothers (n=26) selected exclusive formula feeding at birth and one mother in the UE group chose to formula feed (n=29).

The characteristics of the study population are outlined in Table 4-2 (Chapter 4).

Table 5- 2: Summary of patient attrition

Number of patients	Maternal HIV Status	Reason
28	18 HIV negative 10 HIV positive	Refused further participation
1	HIV positive	Relocation
1	HIV negative	Mother diagnosed with hypertension
1	HIV negative	Mother diagnosed with anaemia
1	HIV negative	Under-age (17 years) Recruitment required consent from parents or guardian
1	HIV positive	Late recruitment
1	HIV negative	Missed 2 week visit and arrived for 6 weeks

5.2 PRESUMED MATERNALLY DERIVED SPECIFIC ANTIBODY LEVELS

Results will be graphically presented using type III vertical bar graph (95% confidence intervals). Time-group interaction vs. LS means for both HEU and UE groups will be used to present the IgG specific responses at different time points. The x-axis on the graph depicts the time points and the y-axis depicts the specific IgG concentration (Figures 5.1 to 5.4).

Mean values, standard deviation (SD) and p-values for the specific IgG responses at different time points will be summarised in Tables 5.3 to 5.6. Statistical significance is indicated when $p < 0.05$.

5.2.1. Pertussis IgG levels at 2 and 6 weeks

A total of 84 samples were evaluated for IgG levels against pertussis toxin at 2 and 6 weeks of age. At 2 weeks, a total of 22 samples in the HEU and 23 samples in the UE groups were processed and at 6 weeks, a total of 19 HEU and 20 UE samples were analysed respectively. Infants who received the 6 weeks vaccination before or on the blood draw day were removed from the analyses.

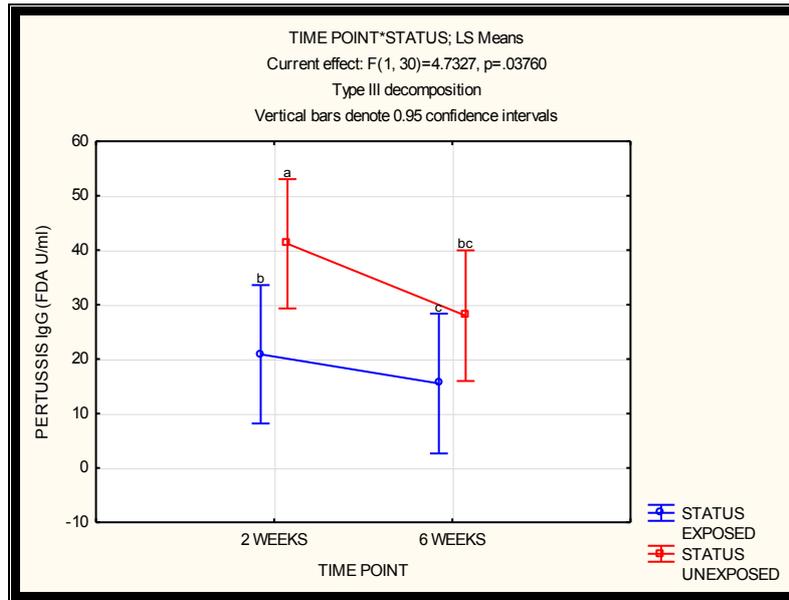


Figure 5- 1: Comparison of pertussis IgG levels (FDA U/ml) from 2 to 6 weeks of age. The time group effect is shown to be significant (p=0.03760).

HEU infants displayed lower mean IgG levels to pertussis (20.80 vs. 38.01 FDA U/ml at 2 weeks and 13.72 vs. 25.05 FDA U/ml at 6 weeks). The differences between the HEU and UE groups reached statistical significance at 2 weeks only. In addition, the lower mean levels of IgG in the HEU group at 2 and 6 weeks corresponded with decreased levels of protection (protective pertussis levels >30 FDA U/ml). However, in the UE group, the mean level at 2 weeks was considered protective, but decreased to non-protective levels at 6 weeks of age (38.00-23.40 FDA U/ml).

At 2 weeks only 5 HEU infants had levels deemed to be protective (22.7%) in comparison to 9 infants in UE group (39.1%). At 6 weeks, no infant in the HEU group had protective levels for pertussis compared to the UE group where a total of 5 infants (25%) had protective levels. Both groups showed a 1.5 fold decrease in the maternal antibody levels from 2 to 6 weeks of age.

Table 5- 3: Pertussis IgG levels at 2 and 6 weeks of age.

PERTUSSIS IgG					
Group	n	Time point	Mean (FDA U/ml)	SD	P-value
HEU	22	2 weeks	20.80	21.43	0.0237
UE	23		38.01	39.72	
HEU	19	6 weeks	13.72	6.45	0.1579
UE	20		25.05	23.51	

5.2.2. Tetanus IgG levels at 2 and 6 weeks

A total of 84 samples were evaluated for IgG levels against tetanus at 2 and 6 weeks of age. At 2 weeks a total of 22 samples in the HEU and 23 samples in the UE groups were

processed and at 6 weeks, a total of 19 HEU and 20 UE samples were analysed respectively. Infants who received the 6 weeks vaccination before or on the blood draw day were removed from the analyses.

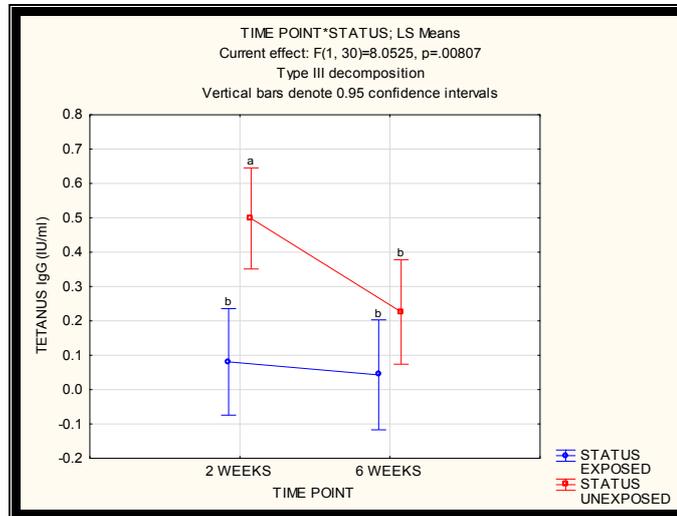


Figure 5- 2: Comparison of tetanus IgG levels (IU/ml) from 2 to 6 weeks of age. The time group effect is shown to be significant (p=0.00807).

HEU infants had lower mean levels for tetanus IgG antibodies compared to UE controls (0.08 vs. 0.53 IU/ml and 0.05 vs. 0.21 IU/ml). A statistical difference between the two groups was noted at both 2 weeks of age. At both time points, HEU infants displayed non-protective mean levels for tetanus IgG (protective tetanus levels >0.1 IU/ml) compared to the UE group that showed protective mean levels at both 2 and 6 week time points.

At 2 weeks only 5 HEU infants had levels deemed to be protective (22.7%) in comparison to 13 infants in UE group (56.5%). At 6 weeks, 3 infants in the HEU group (15.8%) had protective levels for tetanus compared to the UE group where a total of 8 infants (40%) had protective levels. The HEU group showed a 1.8 fold decrease in the tetanus levels from 2 to 6 weeks compared to the UE group that had a 2.5 fold decrease.

Table 5- 4: Tetanus IgG levels at 2 and 6 weeks of age.

TETANUS IgG					
Group	n	Time point	Mean (U/ml)	SD	P-value
HEU	22	2 weeks	0.08	0.12	0.000394
UE	23		0.53	0.62	
HEU	19	6 weeks	0.05	0.05	0.100574
UE	20		0.21	0.31	

5.2.3. Hib IgG levels at 2 and 6 weeks

A total of 84 samples were evaluated for IgG levels against Hib at 2 and 6 weeks of age. At 2 weeks a total of 19 samples in the HEU and 20 samples in the UE groups were processed and at 6 weeks, a total of 24 HEU and 27 UE samples were analysed. Infants who received the 6 weeks vaccination before or on the blood draw day were removed from the analyses.

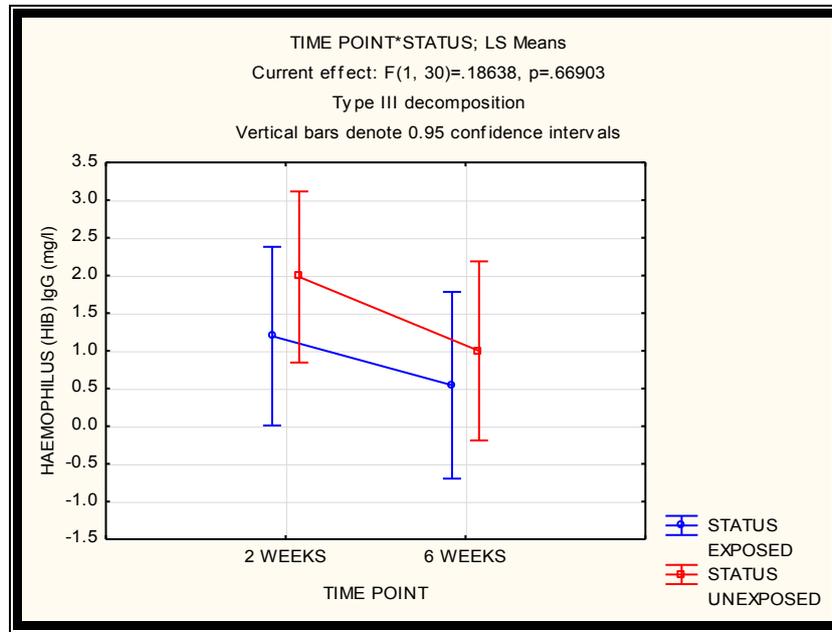


Figure 5- 3: Comparison of Hib IgG levels (mg/l) from 2 to 6 weeks of age. The time group effect is shown to be non-significant ($p=0.66903$).

HEU infants displayed lower mean values for IgG against Hib at both time points (1.21 vs. 2.01 mg/l and 0.70 vs. 1.34 mg/l). The differences between the two groups, however, did not reach statistical significance for either of the time points. At 2 weeks both groups showed protective mean levels for Hib (protective Hib levels >1.0 mg/l), however at 6 weeks, the mean levels for the HEU group decreased to below 1.0mg/l and the UE group remained protective.

At 2 weeks, only 4 HEU infants had levels deemed to be protective (18.2%) in comparison to 9 infants in UE group (39.1%). At 6 weeks, 2 infants in the HEU group (10.1%) had protective levels for Hib compared to the UE group where a total of 6 infants (30%) had protective levels. The HEU group showed a 1.7 fold decrease in the Hib levels from 2 to 6 weeks compared to the UE groups who had a 1.5 fold decrease.

Table 5- 5: Hib IgG levels at 2 and 6 weeks of age.

HIB IgG					
Group	n	Time point	Mean (mg/l)	SD	P-value
HEU	22	2 weeks	1.21	3.53	0.09664
UE	23		2.01	3.61	
HEU	19	6 weeks	0.70	1.61	0.2206
UE	20		1.34	1.96	

5.2.4. PCP IgG levels at 2 and 6 weeks

A total of 79 samples were evaluated for IgG levels against PCP at 2 and 6 weeks of age. At 2 weeks, a total of 22 samples in the HEU and 23 samples in the UE groups were processed and at 6 weeks, a total of 16 HEU and 18 UE samples were analysed respectively. Infants who received the 6 weeks vaccination before or on the blood draw day were removed from the analyses.

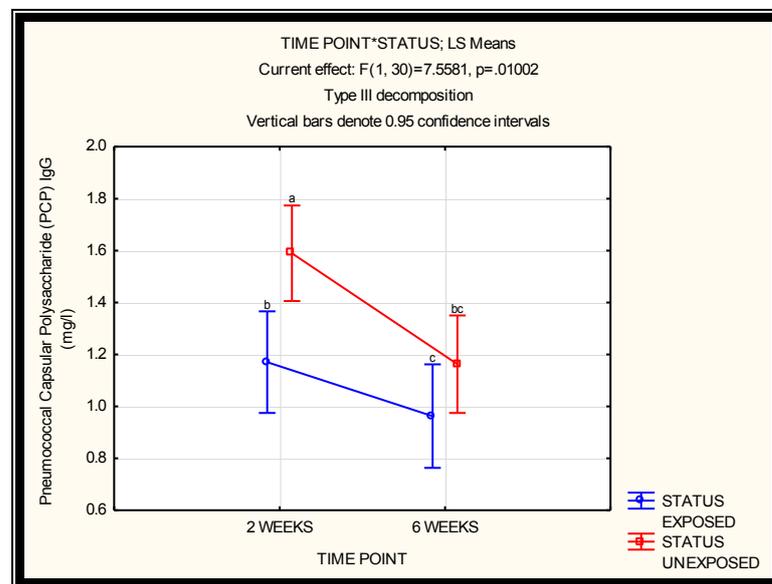


Figure 5- 4: Comparison of PCP IgG levels (mg/l) from 2 to 6 weeks of age. The time group effect is shown to be significant (p=0.01002).

HEU infants displayed lower mean values for IgG against PCP (31.67 vs. 80.77 mg/l and 11.47 vs. 23.31 mg/l). The differences between the two groups at 2 weeks were statistically significant. There are no levels listed for protective immunity for the collective response to pneumococcal serotypes.

Both groups displayed a 2.8 fold decrease in maternal PCP levels from 2 to 6 weeks of age.

Table 5- 6: PCP IgG levels for HEU and UE infants at 2 and 6 weeks of age.

PCP IgG					
Group	n	Time point	Mean (mg/l)	SD	P-value
HEU	22	2 weeks	31.67	53.19	0.003330
UE	23		80.77	107.49	
HEU	16	6 weeks	11.47	14.10	0.097388
UE	18		23.31	25.15	

5.3 POST-VACCINE LEVELS OF SPECIFIC ANTIBODIES

5.3.1 Vaccination Dose

At the 12 week time point, a total of 54 samples were analysed for IgG levels to the specified vaccines. Of these 54 samples, a total of 36 infants (18 HEU and 18 UE) had received 2 doses to the combined pertussis, tetanus and Hib vaccine. The median days from the first dose to the second dose was 41.5 in the HEU group and 40 in the UE group. The median days from the second dose to the 12 week blood draw was 13 in the HEU and 11.5 in the UE group respectively. Thirteen (8 HEU and 5 UE) infants received one dose of the vaccine (median day in HEU was 40 and UE group was 36) and a total of 5 infants (1 HEU and 4 UE) did not receive any doses.

These groups were stratified and statistically analysed by use of log transformation and the employment of an ANOVA analysis, to determine the effect of the administration of 1 or 2 doses on the IgG levels to pertussis, tetanus and Hib. The analysis (by means of noted the F-test p-value) showed that dosage did not have an effect on the pertussis IgG levels ($p=0.61$) at 12 weeks. It however, did have an effect on the IgG levels to tetanus ($p<0.01$) and Hib ($p=0.04$).

Based on these findings, infants who did not receive both dosages at the time of the 12 week blood draw were excluded from the analysis. All HEU and UE infants included in the subsequent analysis (6 and 18 months included) received the required vaccination doses as scheduled. Infants who received a vaccination after the specified blood draw were noted and included in subsequent time points.

At the 6 month follow-up visit, a total of 47 infant samples were analysed for vaccine specific antibodies. Of these, 40 infants (22 HEU and 18 UE) received all 3 required vaccination dosages. The median days from the various doses are listed in Table 5-7 below. A total of 3 infants (2 HEU and 1 UE) received only 2 doses at the time of blood draw and one UE infant received only one dose. Three infants (1 HEU and 2 UE) received no vaccinations prior to the blood draw. Infants who received less than 3 doses were excluded from the analyses. At this time point, 11 infants (7 HEU and 4 UE) received catch-up vaccinations.

Table 5- 7: Median days from various doses for HEU and UE groups at 6 month visit

GROUP	Median day from 1 st dose to 2 nd dose	Median day from 2 nd dose to 12 week blood draw	Median day from 3 rd dose to 6 month blood draw
HEU	41.5	14	77.5
UE	41	13.5	85

At the 12 month follow-up visit, a total of 42 samples were analysed for vaccine specific antibodies. Thirty six babies (20 HEU and 16 UE) received all 3 required vaccination dosages. A total of 2 babies (1 HEU and 1 UE) received only 2 doses at the time of blood draw and one UE infant received only one dose. Three babies (1 HEU and 2 UE) received no vaccination doses prior to the blood draw. Babies who received less than 3 doses were excluded from the analysis. At this time point there had been no documentation of any catch-up vaccinations given to any babies in our study.

At the 18 month follow-up visit, a total of 39 samples were analysed. Of these, 35 (17 HEU and 18 UE) received all 4 required vaccination dosages. The median days from the various doses are listed in Table 5-8 below. One UE baby received only 3 doses and a total of 3 (1 HEU and 2 UE) received no vaccinations prior to the blood draw. Babies who received less than 4 doses were excluded from the analysis. At this time point, 3 babies (1 HEU and 2 UE) received catch-up vaccinations.

Table 5- 8: Median days from various doses for HEU and UE groups at 18 month visit

GROUP	Median day from 1st dose to 2nd dose	Median day from 2nd dose to 12 week blood draw	Median day from 3rd dose to 6 month blood draw	Median day from 4th dose to 18 month blood draw
HEU	41	14	118	29
UE	40	13.5	85.5	14

At the final 24 month follow-up, a total of 37 samples were analysed. Thirty four babies' infants (16 HEU and 18 UE) received all 4 required vaccination doses and one UE baby received only 3 doses. A total of 2 babies (1 HEU and 1 UE) did not receive any vaccinations prior to the blood draw time point. Babies who received less than 4 doses were excluded from the analysis. At this time point none of the babies received catch-up vaccinations.

The median vaccine-to-blood draw time in days for each time point is represented for both groups in Figure 5-5 and Table 5-9 below.

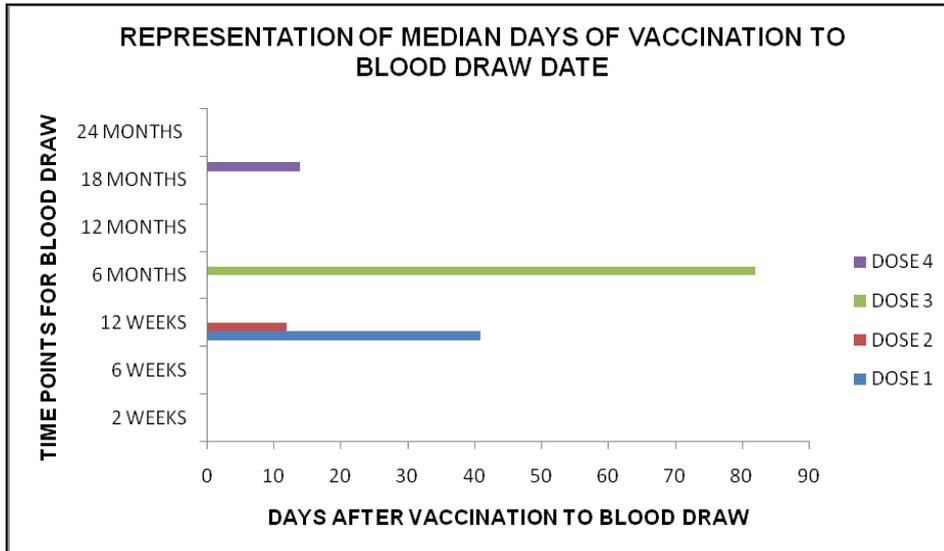


Figure 5- 5: Bar graph representation of the number of days after vaccination on which blood was drawn for the measurement of vaccine specific antibody response. The x-axis depicts the number of days after vaccination and the y-axis represents the various time points at which blood was taken. The median ranges were calculated from the days measured for both groups collectively.

Table 5- 9: Summary of median days from vaccination to blood draws for both groups.

Dose number	Group status	Median days	Median days for both groups	Number of infants
1	HEU	42	41	18
	UE	40		18
2	HEU	13	12	18
	UE	11.5		18
3	HEU	77.5	82	22
	UE	85		18
4	HEU	15	14	17
	UE	14		18

Results for post-vaccination responses will be graphically presented using type III vertical bar graph (95% confidence intervals). Time-group interaction vs. LS means for both HEU and UE groups will be used to present the IgG specific responses at different time points. The x-axis on the graph depicts the time points and the y-axis depicts the specific IgG concentration (Figure 5.6 – 5.8).

Mean values, SD and p-values for the specific IgG responses at different time points will be summarised in Tables 5.10 to 5.12. Statistical significance is indicated when $p < 0.05$.

5.3.2 Post-vaccine IgG levels to Pertussis

A total of 181 samples were evaluated for post-vaccine IgG levels against pertussis toxin at the 12 weeks, 6, 12, 18 and 24 month time point.

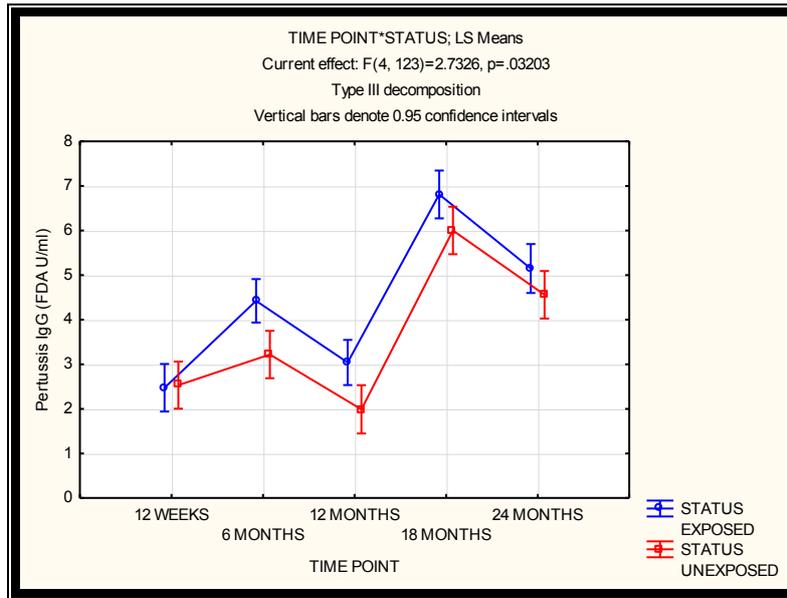


Figure 5- 6: Comparison of pertussis IgG levels (FDA U/ml) from 12 weeks to 24 months. The time group effect is shown to be significant (p=0.03203).

