



Department of Science and Technology/

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**EFFECTS OF HUMAN IMMUNODEFICIENCY VIRUS INFECTION
AND TREATMENT WITH ANTIRETROVIRAL THERAPY ON
IMMUNOLOGICAL RESPONSES TO CHILDHOOD VACCINES**

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DECLARATION

I, Omphile Elizabeth Simani, declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy (Thesis) at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



Signature of candidate

Signed on the 21st day of February 2017

DEDICATION

I dedicate this thesis to my mother, my pillar of strength. For being my source of prayer towards my success and who has been my support system throughout this work.

To my son, Sisa (15) who I love dearly, for so generously given up his mummy and son time in order for me to finish this work.

To wake up jolly
To wake up ready to serve
To wake up with a prepared mind,
To optimize your contribution
To perfect your strategies
To absolute your path...as intended.

These are the things that preoccupy a grand mind.
These are the prayers of the faithful with ambition...

Divine Guide, I pray!

By Xolani Osborne Mnyandu

PUBLICATIONS AND PRESENTATIONS ARISING FROM THE THESIS

Publications:

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Submitted Publications:

Simani, O.E., Izu, A., Nunes, M.C., Violari, A., Cotton, M.F., Van Niekerk, N., Adrian, P.V., & Madhi, S.A. Antibody persistence and booster response to diphtheria-tetanus-pertussis and hepatitis B vaccination among HIV-uninfected and HIV-infected children initiated on early antiretroviral therapy. (Manuscript submission pending)

ABSTRACT

Introduction: HIV-infected and HIV-exposed-uninfected children have a heightened susceptibility to some vaccine preventable disease. There is a paucity of data on immunogenicity of vaccines in these children, including HIV-infected children who are initiated on early antiretroviral therapy (ART). We evaluated the effect of maternal HIV-exposure and timing of ART in HIV-infected children on antibody responses to combined diphtheria-toxoid (DT) -tetanus-toxoid (TT)-whole cell pertussis (wP) and *Haemophilus influenzae* type b conjugate vaccine (HibCV); monovalent hepatitis B vaccine (HepB) and live-attenuated measles vaccine (MV).

Methods: Samples obtained from children aged 6–12 weeks who had been enrolled into the CIPRA-SA study were analysed. Briefly, HIV-uninfected children born to HIV-uninfected (HIV-unexposed) and HIV-infected mothers (HEU). Additionally, we enrolled perinatally HIV-infected children with $CD4+ \geq 25\%$ randomized to deferred-ART (i.e. initiated when clinically or immunologically indicated per the then WHO recommended treatment criteria; ART-Def) or immediate-ART initiation (i.e. initiated on ART immediately upon confirmation of HIV-infection status at 4-10 weeks of age; ART-Immed). Children enrolled in the ART-Immed arm were further randomized to interrupt ART at one-year (ART/12m) or two-years of age (ART/24m). Additionally, a convenience sample of HIV-infected children with $CD4+ < 25\%$ initiated on immediate-ART was enrolled (ART- $CD4+ < 25\%$). Children received a primary series of DTwP-HibCV/HepB at 6, 10 and 14 weeks of age; and MV at 40 weeks of age. Booster dose of DTwP and MV was given at 15-18 months of age. Sampling time-points were: prior to the first dose of vaccine, four weeks after the third dose (18 weeks age), 24 weeks after the third dose (39.3 weeks of age), at the time of the booster dose (15- 18 months age), two to four weeks after the booster dose and at 24 months of age. Samples were analysed for antibodies for DT, TT, PT, FHA, HepB measured by Luminex microbead-immunoassay; and MV antibodies were quantified by an indirect enzyme immunoassay.

Results: Antibody kinetics and response to primary series of DTwP-HibCV/HepB:

Pre-vaccination GMCs were higher in HIV-unexposed than HEU children for TT, but lower for HepB, DT and FHA. Post-vaccination, sero-conversion, sero-protection and GMCs were similar in HEU and HIV-unexposed children for all vaccines. Furthermore, GMCs were higher in HIV-unexposed for TT, DT, HepB and FHA than in ART-Immed children; and for

TT, HepB and PT than in ART-Def children. Nevertheless, there was no difference in proportion of HIV-unexposed and HIV-infected children who developed sero-protective vaccine-specific antibody levels post-vaccination. The timing of ART initiation generally did not affect immune responses to vaccines between HIV-infected groups.

Antibody kinetics and booster responses to DTwP-HibCV/HepB vaccines:

Pre-booster GMCs were generally higher in HIV-unexposed than HIV-infected children for all vaccine epitopes. Post-booster and at 24 months of age the ART-Def group had lower GMCs (except to FHA), and were less likely to have sero-protective antibody levels compared to HIV-unexposed group. Also, post-booster and at 24 months of age, GMC were generally higher in HIV-unexposed than ART-Immed children, and a higher percentage of HIV-unexposed than ART-Immed children maintained antibody levels $\geq 1\text{IU/ml}$ to TT and DT at 24 months of age. The GMCs and percentage of children with sero-protective thresholds were similar pre-booster and at 24 months of age between HIV-unexposed and HEU children.

Antibody kinetics and response to measles virus vaccine:

At 7.3 weeks of age, the proportion with sero-protective titers was higher in HIV-unexposed (65.2%) compared to any HIV-infected group (range: 16.7% to 41.8%); but dropped to <17% in all Groups at age 19.6 weeks. Twenty-eight weeks following the first measles-vaccine, ART/12m were less likely to have sero-protective titers (79.3%) compared to HIV-unexposed (94.8%; $p<0.001$), ART-Def (95.7%; $p=0.003$) or ART/24m (92.1%; $p=0.02$). Although the proportion with sero-protective levels were similar between groups immediately post-booster dose, this was lower in HEU (79.6%; $p=0.002$) and ART/12m (80.3%; $p=0.01$) compared to HIV-unexposed (94.3%) 41-weeks later.

Conclusion: Primary vaccination with DTwP-HibCV/HBV of HIV-infected children initiated on early-ART confers similar immunity compared to HIV-unexposed children. HIV-infected children had poor anamnestic responses, if ART was not initiated prior to primary vaccination. In contrast, the memory response and persistence of antibody to most vaccine epitopes were similar between HIV-unexposed and HEU children. Increased waning of vaccine induced immunity over a 24 month period in ART-Def, ART/12m and HEU children following MV booster-dose; indicating the need for further booster doses after two-years of

age in these children. I recommend close monitoring of HEU children, as this group makes up most children born to HIV-infected mothers and what facets of the immune system have been impacted by maternal exposure to HIV.

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TABLE OF CONTENTS

EFFECTS OF HUMAN IMMUNODEFICIENCY VIRUS INFECTION AND TREATMENT WITH ANTIRETROVIRAL THERAPY ON IMMUNOLOGICAL RESPONSES TO CHILDHOOD VACCINES	i
DECLARATION	ii
DEDICATION	iii
PUBLICATIONS AND PRESENTATIONS ARISING FROM THE THESIS	iv
ABSTRACT	v
ACKNOWLEDGEMENTS	viii
LIST OF FIGURES.....	xv
LIST OF TABLES.....	xvi
LIST OF ABBREVIATIONS	xix
PREFACE	xxii
STUDENT CONTRIBUTIONS	xxiv
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	5
2.1 Overview of immune system	5
2.1.1 Innate immunity	5
2.1.2 Adaptive immunity.....	6
2.1.2.1 Cells involved in Adaptive immunity.....	7
2.1.2.2 T lymphocytes and cell-mediated immunity	8
i) Activation of T lymphocytes by recognition of MHC associated peptides.....	8
ii) T lymphocyte activation through co-stimulation	10
iii) Cytokines and their role in T lymphocyte activation	10
iv) Th1 and Th2 lymphocytes	11
2.1.2.3 Humoral immunity.....	11
i) An introduction to antibodies	12
ii) Antibody function.....	12
iii) B lymphocyte activation.....	15
iv) T-independent antigens.....	15
v) T-dependent antigens	15
vi) Immunopathology	16
2.2 THE HIV PANDEMIC	16
2.2.1 The status of HIV/AIDS pandemic.....	16
2.3 HIV PATHOGENESIS AND IMMUNOLOGICAL ABERRATIONS	17

2.3.1 Structure of HIV	17
2.3.2 Viral entry and life cycle	18
2.2.3 Immune response following HIV infection	19
2.3.4 Immunopathogenesis of HIV infection	20
2.4 ACCESS TO ANTIRETROVIRAL THERAPY	20
2.5 PREVENTION OF MOTHER-TO-CHILD TRANSMISSION (PMTCT) PROGRAMMES	22
2.6 HIV-EXPOSED UNINFECTED (HEU) CHILDREN	23
2.6.1 The increasing number of HEU children.....	23
2.7 PREVENTION OF INFECTION IN EARLY LIFE (MATERNAL ANTIBODIES)	25
2.7.1 Role of maternal antibodies	25
2.7.2 Mechanisms of maternal antibody transfer	26
2.7.3 How maternal antibodies influence the child's immune response	26
2.7.4 HIV and its effects on maternal antibody transfer	29
2.8 VACCINATION FOR THE PREVENTION OF DISEASE	30
2.8.1 The burden of vaccine preventable diseases.....	31
2.8.2 History of childhood immunization.....	32
2.8.3 South African EPI (EPI-SA)	33
2.8.4 Immunology of vaccines	34
i) Immunology of viral vaccines	35
ii) Immunology of bacterial vaccines	36
2.8.5 Early life vaccine antibody responses	37
2.8.6 Correlates of protection.....	38
2.9 SELECTED VACCINES OF THE EPI	39
2.9.1 Vaccination in HIV infected children	39
2.9.2 Non-replicating vaccines	39
2.9.2.1 Diphtheria toxoid vaccine	40
2.9.2.2 Tetanus toxoid vaccine.....	46
2.9.2.3 Pertussis vaccine	54
2.9.2.4 Hepatitis B vaccine	62
2.9.2.5 <i>Haemophilus influenzae</i> type b disease and Hib conjugated vaccine use in HIV infected children.	69
2.9.3 Live attenuated vaccines.....	71
2.9.3.1 Measles vaccine.....	71

2.10 ASSESSMENT OF ANTIBODY RESPONSE TO VACCINES	81
2.11. Chapter summary	82
CHAPTER 3: STUDY OBJECTIVES	85
CHAPTER 4: METHODS	87
4.1 Ethical clearance and approval	87
4.2 Study design and site	87
4.3 Description of study population.....	88
4.3.1 Inclusion criteria	90
4.3.2 Exclusion criteria.....	90
4.4 Antiretroviral therapy administered.....	90
4.5 Study vaccine regimens, administration and duration.....	91
4.6 Specimen collection	92
4.7 MEASUREMENT OF QUANTITATIVE AND FUNCTIONAL ANTIBODY RESPONSES	92
4.7.1 Quantification of antibodies to DT, TT, PT, FHA and HepB using commercially available assays as gold standard assays for comparison with the Luminex assay	93
4.7.1.1 Material.....	93
4.7.1.2 Measurement of antibody to DT by quantitative ELISA	93
4.7.1.3 Measurement of antibody to TT by quantitative ELISA	94
4.7.1.4 Measurement of antibody to PT and FHA by quantitative ELISA.....	94
4.7.1.5 Measurement of antibody to HepB by quantitative ELISA	95
4.7.1.6 Quantitative measurement of antibodies against DTwP and HepB vaccines using Luminex assay	95
i) Coupling of DT, TT, PT, FHA and HBsAg antigens to carboxylated microspheres	96
ii) Reference, controls and serum samples	96
iii) Multiplex immunoassay (MIA) for quantitation of DT, TT, PT, FHA and HepB specific antibodies.....	96
4.8 VALIDATION OF LUMINEX ASSAY	97
4.8.1 Calibration curve and its fitting algorithm	97
4.8.2 Comparison of Luminex assay with ELISA	97
4.8.3 Competitive inhibition studies of Luminex assay	98
4.8.4 Sensitivity of Luminex and ELISA serum IgG	98
4.8.5 Assay Reproducibility.....	98
4.9. MEASUREMENT OF <i>HAEMOPHILUS INFLUENZAE</i> TYPE B ANTI-PRP ANTIBODIES WITH AN IN-HOUSE ELISA	99

4.9.1 Principle of the Method	99
4.10 Measurement of serum bactericidal activity specific for <i>Haemophilus influenzae</i> type b.....	99
4.10.1 Principle of Assay.....	99
4.11 MEASUREMENT OF MEASLES VIRUS ANTIBODY TITRES.....	100
4.11.1 Principle of assay.....	100
4.12 Statistical analysis of antibody kinetics.....	100
CHAPTER 5 RESULTS: VALIDATION AND OPTIMIZATION OF LUMINEX ASSAY	102
5.1 Results	102
5.1.1 Calibration curve and its fitting algorithm.....	102
5.2 Comparison of Luminex assay with ELISA	105
5.3 Competitive inhibition studies of Luminex assay	109
5.4 Sensitivity of Luminex and ELISA serum IgG	109
5.5 Linearity and range of assay	110
5.6 Multiplex assay reproducibility	113
5.7 Concluding comments	113
CHAPTER 6 RESULTS: EFFECT OF HIV-1 EXPOSURE AND ANTIRETROVIRAL TREATMENT STRATEGIES IN HIV-INFECTED CHILDREN ON IMMUNOGENICITY OF VACCINES DURING INFANCY	115
6.1 Results	115
6.1.1 Study population and demographic information	115
6.1.2 Antibody responses in HIV-unexposed compared to HIV-infected children	121
6.1.3 Antibody responses in HIV-unexposed compared to HEU children	128
6.1.4 Antibody responses in HEU compared to HIV-infected children.....	128
6.1.5 Antibody responses in antiretroviral treatment-Immed compared to antiretroviral treatment-Def. children	129
6.2 Correlation of pre-vaccination response with post-vaccination response in study participants.....	130
6.3 Factors associated with response to vaccination at following primary vaccination for all study children.....	131
6.4 Factors associated with immune response to three dose schedules of DTwP and HepB vaccines stratified by maternal HIV status.....	133

6.5 Factors associated with immune response to three dose schedules of DTwP and HepB vaccines, as well as after booster dose of DTwP in children born to HIV-infected woman	135
6.6 Factors associated with immune response to three dose schedules of DTwP and HepB vaccines, in HIV-infected children stratified by ART status	137
6.7 Sensitivity Analysis	139
6.8 Concluding comments	139
CHAPTER 7 RESULTS: ANTIBODY PERSISTENCE AND BOOSTER RESPONSE TO DIPHTHERIA-TETANUS-PERTUSSIS AND HEPATITIS B VACCINATION AMONG HIV-UNINFECTED AND HIV-INFECTED CHILDREN INITIATED ON EARLY ANTIRETROVIRAL THERAPY.....	
7.1 Results	141
7.1.1 Study population and demographic information	141
7.1.2 Pre- and post-booster antibody kinetics to tetanus-toxoid	146
7.1.3 Pre- and post-booster antibody kinetics to diphtheria-toxoid.....	151
7.1.4 Pre- and post-booster antibody kinetics to pertussis antigens (PT and FHA)	155
7.1.5 Persistence and antibody kinetics to hepatitis B.....	159
7.2 Correlation of post-primary vaccination response with post-booster response in study participants.....	161
7.3 Factors associated with response to booster vaccine in all children irrespective of HIV status.....	161
7.4 Factors associated with immune response to booster dose of DTwP in children born to HIV-infected woman	162
7.5 Factors associated with immune response to booster dose of DTwP in HIV-infected children stratified by ART status	163
7.6 Concluding comments	165
CHAPTER 8 RESULTS: EFFECT OF IN-UTERO HIV EXPOSURE AND ANTIRETROVIRAL TREATMENT STRATEGIES ON MEASLES SUSCEPTIBILITY AND IMMUNOGENICITY OF MEASLES-VACCINE ...	
8.1 Results	166
8.1.1 Study population and demographic information	166
8.1.2 Pre-vaccination measles antibody kinetics.....	172
8.1.3 Kinetics of measles antibody post-primary and booster doses of measles vaccine	174
8.2 Correlation of post-primary vaccination response with post-booster response in study participants.....	177