The HEU group displayed higher mean levels when compared to the UE group at each of the time points except at 24 months of age. These differences were statistically significant at 6, 12 and 18 months. Apart from the 12 week time point, these significantly higher levels also corresponded with protective levels in the HEU group. At 12 weeks and 12 months of age, the UE group presented with non-protective mean IgG levels (13.302 and 9.425 FDA U/ml).

At 24 months, both groups had lowered mean IgG levels compared to the 18 month time point. The HEU mean levels were lower compared to the UE; however the difference between the groups did not reach statistical significance. Furthermore, the levels in both groups were considered protective.

A summary of statistical data is shown in Table 5-10 below.

Table 5- 10: Summary of pertussis IgG levels for HEU and UE groups from 12 weeks to 24 months

PERTUSSIS IgG					
Group	n	Time point	Mean (FDA U/ml)	SD	P-value
HEU	18	12 weeks	19.044	1.484	0.876
UE	18		13.302	1.273	
HEU	22	6 months	155.487	200.462	0.00134
UE	18		63.729	119.023	
HEU	20	12 months	26.539	17.252	0.000003
UE	16		8.501	8.435	
HEU	17	18 months	1658.947	1386.499	0.0362
UE	18		792.027	882.172	
HEU	16	24 months	263.181	232.464	0.1549
UE	18		557.867	1747.278	

The outcomes of pertussis IgG in both groups at each time point was not influenced by maternal age. No significant correlations were associated with housing structure, gender, birth weight, racial groups or number of individuals in a household or access to running water.

5.3.3 Post-vaccine IgG levels to Tetanus

A total of 181 samples were evaluated for post-vaccination IgG levels against tetanus at 12 weeks, 6, 12, 18 and 24 month time point respectively.

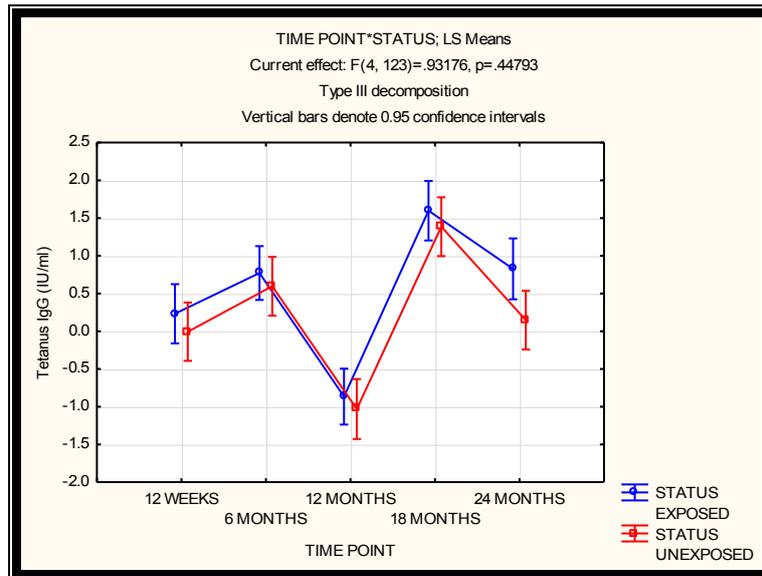


Figure 5- 7: Comparison of tetanus IgG levels (U/ml) from 12 weeks to 24 months. The time group effect is shown to be non-significant (p=0.44793).

At 12 weeks, 6, 12 and 18 months the HEU group had slightly higher mean levels to tetanus compared to the UE group. However, no statistical differences were noted between the groups at any of the four time points. At each time point, both groups showed protective levels for tetanus IgG.

At 24 months, both groups had lowered mean IgG levels compared to the 18 month time point. The HEU mean levels however remained higher than the UE group and this difference was statistically significant (p=0.037). In addition, the levels in both groups were considered protective.

A summary of statistical data is shown in Table 5-11 below.

Table 5- 11: Summary of tetanus IgG levels from 2 weeks to 24 months

TETANUS IgG					
Group	n	Time point	Mean (IU/ml)	SD	P-value
HEU	18	12 weeks	1.484	0.723	0.394
UE	18		1.273	0.986	
HEU	22	6 months	2.587	1.395	0.512
UE	18		2.331	1.529	
HEU	20	12 months	0.568	0.419	0.549
UE	16		0.475	0.364	
HEU	17	18 months	5.845	2.710	0.452
UE	18		5.424	3.596	
HEU	16	24 months	3.277	2.606	0.018
UE	18		1.697	1.617	

The outcome of tetanus IgG in both groups at each time point was not influenced by maternal age, gravidity or gender. However, at 2 weeks of age, UE infants living in informal housing structures had increased levels of tetanus IgG compared to those living in formal structures ($p=0.02267$). Infants who had higher birth weight also had increased IgG levels at 12 weeks of age ($p=0.04$). In addition, the number of individuals in a household was positively correlated with an increase in tetanus levels at 2 and 6 weeks of age ($p=0.01$ and $p<0.01$).

5.3.4 Post-vaccine IgG levels to Hib

A total of 181 samples were evaluated for post-vaccine IgG levels against Hib at 12 weeks, 6, 12, 18 and 24 month time point respectively.

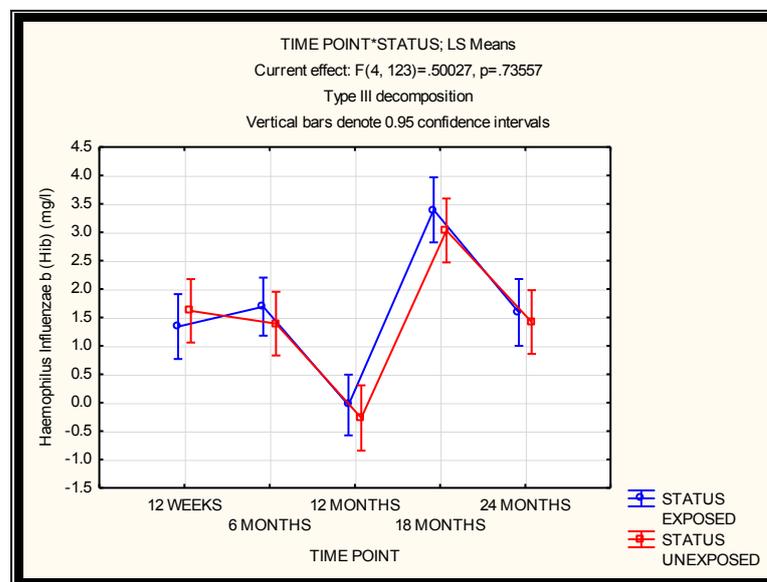


Figure 5- 8: Comparison of Hib IgG levels (mg/l) from 12 weeks to 24 months. The time group effect is shown to be non-significant ($p=0.73557$).

The HEU group displayed slightly lower mean values to Hib compared to the UE group at 12 weeks. However at 6, 12 and 18 months, the mean Hib levels were slightly higher in the HEU group compared to the UE group. At each of these time points described, no statistical significance was noted. Both groups showed protective levels for Hib at each of the four time points.

At 24 months, HEU babies had slightly higher mean values to Hib compared to the UE group, however this difference did not reach statistical significance. The Hib levels in each group were considered protective.

Summary of statistical data is shown in Table 5-12 below.

Table 5- 12: Summary of Hib IgG levels from 12 weeks to 24 months

HAEMOPHILUS INFLUENZAE B					
Group	n	Time point	Mean (mg/l)	SD	P-value
HEU	18	12 weeks	9.313	12.017	0.494
UE	18		10.089	12.562	
HEU	22	6 months	11.867	17.490	0.437
UE	18		8.453	12.883	
HEU	20	12 months	2.113	3.795	0.567
UE	16		1.357	2.588	
HEU	17	18 months	41.798	31.817	0.372
UE	18		34.812	29.734	
HEU	16	24 months	7.200	5.968	0.682
UE	18		5.647	4.286	

An increase in maternal age and gravidity was associated with increased Hib IgG levels at 12 weeks of age ($p < 0.01$ and $p = 0.03$). No significant correlations were found between were associated with housing structure, access to running water or gender.

5.3.5 Summary of Antibody Response Results

In this section of the study the IgG responses to specific vaccines namely; pertussis, tetanus, Hib and pneumococcus were evaluated from 2 weeks to 24 months age in two infant groups, HEU and UE controls. The 2 and 6 week time points represented the presumed maternal antibody transfer and the subsequent time points, represented post-vaccination levels.

The HEU group had decreased mean levels for all measured antibodies at 2 and 6 weeks compared to UE controls. At 2 weeks statistical significance was reached for pertussis, tetanus and pneumococcus, while at 6 weeks, statistical significance was reached only for tetanus and pneumococcus.

IgG levels for pertussis was significantly higher in the HEU group compared to the UE group at 6, 12 and 18 months time points. The response to tetanus was similar in both groups at 12 weeks, 6, 12 and 18 months of age. However at 24 months, the HEU group had significantly higher IgG levels to tetanus compared to the UE group. There were no significant differences between the two groups for Hib IgG levels, however higher mean levels were noted for the HEU group only at 6, 12, 18 and 24 months. At 12 weeks, HEU infants had lower mean levels compared to the UE group.

The HEU group showed robust IgG responses mainly to protein vaccine antigens (pertussis and tetanus) at various time points.

The next set of results examines the levels of immune activation, regulation and apoptosis and B cell memory in the HEU and UE groups at 18 and 24 month follow-up visit.

5.4 CELLULAR IMMUNE MARKERS OF ACTIVATION, APOPTOSIS AND B CELL MEMORY AT 18 MONTHS

5.4.1 Cellular markers on CD4+ T cells at 18 months time point

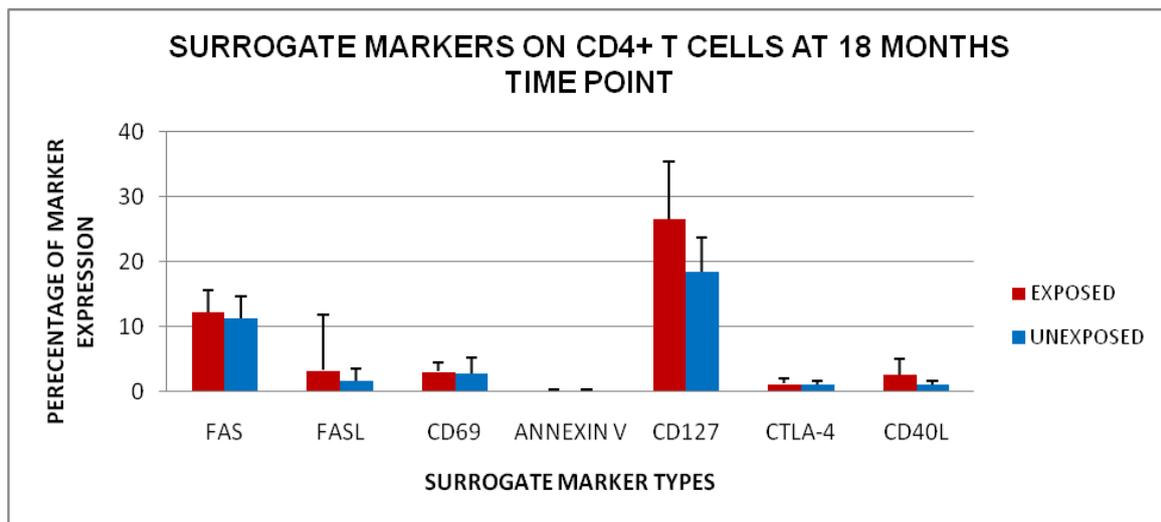


Figure 5- 9: Bar graph (Error bars with SD) representation for cellular markers on CD4+ T cells. The X-axis represents the cellular marker types (Fas, FasL, CD69, Annexin V, CD127, CTLA-4 and CD40L). The Y-axis represents the percentage of marker expression.

The evaluation of cellular markers of activation/proliferation (CD69, CD127 and CD40L) and apoptosis/regulation (FAS, FASL, Annexin V and CTLA-4) on CD4+ T cells was determined at the 18 months time point. A total of 39 samples were analysed for cellular markers FAS, FASL, CD69 and Annexin V and a total of 19 samples were analysed for cellular markers CD127, CTLA-4 and CD40L (Figure 5-9 and Table 5-13).

As depicted in Figure 5-12 and summarised in Table 5-13 below, compared to the UE group, the HEU group displayed an increased mean value for markers of activation (CD69 [3.04 vs. 2.70%], CD127 [26.47 vs. 18.40%] and CD40L [2.50 vs. 1.01%]) on CD4+ T cells. Although an increase in activation markers may be consistent with HIV exposure, a statistical difference between the two groups was only noted for the expression of CD127.

The increase in mean values for markers of activation in the HEU population may possibly correspond with the increase mean values for markers of apoptosis (FAS [12.137 vs. 11.18%] and FASL [3.20 vs. 1.57%]), however these differences did not reach statistical significance.

Annexin V expression in both groups was minimal and the percentage mean was similar between the two groups (HEU [0.14%] and UE [0.15%]). The expression of Annexin V was measured after 16 and 24 hour incubation periods in both groups. Results are listed in Section 5-6 below.)

The expression of CTLA-4 was similar between the two groups (HEU [1.17%] and UE [1.04%]); but no statistical significance was reached.

Table 5- 13: Summary of cellular markers on CD4+ T cells.

Group	n	Marker	Mean (% gated)	SD	p-value
HEU	18	FAS	12.13722	3.453801	0.39
UE	21		11.18143	3.450505	
HEU	18	FASL	3.198333	8.635765	0.41
UE	21		1.567143	1.983734	
HEU	18	CD69	3.041111	1.327921	0.20
UE	21		2.700476	2.519797	
HEU	18	ANNEXIN V	0.144444	0.167901	0.83
UE	21		0.153810	0.105474	
HEU	10	CD127	26.47300	8.970801	0.03
UE	9		18.40111	5.213915	
HEU	10	CTLA-4	1.174000	0.799669	0.69
UE	9		1.042222	0.614609	
HEU	10	CD40L	2.499000	2.424213	0.09
UE	9		1.008889	0.563393	

5.4.2 Cellular markers on CD20+ B cells at 18 months time point

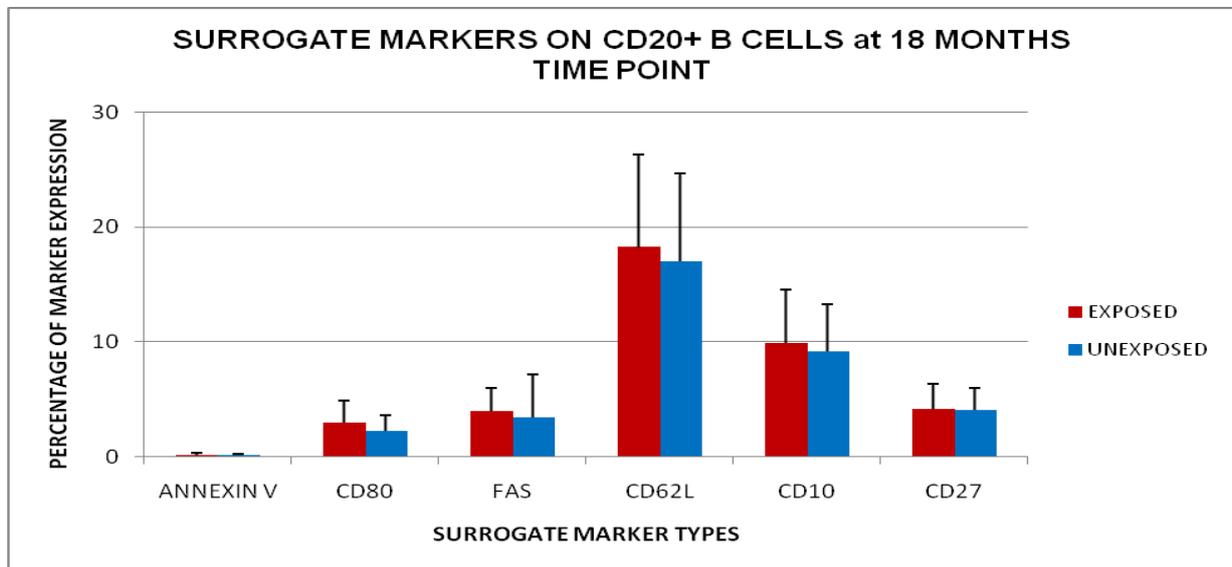


Figure 5- 10: Bar graph (Error bars with SD) representation for cellular markers on CD20+ B cells. The X-axis represents the cellular marker types (Annexin V, CD80, Fas, CD62L and CD27). The Y-axis represents the percentage of marker expression.

The evaluation of cellular markers of activation/proliferation (CD80, CD62L and CD10), apoptosis (Annexin V, Fas) and memory (CD27) was determined at the 18 month time point. A total of 39 samples were analysed for each described marker.

As depicted in Figure 5-10 and summarised in Table 5-14 below, when compared to UE controls, the HEU group displayed increased mean values for certain markers of activation/proliferation on CD20+ B cells (CD80 [2.99 vs. 2.28%]; CD62L [18.25 vs. 17.01%]). However, statistical significance between the two groups was only reached for the cellular activation marker, CD80. The expression of CD10 was similar between the groups (4.23 vs. 4.10%).

The HEU group also showed an increase in mean percentage of the apoptotic marker, FAS (4.04 vs. 3.44%) compared to the UE controls. However, the expression of Annexin V was lower in the HEU than UE groups (0.18 vs. 0.21 %). The expression of the described apoptotic markers did not reach statistical significance between the two groups.

Annexin V expression in both groups was minimal. Therefore, the expression of Annexin V was measured after 16 and 24 incubation periods (“stressed conditions”) in both groups. (Results listed in Section 5-6 below)

The cellular marker for B cell memory (CD27) was similar in both HEU and UE groups (4.22 vs. 4.10 %).

Table 5- 14: Summary of cellular markers on CD20+ B cells.

Group	n	Marker	Mean (% gated)	SD	P-value
HEU	18	CD80	2.986111	1.360744	0.03^a
UE	21		2.279048	1.938845	
HEU	18	CD62L	18.24778	7.882515	0.63
UE	21		17.01762	8.043571	
HEU	18	CD10	9.875556	4.238405	0.63
UE	21		9.162381	4.730180	
HEU	18	FAS	4.037222	3.857614	0.54
UE	21		3.438571	1.971523	
HEU	18	ANNEXIN V	0.180000	0.087313	0.44
UE	21		0.214286	0.168213	
HEU	18	CD27	4.225556	1.959361	0.85
UE	21		4.100476	2.106394	

^aMann-Whiney U test p-value reported which was used a confirmatory tool to the F-test which was non-significant

5.4.3 Cellular markers on CD19+ B cells at 18 months time point

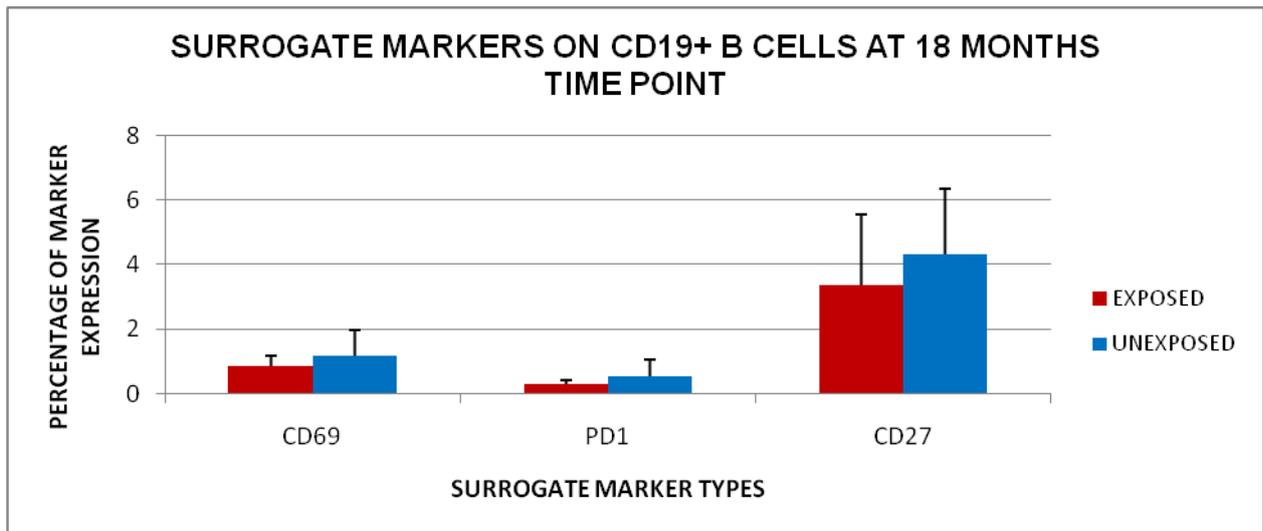


Figure 5- 11: Bar graph (Error bars with SD) representation for cellular markers on CD19+ B cells. The X-axis represents the cellular marker types (CD69, PD1 and CD27). The Y-axis represents the percentage of marker expression.

The expression of cellular markers of activation (CD69), programmed cell death (PD1) and memory (CD27) on CD19+ B cells was determined in 19 subjects.

HEU showed decreased mean values for the expression of CD69 (0.85 vs. 1.19%), PD1 (0.30 vs. 0.53%) and CD27 (3.37 vs. 4.30 %) compared to UE controls. These differences did not reach statistical significance (Figure 5-11 and Table 5-15).

Evaluating the expression of activation markers on CD19+ B cells in comparison to cellular markers described on CD20+ B cells, the expression of certain markers seem to be higher in both groups. The expression of the memory marker, CD27, on CD20+ B cells seems to be similarly expressed on CD19+ B cells; however the HEU group seems to show greater expression of memory on CD20+ B cells as opposed to CD19+ B cells (4.23 vs. 3.37%). In UE controls, there was similar expression on both cell types (4.10 vs. 4.30%).