8.3 Factors associated with response to the two doses of measles virus vaccine administered in the study	178
8.4 Factors associated with immune response to live attenuated measles virus vaccine in children stratified by maternal HIV status	178
8.5 Factors associated with immune response to first dose of live attenuated measles virus vaccine as well as after booster dose in children born to HIV-infected woman	180
8.6 Factors associated with immune response to the first dose of measles vaccine in HIV-infected children stratified by ART status.....	181
8.7 Concluding comments	182
CHAPTER 9: DISCUSSION.....	184
9.1 Validation and optimization of Luminex assay.....	184
9.2 Effects of HIV and treatment of HIV on response to childhood vaccines .	185
9.2.1 Immune response to DTwP and HepB vaccines in HEU children	186
9.2.2 Immune response to DTwP and HepB vaccines in HIV-infected children on ART or not on ART.....	188
9.2.3 Immune response to measles virus vaccine in HIV-infected and HEU children	191
9.3 Limitations of the study.....	194
9.4 Concluding comments	195
CHAPTER 10: REFERENCES	197
CHAPTER 11: APPENDICES.....	222

LIST OF FIGURES

Figure 2.1: T- and B-lymphocyte activation and function: Adaptive immunity (Warrington et al., 2011). APC: antigen presenting cell; MHC: major histocompatibility complex; TCR; T-lymphocyte receptor.	9
Figure 2.2: The dual effects of maternal antibodies on response to immunization by children. The presence of maternal antibodies inhibits B-lymphocyte responses to masked epitope, but not to the generation of memory B-lymphocytes. Secondly, maternal antibodies also play a role in increasing T-lymphocyte responses by enhancing antigen capture and presentation (Lambert et al., 2005).	28
Figure 4.1: Flow diagram of all recruited study participants. N=total number recruited, however, there may have been missing samples at later visits.	89
Figure 5.1: Fitting algorithm of standard curves for observed versus expected concentration in a seven step 4-fold dilution of the reference sera.	103
Figure 5.2: Comparison of the IgG concentrations against DT, TT, PT, FHA and HepB determined by Luminex assay and monoplex ELISA. Sera from 25 patients were quantitated by both Luminex and ELISA methods. Antibody concentrations for all five protein antigens were reported in either IU/ml (DT, TT, PT and FHA) or mIU/ml (HepB).	106
Figure 5.3: Difference between Luminex assay and ELISA using the Bland-Altman plot. The closer the difference is to zero the more alike the methods are; DT: diphtheria-toxoid; TT: tetanus-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; HepB: hepatitis B.....	108
Figure 5.4: Specificity assay: Percentage inhibition on addition to homologous antigen inhibitor. DT=93%, TT= 99%, PT=91%, FHA=85% and HepB= 91% for homologous inhibition per antigen.....	109
Figure 5.5: MFI generated from the pentaplex assay with in-house standard reference sera titration.	110
Figure 5.6: Comparison of MFI generated from monoplex and pentaplex assays for (A) DT, (B) TT, (C) PT, (D) FHA and (E) HepB.	112
Figure 6.1: Stratification of study population and reasons for children being excluded from analysis.	119
Figure 7.1: Reasons for exclusion from analyses at the different study time-points.	144
Figure 8.1: Subject follow-up during the duration of the study.....	169

LIST OF TABLES

Table 2.1: Properties of adaptive immunity (Benjamin, 1996b).	6
Table 2.2: Main functions of human immunoglobulin antibodies (Warrington et al., 2011, Panda and Ding, 2015).	13
Table 2.3: Worldwide reported incidence, estimated worldwide incidence and prevalence, and deaths of diphtheria, tetanus, pertussis, Hepatitis B, <i>H. influenzae</i> type b disease and measles.....	31
Table 2.4: Vaccination schedule for South Africa for the years 2005 and 2013	33
Table 2.5: Comparison of antibody response to diphtheria-toxoid epitope between HIV-infected, HEU and/or HIV-unexposed children.....	42
Table 2.6: Comparison of vaccination response to tetanus-toxoid epitope between HIV-infected children and HEU and/or HIV-unexposed children	48
Table 2.7: Comparison of vaccination response to pertussis epitopes between HIV-infected children and HEU and/or HIV-unexposed children ...	58
Table 2.8: Comparison of vaccination response to antibody to HepB epitopes between HIV-infected children and HEU and/or HIV-unexposed children	65
Table 2.9: Immunogenicity of measles virus vaccine in children with HIV prior to use of ART in HIV-infected children.....	73
Table 2.10: Studies reporting immune response to measles virus vaccination in children born to HIV-infected mothers	76
Table 4.1: EPI vaccine regimen	91
Table 4.2: Specimen collection schedule	92
Table 5.1: Data on percentage recovery and relative error for each standard point	104
Table 5.2: Frequency table of agreement between the Luminex assay and ELISA (antibody concentration classified as above or below sero-protective titers for DT (≥ 0.1 IU/ml), TT (≥ 0.1 IU/ml) and HepB (≥ 10 mIU/ml). While PT was classified by antibody concentration ≥ 20 IU/ml and FHA was classified by antibody concentration ≥ 2 IU/ml).	107
Table 5.3: Lower limit of quantitation of antigen specific IgG antibodies against DT, TT, PT, FHA and HepB antigens determined by ELISA and Multiplex methods, and the –fold difference in sensitivity of Multiplex over ELISA.	110
Table 5.4: Intra- and inter assay variation.....	113
Table 6.1: Demographic and baseline features of participants included in the analysis evaluating the immunogenicity of a three doses primary series of diphtheria toxoid-tetanus toxoid- whole cell pertussis and <i>Haemophilus influenzae</i> type-b conjugate vaccine and hepatitis B vaccine.	117
Table 6.2: Antibody concentrations and proportion of children with sero-protective antibody levels prior to first dose of diphtheria toxoid- tetanus	

toxoid- whole cell pertussis- <i>Haemophilus influenzae</i> type b conjugate vaccine (DTPw-HibCV) and Hepatitis-B vaccines.....	122
Table 6.3: Antibody concentrations and proportion of children with sero-protective antibody levels following primary series of three doses of diphtheria toxoid- tetanus toxoid- whole cell pertussis- <i>Haemophilus influenzae</i> type b conjugate vaccine and hepatitis B vaccines.....	124
Table 6.4: Antibody concentrations and proportion of children with sero-protective antibody levels six months following primary series of three doses of diphtheria toxoid- tetanus toxoid- whole cell pertussis- <i>Haemophilus influenzae</i> type b conjugate vaccine and hepatitis B vaccines (9-months of age).	126
Table 6.5: Correlation of pre-vaccination (Time-point 1) and post-vaccination (Time-point 2) antibody levels.	130
Table 6.6: Correlation of CD4+% with response to primary vaccination (Time-point 2).....	131
Table 6.7: Independent variables and their association to response to primary vaccination at Time-point 2 for DTwP and HepB vaccines	132
Table 6.8: Independent variables and their association to response to primary vaccination at Time-point 2 for DTwP and HepB vaccines by maternal exposure to HIV in their children.....	134
Table 6.9: Independent variables and their association to response to primary vaccination at Time-point 2 for DTwP and HepB vaccines in HEU and HIV-infected children.....	136
Table 6.10: Independent variables and their association to response to primary vaccination at Time-point 2 for DTwP and HepB vaccines in ART-Def and ART-Immed children	138
Table 7.1: Demographic and baseline information of participants who had received three doses of diphtheria toxoid-tetanus toxoid-whole cell pertussis- <i>Haemophilus influenzae</i> type b conjugate vaccine and hepatitis B vaccines and where available for follow-up.....	143
Table 7.2: Percentage of children with sero-protective antibody levels and geometric mean concentrations to Tetanus-toxoid at pre-booster, post-booster and at 24 months of age.....	148
Table 7.3: Independent variables and their association with response to booster vaccination for DTwP-HibCV and DTwP vaccines by HIV status	150
Table 7.4: Percentage of children with sero-protective antibody levels and geometric mean concentration to diphtheria-toxoid at pre-booster, post-booster and at 24 months of age.....	153
Table 7.5: Geometric mean concentration at pre-booster, post-booster and at 24-months of age for pertussis antigens and percentage of children who achieved a 4-fold increase in antibody levels.....	157
Table 7.6: Percentage of children with sero-protective antibody levels and geometric mean concentration to hepatitis B at 15-18 months and 24 months of age	160

Table 7.7: Correlation of primary vaccination and post-booster antibody levels in study participants.....	161
Table 7.8: Independent variables and their association to response to booster vaccination at Time-point 5 for DTwP	162
Table 7.9: Independent variables and their association to response to booster vaccination at Time-point 5 for DTwP vaccines in HEU and HIV-infected children	162
Table 7.10: Independent variables and their association to response to booster vaccination at Time-point 5 for DTwP vaccines in ART-Def and ART-Immed children.....	164
Table 8.1: Demographic information for study groups at separate study time-points	170
Table 8.2: Geometric mean titers and proportion of children with sero-positive and sero-protective levels of measles antibody prior to measles vaccination	173
Table 8.3: Measles-antibody titers following primary and booster-vaccine stratified by HIV-exposure and HIV-infection status	175
Table 8.4: correlation of pre- and post vaccination antibody titers for measles vaccine	177
Table 8.5: Correlation of CD4+ percent with response to primary vaccination (Time-point 4)	177
Table 8.6: Independent variables and their association to response to primary vaccination at Time-point 4 (post-primary) and Time-point 5 (post-booster) for measles virus vaccine.	178
Table 8.7: Independent variables and their association to response to the first dose of measles virus vaccine by maternal exposure to HIV in their children	179
Table 8.8: Independent variables and their association to response to booster vaccination at Time-point 5 for measles virus vaccines by maternal exposure to HIV in children	179
Table 8.9: Independent variables and their association to response to primary vaccination at Time-point 4 for measles virus vaccine in HEU and HIV-infected children	180
Table 8.10: Independent variables and their association to response to booster vaccination at Time-point 5 for measles virus vaccine in HEU and HIV-infected children	181
Table 8.11: Independent variables and their association to response to the first dose of measles virus vaccine in ART-Def and ART-Immed children.....	181
Table 8.12: Independent variables and their association to response to booster vaccination at Time-point 5 for measles virus vaccine in ART-Def and ART-Immed children	182

LIST OF ABBREVIATIONS

°C:	Degrees Celsius
aa:	Amino acid
AIDS:	Acquired immunodeficiency syndrome
aP:	Acellular pertussis vaccine
APC:	Antigen presenting cell
ART:	Antiretroviral therapy
ART-Def:	HIV-exposed, infected children on delayed ART
ART-Immed:	HIV-exposed, infected children on early ART
ART/12m:	HIV-exposed, infected with interrupted treatment at 12 months of age
ART/24m:	HIV-exposed, infected continued treatment till 24 months of age
ARV:	Antiretroviral
BCG:	Bacillus Calmette-Guérin
CCR5:	CC-chemokine co-receptor 5
CD4+<25%:	HIV-exposed, infected children on early ART with a CD4+ <25%
CDC:	Centers for Disease Control and Prevention
CHER:	Children with HIV Early Antiretroviral study
CI:	Confidence interval
CIPRA:	Comprehensive International Programme of Research on AIDS
cfu:	Colony forming units
CTLs:	Cytotoxic T-lymphocytes
CV:	Coefficient of variation
CXCR4:	CX-chemokine receptor 4
DC:	Dendritic cell
DNA:	Deoxyribonucleic acid
Ds:	Double stranded
DTP:	Diphtheria-Tetanus-Pertussis
DT:	Diphtheria toxoid
DTwP:	Diphtheria-tetanus-whole cell pertussis
EDC:	1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl
EIA:	Enzyme immuno assay
ELISA:	Enzyme-linked immunosorbant assay
EPI:	Expanded Programme on Immunization
EPI-SA:	Expanded Programme on Immunization South Africa

FBS: Foetal bovine serum

FDA: Food and Drug Administration

FDC: Follicular dendritic cell

FHA: Filamentous heamagglutinin

FI: Fluorescence intensity

GMC: Geometric mean concentration

GMT: Geometric mean titer

gp: Glycoprotein

HAART: Highly Active Antiretroviral Therapy

HBeAg: Hepatitis B endogenous antigen

HBsAg: Hepatitis B surface antigen

HBV: Hepatitis B virus

HepB: Hepatitis B vaccine

HEU: HIV-exposed, uninfected children

Hib: *Haemophilus influenzae* type b

HibCV: *Haemophilus influenzae* type b conjugate vaccine

HIV: Human immunodeficiency virus

HIV-unexposed: HIV-unexposed, uninfected children

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgM: Immunoglobulin M

IQR: Interqaurtal range

IU: International Units

LLOQ: Lower limit of quantification

LOQ: Lower limit of quantification

LTFU: Loss-to-follow-up

MFI: Mean fluorescent intensity

MHC: Major Histocompatibility Complex

mIU: milli International unit

ml: Millilitre

MTCT: Mother-to-child transmission

MV: Measles vaccine

NA: Not applicable

ND: Not done

ng: nanogram

NS: Not significant

OD: Optical density

OPV: Oral Polio vaccine

PBS: Phosphate buffer saline

PMTCT: Prevention of mother-to-child-transmission

PCR: Polymerase chain reaction

PCV: Pneumococcal conjugate vaccine

PncCV: Pneumococcal polysaccharide-protein conjugate vaccine

PT: Pertussis toxin

r: Spearman's correlation coefficient

RE: Relative error

RMPRU: Respiratory and Meningeal Pathogens Research Unit

RNA: Ribonucleic acid

R-PE: R-phycoerythryn

S. pneumoniae: *Streptococcus pneumoniae*

SD: Standard deviation

Sulpho-NHS: N-hydroxy-sulphosuccinimide

TCR: T-cell receptor

TD: T-lymphocyte dependent

Th: T helper

TI: T-lymphocyte independent

TT: Tetanus-toxoid

ULOQ: Upper limit of quantification

UNFPA: United Nations Population Fund

UNICEF: United Nations Children's Fund

WHO: World Health Organization

wP: Whole-cell Pertussis vaccine

PREFACE

The format presented for this thesis is the “Divided block” as recommended by the University of the Witwatersrand. This thesis is divided into two parts: the first part is a background literature review and objectives, and study method description. The second part consists of results and discussion of the study objectives that are written as separate chapters.

The thesis deals with the effects of human immunodeficiency virus exposure and timing of antiretroviral therapy initiation among HIV-infected infants on their immunological responses to childhood vaccines. Furthermore we aimed to identify factors that affect immune response to childhood vaccine in HIV-infected children.

Chapter 1: The introductory chapter provides a synopsis of the burden of HIV-infection and how it has thus far affected immunization and the burden of vaccine preventable diseases globally and in sub-Saharan Africa. I also review studies that have been conducted and highlight the paucity of data surrounding the objectives of my research.

Chapter 2: The literature review chapter provides an overview of the immune system and the dysfunctions that might result from HIV-infection. Furthermore I discuss ART treatment strategies of HIV in young children. I also discuss the public health of HIV-exposed uninfected children. In addition, the burden of vaccine preventable diseases in children under the age of five years, in particular diphtheria, tetanus, pertussis, measles, hepatitis B and *Haemophilus influenzae* type b are summarised. Also, I briefly describe the effects which the high HIV prevalence in sub-Saharan Africa has had on short and long-term immunogenicity of these vaccines. Furthermore, the detection methods (ELISA) that were historically used to assess immune response to vaccines and well as their shortcomings are discussed. The concluding statement of this chapter is the hypothesis and study rationale.