Table 5- 15: Summary of cellular markers on CD19+ B cells.

Group	n	Marker	Mean (% gated)	SD	P-value
HEU	10	CD69	0.851000	0.322506	0.23
UE	9		1.190000	0.796335	
HEU	10	PD1	0.296000	0.144776	0.18
UE	9		0.532222	0.516909	
HEU	10	CD27	3.370000	2.192127	0.35
UE	9		4.302222	2.055212	

5.4.4 Cellular markers on CD8+ T cells at 18 months time point

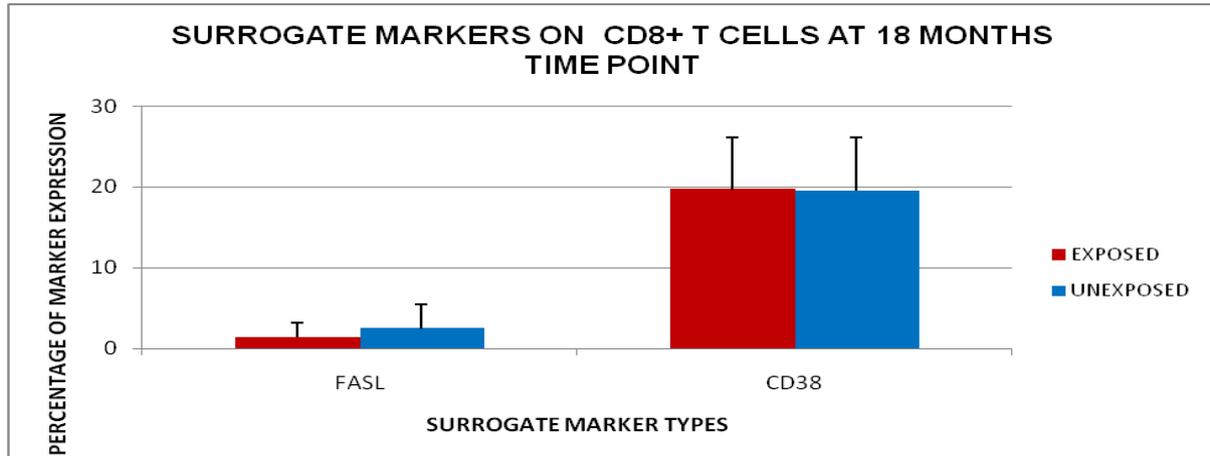


Figure 5- 12: Bar graph (Error bars with SD) representation for cellular markers on CD8+ T cells. The X-axis represents the cellular marker types (FASL and CD38). The Y-axis represents the percentage of marker expression.

The evaluation of cellular markers of activation (CD38) and apoptosis (FASL) was determined at the 18 month time point. A total of 19 samples were analysed for each of the two bio-markers described.

As depicted in Figure 5-12 and summarised in Table 5-16 below, both groups produced similar mean values for expression of the activation marker, CD38 (19.72 vs. 19.55%). In contrast, the HEU infants group showed lower mean values for the expression of the apoptotic marker FASL than the UE controls (1.38 vs. 2.48%). Neither of these markers produced any statistical difference between the groups.

Table 5- 16: Summary cellular markers on CD8+ T cells

Group	n	Marker	Mean (% gated)	SD	P-value
HEU	10	CD38	19.72400	6.450415	0.95
UE	9		19.55111	6.566196	
HEU	10	FASL	1.381000	1.853552	0.34
UE	9		2.482222	2.940314	

5.5 CELLULAR IMMUNE MARKERS OF ACTIVATION, APOPTOSIS AND B CELL MEMORY AT 24 MONTHS

5.5.1 Cellular markers on CD4+ T cells at 24 months time point

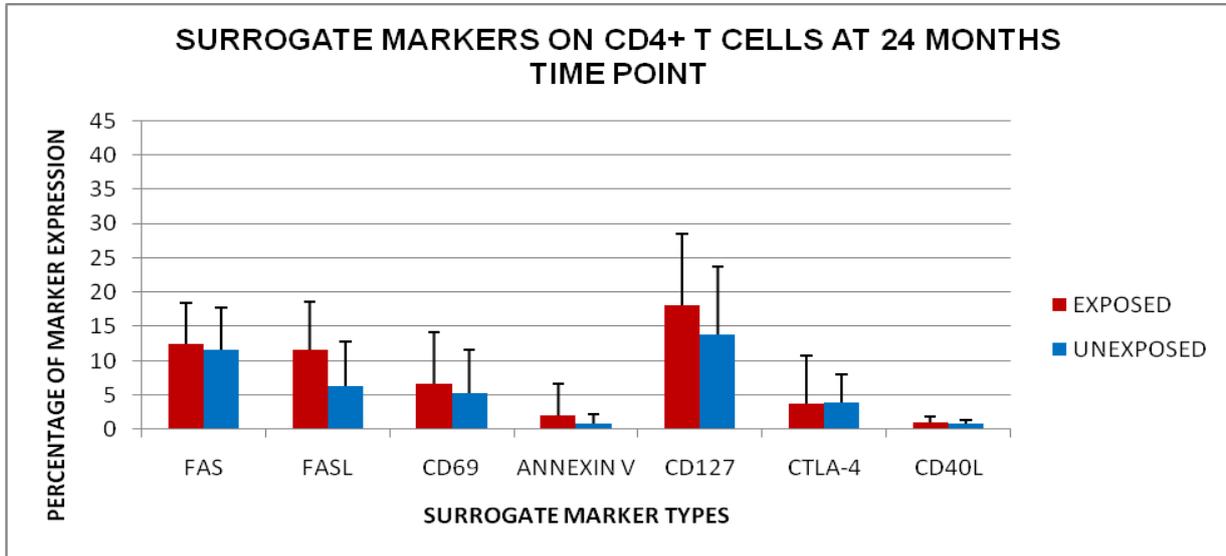


Figure 5- 13: Bar graph (Error bars with SD) representation for cellular markers on CD4+ T cells. The X-axis represents the cellular marker types (FAS, FASL, CD69, Annexin V, CD127, CTLA-4 and CD40L). The Y-axis represents the percentage of marker expression.

The expression of cellular markers of activation/proliferation (CD69, CD127 and CD40L) and apoptosis/regulation (FAS, FASL, Annexin V and CTLA-4) on CD4+ T cells was determined at the 24 months follow-up. A total of 37 samples were analysed for each described cellular marker.

As depicted in Figure 5-16 and summarised in Table 5-17 below, the HEU group produced higher mean values than the UE group for all the markers on CD4+ T cells, except CTLA-4 (CD69 [6.66 vs. 5.28%], CD127 [12.47 vs. 11.68%] and CD40L [0.94 vs. 0.77%]). These differences were not statistically significant (Figure 5-13 and Table 5-17).

Compared to the 18 months time point, HEU and UE infants showed increased expression of CD69. Both HEU and UE groups showed a 2 fold increase in expression of CD69 from 18 to 24 months, however the change was not statistically significant. CD40L in the HEU group decreased by 3 fold compared to the UE group that only showed a 2 fold decrease over the same period. Both HEU and UE groups showed similar levels for the expression of CD127 at each of the two time points.

The HEU group showed increased expression of apoptotic markers Fas (12.47 vs. 11.68%), FasL (11.53 vs. 6.32%) and Annexin V (2.11 vs. 0.93%). The expression of T cell regulation marker, CTLA-4, was similar in both groups (3.78 vs. 3.96%). A significant difference between the expression of these markers between the two groups was reached for FASL and Annexin V.

From 18 to 24 months of age, both HEU and UE groups displayed similar levels of Fas. HEU infants showed a 4 fold increase in the expression of FasL whereas the UE group only showed a 3 fold increase. The change in both groups from 18 to 24 months was statistically significant. The HEU group displayed a statistically significant 15 fold increase in the expression of Annexin V, whereas the UE group only showed a non-significant 6 fold increase. The HEU group further showed a 3 fold increase in the expression of CTLA-4 from 18 to 24 months of age, while a statistically significant 4 fold increase was found in the UE control group.

Table 5- 17: Summary of cellular markers on CD4+ T cells at 24 months of age

Group	n	Markers on CD4+ T cells	Mean (% gated)	SD	P-value
HEU	17	FAS	12.47	5.95	0.69
UE	20		11.68	6.00	
HEU	17	FASL	11.53	7.009	0.03
UE	20		6.32	6.544	
HEU	17	CD69	6.66	7.47	0.55
UE	20		5.28	6.37	
HEU	17	ANNEXIN V	2.11	4.46	0.01
UE	20		0.93	1.20	
HEU	17	CD127	18.09	10.36	0.20
UE	20		13.78	9.87	
HEU	17	CTLA-4	3.78	6.93	0.92
UE	20		3.96	4.04	
HEU	17	CD40L	0.94	0.94	0.50
UE	20		0.77	0.63	

5.5.2 Cellular markers on CD20+ B cells at 24 months time point

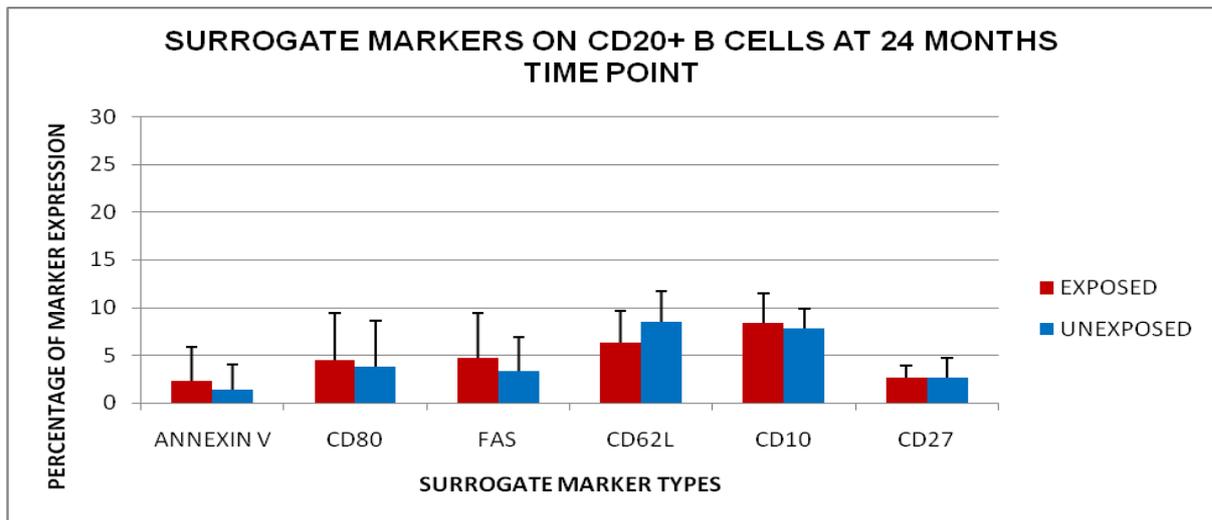


Figure 5- 14: Bar graph (Error bars with SD) representation for cellular markers on CD4+ T cells. The X-axis represents the cellular marker types (Annexin V, CD80, FAS, CD62L, CD10 and CD27). The Y-axis represents the percentage of marker expression.

The expression of cellular markers of activation/proliferation (CD80, CD62L and CD10), apoptosis (Annexin V, Fas) and memory (CD27) was determined at the 24 month time point. A total of 37 samples were analysed for each described marker.

As depicted in Figure 5-14 and summarised in Table 5-18 below, compared to UE controls, the HEU group displayed higher mean values for the expression of cellular markers CD80 [4.48 vs. 3.77%]. The HEU group also displayed lower mean values for the expression of, CD62L [6.35 vs. 8.55%] and CD10 [8.34 vs. 7.79%]. These differences did not however reach any statistical significance.

Both HEU and UE groups showed a 2 fold increase in the expression of CD80 from 18 to 24 months of age. The difference between the two time points only proved to be significant for the UE group. The HEU group showed a 3 fold increase for the expression of CD23 compared to the UE group infants who displayed a 2 fold increase. The expression of CD10 was similar for both groups 18 to 24 months.

At 24 months, the HEU group displayed higher mean values for the expression of cellular marker of apoptosis Fas [4.75 vs. 3.32%] and Annexin V [2.36 vs. 1.36%] than the UE group. These differences did not reach statistical significance.

From 18 to 24 months both groups displayed an increase in the expression Annexin V. The HEU group displayed a 13 fold increase, whereas the UE group displayed a 6 fold increase; both increases were statistically significant ($p < 0.05$) for both groups. The expression of Fas was similar in both groups at both the 18 and 24 month time points.

The expression of the cellular B cell memory marker, CD27 was similar in both groups (2.66 vs. 2.72%). Both groups showed a statistically significant 2 fold decrease in the expression of CD27 from 18 to 24 months.

Table 5- 18: Summary of cellular markers on CD20+ B cells at 24 months of age

Group	n	Marker	Mean (% gated)	SD	P-value
HEU	17	CD80	4.48	3.55	0.49
UE	20		3.77	2.68	
HEU	17	CD62L	6.35	4.93	0.18
UE	20		8.55	4.84	
HEU	17	CD10	8.34	4.65	0.69
UE	20		7.79	3.59	
HEU	17	FAS	4.75	3.27	0.19
UE	20		3.32	3.2	
HEU	17	ANNEXIN V	2.36	3.14	0.42
UE	20		1.36	2.09	
HEU	17	CD27	2.66	1.31	0.91
UE	20		2.72	1.98	

5.5.3 Cellular markers on CD19+ B cells at 24 months time point

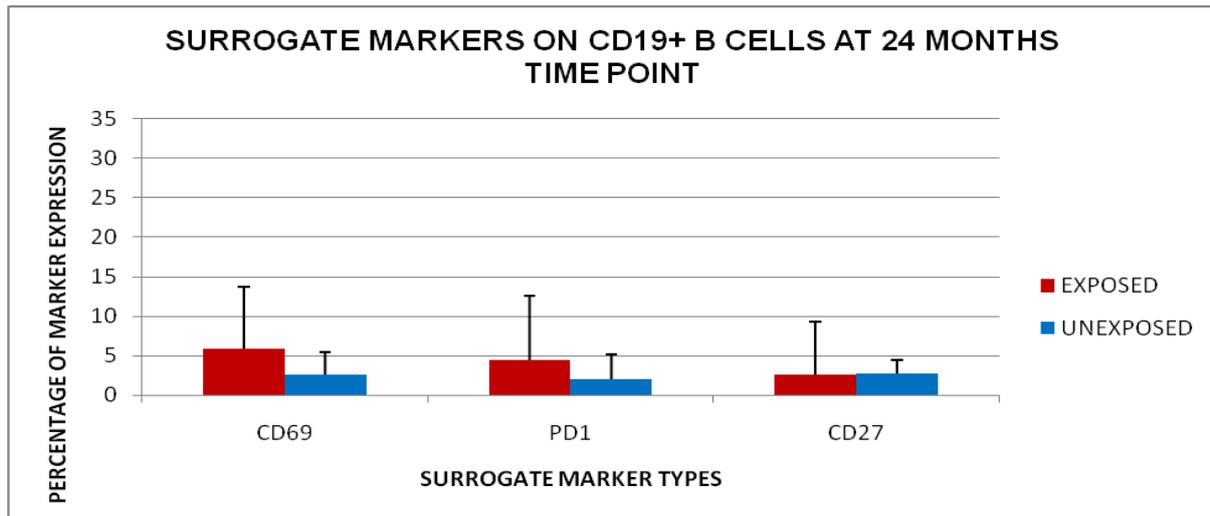


Figure 5- 15: Bar graph (Error bars with SD) representation for cellular markers on CD19+ B cells. The X-axis represents the cellular marker types (CD69, PD1 and CD27). The Y-axis represents the percentage of marker expression.

The expression of cellular markers of activation (CD69), programmed cell death (PD1) and memory (CD27) on CD19+ B cells was determined in 35 subjects. Blood was insufficient for two subjects (one in each group).

The HEU group showed increased mean value for the expression of the cellular markers of activation (CD69 [5.94 vs. 2.65%]), programmed cell death (PD1 [4.45 vs. 2.09%]) and memory (CD27 [4.03 vs. 2.61%]). Statistical significance was only reached for CD69 expression ($p < 0.01$) (Figure 5-15 and Table 5-19).

Both groups displayed increased expression of CD69 and PD1 from 18 to 24 months of age. However, in the HEU group there was a 7 fold increase compared to a 2 fold increase in the UE group for the expression of CD69. Statistical significant was reached for the HEU group only. This correlated with a 15 fold increase in the expression of PD1 in the HEU group compared to a 4 fold increase in the UE group. Furthermore, the HEU group displayed similar expression levels of CD27 from 18 to 24 months of age (3.37 to 4.03%), compared to the UE group who conversely displayed a 2 fold decrease in the expression of CD27 (4.20 to 2.78%). The differences between the expression of CD27 from 18 to 24 months between the two groups were not statistically significant.

Table 5- 19: Summary of cellular markers on CD19+ B cells at 24 months of age

Group	n	Marker	Mean (% gated)	SD	P-value
HEU	16	CD69	5.94	7.83	0.019
UE	19		2.65	2.82	
HEU	16	PD1	4.447	8.17	0.69
UE	19		2.09	3.08	
HEU	16	CD27	4.03	6.67	0.54
UE	19		2.61	1.70	

5.5.4 Cellular markers on CD8+ T cells at 24 months time point

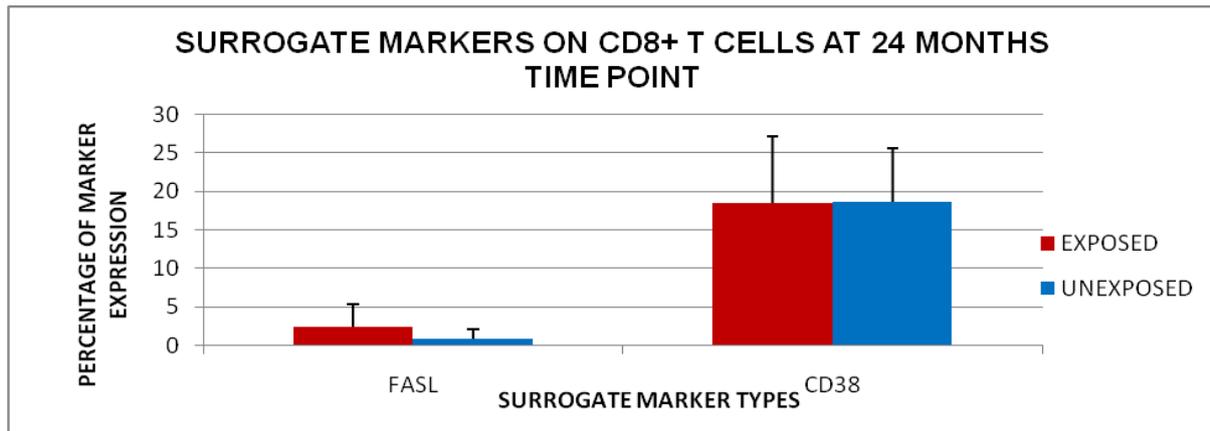


Figure 5- 16: Bar graph (Error bars with SD) representation for cellular markers on CD8+ T cells. The X-axis represents the cellular marker types (FasL and CD38). The Y-axis represents the percentage of marker expression.

A total of 37 samples were analysed for each of the two markers represented above.

As depicted in Figure 5-16 and summarised in Table 5-20 below, both groups displayed similar expression of the activation marker, CD38 (18.51 vs. 18.65%). Conversely, the HEU group displayed increased expression levels of apoptotic marker FasL when compared to the UE group (2.44 vs. 0.86%). Statistical significance was reached for the expression of FasL.

Both groups showed similar levels of CD38 expression at both 18 and 24 months of age.

Compared to the 18 months time point, the HEU group showed a 2 fold increase in the expression of FasL at the 24 month time point, in contrast to the UE group who showed a 3 fold decrease in expression. The change in levels for FasL from 18 to 24 month of age was not statistically significant for either group.

Table 5- 20: Summary of cellular markers on CD8+ T cells at 24 months of age

Group	n	Marker	Mean (% gated)	SD	P-value
HEU	17	FASL	2.44	2.85	0.03
UE	20		0.86	1.32	
HEU	17	CD38	18.51	8.55	0.96
UE	20		18.65	6.96	

5.6 MEASUREMENT OF *IN VITRO* SPONTANEOUS APOPTOSIS

To measure the expression of early apoptotic marker Annexin V as an opportunity to explore the concept of spontaneous apoptosis, samples were placed under timed (16 and 24 hour) incubations at 37°C in the presence of cell culture media (RPMI + 10% Foetal Calf Serum [FCS]) and CO₂ (Refer to Chapter 4 for methodology).

At 18 months, the expression of Annexin V was measured on CD4+ T and CD20+ B cells at both 16 and 24 hour time periods. At 24 months, Annexin V expression was measured only at 16 hours on the same cell types. The expression of Annexin V on CD4+ T and CD20+ B cells described previously represented the *ex vivo* (after blood draw) expression.

5.6.1 Spontaneous apoptosis at 18 months (16 and 24 hour intervals)

5.6.1.1 CD4+/Annexin V+

A total of 13 samples (5 HEU and 8 UE) were analysed for the expression of Annexin V on CD4+ T cells at 16 and 24 hour periods. The expression levels measured at these time points were subsequently compared to the levels expressed within an hour of blood draw (*ex vivo*).

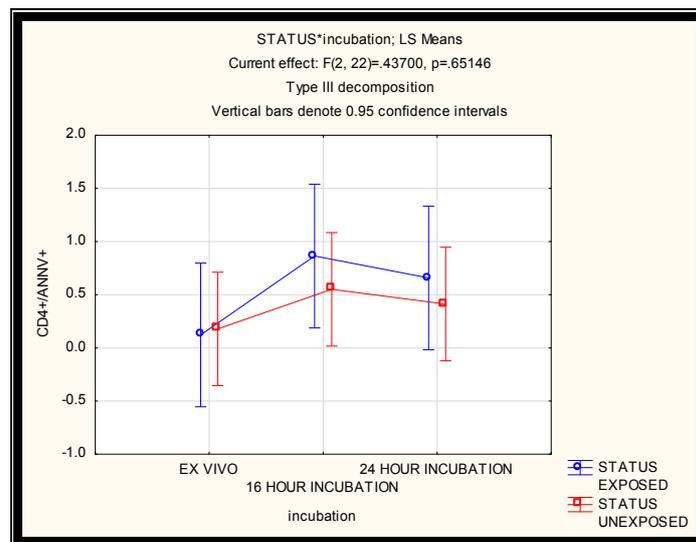


Figure 5- 17: Summary of Type III vertical bar graph (95% confidence intervals) representing the time-group interaction vs. Least Square (LS) means for both HEU and UE infants for Annexin V expression on CD4+ T cells expression at zero (*ex vivo*), 16 and 24 hour time points. The x-axis depicts the time points and the y-axis depicts the expression percentage of Annexin V (p= 0.65146).