Chapter 3: This chapter lists the study aims and details the primary and secondary objectives of the study.

Chapter 4: This chapter details the methods of the research. To address the study objectives, the study was divided into two phases: Phase 1: I developed, validated and optimized the Luminex microbead immunoassay by using samples from a previous cohort (Madhi et al., 2009), to determine which reference standard to use, the limits of detection, controls, required sample volume, antigen coating concentration, incubation times, sensitivity and specificity.

Chapters 5 to 8 present the results and brief discussion of my work. In chapter 5, the validation and optimization of Luminex microbead immunoassay is presented and answers objective (i). The results are a comparison of the conventional ELISA vs. the Luminex microbead immunoassay. Also, I show the superiority of the microbead immunoassay over the conventional ELISA. In Chapter 6, I present results of response to primary vaccine (Objective ii) and the short term persistence of antibodies to DTwP/HepB vaccines in HEU and HIV-infected either on immediate ART or not. I also show factors that may be responsible for response to primary vaccination in children born to HIV-infected mothers. In Chapter 7, the effects of HIV-infected and ART treatment strategies on booster responses and persistence of antibodies to DTwP/HepB are presented (Objective iii). Furthermore, factors that may be associated with response to booster vaccination are analysed by mixed linear regression model. In chapter 8, the effects of HIV-exposure and ART treatment on immune response to measles vaccine are presented (Objective iv). In this chapter I evaluate immune responses to primary and booster vaccination, as well as factors associated with response to measles vaccine.

Chapter 9: This chapter provides a summary of my research findings, a description of the study-limitations, and the implications of my research findings in formulating health policy.

The thesis concludes with a summary of the findings and a conclusion.

STUDENT CONTRIBUTIONS

Protocol and ethics: The draft of the protocol were written and prepared by Omphile Elizabeth Simani (OES). OES was also responsible for the submission and obtaining ethical approval for all components of the study.

Recruitment and follow-up: The first phase of the study which is the development, validation and optimization of the Luminex microbead immunoassay, participants were previously enrolled in a study on "Durability of antibody response and measurement of anamnestic responses to monovalent Pneumococcal Conjugate Vaccine among HIV-infected and uninfected children" (HREC reference number: 031013) (Madhi et al., 2009).

For the second phase of the study which was the assessment of immune response to select vaccines (DTwP-HibCV/HepB and measles vaccines) in HIV-infected, HIV-exposed uninfected and HIV-unexposed uninfected children, participants were recruited by staff of RMPRU and PHRU. HIV-infected children were recruited within the "Children with HIV Early Antiretroviral (CHER)" study (HREC reference number: 040703) Under the Trial Title: A randomized trial to evaluate strategies for providing antiretroviral therapy to infants shortly after primary infection in a resource poor setting. The rest of the children were recruited in parallel within the CIPRA 4 cohort (HREC reference number: 040704) under Trial Title: Evaluation of quantitative and qualitative antibody responses to *Streptococcus pneumoniae* and *Haemophilus influenzae* type B conjugate vaccines among HIV-1-exposed-infected children that are receiving vs. those not receiving antiretroviral therapy, as well as among HIV-1-exposed-uninfected children and HIV-1-unexposed-uninfected children.

OES was responsible for sourcing out of these archived samples from long-term storage at -70°C freezer. OES was also responsible for verification of the sample number that was on the storage tube with the children's file and that information captured in the file was correct. As well as ensuring that there was sufficient sample to perform assays.

Laboratory component: Assay development, validation and optimization of the Luminex microbead immunoassay was carried out by OES. The antibody assays were conducted by OES and overseen by my co-supervisor (Dr Peter Adrian).

Commercial ELISAs for TT, DT, PT and FHA were validated by OES. Antibody concentrations were determined by OES.

Commercial EIAs for measles virus were validated by OES. Antibody concentrations were determined by OES.

Haemophilus influenzae type B antibodies and bactericidal activity were conducted by laboratory technicians and overseen by Dr Peter Adrian and Ms Nadia van Niekerk (Analysed separately to this thesis).

Statistical analysis: All statistical analysis was conducted by OES (100%), the analyses was overseen by the RMPRU statistician, Dr Alane Izu.

Compilation and completion of thesis and publications arising from this research: OES was responsible for 100% of the compilation and completion of the thesis, as well as the resulting publications from this research.

CHAPTER 1: INTRODUCTION

The burden of childhood mortality still remains one of the biggest challenges facing sub-Saharan Africa, with the United Children's Fund most recent estimates indicating that one in every nine children dies before the age of five years (United Nations, 2015). In view of these recent developments, the majority of sub-Saharan African countries have not met the United Nations Millennium Development Goal (MGD) 4 aimed at reducing the mortality of children under the age of five years by 2015 compared to 1990 levels (United Nations, 2015). In South Africa, human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) was identified as one of the five major causes of mortality in children under the age of five years in 2013, in addition to diarrhoeal diseases, pneumonia, preterm birth complications and birth asphyxia (Nannan et al., 2012, Massyn et al., 2015).

One in three women aged 25 to 29 years, the biggest group of woman of child bearing age, were at risk of contracting HIV in 2008 in South Africa (Shisana et al., 2009). Furthermore, there has been an epidemiological change between 2008 to 2012, which showed that the peak HIV prevalence for females had shifted from 25 to 29 year age group in 2008 to the 30 to 39 year age group in 2012 (Shisana et al., 2014). Furthermore, in 2014/15 an estimated 91% of South African pregnant woman living with HIV were receiving some form of antiretroviral treatment (ART) for the prevention of mother-to-child-transmission (PMTCT) (Massyn et al., 2015). The steady increase in access to ART has resulted in HIV mother-to-child-transmission rates dropping to <2% in 2014/15 (Massyn et al., 2015), however the prevalence of children born to HIV-infected women has remained unchanged over the past decade in South Africa. This has resulted in approximately one-third of newborns in South Africa being HIV-exposed *in utero*, albeit the majority are HIV-uninfected (Massyn et al., 2015).

HIV-infected children demonstrate aberrations of both humoral and cell mediated immune responses (Resino et al., 2004, Letvin and Walker, 2003), and several abnormalities in B-lymphocytes (Resino et al., 2004, Shearer et al., 2000). The dysfunction of the immune response is evident early on during the course of their HIV-infection, when individuals show a gradual loss of their T-lymphocytic proliferative responses (Gona et al., 2006, Letvin and Walker, 2003), progressive decline in total B-lymphocytes, with polyclonal hyper-gamma

globulinemia (Shearer et al., 2000), and loss of pathogen specific antibodies (Pensiero et al., 2009). This immunodeficiency state could impair immune responses to vaccines.

With improving survival rates in children with HIV being observed as a result of increased access and early initiation of ART (Violari et al., 2008), the vaccine responses in this population of children needs to be considered (Miller and Rathore, 2012). It is important to understand the degree of immune compromise and prospective immune recovery of these children, to gain guidance in vaccine administration for these children (Miller and Rathore, 2012). Vaccines have generally been found to be safe in HIV-infected children, with the notable exception of live-attenuated *Bacillus Calmette-Guérin* (BCG) (Jones et al., 2011). Although caution is raised with other live-attenuated vaccines, including oral polio vaccine and measles vaccine, there has been no documentation indicating an adverse safety profile for these vaccines in HIV-infected children (Moss et al., 2003). Immune response to vaccines among HIV-infected children in the pre-ART era was generally poor following primary vaccination, for diphtheria (10-88% sero-protection) (Ryder, 1993, Borkowsky et al., 1992, Borkowsky et al., 1987, Tejiokem et al., 2007), tetanus (25-82% sero-protection) (Essajee et al., 1999, Zaccarelli-Filho et al., 2007, Tejiokem et al., 2007, Borkowsky et al., 1987, Pensiero et al., 2009, Farquhar et al., 2009, Barbi et al., 1992, Rosenblatt et al., 2005), pertussis (23%-75% sero-positivity) (Tejiokem et al., 2009, de Martino et al., 1997, Tejiokem et al., 2007), hepatitis B (0-78% sero-protection) (Schoub, 2002, Bruguera et al., 1992, Zaccarelli-Filho et al., 2007, Zuin et al., 1992, Simani et al., 2009, Zuccotti et al., 1994) and measles (16-77% sero-protection) (Oxtoby et al., 1989, Krasinski and Borkowsky, 1989, Thaithumyanon, 2000, Rudy et al., 1994, Moss et al., 2007, Tejiokem et al., 2007, Palumbo et al., 1992). The lesser likelihood of inducing sero-protective antibody concentrations among HIV-infected children was particularly evident among those with low CD4+ lymphocyte counts; with humoral and cellular immune responses often correlated with CD4+ lymphocyte count (Tejiokem et al., 2007). In those children who demonstrated response, there was a greater waning of immunity to diphtheria-toxoid, tetanus-toxoid, whole-cell pertussis and measles vaccine over time when compared to their HIV-uninfected controls (Tejiokem et al., 2007, Rosenblatt et al., 2005). Consequently, the high burden of HIV-infection in South Africa and other sub-Saharan African countries could increase the number of vaccine failures (either primary or secondary) and reduce the effectiveness of these vaccines (Burnett et al., 2005).

Despite the success of reducing mother-to-child HIV transmission, HEU compared to HIV-unexposed children in developed and developing countries have been found to have lower concentrations of specific antibody to vaccine-preventable diseases including to *H. influenzae* type b, pneumococcus, tetanus and pertussis (Gaensbaier et al., 2014, Jones et al., 2013, Madhi et al., 2013a, Jones et al., 2011, Madhi et al., 2005, Madhi et al., 2013b). The decreased antibody concentration observed in HEU children could be due to low concentration of specific antibodies in the HIV-infected mothers, compounded by reduced transplacental transfer of antibody from mother-to-child (Jones et al., 2013, Jones et al., 2011, Cumberland et al., 2007, de Moraes-Pinto et al., 1998, de Moraes-Pinto et al., 1996). On the other hand, response to primary vaccination in HEU children are similar (for diphtheria, tetanus, *H. influenzae* type b and BCG) (Jones et al., 2011, Reikie et al., 2013, Hesseling et al., 2015) or even increased (for pertussis, pneumococcal capsular polysaccharide, and measles) (Jones et al., 2011, Madhi et al., 2013a, Madhi et al., 2010) compared to HIV-unexposed children. Also, high percentage (>97%) of HEU children have serological evidence of protection after combined measles, mumps and rubella immunization (Siberry et al., 2015). In addition, the fold-increase of antibody concentration from pre- to post vaccination is increased in HEU for pertussis (PT and FHA) and pneumococcus vaccine epitopes when compared to HIV-unexposed children (Jones et al., 2011). The robust immune response to vaccine epitopes observed in HEU children could be explained by the reduced interference of maternal antibody on the child immune response to vaccines, which has been reported for multiple vaccines.

In contrast to the above, other studies have shown decreased antibody response in HEU children for hepatitis B, oral polio and BCG vaccines when compared to HIV-unexposed children (Abramczuk et al., 2011, Mazzola et al., 2011, Van Rie et al., 2006). Possibly due to ART exposure for the prevention of mother-to-child transmission at birth, and *in utero* exposure to HIV virion particle may affect the cytokine profile that is necessary for vaccine response and lower total B-lymphocyte population in HEU children (Hesseling et al., 2015, Kakkar et al., 2014, Abramczuk et al., 2011). Reduced thymus lymphocyte output and functional defects of antigen presenting cells have also been reported in HEU children (Desmonde et al., 2016)

Although these studies indicate significant differences and trends in vaccine specific responses in children born to HIV-infected woman, they have mainly evaluated immune

responses following the primary series of vaccines, without further follow-up. Others have mainly assessed re-vaccination in HIV-infected children while receiving ART, without evaluating the responses following the primary series (Zaccarelli-Filho et al., 2007, Rigaud et al., 2008, Ching et al., 2007, Fernandes et al., 2008, Abzug et al., 2009, Aupibul et al., 2006). In all these studies, none evaluated the effect of the timing of initiation of ART among HIV-infected children on immune responses to the primary series of vaccines; and longitudinal follow-up of the HIV-infected children to determine the specific effects of timing of ART initiation or interruption thereof on persistence of antibody and response to booster doses of vaccines has been limited. Furthermore, there is a paucity of data on immune response to childhood vaccines with regards to HEU children, especially in Africa.

We set out to investigate vaccine specific immunoglobulin G (IgG) responses to diphtheria-toxoid, tetanus-toxoid, whole cell pertussis, Hib conjugate vaccine (Analysed in a separate study and not part of this thesis), hepatitis B and measles vaccine in HIV-infected children initiated on ART immediately upon being diagnosed as being HIV-infected within 4-10 weeks of age (ART-Immed) or in whom it was only initiated following progression to the then WHO recommended criteria for ART initiation based on clinical or immunological progression. Also, we evaluated immune responses to these vaccines among HEU children and compared all groups to the immune responses observed in the cohort of HIV-unexposed uninfected children. These children had been enrolled into a longitudinal cohort study in which the immune responses to 7-valent pneumococcal conjugate vaccine had been evaluated (Madhi et al., 2010), and the HIV-infected children were also participants in the CHER study in which the different ART regimens was evaluated (Violari et al., 2008).

CHAPTER 2: LITERATURE REVIEW

2.1 Overview of immune system

The immune system is composed of proteins and cells whose major role is to protect the skin, respiratory tract, intestinal tract and other areas of the body from bacteria, fungi, parasites, viruses, cancer cells and toxins (Warrington et al., 2011). Immunity is the ability of the human body to respond to infection and maintain resistance to infectious diseases. Synchronization of the immune system, tissues and organs against potential pathogens is defined as an immune response. Therefore, the major role of the immune system is to confer protection by prevention and elimination of invading pathogens (Warrington et al., 2011).

There are two major arms of the immune system, innate and adaptive immunity, that act as the host line of defence (Warrington et al., 2011). Innate immunity (non-specific) represents the first line of defence to potential pathogens. This defence mechanism is antigen-independent and is triggered by the host immediately or within hours of antigen exposure. Furthermore, it does not result in immunologic memory (Turvey and Broide, 2010). On the other hand, adaptive immunity is antigen-dependent and antigen-specific and therefore involves a lag time between exposure to the antigen and optimal immune response. This arm of immunity, could result in immunologic memory, which allows the host to mount a more rapid and efficient immune response to subsequent homotypic antigen exposure (Bonilla and Oettgen, 2010). The innate and adaptive arms of immunity are inter-dependent as host defence mechanisms, with defects in either increasing susceptibility to infectious diseases (Warrington et al., 2011).

2.1.1 Innate immunity

Innate immunity plays a key role (Fearon and Locksley, 1996) by recruiting immune cells to the site of infection and inducing an inflammatory response through production of cytokines (Warrington et al., 2011). The effectiveness of the immune response is dependent on information about the common pathogenic molecular patterns of pathogens that the innate immune response encounters (Kalinski et al., 2001, Bonilla and Oettgen, 2010). The availability of the common pathogenic molecular patterns allows the pathogen to be targeted selectively and for the effector

mechanisms to be targeted against the particular pathogen and not harmful to the affected host tissue (Kalinski et al., 2001).

Exposure of the innate system to a pathogen's antigen results in its activation and stimulates an adaptive immune response (Ploegh, 1998, Turvey and Broide, 2010). Many organisms have evolved mechanisms to overcome the defences of innate immunity, thereby prompting the need for more adaptive defences.

2.1.2 Adaptive immunity

Adaptive immunity may be humoral or cell mediated (Bonilla and Oettgen, 2010), and develop over the lifetime of an individual in response to different antigen exposures, including that of potential pathogens. Adaptive immunity is more specific in defence against infection and disease progression than innate immunity (Benjamin, 1996b), and can induce immunological long-term memory that offers protection against re-infection (Bonilla and Oettgen, 2010).