At 16 hours, the HEU group displayed increased levels of Annexin V compared to the UE group (0.86 vs. 0.55%). Similarly, at 24 hours, the HEU group also displayed increased levels of Annexin V compared to the UE group (0.66 vs. 0.41%). These differences were not statistically significant. Both groups however demonstrated an increase in expression from *ex vivo* to 16 and 24 hours. The expression of Annexin V seemed to decrease from 16 to 24 hours in both groups (HEU [0.86 to 0.66 %] and UE [0.55 to 0.41%]) (See Figure 5-17 and Table 5-21).

In the HEU group, the increase from *ex vivo* to 16 hours was statistically significant and resulted in a 7 fold increase. However, the increase from *ex vivo* to 24 hours (5 fold increase), as well as the decrease from 16 to 24 hours (1.3 fold decrease), did not reach statistical significance.

In the UE group, the increase from *ex vivo* to 16 hours represented a 3 fold increase, compared to HEU with a 7 fold increase. The fold increase from *ex vivo* to 24 hours was 2.3. The fold decrease from 16 to 24 hours was also 1.3 as displayed by HEU group.

Table 5- 21: Summary of expression of Annexin V on CD4+ T cells at *ex vivo*, 16 hour and 24 hour time periods at 18 months of age.

Group	n	Factor	Mean (% gated)	SD	p-value
HEU	5	<i>EX VIVO</i>	0.122000	0.043243	0.892533
UE	8		0.178750	0.082365	
HEU	5	16 HOURS	0.864000	1.429066	0.459313
UE	8		0.551250	0.813115	
HEU	5	24 HOURS	0.658000	0.778569	0.560384
UE	8		0.412500	0.564415	
HEU					
	5	<i>EX VIVO</i>	0.122000		0.035039
	5	16 HOURS	0.864000		
	5	<i>EX VIVO</i>	0.122000		0.118873
	5	24 HOURS	0.658000		
	5	16HOURS	0.864000		0.539242
	5	24 HOURS	0.658000		
UE					
	8	<i>EX VIVO</i>	0.178750		0.167745
	8	16 HOURS	0.551250		
	8	<i>EX VIVO</i>	0.178750		0.380375
	8	24 HOURS	0.412500		
	8	16HOURS	0.551250		0.600487
	8	24 HOURS	0.412500		

5.6.1.2 CD20+/Annexin V+

A total of 13 samples (5 HEU and 8 UEs) were analysed for the expression of Annexin V on CD20+ B cells at 16 and 24 hour periods. The expression levels measured at these time points were subsequently compared to the levels expressed within an hour of blood draw.

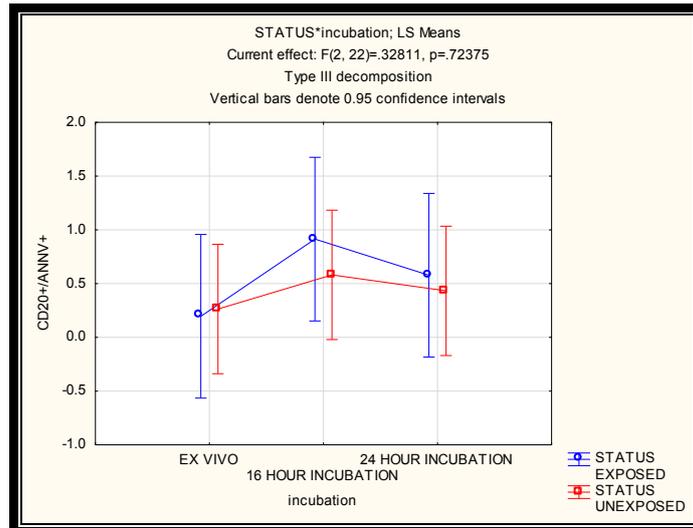


Figure 5- 18: Summary of Annexin V expression on CD20+ B cell expression at zero (*ex vivo*), 16 and 24 hour time points. The x-axis depicts the time points and the y-axis depicts the expression percentage of Annexin V ($p=0.72375$).

At 16 hours, the HEU group displayed an increased mean value of Annexin V compared to the UE group (0.91 vs. 0.58%). Similarly, at 24 hours, the HEU group also displayed an increased mean value for Annexin V expression compared to UE (0.58 vs. 0.43%) on CD20+ B cells. These differences however, did not reach statistical significance. Both groups display an increase in expression of Annexin V from *ex vivo* to 16 and 24 hours. In addition, as with the expression on CD4+ T cells, the expression of Annexin V tends to decrease from 16 to 24 hours in both groups (HEU [0.91 to 0.58%] and UE [0.58 to 0.43%]) (See Figure 5-18 and Table 5-22).

In the HEU group, a 5 fold increase was displayed for the expression of Annexin V from *ex vivo* to 16 hours. Conversely, the increase from *ex vivo* to 24 hours resulted in a 3 fold increase. As previously noted with the Annexin V expression on CD4+ T cells, a fold decrease of 1.6 was noted from 16 to 24 hours.

In the UE group, the increase from *ex vivo* to 16 hours resulted in a 2 fold increase (compared to HEU with a 5 fold increase). The fold increase from *ex vivo* to 24 hours was 1.6. The fold decrease from 16 to 24 hours was also 1.3 as similarly displayed by the HEU group.

Table 5- 22: Summary of Annexin V expression on CD20+ B cells at *ex vivo*, 16 hour and 24 hour time periods at 18 months of age.

Group	n	Factor	Mean (% gated)	SD	p-value
HEU	5	<i>EX VIVO</i>	0.196000	0.046690	0.886171
UE	8		0.263750	0.171958	
HEU	5	16 HOURS	0.914000	1.419165	0.484387
UE	8		0.581250	1.161962	
HEU	5	24 HOURS	0.578000	0.722336	0.758716
UE	8		0.432500	0.587045	
HEU	5	<i>EX VIVO</i>	0.196000		0.077795
	5	16 HOURS	0.914000		0.335694
	5	<i>EX VIVO</i>	0.196000		
	5	24 HOURS	0.578000		
	5	16HOURS	0.914000		0.395983
	5	24 HOURS	0.578000		
UE	8	<i>EX VIVO</i>	0.263750		0.312015
	8	16 HOURS	0.581250		0.587875
	8	<i>EX VIVO</i>	0.263750		
	8	24 HOURS	0.432500		
	8	16HOURS	0.581250		0.632613
	8	24 HOURS	0.432500		

5.6.2 Spontaneous apoptosis at 24 months (16 hour interval only)

The spontaneous apoptosis assay was repeated at the 24 month time point. However, only the *ex vivo* (after blood draw) versus the 16 hour time periods were assessed. Due to sample volume limitations and deterioration in the expression of Annexin V after 16 hours (noted previously), the 24 hour time point was not included at this time point.

5.6.2.1 CD4+/Annexin V+

A total of 37 samples (17 HEU and 20 UE) were analysed for the expression of Annexin V on CD4+ T cells at 16 hours. The expression levels measured at these time points were subsequently compared to the levels expressed within an hour of blood draw (*ex vivo*).

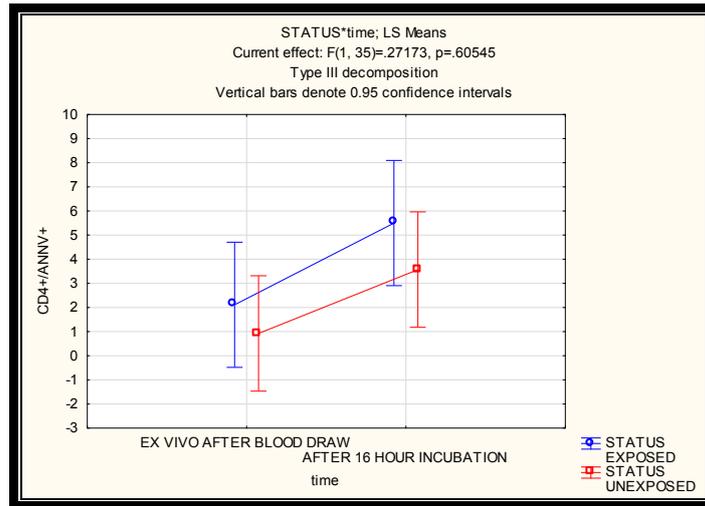


Figure 5- 19: Summary of Annexin V expression on CD4+ T cell expression at zero (*ex vivo*), 16 and 24 hour time points. The x-axis depicts the time points and the y-axis depicts the expression percentage of Annexin V ($p= 0.6054$).

At 24 months of age, the HEU group displayed an increased mean value for the expression of Annexin V (*ex vivo*) on CD4+ T cells compared to the UE group (2.11 vs. 0.93%). These differences were not statistically significant. Both infant groups however showed an increased mean value from *ex vivo* to 16 hours (HEU [2.11 to 23.77%] and UE [0.93 to 0.3.57%]) (See Figure 5-19 and Table 5-23).

In the HEU group, the mean increase from *ex vivo* to 16 hours was highly significant ($p<0.01$) with a fold increase of 3. Similarly, in the UE group, the mean increase from *ex vivo* to 16 hour was also highly significant, resulting in a fold increase of 4.

Table 5- 23: Summary of means, standard deviations (SDs) and p-values for both HEU and UE groups for expression of Annexin V expression on CD4+ T cells at *ex vivo*, 16 hour and 24 hour time periods at 18 months of age.

GROUP	FACTOR	MEAN	SD	P-VALUE	N
HEU	EX VIVO	2.11	4.46	0.50	17
UE		0.93	1.20		20
HEU	16 HOURS	5.50	9.20	0.27	17
UE		3.57	3.56		20
HEU	EX VIVO	2.11		0.002630	17
	16 HOURS	5.50			17
UE	EX VIVO	0.93		0.00951	20
	16 HOURS	3.57			20

5.6.2.2 CD20+/Annexin V+

A total of 37 samples (17 HEU and 20 UE) were analysed for the expression of Annexin V on CD20+ B cells at 16 hours. The expression levels measured at these time points were subsequently compared to the levels expressed within an hour of blood draw (*ex vivo*).

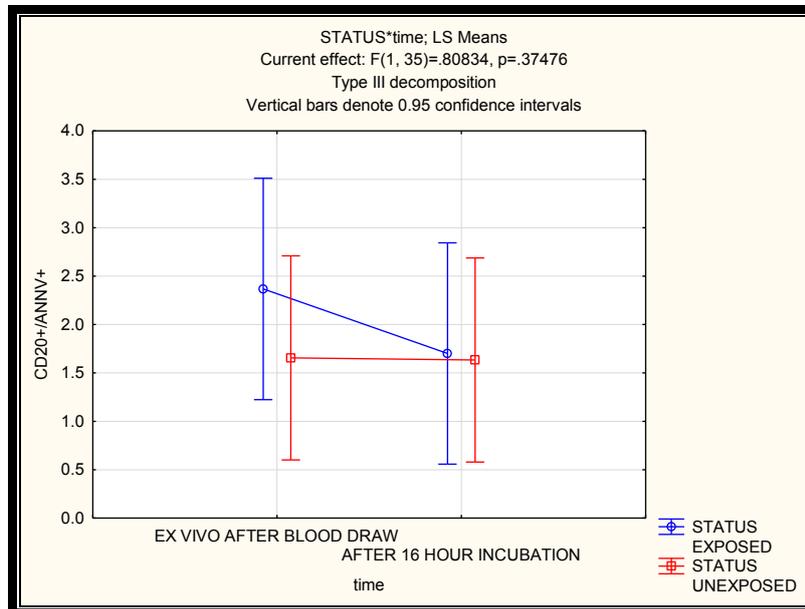


Figure 5- 20: Summary of Annexin V expression on CD420+ B cell expression at *ex vivo*, 16 and 24 hour time points. The x-axis depicts the time points and the y-axis depicts the expression percentage of Annexin V ($p= 0.37476$).

At 24 months of age, the HEU group displayed an increased expression of Annexin V (*ex vivo*) on CD20+ B cells compared to the UE group (2.37 vs. 1.66%). These differences were not statistically significant. The HEU group also showed a decreased mean value from *ex vivo* to 16 hours (2.37 to 1.70%). The UE group displayed similar levels of Annexin V from *ex vivo* to 16 hours (1.66 to 1.33%). These differences were not statistically significant for any of the groups (See Figure 5-20 and Table 5-24).

Table 5- 24: Summary of means, standard deviations (SDs) and p-values for both HEU and UE groups for expression of Annexin V on CD20+ B cells at ex vivo, 16 hour and 24 hour time periods at 18 months of age.

GROUP	FACTOR	MEAN	SD	P-VALUE	N
HEU	EX VIVO	2.37	3.13	0.359	17
UE		1.66	2.09		20
HEU	16 HOURS	1.70	2.39	0.930	17
UE		1.63	1.54		20
HEU	EX VIVO	2.37		0.210	17
	16 HOURS	1.70			17
UE	EX VIVO	1.66		0.965	20
	16 HOURS	1.63			20

5.7 ROUTINE LYMPHOCYTE COUNTS

Routine lymphocyte counts were carried out as described in previous sections at each of the indicated time points. The percentage of CD3/CD4 and CD8 counts are listed and displayed in Table 5-25 below. In addition, the CD4:CD8 ratio is shown in the indicated table.

Table 5- 25: Summary of means, standard deviations (SDs) and p-values for both HEU and UE of routine lymphocyte counts (CD3/CD4/CD8 and CD4:CD8 ratio) from 2 weeks to 24 months of age

Time point	Status	%CD3 Mean	SD	p-value	%CD4 mean	SD	p-value	%CD8 mean	SD	p-value	CD4:CD8 Ratio	SD	p-value	N
2 weeks	HEU	74.94	6.94	0.12	53.84	54.21	0.68	20.72	20.75	0.10	2.84	2.88	0.11	24
	UE	71.48	7.40		52.91	53.04		17.94	17.64		3.32	3.38		28
6 weeks	HEU	63.23	8.55	0.84	43.42	43.28	0.45	18.56	18.64	0.22	2.77	2.77	0.55	25
	UE	62.78	7.34		45.14	45.04		16.47	16.33		2.96	2.98		27
12 weeks	HEU	56.95	9.76	0.09	39.74	39.84	0.74	15.27	15.28	0.02	2.97	2.99	0.08	25
	UE	60.67	7.69		40.49	40.22		19.13	19.15		2.44	2.44		27
6 months	HEU	56.56	10.79	0.09	36.68	37.00	0.16	18.06	17.72	0.70	2.30	2.39	0.74	25
	UE	60.43	7.34		39.97	39.52		18.73	18.65		2.42	2.40		23
12 months	HEU	61.16	8.04	0.60	38.26	38.91	0.86	20.79	20.39	0.30	2.11	2.19	0.63	23
	UE	62.39	7.44		37.84	36.90		22.67	22.85		1.96	1.86		21
18 months	HEU	61.19	7.70	0.44	38.46	40.78	0.50	20.10	19.33	0.04	2.006	2.25	0.41	18
	UE	63.11	6.60		36.89	35.95		24.05	24.38		1.72	1.63		21
24 months	HEU	63.45	8.96	0.58	35.97	3.71	0.75	24.70	24.12	0.33	1.54	1.31	0.73	17
	UE	64.84	6.48		35.18	34.70		26.57	26.80		1.43	1.36		20

Results of the lymphocyte counts show no statistically significant differences between the HEU and UE groups respectively over each time point except for the %CD8 counts at 12 weeks and 18 months of age, where the HEU group presented with decreased %CD8 counts at each of the indicated time points.

CHAPTER 6

DISCUSSION

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6.1 OVERVIEW

The impact and consequences underlying the effects of *in utero* HIV exposure on infant immune responses have not been extensively characterised. Clinical follow-up of these HEU infants reveals increased infectious morbidity and mortality (Filteau, 2009)

This study aimed to investigate the potential immunological mechanisms for the increased incidence of infectious morbidity and mortality in HEU infants in a longitudinal multi-collaborative pilot study. The various properties of adaptive immune responses were explored and evaluated.

The results investigation highlighted important differences in specific adaptive immune responses in the HEU group when compared to their UE counterparts. We show that HEU infants acquire significantly fewer antibodies from their mothers to specific vaccine antigens; however subsequently mount robust responses to some routine vaccinations.

This investigation also documents the persistence of adaptive immune differences in HEU infants beyond the initial vulnerable phase of the first 6 months of life of acquiring infection. Our data showed the upregulation of certain cellular markers of activation and apoptosis in both T and B cell components of adaptive immunity.

In keeping with our formulated hypothesis, we demonstrate that HEU babies in comparison to UE controls, showed differences in both the cellular and humoral arms of the adaptive immune system. These observations may thus be related to HIV exposure and possibly explain long-term immunological aberrations.

6.2 IgG SPECIFIC ANTIBODY RESPONSES

6.2.1 Maternal antibody levels

The placental transfer of maternal antibodies plays a key role in protecting infants from infection during the first stages of life before receiving vaccinations and prior to developing their own adaptive immune responses (Mackay, 2006).

Analyses of our data show that the HEU infant group had significantly lower levels of maternally derived antibodies to pertussis, tetanus and pneumococcus at 2 weeks of age when compared to the UE group. The results of this observation were consistent with a recent study evaluating the association of maternal HIV infection on uninfected infant vaccine specific antibody levels. Jones *et al* (2011) noted significantly lower levels of antibodies to pertussis, tetanus, Hib and pneumococcus at birth in HEU infants compared to their UE group. Our evaluation in comparison to this study did not measure the specific antibody levels in the mothers. Observations by Jones *et al* showed that HIV infected mothers have significantly lower levels of antibodies to Hib and pneumococcus; however no differences were noted for pertussis and tetanus. In contrast to Jones *et al.*, the maternal groups for both HIV infected and uninfected mothers in our study were similar for age and gravidity characteristics (Jones *et al.*, 2011).

Studies evaluating viral antigens found similar trends in the deficient transfer of antibodies from an HIV infected mother to her infant. A Zambian study evaluated the influence of HIV-1 exposure on the levels of passively acquired antibodies and showed that infants born to HIV positive mothers had lower antibody levels to measles than infants born to HIV negative women. Levels were even lower in infants infected with HIV. This suggests that prior to vaccination, HEU and HIV infected infants are at greater risk for contracting measles (Scott *et al.*, 2005). The findings of this study was further supported by other investigations in Kenya and Brazil, who showed a decrease of measles antibodies in cord blood samples of infants from HIV positive women (Scott *et al.*, 2005, de Moraes-Pinto *et al.*, 1993).

In addition to HEU infants receiving lower levels of antibodies from their maternal source, a small group of infants received levels deemed to be protective. We also observed that despite UE infants receiving higher levels of maternal antibodies compared to HEU infants, our data highlighted that more than half of these UE infants did not receive levels that are considered protective for pertussis and Hib. A plausible explanation for this may be the fact that HIV negative women also do not have sufficient antibody levels to transfer, which may be due to their own waning immunity. Our speculation may be supported by similar studies that found that HIV uninfected women generally have low levels of specific antibodies and hence may lead to the lack of transfer of sufficient levels (Jones *et al.*, 2011, van den Berg *et al.*, 2010).

In contrast to the findings of Jones *et al* (2011), the maternal antibody levels to Hib were similar between the two groups at the 2 and 6 week time points. Although a significant trend at 2 weeks ($p=0.09764$) was observed. A large proportion of infants in both groups did not acquire protective levels to Hib. This could imply that HIV uninfected mothers have deficient levels of Hib antibodies also due to waning immunity. Studies investigating the transfer of maternal Hib antibodies in infants showed that the maternal levels are generally low, even with effective transfer to the infant (de Voer *et al.*, 2009).

Another possible explanation for the deficient transfer of Hib titres in the absence and presence of HIV infection may be related to the mechanism and structural components of placental transport of Hib antibodies. Transportation of IgG is dependent on placental receptors that are specific for the Fc γ R region. Fc γ R I and III have a predilection for IgG1 and IgG3, but show low affinity for IgG2 (Takizawa *et al.*, 2005). This impacts the transfer of antibodies to polysaccharide antigens across the placenta. Polysaccharides are likely to elicit more IgG2 antibodies in contrast to protein antigens that elicit IgG1 and 3 subclasses respectively, which are more favourable to crossing the placenta. Other factors that may individually relate to the transfer of antibodies to the infant may be associated to gestational age at birth, placental defects, as well as the IgG subclass concentration in the mother (Findlow *et al.*, 2006).

Similarly, a study conducted in the Netherlands, investigated the seroprevalence and placental transportation of maternal antibodies specific for Hib, diphtheria, tetanus and pertussis. De Voer *et al* (2009) showed that in contrast to antibodies elicited by proteins, such as diphtheria, tetanus and pertussis antigens, antibodies directed to polysaccharides, such as Hib, were transferred less effectively. In addition, De Voer *et al* (2009) also noted that even with effective antibody transfer to the neonate, the antibody concentrations in the mothers were relatively low, potentially leaving infants at increased risk of infection before vaccination. They observed that almost all diphtheria and tetanus titres in cord blood serum samples were above the generally expected protection level of 0.01 IU/ml (de Voer *et al.*, 2009).

A number of studies have alluded to the decreased level of passively transferred antibodies from an HIV infected mother to her infant. However, very few studies have addressed the immunological mechanism responsible for this. A possible explanation may be due to lower maternal antibody levels or impairment in the transfer from an HIV infected mother (Scott *et al.*, 2007, Jones *et al.*, 2011). Jones *et al* found a 23% reduction in the placental transfer of antibodies for Hib, 40% for pertussis and 27% for tetanus, but they could not show any association between maternal CD4 count or HIV viral load and placental transfer. This goes in contrast to a study by Farquhar *et al.*, who showed a positive correlation with maternal viral load and reduced transfer of maternal antibodies (Farquhar *et al.*, 2005). It is therefore still unclear whether HIV viral load may interfere with the mechanism of transfer.

A further possible explanation for the decreased efficacy of transfer may be related to the concentration of HIV-1 specific antibodies such as gp41 circulating in maternal blood during the final trimester. These HIV-1 specific antibodies may block hFcRn receptors and inhibit the transport of specific antibodies (such as measles) from the mother to infant. Therefore, it can be hypothesised that severity of maternal HIV disease may place infants at risk of

contracting diseases despite being HIV uninfected. Based on these observations, it could be suggested that the effective control of maternal viral load by initiation of early antiretroviral therapy preceding the period when the bulk of IgG is transferred from the maternal source (4 weeks of gestation), may play a role in the transfer of antibodies from an HIV infected mother (Farquhar *et al.*, 2005).

Polyclonal hypergammaglobulinemia is commonly associated with HIV infection in both symptomatic and asymptomatic patients. Brambell, a British medical scientist, was the first to associate maternal hypergammaglobulinemia with the transfer of IgG across the placenta during the 1960s. It is believed that an excess of immunoglobulin in the mother may cause saturation of Fc γ receptors on placental elements, such as syncytiotrophoblasts which may limit transfer of antibodies across the placenta (Brambell, 1966, de Moraes-Pinto *et al.*, 1996, Chucuri *et al.*, 2010). This concept was further supported by a Brazilian study that showed that maternal HIV infection was associated with reduced placental transfer of antibodies to tetanus toxoid, measles and *S. pneumoniae*. The researchers showed that for some antibodies the efficiency of placental transfer decreased as the respective maternal levels increased with advancing HIV and related to nonspecific polyclonal antibodies (de Moraes-Pinto *et al.*, 1996).

In addition, a Malawian study showed that maternal HIV infection and hypergammaglobulinemia was associated with a reduction in transfer of IgG antibodies to *S. pneumoniae*. However, in contrast to our findings, they found no difference in the transfer of tetanus toxoid antibodies. Although these infants may be considered protected against neonatal tetanus, it has been shown that infection occurs despite having adequate antibody level (de Moraes-Pinto *et al.*, 1998). We therefore concluded that the susceptibility of HEU infants to tetanus is still unclear. In contrast to the latter study a Brazilian study showed a reduction of tetanus IgG levels in neonates which was associated with maternal HIV infection (de Moraes-Pinto *et al.*, 1996).

A study conducted in Kenya in addition showed that maternal HIV infection was associated with a 52% reduction of tetanus antibodies in infants after compensating for maternal vaccination. The placental transfer was measured using the ratio of levels in cord blood serum to that in serum of the mothers i.e. CMR (cord: maternal ratio). They also found that 5.3% of the mothers and 7.8% of the newborns were classified as being seronegative for tetanus. The study concluded that HIV interferes with the transfer of maternal antibodies (Cumberland *et al.*, 2007).

Although several studies have supported the lack in transfer of maternal antibodies in the presence of HIV infection, only a few studies have alluded to the rate of decreased levels of passively acquired neutralising antibodies during infancy (Cabau *et al.*, 1974).

At 6 weeks of age the levels between HEU and UE infants in our study were similar, although HEU infants still had a lower mean level compared to the UE group. As HEU infants initially starting with a lower level of antibodies to various antigens, their levels were depleted before those of UE infants. Similar findings were confirmed by Cabau *et al* who reported a significant decay in maternally acquired antibodies to measles and *S. pneumoniae* in a HEU population (Cabau *et al.*, 1974).

The time spent *in utero* may also affect the levels of passively acquired antibodies. Investigations have shown that the level of transport of IgG from the placenta is dependent on the gestation period. A study evaluating the transport of IgG maternal antibodies to pertussis, diphtheria, tetanus, Hib and Neisseria meningitis C showed that levels in preterm infants born at a gestational age less than 32 weeks had significantly lower levels of maternal antibodies to the above listed antigens (van den Berg *et al.*, 2010).

In our cohort, 4 infants with prematurity had a gestational age less than 34 weeks (3 HEU and 1 UE). At 2 weeks of age, 2 of these infants had non-protective levels to pertussis (one presented with borderline levels). None of the infants had protective levels to tetanus and only one infant displayed protective levels to Hib.

In a review compiled by Cáceres *et al* (2000), gestational age was directly linked to the degree of placental antibody transfer, with prematurity having an adverse effect. Hence they questioned whether the increased frequency of lower gestational age births often documented in developing countries contributes to a number of newborns with decreased levels of antibodies (Caceres *et al.*, 2000).

Our observation, along with documentation by other researchers, showed that infants may have decreased levels of maternally derived antibodies, irrespective of HIV exposure and should be addressed. Maternal re-immunisation should be considered in addition to defining good predictors for correlates of protection. Previous studies have shown direct correlations between antibody concentrations in mothers and infants who had increased levels due to maternal history of pertussis or pertussis immunisations (Van Rie *et al.*, 2005). These authors also emphasised the difficulty in determining the proportion of infants that are born protected to pertussis, because of the absence of definite serological correlates of protection.

There is a great need to evaluate effective interventions to increase maternal and neonatal immunity. An approach towards neonatal immunisation in all infant populations should perhaps be considered in order to prevent infections in early life.

The possibility of protecting young infants against diseases by immunising their mothers during the third trimester of pregnancy has been investigated since the 1930s. Vaccination during pregnancy may be considered as an effort to limit maternal and infant morbidity; however, this raises a number of concerns with regard to impairment of infant vaccination responses (Siegrist, 2001).

A study by Englund *et al* showed that mothers immunised with conjugated Hib vaccines at some stage in the third trimester of pregnancy, conveyed higher levels to their infants than those mothers that were not immunised. Pregnant mothers, immunised at 32 weeks gestation, transferred 82% of antibodies compared to a 92% transfer in mothers immunised at 36 weeks gestation (Englund *et al.*, 1995b). More recent studies have indicated that the efficiency of placental transfer depends on a number of factors, that include the integrity of the placenta, concentration of IgG, type of vaccine and gestational age (Englund, 2007). Polysaccharide vaccines, such as Hib and *S. Pneumoniae*, are generally considered safe and immunogenic during pregnancy (Healy and Baker, 2006).

Other studies support the need for maternal immunisation against pertussis, as higher concentrations of maternally derived antibodies to pertussis were related to a weaker pertussis toxin antibody response to whole cell pertussis vaccine, but not cellular pertussis vaccines (Edwards, 2003). Further studies showed that after maternal immunisation, concentrations of pertussis antibodies in infants ranged from 50% of maternal titres to equal titres in both mother and infant pairs (Van Rie *et al.*, 2005).

Rich and Abbud concluded that infants who received protective antibody titres to Hib were born to mothers that received a constant low-dose of Hib. Furthermore, they suggested that adequate binding capacity of the Fc receptor in the placenta is associated with antibody affinity and low avidity complexes which are achieved by low-dose. Similar effects are found with infection. These complexes can be effortlessly cleaved by the reticuloendothelial system and can thus be more readily available for transport via the Fc receptors (Rich, 1996).

Additional plans to prevent infection during the neonatal period include strategies such as “cocoon” immunisation. This is the process whereby the individuals who are in contact with the neonate, are vaccinated. Neonatal immunisation would be of a great advantage and some of the benefits would include high vaccination coverage as more healthcare contacts are available at birth and offering earlier protection prior to the exposure to pathogens (Wood and Siegrist, 2011).

With the increasing number of infants born to HIV infected mothers, assessing the efficiency and effect of maternally derived antibodies is essential for determining the factors that may be related to the increased infectious morbidity in the HEU population. The evaluation of our comparative study showed that HEU infants derive lower levels of maternal antibodies when compared to UE infants. This highlights their potentially related susceptibility to contracting severe diseases at an early age. In addition, we also documented that despite UE infants receiving higher levels of antibody from their HIV uninfected maternal source, in a large portion of these cases, levels were still not considered protective. This indicates that both HEU and UE infants in our group may be vulnerable to infection. Furthermore, our data suggests that HEU infants may need to be vaccinated earlier than their unexposed counterparts. Currently the vaccination schedule is not differentiated for HEU and UE infants. Despite the high levels of maternally derived antibodies to pneumococcus in our cohort, a number of infants are diagnosed with pneumonia in early infancy.

Lastly, our study, along with others illustrated the need for determining the mechanisms that may be responsible for the defective transfer of IgG antibodies to infants with maternal HIV infection. Through this understanding of potential factors that influence the passage of transfer during gestation we could subsequently be able to devise preventative measures for vulnerable infants of disadvantaged communities and improve protection against infections.

6.3 POST-VACCINATION LEVELS

A number of factors influence the quality and quantity of an infant’s antibody response to vaccination, some of which include the stage of development of their immune system, the type of vaccine administered along with its immunogenicity, the number of doses and intervals between doses and lastly the effect of maternal antibodies (Wood and Siegrist, 2011).

Based on these characteristics of vaccination, our aim was to further assess whether HEU infants mount effective immune responses to vaccinations of the EPI-SA programme when compared to UE control infants.

6.3.1 Vaccination response to pertussis

After the second pertussis vaccine dose measured at 12 weeks of age, both HEU and UE control infants displayed similar responses and only a few produced levels that were considered protective. However, after the third vaccination HEU infants mounted significantly higher antibody levels to pertussis compared to the UE control group. Our observation is consistent with a South African study that showed significantly higher IgG levels of pertussis in HEU infants after 3 vaccine doses (Jones *et al.*, 2011). We further showed that this significant increased level persisted until 18 months of age after the receipt of the booster dose. At 24 months of age, the levels in the HEU group were lower than those of the UE group; however, all the HEU infants displayed protected levels compared to 89% in the UE group.

The observed increased response to the pertussis vaccine may potentially be related to the decreased maternally derived antibodies in HEU infants seen at both 2 and 6 weeks of age. A number of studies have alluded to the potential interference of maternal antibodies with the infant's vaccination response (Scott *et al.*, 2007). There is limited understanding around how maternal antibodies inhibit vaccine responses in infants. However, it is believed to be related to the maternal antibody to vaccine antigen ratio during the time of immunisation which subsequently results in epitope masking. Epitope masking refers to the concept whereby maternal antibodies "disguise" vaccine antigenic epitopes, therefore preventing the binding of infant B cells. In addition, it is suggested that a potential means of avoiding such immunological conditions would be to increase the dose of vaccine antigens (Siegrist, 2003). Similar studies have described maternal antibody interference with infant vaccine responses to measles and tetanus vaccines (Sarvas *et al.*, 1992, Albrecht *et al.*, 1977)

Jones *et al* (2011) found that higher maternal antibodies had influenced the infants' response to the first vaccination dose, but played no role in the response to subsequent doses (Jones *et al.*, 2011). However, despite this observation by Jones *et al* (2011) we observed that some infants in the control group with relatively high maternal antibody levels at 2 and 6 weeks still mounted a lower response after the third vaccination dose than infants who had a relatively low maternal antibody level. Further research in support of the study by Jones *et al* confirmed that increased maternal antibody levels did not inhibit the infants' response to specific vaccines, such as tetanus and Hib after the second dose (Kurikka *et al.*, 1996, Saffar *et al.*, 2007). Both the studies by Jones *et al* and Kurikka *et al* is contradicted by a Danish study that showed an inhibitory effect of maternal antibodies on infants response to vaccination (Sarvas *et al.*, 1992). We did however; observe that UE infants mounted an adequate response only after the fourth dose (booster). This corresponds with the findings of Glezen (2003) who observed that during early infancy, passively acquired antibodies suppressed antibody response to active immunisation, but did not affect priming for later boosting (Glezen, 2003). Therefore, some studies suggest that maternal antibodies have no effect after the administration of the first and second vaccination. We have however noted that in UE infants the potential interference of maternal antibody levels seems to diminish only upon the administration of the fourth vaccine dose.

Considering that maternal antibodies play no role in the infants' immune response after the second dose does not explain why after the third and fourth dose, HEU infants still mount an antibody response that is significantly greater than the UE group for pertussis. Perhaps the reason for the increased pertussis levels noted in HEU may potentially be due to a certain degree of immune activation of T and B cells upon the administration of immunisation. This concept was explored by Obaro *et al.*, however during the evaluation of the immunogenicity of childhood vaccines in HIV infected children. The concern was around immunisation and its effects on the proliferation of T cells and subsequent release of cytokines (Obaro, 2004). The same activation of the immune system may occur in HEU infants given the fact that they have been exposed to a highly antigenic environment. However, unlike the HIV positive infants addressed by Obara *et al.*, immunisation does not lead to viral replication in HEU infants, thereby limiting adverse impairment of the immune response. A study by Bunders *et al.*, investigating the impact of maternal HIV infection on immunoglobulin levels in HEU children, showed a significant impact of maternal HIV infection on the B cell compartment of the HEU immune system, which further suggests that it may be due to HIV exposure. Researchers documented that hyperglobulinaemia is a common feature in HIV infection, which also persists in HEU infants until at least 24 months of age. They further concluded that infants born to HIV positive mothers have an altered humoral response (Bunders *et al.*, 2010).

An effective immune response to vaccination should result in the effective priming of both T and B cell compartments of the immune system. Responses to whole cell pertussis and the influence of maternal antibodies are determined specifically by B cells and do not influence the infant's T cell responses. Neonates mount weak cytotoxic and T cell responses and the help for B cell differentiation is limited. Pertussis antibody-secreting as well as memory cells are produced despite immunisation of neonates mounting a rapid antibody response (Siegrist, 2003).

Early findings related to vaccination with pertussis indicated that giving the vaccine at birth produced a decreased immune response. However, studies using the mouse model found that the temporary dampened immune response observed in neonates is antigen specific (type of vaccine administered) and that the underlying reason behind the reduced response may be due to the immaturity of the neonatal immune response along with the inhibitory effects of competing maternal antibodies. Infants immunised with whole-cell pertussis seemed to have lowered antibodies in relation to having increased maternal antibodies. However, in infants immunised with acellular pertussis vaccine, maternal antibodies did not confer interference, thus indicating the vaccination with acellular pertussis allows for a better immune response and may be explained by evaluating T-cell mediated immunity (Van Rie *et al.*, 2005). Similar studies have observed a decrease in antibody response of up to 56% in infants immunised with whole- cell pertussis vaccine as opposed to acellular pertussis (Glezen, 2003).

Only one out of the three HIV positive infants in our study displayed non-protective levels to pertussis after receipt of the primary vaccination. At 12 months of age, none of the HIV positive infant's maintained levels deemed to be protective. However, after the fourth vaccination dose given at 18 months of age, HIV positive infants had increased levels of IgG to pertussis, which was maintained at 24 months of age. A study by Tejiokem *et al* showed that after 3 doses of the whole-cell pertussis vaccine, only 19% of HIV positive children and

48% of HEU children possessed detectable antibodies. The initial antibody response was much lower in HIV-infected infants than exposed infants; however, it is unclear whether HIV-infected children may be at risk of severe or more frequent pertussis disease relative to HEU infants (Tejiokem *et al.*, 2009).

There was one documented pertussis infection in a control infant in our study. At 2 weeks of age the infant had a maternal antibody level of 50.94 FDA U/ml and at 6 weeks of age this level decreased to 21.0 FDA U/ml. After 3 doses of the pertussis vaccine this infant did not mount any response and at 12 months of age, had the lowest documented level of 0.9 FDA U/ml. At the 18 month time point the levels increased significantly to 327.16 FDA U/ml. At 24 months the child did not have protective levels to pertussis (24 FDA U/ml) of which during this period was diagnosed with pertussis. It is likely that due to receiving high maternal antibodies, this infant could not respond adequately to vaccination due to maternal antibody interference. The heightened response to the vaccine at the 18 months may have resulted due to diminished maternal antibody levels. It is interesting to note, that even after infection the infant did not display pertussis levels that were considered protective even after one month of natural infection. These observations may suggest primary immunodeficiency and indicate the need for further evaluation (Gaspar and Conley, 2000).

In South Africa the efficacy of both whole-cell and acellular pertussis vaccines have been shown to be in the ranges of 83-98%. However, it is mentioned that the extent of the immune response to pertussis is proportionate to the number of doses administered (SAVIC, 2009b). Our findings suggest that infants in both the HEU and UE groups mount the highest response to pertussis after the fourth vaccination dose. Similar studies have confirmed this finding (Torbicka *et al.*, 1995). UE infants in our study mounted the highest protective response to pertussis only after the fourth dose where 94% of infants had protective levels. Our findings are in contrast to a study conducted in Taiwan where infants vaccinated at 2, 4 and 6 months showed protective antibody titres against pertussis at each of these time points (Lin *et al.*, 2003).

A study in Finland observed the effects of genetic divergence between whole-cell and acellular pertussis vaccines. It was stated that *B. pertussis* population undergoes continuous and rapid changes, however despite this molecular observations, it was concluded that both types of vaccines still offered protection against pertussis. The study advised administering the vaccination as early as possible, and furthermore highlighted the importance of booster immunisation (Elomaa *et al.*, 2009).

No correlates of protection for pertussis have thus far been established. It is suggested that protection induced against pertussis may depend on a number of immunological factors relating to the humoral and cell-mediated responses. Bearing in mind the decay of maternal antibodies, a definitive vaccine response represents a 4-fold increase in antibody concentration between the pre- and post-vaccination samples (Godfroid *et al.*, 2004). Similarly, Capeding *et al.* (2008) described pertussis seroconversion as a four-fold increase in antibody titres that occurred in 95.3% of their study population (Capeding *et al.*, 2008).

There is much concern regarding the re-emerging profile of pertussis. The effects of adapted epidemiology show increased incidences in infants, adolescents and adults, which may be due to a number of reasons including increased efficacy of diagnostic techniques. Other

factors, such as demographic changes, waning immunity, changes in vaccine coverage and perhaps a decrease in vaccine efficacy due to drifts in isolates and strains, may all be related to the increase in incidence rates. Studies have shown that immunisation and infection during childhood are not producing sufficient immunity (Edwards and Freeman, 2006, Cherry, 2005, Bamberger and Srugo, 2008, Campins-Marti *et al.*, 2001). A Brazilian study has confirmed that immunity in infants immunised with 3 doses of whole-cell pertussis produced high levels of antibodies. The researchers concluded that the vaccine induces effective immunological memory that may only last for 2 years subsequent to the completion of the immunisation schedule (Pereira *et al.*, 2010). A study conducted in France showed that infants produced an adequate response to pertussis whole-cell vaccines, but these responses diminished without a booster dose. However, 6 years after vaccination, responses increased suggesting exposure to infected persons and persistence of pertussis strains with unfamiliar circulating strains. The researchers highlighted that infection is more likely to occur within 6 years of receiving their last vaccination.

These findings therefore support the need to further evaluate the disease causing isolates in more detail and promote the implementation of additional booster vaccinations, either in children at pre-school age and/or adolescents to prevent the possibility of an outbreak.

6.3.2 Vaccination response to *Clostridium Tetani*

After the second vaccination dose, both HEU and UE infant groups responded adequately to the tetanus vaccine and both groups produced 100% antibody protection. After the third vaccination dose, infants were still able to maintain adequate levels to tetanus. There seemed to be no difference in the antibody levels in HEU and UE infants from 12 weeks to 18 months of age. Our data follows that of Jones *et al.*, who showed no differences in the vaccine responses in HEU and UE after receiving the third dose (Jones *et al.*, 2011). Despite receiving significantly low maternal antibody levels at birth, there was no increase in the antibody response to the vaccine after 2, 3 or 4 doses. Our longitudinal data show that differences in HEU and UE infants appear to emerge at 24 months of age. Despite HEU infants having an increased IgG level to tetanus at 24 months, infants in both groups displayed protective levels.

Protective responses are based on both the levels of IgG antibodies, which are mainly IgG1 responses, as well avidity aspects. A study conducted in Gabon measured the IgG levels in a group of children originating from rural and urban areas respectively. Researchers showed increased level of Ig1 anti-tetanus antibodies were present in rural children after vaccination when compared to semi-rural children. The avidity was similar amongst the two groups (van Riet *et al.*, 2008). It could be speculated that the increased IgG specific antibodies to tetanus in the HEU group at 24 months may be due to our HEU group (compared to our UE group) differing significantly with regards to social characteristics. More infants in the exposed group live in informal housing structures thus may be pre-exposed to tetanus antigens that may be contained in soil. In addition, may be due to exposure to unhygienic environments. This may have resulted in the heightened response represented at the 24 months time point.

There is some speculation with regards to the correlates of protection for tetanus. There have been a number of tetanus infections where individuals presented sufficient levels expected to confer protection (Borrow *et al.*, 2003). Therefore, despite having adequate

levels to tetanus in both HEU and UE groups after vaccination, does not imply that these infants are not susceptible to infection.

A recent Brazilian study evaluating the vaccine response in HEU infants to a number of antigens including tetanus, reported that although HEU infants had lower mean IgG levels to tetanus in comparison to UE infants, a statistically significant difference between the two groups was not achieved (Abramczuk *et al.*, 2011) which is in keeping with our findings. The median age however in the HEU group was just over 7 months. There were no reports on the tetanus levels prior to the 7 month time point. Abramczuk *et al* found that a large portion of HEU infants were non responders to Hepatitis B vaccine and concluded that HIV infection is not largely associated with failure to respond to tetanus or diphtheria, but more likely to be associated with aberrant Hep B responses. Based on this speculation, we have observed that all HIV positive infants in our study had protective levels to tetanus and therefore support no association with HIV exposure or infection with different responses to tetanus and further speculate that the increased response is possibly due to environmental antigen exposure. Another study showed that 75% of their HIV positive cohort responded to the tetanus vaccination (Melvin and Mohan, 2003). Therefore, the significantly higher response depicted in HEU infants at 24 months of age in our study might not directly be due to HIV exposure but perhaps rather to greater antigenic exposure.

Abramczuk *et al* (2011) also found no association between infant CD4 counts and IgG levels to tetanus, which corresponded to another study that showed similar responses in HEU individuals with lower CD4+ T cell responses (Gesner *et al.*, 1994). Based on these observations, it is believed that the differing humoral immune responses in HEU infants be due to functional changes in T lymphocytes and aberrant functional abilities related to antigen presentation (Abramczuk *et al.*, 2011)

It is unlikely that maternal antibody levels would have had an influence on the heightened response observed for HEU infants at 24 months of age. According to Kurika *et al.*, maternal antibody interference to tetanus IgG levels in infants are diminished after the second dose (Kurikka *et al.*, 1996).