In the event that adaptive immunity is compromised, illness or disease may occur. This could occur due to an overactive immune response (known as hypersensitivity reactions), an inappropriate reaction to self (known as autoimmunity) or ineffective immune responses (known as immunodeficiency) (Warrington et al., 2011). There are five main properties that define the adaptive immune responses, as listed in Table 2.1.

Table 2.1: Properties of adaptive immunity (Benjamin, 1996b).

Property	Significance for immunity to microbes
Specificity	Ability to discriminate among different antigenic epitopes presented to it and respond specifically to those epitopes
Adaptiveness	Ability to respond to antigenic epitopes to which the immune system is naïve
Discrimination between self and 'non-self' antigens	Ability of the immune system to recognize and respond only to non-self epitopes and avoid immune responses to the host antigenic structure
Clonal Expansion	The capacity to compete with rapidly proliferating microbes
Memory	Ability of memory cells being induced following antigenic exposure, which are able to elicit more rapid and robust response upon subsequent exposure to the same epitope

2.1.2.1 Cells involved in Adaptive immunity

Lymphocytes are the major players in adaptive immunity. These cells include the effectors of cellular immune responses, the T-lymphocytes which mature in the thymus, and antibody producing B-lymphocytes which originate from the bone marrow (Bonilla and Oettgen, 2010). After their development in the primary lymphoid organs (thymus and bone marrow), they are transported to secondary lymphoid organs, which include lymph nodes and the spleen, and serve to capture circulating antigens from the lymph and blood, respectively (Bonilla and Oettgen, 2010, Ploegh, 1998). These cells play an important part in defining the adaptive immune response; and are produced and/or activated often under the direction of the innate immune system signals (cytokines and chemokines) stimulated directly or indirectly by circulating antigens; or by pathogen activated cutaneous or mucosal antigen-presenting cells (APCs, for example macrophages, dendritic cells (DCs) and B-lymphocytes) (Bonilla and Oettgen, 2010, Ploegh, 1998).

In the presence of antigen, T- and B-lymphocytes are activated, and after proliferation and maturation become effector cells. Effector B-lymphocytes produce antibodies, upon maturation they become plasma cells. On the other hand, effector T-lymphocytes produce a wide array of cytokines, which serve as signalling proteins that act to mediate the type of immune response required (Janeway et al., 2001b). T-lymphocytes are divided into three main classes. 1) Cytotoxic T-lymphocytes (CTLs), which are involved in the direct killing of infected host cells. CTLs that express the CD8⁺ molecule on their surfaces are referred to as CD8⁺ T-lymphocytes. These lymphocytes recognize intracellular antigen peptides presented on the MHC class I molecule, such as viruses, which results in the activation and killing action of these lymphocytes. The CTL killing action is through the release of various toxic molecules, such as perforin and granzymes, which induce apoptosis or cell death (Janeway et al., 2001b, Bonilla and Oettgen, 2010). 2) T helper (Th) lymphocytes, which are involved in the activation of APCs. Th- lymphocytes are also known as CD4⁺ T-lymphocytes. They interact with major histocompatibility complex (MHC) class II molecules on antigen presenting cells (Janeway et al., 2001b). Two main classes of these cells exist. The first being Th1 cells, which are involved in mediating inflammatory responses, and the second is Th2 cells, which play an important role in humoral immunity through the activation of B-lymphocytes (Bonilla and Oettgen, 2010, Ridge et al., 1998, Rissoan

et al., 1999). 3) T regulatory (Treg) or suppressor lymphocytes, are involved in the control and regulation of the immune response (Romagnani, 2005).

Two major forms of adaptive immunity exist, i.e. cell mediated and humoral immunity. Both forms are mediated by various cells and proteins and are intended to provide defence against extra- and intracellular organisms (Bonilla and Oettgen, 2010).

2.1.2.2 T-lymphocytes and cell-mediated immunity

Intracellular pathogens are eliminated from the host through cell mediated immunity, and this process is mediated by T-lymphocytes.

i) Activation of T-lymphocytes by recognition of MHC associated peptides

Mature T-lymphocytes express a unique antigen binding receptor on their membrane, known as the T-cell receptor (TCR) which is comprised of an α and β chain, and requires the action of APCs to recognize specific antigens (Warrington et al., 2011). TCRs recognize displayed peptides and residues located around the peptide-binding cleft of MHC molecules. MHC molecules are expressed on the surface of APCs, and are divided into MHC class I which are found on all nucleated cells, or class II which are found on certain cells of the immune system, including DCs, macrophages and B-lymphocytes (Warrington et al., 2011). Extracellular antigens are taken up by either phagocytosis or endocytosis into vesicles of APCs, which process them into peptides, which are then displayed by MHC class II complexes (Figure 2.1). Antigens that are present within the cytoplasm are processed and displayed by MHC class I molecules (Janeway et al., 2001b).

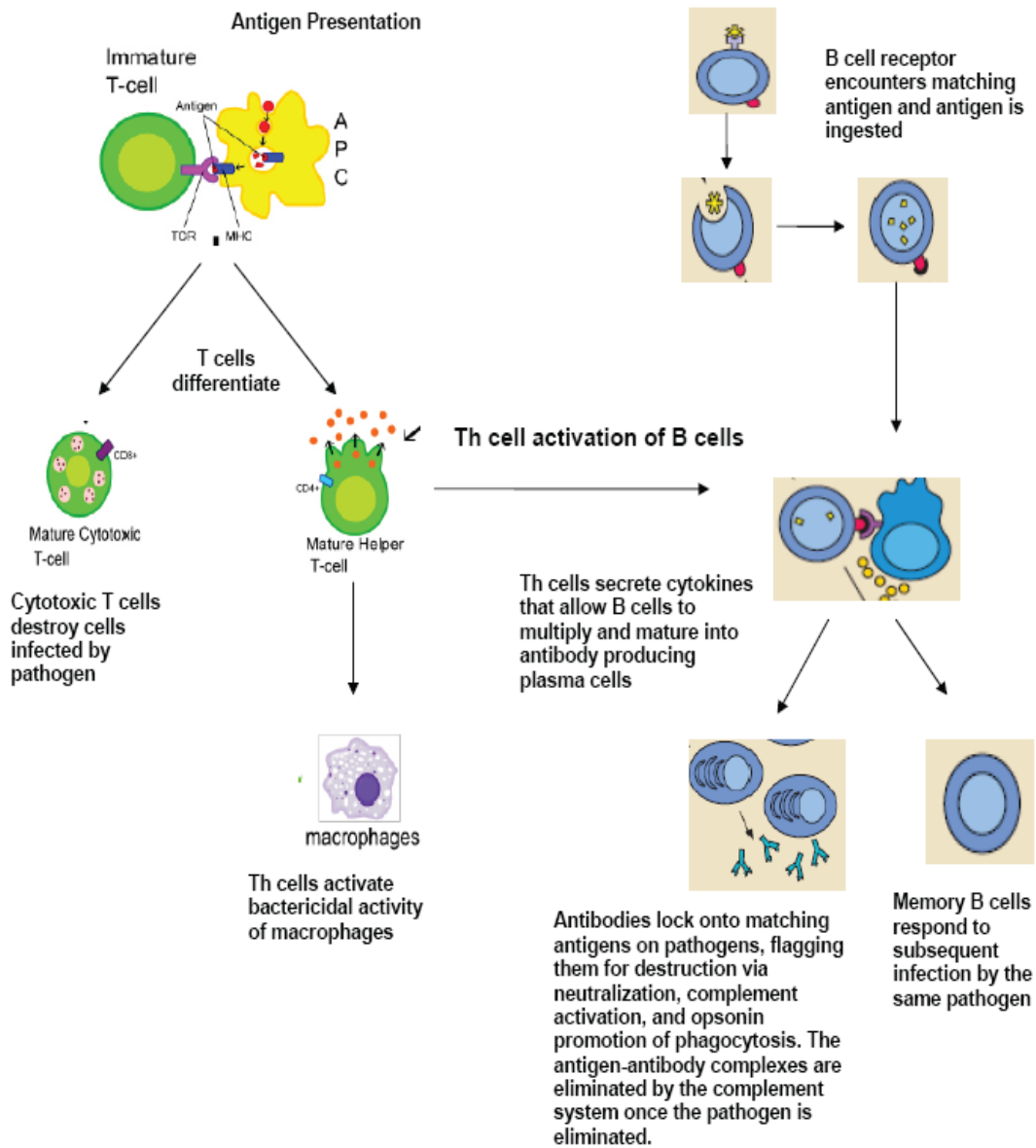


Figure 2.1: T- and B-lymphocyte activation and function: Adaptive immunity (Warrington et al., 2011). APC: antigen presenting cell; MHC: major histocompatibility complex; TCR; T-lymphocyte receptor.