6.3.4 Vaccination response to *Haemophilus Influenzae b*

In contrast to the protein vaccines described above, HEU infants showed no significant differences compared to the UE group at each of the post-vaccination time points. At 12 weeks of age, they still had lower levels of Hib IgG compared to UE control infants and only 67% of these infants had protective levels in comparison to 100% of infants in the UE group. After 3 vaccine doses, measured at the 6 months time point, both HEU and UE infants displayed similar levels antibodies and protection.

Our findings are consistent with Jones *et al.*, who found similar responses in both HEU and UE infant groups after 3 doses of the Hib vaccine (Jones *et al.*, 2011). Researchers observed that HEU infants do not demonstrate robust (but similar) response to the Hib vaccine in comparison to UE infants. This finding may be related to the lack of competing maternal antibodies as both infant groups (HEU and UE) seem to inherit limited amounts of maternal antibodies. The findings of Jones *et al* showed that HEU infants had significantly lower levels to Hib at birth however still did not mount a significantly higher response to the

vaccine after 3 doses compared to other vaccines, such as pertussis and pneumococcus. This may indicate other potential reasons for why HEU infants have similar responses to the Hib conjugate vaccine.

Our data shows that, despite having relatively high levels of protection after the third vaccine dose, a small percentage of infants in both HEU and UE groups were able to maintain these levels at 12 months of age. Although the levels in the HEU group seemed faintly higher than the controls, they were only able to maintain 30% of their protective levels to Hib. In addition, only 25 % of the UE infants had levels deemed to be protective. This implies that at 12 months of age, both HEU and UE infants in our cohort were susceptible to the major causes of diseases, such as meningitis and pneumonia due to Hib.

In 2010, a total of 82 cases of Hib were reported amongst children less than 5 years of age. According to the GERMA-SA audit, a total of 91 Hib disease cases were reported in South Africa of which the Western Cape represented the third highest (21 cases). The highest incidence was in the Eastern Cape, followed by Gauteng (GERMS-SA, 2010). There had been no documented incidence of Hib related infections in our cohort even during the vulnerable period at 12 months when the levels of protection was relatively low.

A study by Goldblatt *et al* demonstrated an increase in Hib levels after 3 doses of the vaccine. This seemed to be effective in inducing immunological memory; thus, with the primary schedule sufficient memory associated with long term protection would be generated upon infection even though the circulating antibody titres are low (Goldblatt *et al.*, 1998). This observation can therefore be related to the incidence in both HEU and UE control infants having decreased levels to Hib, which according to Goldblatt *et al.*, does not make them vulnerable to infection due to the initiation of long-term memory associated with Hib conjugate vaccines. In addition, infants in our study who received only 3 doses of the Hib vaccine still maintained protective IgG levels at 18 and 24 months of age.

A review by Plotkin outlined the importance of defining correlates of protection and highlighted the underlying mechanism of protection may not be related to the mechanisms involved in the recovery of infection. Rather, Plotkin states that correlates of protection differ both in quantity and quality and thus depends on the purpose of wanting to prevent infection (systemic or mucosal) or to prevent disease (Plotkin, 2010). For pathogens such as Hib, protection should be defined by opsonophagocytic or bactericidal antibodies (functionality), however antibody binding is considered as useful substitutes. The protective ranges relating to antibody concentration differ amongst countries and research groups. Three important points that have emerged with regards to adequate protection against Hib: (1) Antibodies should be present at the time of exposure (2) those antibodies must demonstrate higher levels of avidity to propose protection and (3) protein conjugates reduce T cell dependent responses and protection may be offered by lower levels of antibody (Plotkin, 2010).

Hib IgG levels subsequent to 12 months were similar between the two groups and both HEU and UE infants displayed protective levels above 80%. By the age of 2 years both groups had adequately protective levels to Hib. However, four infants in our study (2 HEU and 2 UE) displayed IgG levels $<0.1\mu\text{g/ml}$. Only one of these four infants mounted an adequate response to the first two doses of the Hib vaccine. After the third vaccine dose all infants mounted adequate levels to Hib. At 12 months of age, only one of the 4 infants had levels

>0.1µg/ml. This same infant did not mount a response to the booster vaccination at 18 months, compared to the other three who had levels deemed to be protective. A common trend that seems to be emerging with this group of infants is that after each vaccination, most of these infants were not able to maintain protective levels.

There is a relative amount of debate regarding the immunological response related to capsular polysaccharide elicited by B cells. It is often reported that the response is carried out independent of T cell help; however increasing evidence suggests a potential role for CD4+ T cells. Suggestions indicate that the conjugation of bacterial polysaccharides to a carrier protein provides foreign peptide antigens that are presented to the immune system and subsequently recruits antigen specific CD4+ T cells (Siegrist, 2001, Clerici *et al.*, 2000). Matos outlined however that the response to conjugated vaccines is known to be T cell independent. Molecules such as PRP are T cell independent antigens that can activate only mature B cells and prevent infants from producing antibodies against polysaccharide epitopes. T-independent mechanisms develop over age. Due to this a number of conjugated vaccines have been developed to enhance the immune response to PRP, some of which include the conjugation to diphtheria toxoid, tetanus toxoid and the outer membrane protein of *Neisseria meningitidis* (Matos *et al.*, 2009).

There are some concerns surrounding the efficacy of vaccines used in combination with others. Despite having many advantages, such as lowered cost, few injections given and better conformity, concerns have been raised about the increased reactogenicity and interfering immunogenicity. In our cohort, Hib was given in combination with diphtheria, tetanus and pertussis (DTP-Hib; Sanofi Pasteur). Studies evaluating the effect of combination vaccines found similar responses to all vaccines given in combination or individually. Even newer combination vaccines, such as DTaP-IPV//Hib, induce protective antibody titres against all antigens covered in the vaccine (Lin *et al.*, 2003). Other studies have found lower antibody levels to combination vaccines in comparison to separate vaccinations, but thereafter concluded that functional characteristics were similar and resulted in adequate immunological memory (Poolman *et al.*, 2001).

In a Brazilian study designed to evaluate the functional activity of Hib antibodies elicited in a group of infants immunised with the diphtheria-tetanus-pertussis vaccine combined with Hib, it was found that a strong anti-PRP response ($\geq 1.0\mu\text{g/ml}$) was generated. It was further observed that 97% of the infant's immunised with DTP-Hib produced high levels of PRP IgG antibodies that were considered to be a serological substitute of long-term protection. Antibody levels of 0.15µg/ml of anti-PRP were considered as being the minimum level for eliciting short-term protection (Matos *et al.*, 2009).

In a Nigerian study, the response to the Hib vaccine varied in different population groups according to age at which immunisation was administered, the type of conjugate vaccine given, whether the vaccine was administered in the same syringe, pre-existing antibodies and even nutritional elements. Despite these factors 86% of the infants in their study achieved antibody levels >1.0µg/ml four weeks after two or three doses of the vaccine and 95% of their cohort maintained these levels until 9 months of age (Campagne *et al.*, 1998).

HIV-infected individuals have a 6-8 fold increased risk of invasive Hib disease. The initial immunological response in HIV-infected individuals is 54% as opposed to 90% in uninfected

individuals and by 15 months of age there is sequential loss of memory to a protective efficacy of just 30% (Jeena, 2008). HIV positive infants in our study were able to mount adequate responses to Hib after the second vaccine dose, however out of the 3 HIV positive infants in our study, only 2 were able to mount an effective response to Hib after the third vaccine dose. At 12 and 18 months of age, all HIV positive infants had protective levels to Hib, however only 1 infant retained protective levels at 24 months.

Madhi *et al* (2005) investigated both the quantity and quality of the Hib conjugate vaccine in both HIV-infected and uninfected infants. In this study only a few HIV positive infants were likely to have antibody concentrations above the indicated protective levels and were also lower compared to uninfected infants. In addition, a limited proportion of HIV infected infants had antibody levels considered to be functional. The concentration of antibody required for 50% killing of Hib bacteria was much higher in HIV infected infants. The risk for vaccine failure was much higher for HIV infected infants than HIV uninfected infants (Madhi *et al.*, 2005). In contrast, Read *et al* showed no correlation between antibody levels to Hib and viral load (Read *et al.*, 1998). However, similar studies have shown that robust responses to Hib were associated with higher CD4+ T cell counts (Steinhoff and Goldblatt, 2003). In the same study by Madhi *et al* (2005), 95.3% of HIV uninfected infants produced antibody levels greater than 0.1µg/ml after the third dose. Similarly, the findings in our study showed that 89% of uninfected infants mounted adequate responses.

Rustein *et al.*, found that only 46% of HIV infected infants mounted adequate Hib responses compared to 79% of infants in their HEU group. After a booster dose at 15 months, the differences between the group were no longer comparable (Rustein *et al.*, 1996). It was therefore recommended that HIV infected infants receive a booster dose after 2 years of age as Hib is considered a significant pathogen in HIV-infected adults (Mangtani *et al.*, 2010).

6.3.5 Response to PCV₇ vaccination

Approximately 1 million children under the age of 5 years die of pneumococcal infections each year (Scott, 2007). *Streptococcus pneumoniae* infection is classified as the major cause of pneumonia and meningitis (Quiambao *et al.*, 2007). Immune compromised patients such as those infected with HIV are more susceptible to contracting diseases, including those related to *S. pneumoniae* infection.

Two randomised placebo control trials evaluated pneumococcal vaccine (PCV₉-CRM) in South Africa and Gambia. The South African trial conducted from 1998 to 2001 demonstrated 65% and 83% efficacy against pneumococcal disease in both HIV infected and uninfected children respectively (Klugman *et al.*, 2003). Although a number of studies have measured the responses to PCV vaccination in HIV infected infants, a paucity of studies have addressed the immunogenicity in HEU infants

The approval of the pneumococcal vaccination into the EPI-SA schedule occurred during April 2009, the same time the recruitment of our cohort commenced. However, due a number of delays with the roll-out of the vaccine programme, a number of infants in our study did not receive the required vaccine dosage according to schedule. In addition, there had been no catch-up programme during the same year. Although the IgG levels to pneumococcus were measured for all infants at each at the indicated time points of the

study, the data could not be collated due to high variability of missed dosages in the limited amount of infants who did receive the vaccine. This section, will therefore only focus on the antibody levels of a few infants who received the first vaccine dose according to the schedule.

As most of our infants have not received the vaccine, we can only speculate the impact of maternal levels on the infants' vaccine responses. Jones *et al* documented robust responses to pneumococcal vaccination in HEU infants compared to UE controls who had received significantly higher levels of maternal antibodies in comparison to the exposed group (Jones *et al.*, 2011). Considering that the population of mothers and infants were from the same region and maternal age groups, we assume that the response in our cohort of HEU and UE controls would have been similar. Madhi *et al* (2010) also showed higher IgG antibody concentrations after vaccination in HEU infants which was indicative of a more vigorous response. Furthermore, Madhi *et al* also considered the contributing factor of maternal antibodies as interference (Madhi *et al.*, 2010).

At the 12 week visit, 12 of our infants (5 HEU and 7 UE) received one dose of the PCV₇ vaccine. Quantitative measurement of the specific IgG levels show that HEU infants had a higher median level of antibodies in comparison to the UE group. These results are consistent with the general findings of Jones *et al* (2011) and furthermore promote the reason for robust responses to vaccination in HEU infant's due to the lack of competing maternal antibodies. Infants who received only one dose of the vaccine presented with sufficient levels considered to be protective. These findings are consistent with another South African study observing the effective immunogenicity of PCVs after one dose (Huebner *et al.*, 2004). Other studies have found immunogenicity to only some of the pneumococcal serotypes after one vaccine dose whereas different studies documented immunogenicity to all serotypes after 3 vaccine doses (Rennels *et al.*, 1998, Leach *et al.*, 1996).

A South African study measured both serotype-specific antibody concentrations (quantity) and OPA (quality) properties after 3 vaccine doses in a subgroup of infants born to HIV positive and negative mothers. Results of this study showed that both HEU and UE infants had antibody concentrations greater than 0.35µg/ml for each serotype measured. However, HEU infants showed a greater concentration for serotype 6B and in addition required higher concentrations of antibody for 50% killing activity against serotype 19F. These findings indicate that antibody levels to PCV in its entirety should not be the only determining factors of correlates of protection, but evaluation of functionality will serve as better determinants of vaccine efficacy. In addition, this functional observation may advocate faint changes in the immune response to vaccines in HEU infants which could contribute to increased morbidity related to pneumococcal disease in this population (Rodgers and Klugman, 2011, Madhi *et al.*, 2010).

Madhi *et al* (2010) further highlighted the importance of using OPA as a means of evaluating protection against pneumococcal disease in addition to antibody concentrations given the fact that serotype-specific antibodies confers protection against disease by opsonising and initiating phagocytosis. In addition, Madhi *et al* states single measurements of IgG levels may result in an over-estimation of protection offered by PCV (Madhi *et al.*, 2010).

Given that PCV₇ contains the most common serotypes known to be resistant to antibiotics, a decrease in both antibiotic resistance and treatment has been accepted after routine use in immunisation programmes. A large portion of infants in our study, were not vaccinated against pneumococcal disease thus potentially leaving them vulnerable to infection. It is therefore recommended that extra care be taken to increasing the coverage and administration of PCV₇ in low-resource settings such as South Africa. In addition, the initiation of catch-up programmes would serve well considering the delay in the vaccine roll-out plan

On a global level, the implementation of PCV₇ into the national immunisation schedule in over 50 countries has shown decreased rates of mortality, pneumonia and otitis media. Furthermore infection rates have decreased in areas where children are known to be affected by HIV (Rodgers and Klugman, 2011).

6.4 EVALUATION OF SPECIFIC CELLULAR BIOMARKERS ON T AND B LYMPHOCYTES BY FLOW CYTOMETRY

Cellular of immune activation, regulation and apoptosis were investigated in HEU children at 18 and 24 months of age and compared to UE controls. The aim of this part of the study was to evaluate these as possible consequences of HIV exposure and whether they persisted as potential long term immunological aberrations.

HEU infants have differences in immune function that had been shown in their responses to vaccination and may not necessarily be linked to the significantly decreased levels of maternal antibody acquisition.

Based on the preliminary findings of vaccine hyper-responsiveness our pilot cohort we hypothesised that those infants born to HIV positive may also experience a prolonged state of immune activation.

This could possibly be related to an antigen loaded environment as well as maternal factors including ARV therapy.

6.4.1 Expression of cellular markers of immune activation and regulation on CD4+ and CD8+ T lymphocytes

Uninfected infants of HIV positive mothers showed evidence of antigenic stimulation and subsequent immune activation of CD4+ lymphocytes (Clerici *et al.*, 2000). These responses have been shown to be HIV specific and appear after birth. In addition, HIV infected women are known to be at higher risk of infections than HIV uninfected women, subsequently exposing their infants to a larger number of antigens therefore leading to increased immune activation in their infants (ter Kuile *et al.*, 2004, Cotton *et al.*, 2008).

A recent study by Borges-Almeida *et al* detected several differences in the cord blood lymphocyte sub-populations in HEU infants compared to UE controls. Newborns exposed to HIV not only showed a decrease in IL-4 production, but more importantly increased levels of IFN- γ . This reflects a skewing towards Th1 responses (more inflamed as such). These observations were more profound in infants born to mothers on HAART, thereby indicating that the disease stage of an HIV positive mother plays a role in the immunological response of their infants, even though HIV negative (Borges-Almeida *et al.*, 2011). Interestingly,

studies have shown that despite adequate antiretroviral therapy, immune activation and inflammation persist (Hunt *et al.*, 2011) as determined by the levels of CD38 expression on CD8 T cells. This has been attributed to several factors such as ongoing leakage of microbial products, such as lipopolysaccharide (LPS) across the gut epithelium (Lee *et al.*, 2009), reactivation of CMV (Naeger *et al.*, 2010) and response to lymphopenia itself (Lee *et al.*, 2009). Therefore, these infants may similarly have been exposed to such pro-inflammatory stimuli, despite administration of maternal ART.

HEU infants display significant alterations compared to UE infants due to intrauterine exposure to HIV and ARV drugs. Some alterations include mitochondrial dysfunction, abnormal haematological features and abnormal maturation of T and B lymphocytes (Bunders *et al.*, 2005b).

During the period of foetal development, HEU infants are exposed to HIV particles and proteins. Evidence of exposure to HIV is illustrated by documented levels of immune activation, the presence of HIV-specific T cells, increased detection of HIV-specific CD4+ T cells and low levels of HIV-specific CD8+ T cells. In addition, treatment with ARV in HIV positive women is linked to the inhibition of bone marrow function (Rowland-Jones *et al.*, 1993, Clerici *et al.*, 2000).

It is well documented that HIV results in the inhibition of progenitor cell function and causes cell apoptosis which leads to immunological and haematological deficiencies (Clark *et al.*, 2000). In HIV positive women, an imbalance between Th1 and Th2 cytokine expression has been described, which results in cytokine imbalances in the infant, leading to abnormal immunological functions (Klein *et al.*, 1997).

A study by Nielsen *et al* showed that HEU infants have impaired progenitor cell function and therefore presented with low CD4 counts. Both numbers of naïve CD4+ T cells and T cell receptor circles (TREC) frequency were lower in infants of HIV positive mothers, strongly suggesting a reduced thymic output. These researchers hypothesised that impaired progenitor cell function in HEU may be due to exposure to HIV particles or HIV proteins (Nielsen *et al.*, 2001).

Several studies have addressed a number of immune abnormalities that persist in HEU infants during the neonatal period; however few studies have examined the effects of HIV exposure subsequent to this time. Clerici *et al.*, observed composite defects such as reduced CD4/CD8 ratios, decreased percentages of both CD4+ and CD8+ naïve T cells as well as increased activation on CD8+ T cells on HEU newborns and further concluded that such defects persisted over time in older exposed children (Clerici *et al.*, 2000).

In this study we evaluated the expression of markers of immune activation (CD69 and CD40L) and proliferation (CD127) on CD4+ T cells, as well as immune activation (CD38) on CD8+ T cells. Analysis of our data showed that HEU babies had increased expression levels for CD69, CD127 and CD40L on CD4+ T cells at both 18 and 24 months, although statistically significant differences were only reached for CD40L and CD127 at 18 months of age.

A study by Nielsen *et al* showed a correlation between lymphocyte proliferation and expression of CD69 in a group the HIV positive individuals (Nielsen *et al.*, 1998). These finding may relate to our observation seen in HEU infants where an increase in both CD69 and IL-7 receptor (involved in generation, activation and homeostasis of T cells), CD127 was observed. CD69 is classified as an early marker of immune activation, which is up regulated in response to antigenic stimulation. Some studies have indicated impairment in the expression of CD69 in patients who are in the advanced stages of HIV disease progression. These patients do not show increased CD69 expression upon stimulation with an antigen, such as Staphylococcus enterotoxin B (Pitsios *et al.*, 2008). The increase in CD69 observed in our HEU population at both 18 and 24 months of age may be indicative of chronic antigenic stimulation.

It is interesting to note that an increase in the expression of several markers seems to be higher at 24 compared to 18 months in both HEU and UE groups. This may indicate that both infant groups have increased antigenic exposure at 24 months in comparison to 18 months. The higher levels in HEU infants at 24 months may be related to excessive exposure. Expression levels in both infant groups at 24 months seem to be within similar ranges to adult control patients in the study by Nielsen *et al* (1998). This would reflect maturation in the development of their immune systems

Reviewing our data on the small group of HIV infected children not included in this study, we showed that at 18 months of age, the expression levels of CD69 were closer to those levels in the HEU group and further, that at 24 months of age, the activation marker was double that seen in both HEU and UE infants. This may have been due to the even greater antigenic exposure of their immunodeficiency (HIV infection)

CD40 ligand is mostly expressed on activated CD4+ T cells and plays a role in B-cell proliferation and immunoglobulin class switching. The interaction of CD40 with CD40L is also responsible for the activation of the immune response (Romano *et al.*, 2006). Our study showed that HEU infants display increased levels of CD40L (CD154) on CD4+ T cells. Although these differences were not significant, there seems to be a significant trend for the expression of this molecule in the HEU population on CD4 T cells at 18 months of age. Ramano *et al* similarly investigated the expression of the molecule by use of a CD45 PanLeucogating approach to study a group of HEU infants and were able to confirm an increase of the expression CD40L on both CD4 and CD8 lymphocytes. In addition, the initial hypothesis of their study was to prove that ARV exposure had a potential immunostimulatory effect on the infant's immune system. Although their study lacked a control population who were not subjected to ARV, they did not find a correlation of CD40L expression related to maternal viral load or CD4 count, gender, gestational age or feeding methods. Based on this they were able to indirectly relate the effect of ART exposure on the increased expression of CD40L (Romano *et al.*, 2006). However, it is more likely that the chronic pro-inflammatory environment despite ART, promoted the upregulated expression of CD40L and not the drugs themselves.

The positive effects of ARV drugs are not only to reduce viral replication, but also to promote antiviral immunity. Similar observations were made in both HIV and non-HIV cases where

anti-virals were administered (Sipsas *et al.*, 2002). In addition, treatment with nucleoside analogs was also found to increase the expression of CD40L (Bergamini *et al.*, 2002).

The binding of CD40L on activated T cells to the CD40 receptor on B cells leads to the increased expression of co-stimulatory molecules, such as CD80 on other antigen presenting cells (O'Gorman *et al.*, 2001). This may explain the increased expression of CD80 on B cells (described below) that significantly increased at 18 months of age in the HEU group of our study.

Altered expression of CD40 ligand can lead to dysfunctions in both the T and B cell compartments of the immune system. O'Gorman *et al.* showed that the levels of CD40L were aberrantly lower in a group of HIV infected children compared to uninfected HIV exposed children. It is hypothesised that this could be due to the loss of a sub-population of T helper cells which upregulate CD40L upon stimulation (O'Gorman *et al.*, 2001). Similarly in our study, we observed that the two HIV infected children showed lower expression of CD40L at 24 months of age.

As with the study by Romano *et al.*, the researchers showed that there was no relationship between the viral load and the expression of CD40L, therefore suggesting that children with the highest viral loads do not necessarily present with the most impaired levels of CD40L (O'Gorman *et al.*, 2001). Further studies are therefore required to describe the reasons behind the abnormal expression of CD40L and whether these levels can be restored with antiretroviral therapy.

Ramano *et al.*, furthermore suggests that strategies that support the expression of CD40L on expanding CD4+T cells may be helpful in complementing the immunity of the host against HIV (Romano *et al.*, 2006).

According to Clerici *et al.*, immune defects observed in HEU infants may not only be a consequence of ARV drug exposure. Similar abnormalities are also observed in older children born to HIV infected mothers who were not exposed to ARVs (Clerici *et al.*, 2000).

CD127 is an interleukin 7 (IL-7) receptor that plays an important role in thymopoiesis (thymic development), enhancement of CTL function, promotion of T cell survival as well maintenance of T cell homeostasis. IL-7 is also essential for T cell development, reconstitution and proliferation through the upregulation of Bcl-2 (Hassan and Reen, 1998, Young and Angel, 2011).

We observed increased levels of CD127 expression at both 18 and 24 months in the HEU group, although only significant at 18 months of age. To our knowledge there are currently no other studies that have observed the expression of CD127 in HEU infants. However, Clerici *et al.* measured the levels of serum IL-7 on ELISA and found increased levels in both newborns and older children born to HIV infected mothers. They conclude that the increased levels of serum IL-7 are consistent with the impairment of T-cell homeostasis previously documented in the same study (Clerici *et al.*, 2000).

Thymic function is altered during with HIV infection and characterised by dysregulation of the thymic epithelial network, reduced thymic output and ultimately an impaired naïve T cell

pool. The IL-7 or IL-7 receptor signalling pathway is critical for the maturation and differentiation of thymocytes. HIV infection is associated with decreased CD127 expression and impaired CD127 signalling (Paiardini *et al.*, 2005). The HIV infected babies in our study displayed much lower levels of CD127 at 24 months of age, which is consistent with the latter findings. However, a recent study by Young and Angel (2011) found that HIV infection alters the activity of IL-7 responses, however not through the down-regulation of CD127. We observed a significant increase in the expression of CD127 in the HEU group at 18 months of age, but without evaluating levels of IL-7. This would have generated data that is more comparable with larger studies.

For successful clearance of a pathogen, a fine balance between inflammation and control of excess immune activation is required. Immune regulatory molecules, such as CTLA-4 or CD152, are such regulatory elements of the immune system that control immune activation, thereby preventing immunologically mediated damage and exhaustion (Kaufmann and Walker, 2009). The expression of CTLA-4 in our cohort was similar in the HEU and UE groups. A recent study evaluated the expression of CTLA-4 along with other molecules for immune regulation in groups of HIV controllers and HIV progressors in an attempt to evaluate mechanisms of viral control. They found an increase in expression of CTLA-4 along with a decrease in CD40L (implying prior activation) on CD4+ T cells in the HIV progressor group compared to the controllers (Whittall *et al.*, 2011). Our findings show a trend (no significance) in the increased expression of CD40L and no upregulation of CTLA-4 in comparison to UE control group. This may indicate that due to controlled immune activation on CD4+ T cells, the expression of CTLA-4 was regulated additionally. Another study showed a greater increase in expression of CTLA-4 in an untreated HIV infected group rather than the HEU infants (Nqoko *et al.*, 2009). The HIV infected infants in our study showed similar levels of CTLA-4 expression to both HEU and UE groups; this may be due to adequate immune control by ARV treatment.

Evidence of exposure to HIV in HEU infants is illustrated by the presence of both HIV-specific T helper and cytotoxic T cells. It has been suggested that these types of observations may be linked to correlates of protection against HIV (Clerici *et al.*, 1993, Bernard *et al.*, 1999).

Clerici *et al* (2000) further showed an increase in the activation marker CD38 on CD8+ T cells in children (approximately 7 years of age) born to HIV positive mothers. This was interpreted as further evidence for the persistent immune abnormalities in HEU individuals. In contrast to the latter findings, we did not find differing levels for the expression of CD38 in our HEU group compared to the UE group. However at both the 18 and 24 months time points, the mean CD8+ T lymphocyte counts were also lower in the HEU group compared to the UE controls. However, the children in the study by Clerici *et al* were not on any form of ARV treatment, whereas our HEU infants received a minimum of NVP shortly after birth. Based on these observations we expect that children born to HIV positive mothers and who were treatment naïve may experience increased levels of immune activation than HEU children exposed to PMTCT drugs. Although the benefits of ARV far outweigh the side-effects, ARV exposure may have further disadvantages. Bunders *et al* (2005) has shown that exposure to ART in foetal and infant life is associated with long-standing effects, such as decreased levels of CD8 cell counts (Bunders *et al.*, 2005a). Similarly, HEU infants in our

study present with decreased CD8 cell counts compared to UE infants from 12 weeks of age to 24 months; however these differences were not statistically significant. Importantly, the study presented by Bunders *et al.*, was conducted in children of around 8 years of age. Further studies are required to evaluate whether this effects of ARV exposure may persist later in life to an extent where the differences between HEU and UE individuals would become significant.

The expression of CD38 on CD8+ T cells is a strong predictor of HIV disease progression in combination with viral load and CD4 T cell counts. A study evaluating the levels of CD38 in HIV positive children showed that children with undetectable viral loads had decreased levels of CD38 compared to those with increased levels associated virological failure (Resino *et al.*, 2004). Although the levels of CD38 expression were much higher in our HIV infected children compared to our HEU infants, the levels were different from those described by Resion *et al.* HIV positive infants in our study presented with undetectable viral loads (but reactive HIV antibodies) at 24 months of age, which may be indicative of adequately suppressed viral replication.

6.4.2 Expression of cellular markers of apoptosis on CD4+ and CD8+ T lymphocytes

Apoptosis is a process by which cells undergo DNA degradation and subsequent death. Cell death is an important component of physiological processes for maintenance of immune homeostasis. However, in the case of HIV infection, apoptosis results in the loss of both infected and uninfected CD4 T cells, the pathogenesis for this continues to be unclear. Since vertical transmission occurs at the time of development of the immune system of the exposed infant, , apoptosis could be responsible for the depletion of mature and immature T lymphocytes (Hosaka *et al.*, 2000).

HIV proteins, such as gp120, *nef* and *tat* have all been described to induce cell apoptosis. Many studies have documented increased levels of apoptosis in HIV infected paediatric populations (Debatin *et al.*, 1994, Finkel *et al.*, 1995), but limited studies have addressed apoptotic characteristics in the HEU population. Importantly, it has been documented that HIV proteins in combination with bound antibodies may result in CD4 anergy and induce apoptosis without direct HIV infection of CD4+ T cells (Banda *et al.*, 1992).

Scruggs and Naylor (2008) suggested that mitochondrial toxicity due to the administration of zidovudine may lead to the reduction of mitochondrial DNA, oxidative stress and the reduction in L-carnitine levels. These factors collectively or individually activate the apoptotic pathway (Scruggs and Dirks Naylor, 2008, Blanche *et al.*, 1999).

It is plausible that the drug toxicities observed in HIV infected patients occur also in HEU individuals exposed to ARVs. HEU patients may also persist with increased apoptotic levels and low mitochondrial DNA numbers after the conclusion of prophylactic medication.

We described the apoptotic characteristics by measuring the expression of death pathway markers on CD4+ (Fas, FasL and Annexin V) and CD8+ (FasL) T cells in both HEU and UE groups and found increased expression of these markers in the HEU group. This may be a result of HIV exposure, increased antigenic stimulation, ARV exposure or protective mechanisms to HIV infection. Our HEU group showed increased levels of expression for

death signalling markers, Fas, FasL and the marker of early apoptosis, Annexin V on CD4+ T cells and an increased expression of FasL on CD8+ T cells.

A study by Economides *et al* (1998) was the first to measure and compare the levels of apoptosis in CD4+ and CD8+ T cells of cord blood samples of HIV infected, in HEU individuals and UE newborns. They found that HEU newborns showed significantly increased apoptosis after one day of *in vitro* culture than those obtained from UE newborns. In comparison to both uninfected groups, the one HIV infected infant in the study, showed the highest levels of apoptosis in CD4 and CD8 lymphocytes.

Studies have suggested that apoptosis may be as a result of immune activation (Hosaka *et al.*, 2000). It has been documented that exposure to HIV increases immune activation. In the HIV population, a stronger correlation is observed between apoptosis and inflammation rather than severity of disease. In addition, studies of HIV infected adults and children show that apoptosis also occurs in HIV uninfected cells (Finkel *et al.*, 1995). Other studies have found increased apoptosis despite immune activation (Miyamoto *et al.*, 2010)

To support these observations, the increased levels of apoptotic markers may be linked to the increased levels of activation (CD40L and CD69) previously described in our population of the HEU group.

In keeping with the observations of Economides *et al* (1998) who found the highest level of apoptosis in HIV infected individuals, the HIV positive infants in our study also showed the highest levels of apoptotic markers (Fas and FasL) on CD4+ T cells. Although the method of evaluating apoptosis and sample types differed between Economides *et al* and our study, we show similar findings.

Hosaka *et al* (2000), showed a correlation with an increase in soluble Fas/FasL and a decline in CD4 counts. In addition, they found a positive correlation with the levels of sFas and increase viral load indicating an association with disease progression. The same study evaluated these parameters in HEU infants, however found no correlation between CD4 counts and levels of sFas/FasL.

Apoptosis has also been described in other forms of viral infection not related to HIV. This may be the reason for the inconsistent trend observed in our HEU group who showed decreased FasL on CD8 T cells at 18 months and significantly higher expression at 24 months. It is possible that the increased apoptotic markers seen in the HEU group may be due to increased exposure to a number of viral and other infections. In addition, infants in our HEU group had significantly higher social morbidity than their unexposed counterparts, which could potentially expose them to more environmental pathogens.

As previously mentioned, exposure to antiretroviral therapy may lead to increased cell death. All exposed infants in our study had exposure to ARV's either during foetal stages, shortly after birth or during both time periods. Although this study was not designed to address ARV therapy and its effects, it is clinically relevant to add to the context of HIV exposure and the association with immunological defects. In contrast to investigations by Scruggs and Naylor (2008) who suggested a potential link between increased apoptosis and ARV therapy,

another study (Miyamoto *et al.* 2010) have observed increased activation and apoptosis in HEU children and adolescents not exposed to ARV drugs during pregnancy or early infancy.

Apoptosis has also been described as an immunological mechanism that may assist in conferring protection against HIV infection. A study by Velilla *et al* (2005) suggested that this may be supported, considering that they observed higher spontaneous apoptosis in monocytes in adults exposed to HIV than the control group. They also measured the *in vitro* spontaneous expression of Annexin V in response to p24 antigens on monocytes and PBMCs in three groups consisting of HIV exposed, HIV positive and negative individuals. As with the babies in our study, their results indicated that when compared to UE individuals, HEU individuals had a higher susceptibility to spontaneous apoptosis in both monocytes and PBMCs. In addition, lower p24 levels were found in monocyte cell cultures. Since monocytes serve as key targets for HIV infection, exploring HIV resistance by means of apoptosis deserves further research bearing in mind that these findings may be applicable to only a limited number of HEU individuals (Velilla *et al.*, 2005).

At 18 months of age, we measured the levels of Annexin V *ex vivo* and at 16 and 24 hour intervals as a means of evaluating levels of spontaneous apoptosis. The expression of Annexin V was minimal at baseline (*ex vivo*): similar levels were found at 18 months of age in both our groups. However, after the 16 hour incubation we found a significant increase in the levels of Annexin V in HEU infants compared to UE controls. Based on the studies by Velilla *et al* (2005), this may indicate that the CD4 T cells of HEU babies are more susceptible to undergoing apoptosis (Velilla *et al.*, 2005). In contrast to these studies, we did not measure the expression of Annexin V in the presence of the HIV antigen. We illustrated susceptibility to apoptosis by incubating cells in culture medium over time. At 24 hours we saw a decreased level of Annexin V, indicating a saturation point of the apoptotic process and a subsequent uptake by phagocytes and increase in cell debris.

At 24 months of age we found an increase in Annexin V expression in both HEU and UE groups after 16 hour incubation periods. This observation can be linked to an error in the experimental process that did not occur at the 18 months time point. After laboratory processing, samples were batched and stored at 4°C until flow cytometric acquisition. We found that samples left for longer than two days at this temperature had increased levels of Annexin V expression than those that were left at a shorter period. We conclude that because we did not add a fixative to preserve cells, continuous expression of Annexin V had occurred. However, considering that our control group was also subjected to same error, we additionally found, in keeping with our hypothesis, that the HEU group had higher levels of Annexin V expression compared to the UE group.

6.4.3 Expression of surrogate markers of immune activation on CD19+ and CD20+ B lymphocytes

The *in utero* development of an infant of an HIV positive mother experiences an environment supplemented with viral constituents. In view of the fact that HIV uses CD4 T cells as its main targets, it may be expected that immune aberrations are mainly associated with T rather than B lymphocytes. Based on this hypothesis set out by Clerici *et al* (Clerici *et al.*, 2000), we aimed to explore potential differences in the B cell compartment that may exist in

HEU infants, given the fact that we had detected significant variation in vaccine specific antibodies compared to UE controls.

We observed a significant increase in activation markers CD80 and CD69 on CD20+ and CD19+ B cells at 18 and 24 months of age.

A number of B lymphocyte abnormalities have been described in HIV infection, some of which include polyclonal B cell activation and hypergammaglobulinemia. *In vitro* studies have also shown that viral antigens tend to induce differentiation of B cells and have inhibitory effects on B cell function through the destruction of CD4+ T cell help (De Milito, 2004). A study by Bunders *et al* (2010), showed persistent hypergammaglobulinemia in children born to HIV positive mothers.

We propose that T cell activation, potentially due to HIV exposure, as well as the chronic pro-inflammatory milieu, may influence the activation of B cells. In contrast, during HIV infection, a decreased expression of CD80 and CD86 has been described. It is believed that this down-regulation may thus be responsible for the defective communication between B lymphocytes and CD4+ T cells (Malaspina *et al.* 2003). We noted however an increase in CD40L expressed on CD4+ T cells; we presume that this type of activation may lead to co-stimulation of B cells in an appropriate communication between B and T cells in HEU babies. In addition, this may account for the increased antibody responses previously described.

Another study evaluated the role of T and B cell interactions and its potential relationship with increasing HIV viral replication. They showed that CD80 down regulated p24 and TNF α production and did not decrease T cell proliferation. In contrast, CD86 was shown to favour promote the replication of HIV-1 (Krzysiek *et al.*, 1998).

Other studies have observed changes in B lymphocytes in cord blood of HEU infants born to HIV positive mothers on HAART. It was further observed that these abnormalities persisted in HEU children up to 2 years of age that related to residual HIV-mediated immune abnormalities (Borges-Almeida *et al.*, 2011).

Our study has also observed an increase (although not significant) in the expression of CD10 and CD23 on CD20+ B cells. CD10 expression occurs mainly on B and T cell precursors and CD23 is expressed on mature B cells. Studies have shown that an increase in the levels of soluble markers of B cell activation, such as CD23, is associated with the occurrence of AIDS-associated B cell lymphomas (De Milito, 2004). Other studies have associated increased expression of CD10 on immature B cells with the development of autoimmune disorders that may be related to the ability of HIV proteins to enhance B cell proliferation (Malaspina *et al.*, 2006). A study of HIV positive infants in comparison to HEU and control infants showed that HIV positive infants had significantly lower levels of CD23 on CD19 B cells compared to HEU and control infants. Also, the levels of CD10 expression was both higher in the HIV positive and HEU infants in comparison to the controls. In contrast to our findings, this study showed no expression of early activation marker CD69 on B cells in any population, however it is worth noticing that their HEU infants were 10 months of age and their control group was over 2 years of age (Rodriguez *et al.*, 1996).

In comparison to UE children in our study, HEU children showed increased levels of expression of CD62L (function in leukocyte adhesion and homing) at 18 months of age, however decreased levels at 24 months of age. These differences were not significant. Rodriguez *et al* (1996), showed decreased expression of this marker in HIV positive children compared with exposed and controls who showed similar levels. However, CD62L was higher in cord blood of HIV positive infants and no differences were noted for HEU and UE cord blood samples (Rodriguez *et al.*, 1996). CD62L and CD23 are expressed upon antigen stimulation; therefore the increase in CD62L observed at 18 months may be as a result of vaccination. Both HEU and UE groups showed a greater increase in CD62L expression at 18 months in comparison to 24 months (Rodriguez *et al.*, 1996).

We found discrepant literature surrounding the various markers described above. Reports however state that, phenotypic characteristics measured by the expression of cell surface markers differ with age and immunological features (Rodriguez *et al.*, 1996).

6.4.4 Expression of markers of apoptosis on CD19+ and CD20+ B lymphocytes

Gp120 also binds to uninfected B cells and may cause depletion through a super-antigen effect (Weinhold *et al.*, 1989). Another study conducted in South Africa evaluated the severity of infections that exist within a group of 8 HEU infants. Researchers observed 14 episodes of primary infection and 4 nosocomial infections. Interestingly, low CD4+ T cells and B-cell depletion were noticed in some of these infants (Slogrove *et al.*, 2009).

At 18 and 24 months of age, we observed increased levels of expression of Fas on CD20+ B cells in HEU infants compared to UE controls, although these differences did not reach statistical significance. In addition, we also found that CD20+ B cells in the HEU group showed a statistical trend of increased susceptibility to undergoing spontaneous apoptosis through the expression of early apoptotic marker Annexin V.

Observations by Miyamoto *et al* (2010) similarly reported a higher percentage of B cell apoptosis in a group of HEU children compared to UE controls. In addition, when the HEU group was separated into groups who had and had not been subjected to ARV exposure during gestation, no significant difference was found, although there was a trend towards higher lymphocyte apoptosis with ARV exposure.

As mentioned earlier, Scruggs and Naylor (2008) pointed out the consequences of ARV drug exposure, such as zidovudine administration leading to mitochondrial toxicity and depletion. Collectively these factors contribute to the induction of apoptosis in the immune system.

The increased expression of Fas and FasL has been observed in B lymphocytes of HIV infected patients thus leading to increased B cell abnormalities (De Milito, 2004)

There are only a limited number of studies relating to B cell apoptosis in the HEU population. Further studies are required to assess the effects of ARV exposure on B cell apoptosis.

6.4.5 Evaluation of PD-1 expression on CD19+ B cells

PD-1 is classified as a transmembrane protein which forms part of the immunoglobulin superfamily and is related to CD28 and CTLA-4 regulatory proteins. The expression of PD-1

on lymphocytes is generally upregulated following antigen binding of the immunoglobulin or T cell receptor and its expression is thought to play a role in dampening or limiting lymphocyte activation. Thus the expression of PD-1 mainly occurs on activated T and B cells and also macrophages (Agata *et al.*, 1996).

In the presence of PD-1, B and T cell stimulation and subsequent proliferation are diminished thereby facilitating decreased responses to antigen stimulation (Nishimura *et al.*, 1998). PD-1 has also been associated with diminished T cell function during chronic viral infections (Barber *et al.*, 2006). In murine models, deficient expression of PD-1 has been associated with multiple autoimmune features (Freeman *et al.*, 2000).

The evaluation of PD-1 expression has mainly been described on T cells in the context of HIV infection (Kaufmann and Walker, 2009, Nakayama *et al.*, 2011, D'Souza *et al.*, 2007). A recent study evaluated the various factors that contribute to increased T cell activation and PD-1 expression in HIV infected children and showed that the increased expression of PD-1 as well as increased immune activation were related more to the generalised immune response to HIV infection rather than specifically to the viral load (Prendergast *et al.*, 2011). Another study showed an association of PD-1 expression in HIV infection with T cell exhaustion resulting in disease progression (Day *et al.*, 2006). Day *et al.* (2006) reported an upregulation of PD-1 on CD8+ T cells which correlated with HIV specific CD8+ T cell functioning and in addition, was associated with disease progression. In contrast to Prendergast *et al.* (2011), this study showed a positive correlation with the expression of PD-1 on CD4+ T cells and viral load (Day *et al.*, 2006).

We evaluated the expression of PD-1 on CD19+ B cells in both HEU and UE infant groups at 18 and 24 months of age. To our knowledge, there has been no study to date that has evaluated the expression of PD-1 on either T or B lymphocytes in HEU infants or children. We showed that the expression of this marker on CD19+ B cells was not statistically significant at any of the time points measured, however at 24 months of age, the levels in the HEU group were higher than those of the UE group. We speculated therefore that the expression of PD-1 is related to the increased expression of cellular activation marker CD69 on CD19+ B cells and may serve to control or regulate lymphocyte proliferation in response to antigenic stimuli. In addition, we found that the expression of PD-1 was decreased in HEU infants at 18 months of age which was likely to correspond to the levels of CD69 which were also decreased.

Although PD-1 has been shown to play a role in the regulation of B cell survival in the murine model (Nishimura *et al.*, 1998), the knowledge of its role during HIV infection is limited. A recent study conducted with *Rhesus macaques* aimed to investigate the function of the PD-1 pathway in B cell dysfunction during SIV infection using both *in vitro* and *in vivo* experimental approaches (Titanji *et al.*, 2010). Researchers of this study found that the highest expression of PD-1 occurred mainly on activated memory B cells when compared with the other B cell subsets measured. The increased expression of PD-1 on activated memory B cells may relate to their intrinsic nature of having the lowest threshold of activation and thus would require intense regulation. The fact that their study did not find a loss in activated memory B cells expressing PD-1, may relate to the role of these cells in preventing rapid disease progression (Titanji *et al.*, 2010). Titanji *et al.* further postulated that individuals with increased activated memory B cells may be at risk for rapid disease progression.

The findings of the latter study therefore prompted further studies to evaluate PD-1 expression on B cells as potential predictors of disease progression in human HIV infected populations. Furthermore, the study highlighted that the expression of PD-1 on activated memory B cells may represent the host response to preventing rapid disease progression. Although we did not find a significant difference between HEU and UE groups for the expression of PD-1 on CD19+ B cells, we postulated that higher levels may be related to a regulatory mechanism and may play a role in preventing autoimmunity.

6.4.6 Evaluation of cellular markers of B cell memory

CD27 is important in regulating interactions between B and T lymphocytes and is also critically involved in the process of B cell differentiation and antibody production (De Milto 2004). More importantly, CD27 is a well described marker of B cell memory, which is an element in adaptive immunity and the response to vaccination (Carsetti and Tozzi, 2009).

In adaptive immunity, the importance of serological protection emerges after the exposure to pathogens or immunisation. Impairment of the humoral immune response in terms of responding to vaccination is a common feature in the HIV infected population. Decreased responses to important vaccinations, such as pneumococcus, tetanus and Hib have been reported along with decreased memory pools (Hart *et al.*, 2007). In the HIV infected population, studies have suggested that memory B cell sub-populations may be a better predictor for the risk of being susceptible to diseases, such as pneumococcus (D'Orsogna *et al.*, 2007).

We found that the HEU group had similar expression of CD27 on both CD20 and CD19 B cells compared to UE controls. In support of our findings, Miyamoto *et al* (2010) also found the expression of CD27 to be similar in both control and HEU groups. This observation is in contrast to the patterns observed during HIV infection. The adequate expression of CD27 in the HEU group serves well in the process of responding to immunisation and may explain the vaccine-specific robust responses observed in this cohort. Further studies are required to address whether these levels of protection will persist in years to come following future encounters with specific pathogens.

Although memory B cells are generally characterised by the expression of cellular surface marker CD27, it has also been described as a cell marker linked to B cell activation and differentiation. In the context of HIV infection, such characteristics are prominent thus according to Moir and Fauci do not represent true memory. It is therefore recommended that cellular markers such as CD21 that differentiate between resting memory (CD21^{hi}) and activated or differentiated (CD21^{lo}) subpopulations B cells, should be included when evaluating B cell memory in the context of HIV. In addition, other features that are observed in HIV patients with high viral load is the fact that B cells undergo terminal differentiation which leads to the loss of expression of CD20 and are further characterised by decreased levels of CD19. Due to this, the levels between patients with high viral loads are similar to that of HIV negative patients. In addition, the number of B cells that express CD27 remain low during ARV treatment and it is further suggested that ARV treatment may prevent the loss of memory B cells (Moir and Fauci, 2008). Based on these observations and suggestions, the same principles could be followed when evaluating memory B cell expression in HEU infants and children. Furthermore, early ARV exposure in these infants

may have contributed to preserving the memory B cell compartment, thus leading to similar levels as the UE group. Perhaps it would also be worth evaluating memory B cell characteristics in early infancy where immune activation would be more prominent.

6.5 SUMMARY AND CONCLUSION: CELLULAR BIOMARKERS

We observed that HEU infants show variability in the expression of certain markers of activation, regulation and apoptosis compared to UE controls on both T and B cell populations at 18 and 24 months of age. We showed that HEU infants have increased expression of certain markers on both T and B cell compartments; however these observations proved to be greater in variance on the T cells. This may be indicative of long-term effects of either *in utero* exposure to HIV or possibly exposure to ARV drugs during foetal development and early infancy. It is possible that the upregulation of certain markers is due to frequent pathogen exposure, based on the differences of social morbidity in the HEU cohort, (which results in the up regulation upon repeated encounters with infectious agents), in addition to the effects of exposure to an HIV infected mother.

Although we do not have evidence for the expression of these markers in early infancy, we can speculate based on observations of other studies that the effects of HIV exposure seems to be continue through to childhood and these children can be distinguished immunologically from control individuals even after the period of vulnerability to infection. It is therefore not appropriate to classify HEU children with immunologically normal controls.

A number of studies have alluded to impairments of both CD4+ and CD8+ T lymphocyte subsets in early infancy that continue into adolescent years. These observations raise concerns that these children may be more susceptible to infection and autoimmune conditions.

We have also observed similar expression of surrogate markers of B cell memory in HEU infants in comparison to UE controls. However, further observations are required to determine if the same levels persist or decay over time.

These findings highlight the need for further studies and evaluation of the clinical implications related to these observations in the HEU population that seems to be increasing in number and age.

6.6 STUDY LIMITATIONS AND FUTURE RECOMMENDATIONS

A major limitation of this study was the relatively small sample size of our study population that may have influenced the statistical outcomes of the analyses. Despite this we were able to observe significant trends in some of the questions we raised. However, further larger longitudinal studies are required to confirm our findings.

We alluded to the presumed maternal antibody transfer in infants. It would have been of greater advantage to measure the levels of specific antibody in the maternal source in order to determine the levels of circulating antibodies. In future, it would also be of great value to measure the maternal total IgG levels as a marker of disease progression, which could be related to the degree of transfer of antibodies to the infant.

As this study formed part of a larger cohort study, the primary hypothesis was not to address the adaptive immune response of HEU infants, but rather to assess the innate immune response. This was thought to be the major contributing factor to the infectious morbidity in early life. As a result of this, we did not originally have a standardised method of collecting vaccination data and ensuring the receipt of immunisation in infants. Therefore, a large proportion of infants were excluded due to non-timely administration of vaccination. Not only does this prompt a greater intervention to ensure vaccination in larger studies, but also serves to highlight the lack of awareness surrounding immunisation as a preventative tool for contracting disease. It would appear that vaccination education would benefit less educated communities.

Owing to the delay in the implementation of the pneumococcal vaccination, a large number of infants in our study did not receive the prescribed dosages. The small proportion that received the vaccination did not complete the schedule. Thus, we were unable to document a response to the pneumococcal vaccination in relation to HIV exposure.

Based on the findings observed with HEU infant response to vaccination, future studies addressing the functionality of these antibodies will ensure better understanding of the correlates of protection for vaccine responses.

To our knowledge this is the only first longitudinal study that evaluates the influence of maternal HIV infection in the first 2 years of life.

A number of limitations with the evaluation of cellular immune biomarkers are also acknowledged. We only evaluated these markers at 18 and 24 months and can therefore not relate these findings with the expression in early infancy. As a result, we are unable to comment on whether the levels of immune activation decreased with age. There is also minimal literature on some of these markers, therefore limiting the comparable nature of our findings.

The flow-cytometric analyses for the evaluation of CD38 expression were determined using quadrant gates. This may have resulted in inadequate analyses, due to the “dribble-over” effect often noted with CD38 cell populations. For future studies, CD38 populations would best be represented or quantified using the mean fluorescence intensity (MFI) of which accuracy will be valid by using standardised quality controls such as Quantibrite™ beads.

Similarly to the accurate flow-cytometric analysis for CD38 quantification, CD127 populations are not best represented using quadrant gates. For future studies, it is recommended that CD127 be characterised as negative, low or high rather than just present or absent. CD127 is generally expressed on Tregs and thus, Tregs that express FOXP3 have been shown to lie in the CD127 low population subset. Therefore, the accurate representation of CD127 should be shown as a percentage of total CD4 populations.

Although this study merely aimed to evaluate and observe the levels of expression for various cellular markers of immune activation and apoptosis, the direct question of whether immune activation correlated with cell death was not addressed. For prospective studies it would be of interest to include a flow cytometric panel of both markers of activation and apoptosis (e.g. CD69 and Annexin V) after *in vitro* cell activation with a suitable stimulant.

The assessment of the immune regulatory properties of T and B cells were evaluated by measuring the cellular expression of specific cellular regulatory markers such as CTLA-4 and CD127 on CD4+T cells as well as PD-1 on CD19+B cells. Although these markers are not extensively evaluated in HEU populations, they are often described during HIV infection thus making them ideal for evaluating immune regulation during HIV exposure. Other markers of immune regulation such as FOXP3 would serve valuable markers for future research.

Lastly, our infant groups (HEU and UE) were not from similar social backgrounds or ethnic groups. These serve as confounding factors for the immunological differences found between HEU and UE infants. Due to HEU infants originating from a background of high social morbidity, the upregulation of immune markers of activation and apoptosis may be due to constant environmental antigenic exposure.

Furthermore, it has been documented that differences in haematologic parameters are related to ethnicity (Lisse *et al.*, 1997). Therefore, it would be of value to match cohorts in terms of ethnicity and social backgrounds in order to have comparable data. With that said, obtaining matching populations are extremely difficult in a setting where the burden of HIV resides in particular groups.

CHAPTER 7

CONCLUSION

This study aimed to evaluate characteristics of the adaptive immune system with the proposed hypothesis that HEU infants display different features to those of UE controls.

Our pilot study longitudinally evaluated and described specific antibody levels in infants born to HIV infected and uninfected mothers from 2 weeks to 2 years of age before and after routine vaccinations as outlined by the EPI-SA.

We concluded that infants born to HIV infected mothers received significantly less, and in addition, non-protective levels of maternally derived antibodies to specific pathogens. Although UE control infants had higher levels of maternally derived antibodies, we showed that a large proportion of these antibodies were also not considered protective.

Larger prospective studies are required to confirm these findings as potential contributing factors to increased infectious morbidity in HEU infants. Based on the findings of this pilot work we suggest that a targeted vaccine approach be considered in HIV infected women and exposed infants. This study also highlighted the need to address re-vaccination of adolescents and women of child bearing age to increase their own levels and also the transfer of maternal antibodies; however the potential effect of interference of maternal antibodies with the infant's vaccination response has to be evaluated carefully. Therefore, the timing and dosage need to be defined. Protective levels for a range of routine childhood vaccines, as well as correlates of protection within the South African setting of HIV exposure are required.

We showed that HEU babies mount robust responses to certain vaccine antigens. We concluded that this may be due to the lack of maternal competing antibodies or alternatively due to the influence of HIV on B cell activation (polyclonal activation), alternatively via indirect T cell activation. The functionality of these antibodies and the ability to clear infection needs to be investigated.

In addition, we highlighted that a significant proportion of infants did not receive their vaccination according to schedule. A number of vulnerable infants in both HEU and UE groups in our cohort did not receive PCV₇ according to maternal questionnaire despite a relatively high coverage documented. There is also a need to clearly define the levels of protection in combination with functionality. Furthermore, assays should be designed more effectively to address specific serotypes of PCVs, due to the fact that serotype prevalence differs amongst various population groups.

This pilot study also evaluated the expression of cellular markers of immune activation, regulation and apoptosis in both T and B cell compartments of the immune system at 18 and 24 months of age, after the vulnerable phase for infection which was documented in the first 6 months of life. We hypothesised that HIV exposure during foetal development adaptive immune responses that are different to those infants born to HIV uninfected mothers and persist after infancy.

We demonstrated that HEU babies displayed increased levels of immune activation and apoptosis mainly of the T cell compartment even at 2 years subsequent to *in utero* exposure. Immune activation of the B cells may represent stimulation by T cell interaction. Our evaluation is however limited to later time points of infant development and we were unable to compare the levels of immune activation prior to 18 and 24 months. We concluded that the increased immune activation and apoptosis in HEU infants may either be related to persistent activation that may be attributable to prior HIV exposure or due to more rapid upregulation in response to excess antigen stimulation of their environment.

Although these findings could be consistent with a differently primed immune system, larger studies are required to confirm these observations in relation to the clinical outcomes and to further assess whether these differences persist into later childhood. A confounding factor in this study was difference of social background between our two groups. We observed that HEU infants came from a more socially disadvantaged environment that may be contributing to chronic immune stimulation.

The implications and consequences of the immunological differences described in this study are unknown. Larger cohort studies are required to confirm whether HIV negative children of HIV positive mothers are at increased risk of serious infections and whether they suffer long lasting impairment of their immune systems. Early priming of their immune systems may in fact “equip” HEU infants to upregulate mechanisms of defence long term; however concerns are raised about the development of autoimmunity also. Further studies of HEU individuals will assist in defining the immunological changes related to exposure and also re-exposure to HIV and assist in structuring a more holistic and successful care of children infected with and affected by HIV

HEU infants are ideal candidates for evaluating appropriate models for effective clearance of the HIV virus. This study population not only provides insight into immune mechanisms of response to HIV exposure but it may serve as a potential study model that is classified as being “vaccinated” against HIV.

“There are data to support the notion that, among infants exposed to HIV in utero, at least one-third develop seemingly protective cellular immune responses bodes well for development of effective vaccines that could be used among infants.”

(Kuhn *et al.*, 2002)

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ADDENDUM

ADDENDUM A: Ethical approval for pilot study (March 2009)



UNIVERSITEIT • STELLENBOSCH • UNIVERSITY
jou kennisvennoot • your knowledge partner

13 March 2009 **MAILED**

Dr M Esser
Department of Pathology
Stellenbosch University
P O Box 19063
Tygerberg
7505

Dear Dr Esser

"Pilot Study of Innate Immune Abnormalities in HIV exposed Uninfected infants."

ETHICS REFERENCE NO: N08/10/289

RE : URGENT

At a meeting of the Committee for Human Research that was held on 10 November 2008 the above project was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 12 March 2009 for a period of one year from this date. This project is therefore now registered and you can proceed with the work. We do however urgently request that you submit the following outstanding documents:

- The signed MTA form (before 1 April 2009)
- Translated Xhosa Information Consent Form (when available)
- Please place a note on the Information Consent Form that the local anesthetic (a cream) should be applied 20-30 min before venepuncture - not "if necessary". (submit changed information consent form to this office before 1 April 2009)

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report (obtainable on the website of our Division: www.sun.ac.za/knowledgepartner/committees_CHR.htm) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit. Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239
The Committee for Human Research complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Kind regards

13 March 2009 11:51 Page 1 of 2



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Tel.: +27 21 938 9075 · Faks/Fax: +27 21 931 3352



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Approval Date: 12 March 2009

Expiry Date: 12 March 2010

041422

Yours faithfully

MRS MERTRUDE DAVIDS

RESEARCH DEVELOPMENT AND SUPPORT

Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za

Fax: 021 931 3352

Dear Dr Ester

"Pilot Study of Intra-uterine Administration of HIV exposed Uninfected Infants"

ETHICS REFERENCE NO: N0810729

RE: URGENT

At a meeting of the Committee for Human Research that was held on 10 November 2008 the above project was approved on condition that further information that was required, be submitted.

This information was supplied and the project was finally approved on 12 March 2009 for a period of one year from this date. This project is therefore now registered and you can proceed with the work. We do however urgently request that you submit the following outstanding documents:

- Translated Informed Consent Form (with a translation)
- Please place a note on the Information Consent Form that the local anesthesia (if given) should be applied 20-30 min before vasopuncture - not "if necessary" (under checked information consent form, to this office before 1 April 2009)

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report sustainable on the website of our Division: www.sun.ac.za/healthsciences/research/ethics (if a report) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary) annually - number of projects may be selected randomly and subjected to an interim audit. Translations of the consent document in the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IP00-00200
The Committee for Human Research complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee is also by the ethical codes and standards for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the research ethics principles as outlined in the National Health Act 2003 (Department of Health).

Kind regards

13 March 2009 11:51

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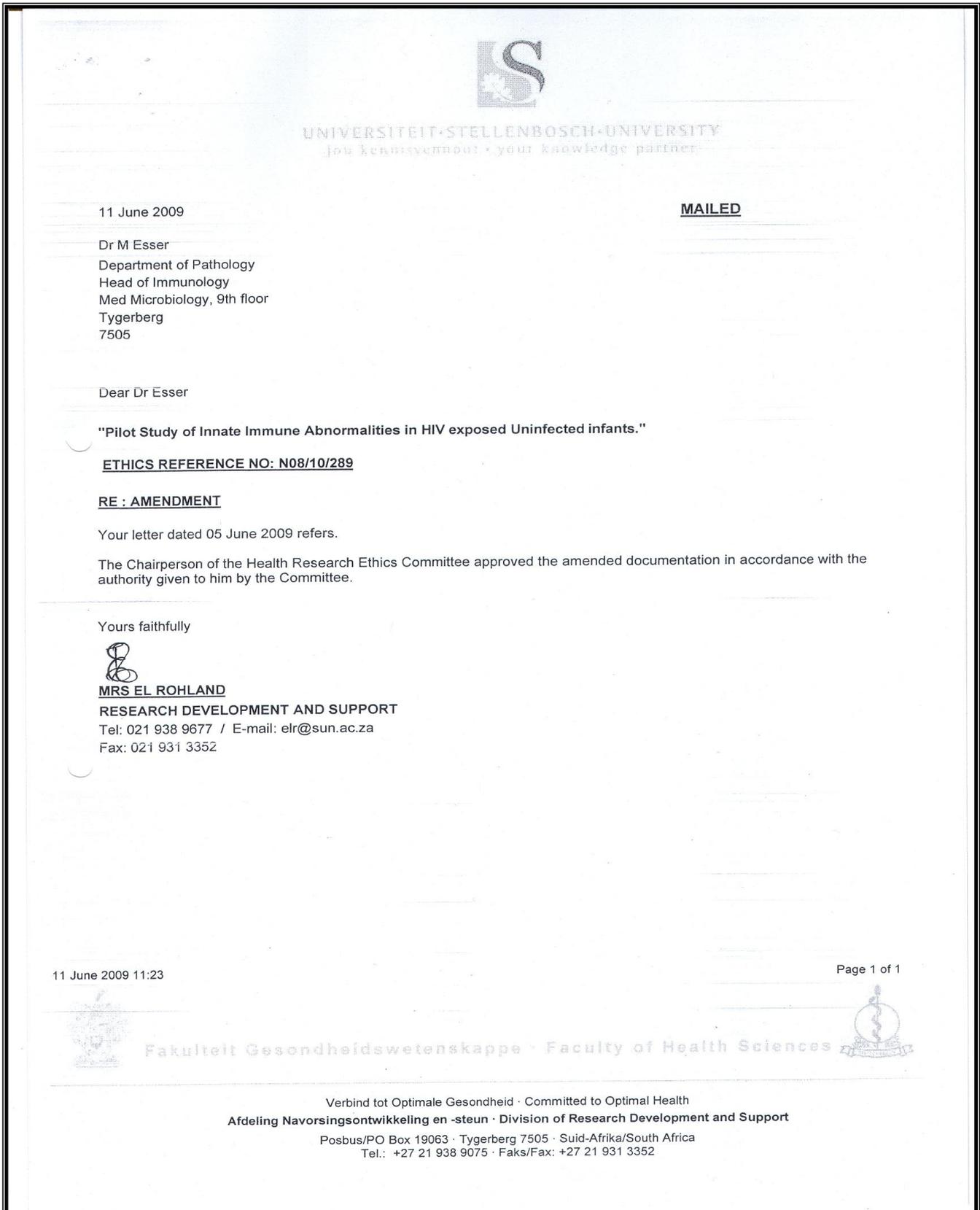


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ADDENDUM B: Ethical approval for extension of larger pilot study (June 2009)



ADDENDUM C: Ethical approval for antibody study (September 2009)



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02 September 2009

MAILED

Dr C de Beer
Department of Pathology
6th Floor, clinical building
Stellenbosch University
Tygerberg Campus
7505

Dear Dr de Beer

"Post vaccine levels of specific antibiotics in HIV exposed uninfected and healthy infants."

ETHICS REFERENCE NO: N08/09/262

RE : APPROVAL

At a meeting that was held on 10 November 2008 the Health Research Ethics Committee considered your application for the registration and approval of the abovementioned project. The Committee referred the project back to you awaiting further information that was required.

This information was supplied and the project was finally approved on 1 September 2009 for a period of one year from this date.

Notwithstanding this approval, the Committee can request that work on this project be halted temporarily in anticipation of more information that they might deem necessary to make their final decision.

Please quote the above-mentioned project number in ALL future correspondence.

Please note that a progress report (obtainable on the website of our Division) should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit. Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372

Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Approval Date: 1 September 2009

Expiry Date: 1 September 2010

02 September 2009 09:14

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Yours faithfully

MRS MERTRUDE DAVIDS

RESEARCH DEVELOPMENT AND SUPPORT

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02 September 2009 09:14

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ADDENDUM D: Ethical approval for pilot study extension (April 2010)



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09 April 2010

MAILED

Dr M Esser
Department of Pathology
Head of Immunology
Med Microbiology, 9th floor
Tygerberg
7505

Dear Dr Esser

"Pilot Study of Innate Immune Abnormalities in HIV exposed Uninfected Infants."

ETHICS REFERENCE NO: N08/10/289

RE : AMENDMENT 3

Thank you for your letter of 26 March, requesting ethical approval for amendment 3 and submitting revised documentation.

Amendment 3 was considered by the chairperson

On behalf of the Committee, I am pleased to confirm an approval for the amendment on the basis described in the documentation as revised.

Yours faithfully

MS CARLI SAGER

RESEARCH DEVELOPMENT AND SUPPORT

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09 April 2010 15:39

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ADDENDUM E: Ethical approval for evaluation of biomarkers at 18 months (September 2010)



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23 September 2010

MAILED

Dr M Esser
Department of Pathology
Head of Immunology
Med Microbiology, 9th floor
Tygerberg
7505

Dear Dr Esser

"Pilot Study of Innate Immune Abnormalities in HIV exposed Uninfected infants."

ETHICS REFERENCE NO: N08/10/289

RE : AMENDMENT

Your letter dated 14 September 2010 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

The following amendments were approved:

- Study extension to include additional neurodevelopmental assessments at 18-24 months.
- Expanded from cytometry and biomarkers at 18 months.

Yours faithfully

MRS MERTRUDE DAVIDS

RESEARCH DEVELOPMENT AND SUPPORT

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23 September 2010 09:17

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**ADDENDUM F: Ethical approval for evaluation of vaccination levels at 18 and 24 months
(September 2010)**



UNIVERSITEIT • STELLENBOSCH • UNIVERSITY
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30 September 2010 **MAILED**

Dr C de Beer
Department of Medical Virology
8th Floor, clinical building
Stellenbosch University
Tygerberg Campus
7505

Dear Dr de Beer

"Post vaccine levels of specific antibiotics in HIV exposed uninfected and healthy infants."

ETHICS REFERENCE NO: N08/09/262

RE : AMENDMENT

Your letter dated 22 September 2010 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee.

The following amendments were approved:
The extension of the follow-up time of this study by adding two additional assessments at 18 and 24 months.

Yours faithfully


MRS MERTRUDE DAVIDS
RESEARCH DEVELOPMENT AND SUPPORT
Tel: 021 938 9207 / E-mail: mertrude@sun.ac.za
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30 September 2010 10:07 Page 1 of 1



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