Mature MHC restricted T-lymphocytes express either CD4⁺ or CD8⁺ cell surface markers, which function as co-receptors of TCR and MHC binding. CD4⁺ T-lymphocytes recognize extracellular microbial antigens displayed on MHC class II molecules, and CD8⁺ T-lymphocytes recognize intracellular peptides displayed on MHC class I molecules (Janeway et al., 2001b).

ii) T-lymphocyte activation through co-stimulation

Expression of B7.1 (CD80) and B7.2 (CD86) ligands (co-stimulatory molecules) appears to be crucial to the ability of professional APCs (DCs, B-lymphocytes and macrophages) to activate T-lymphocytes (Lenschow et al., 1996), their expression is increased during APC encounter with microbes. These ligands are recognized by a receptor expressed on T-lymphocytes, called CD28. Ligation of CD28 (on T-lymphocytes) results in the production of an essential co-stimulatory signal that activates resting T-lymphocytes. B7-CD28 interaction co-stimulates the induction of interleukin (IL)-2, autocrine T-lymphocyte growth factor that rescues TCR-triggered cells from activation induced cell death and allows for their proliferation and acquisition of effector functions (Lenschow et al., 1996). In addition, another set of molecules that provide increased co-stimulatory signals for T-lymphocytes are CD40 Ligand (CD40L) that bind to CD40 on APCs. This binding, results in stimulation or activation of APCs to express more B7 co-stimulation and secretion of cytokines, such as IL-12, which further enhances T-lymphocyte differentiation (Janeway et al., 2001b).

CD8⁺ T-lymphocytes recognize antigen peptides from cytoplasmic proteins, such as viral proteins, in any nucleated cell. During intracellular infection, infected cells are taken up by specific APCs, like DCs, after which antigens are “cross-presented”. The same APCs may present viral antigens from the cytosol in MHC class I. Furthermore, CD4⁺ T-lymphocytes secrete cytokines that play a role in the activation of CD8⁺ T-lymphocytes leading to their clonal expansion and differentiation into effector and memory CTLs. Activation of CD8⁺ T-lymphocyte may explain the defective CTL responses observed in persons with HIV-infection that result in the destruction of CD4⁺ T-lymphocytes and not necessarily CD8⁺ T-lymphocytes. However this event is not necessarily true for all viral infections (Janeway et al., 2001d).

iii) Cytokines and their role in T-lymphocyte activation

Cytokines are a large set of proteins that function as mediators of immunity and inflammation; they are produced by CD4⁺ T-lymphocytes in response to antigen exposure and co-stimulation. Following CD4⁺ T lymphocyte activation, IL-2 is produced. The IL-2 cytokine up-regulates the ability of T-lymphocytes to proliferate and respond by regulating the expression of the IL-2 receptor. CD4⁺ T-lymphocytes are dependent on IL-2 production, while CD8⁺ T-lymphocytes

do not produce IL-2 in response to antigen stimulation. It is suggested that antigen recognition rather than IL-2 production drives the proliferation of CD8⁺ T-lymphocytes (Janeway et al., 2001d).

iv) Th1 and Th2 lymphocytes

CD4⁺ helper T-lymphocytes have an important role in establishing and maximizing the immune response, through the production of distinct sets of cytokines that possess certain functions. These lymphocytes have no cytotoxic or phagocytic activity, and are unable to kill infected cells or clear pathogens. Two main subsets of Th lymphocytes exist, Th1 and Th2. Th1 lymphocytes are involved in cell mediated responses, activating mononuclear phagocytes, natural killer cells and virally infected cells. This response is characterized by the capacity to make interferon gamma (INF γ) and IL-2, which activates the bactericidal activity of macrophages (Bonilla and Oettgen, 2010). An additional function of Th1 lymphocyte subset is to release cytokines that induce B-lymphocytes to make opsonizing and neutralizing antibodies. Furthermore, INF γ stimulates the expression of MHC class II molecules and B7 co-stimulators on APCs, thus leading to increased T-lymphocyte responses (Janeway et al., 2001d, Warrington et al., 2011).

The Th2 lymphocyte response is characterized by its involvement in humoral immunity by enhancement of B-lymphocyte production, as well as hypersensitivity and parasite-induced immune responses. Cytokines released by this subset of lymphocytes include IL-4, IL-5, IL10 and IL-13 (Bonilla and Oettgen, 2010). IL-10 produced by Th2 lymphocytes plays a role in inhibition of macrophages. Furthermore, IL-4 and IL-10 regulate or suppress Th1 lymphocyte types (Janeway et al., 2001d).

2.1.2.3 Humoral immunity

Humoral immunity is an antibody mediated specific immunity, and functions to eliminate extracellular microbes. Humoral immune responses follow either direct recognition by B-lymphocytes through *de novo* recognition of antigenic epitopes, or following presentation of the epitope after processing and presentation thereof by Th-lymphocytes to B-lymphocytes (Bonilla

and Oettgen, 2010). One of the roles of humoral immunity is to defend the host against bacterial capsules rich in polysaccharides and lipids (Janeway et al., 2001d).

i) An introduction to antibodies

Activated B-lymphocytes produce antibodies which together with components of the complement pathway flag pathogens for recognition by T-lymphocytes for destruction (Ploegh, 1998).

Antibodies are depicted by a Y-shaped molecule, and found in the components of blood or plasma, as well as extracellular fluid. An antibody is composed of two distinct regions: the constant region that holds one of five biochemically distinguishable forms, and a variable region that is composed of an unlimited variety of different amino acid (aa) sequences that vary in structure and allow for antibodies to bind to a wide range of antigens (Janeway et al., 2001c).

The variable region determines the binding specificity of an antibody. It consists of two identical regions, as a result has two identical antigen binding sites (Fab region). The constant region engages the effector functions of the antibody molecule and determines how the antibody will process the antigen once bound (Fc region). Furthermore, antibodies are constructed from paired heavy and light polypeptide chains, and the generic term immunoglobulin (Ig) is used to describe them. These polypeptide chains contribute to the variable and constant region, thus allowing the antibody molecule to have a two-fold symmetry. These chains combine to form the antigen-binding specificity of the antibody molecule (Janeway et al., 2001c).

ii) Antibody function

There are three main ways in which antibodies can participate in host defence: neutralization, opsonization and complement activation (Janeway et al., 2001d). To enter cells, pathogens bind to specific molecules on target cell surfaces, antibodies can bind to the pathogen and prevent entry by neutralizing the pathogen. Neutralization is important in preventing bacterial toxins or viruses from entering the target host cell. Antibodies can also protect against bacteria that multiply extracellularly, by facilitating their uptake by phagocytes in two ways. In the first, bound antibodies coating the pathogen are recognized by Fc receptors on phagocytic cells that bind to the Fc region, which enhances phagocytosis through a process known as opsonization. Secondly, antibodies binding to the surface of the pathogen can activate proteins of the

complement system. Complement activation results in complement proteins binding to the pathogen surface, also leading to opsonization and subsequent phagocytes and lysis of the pathogen (Janeway et al., 2001b). In addition to these functions, antibodies can participate in the process of antibody dependent cellular cytotoxicity, where natural killer cells bind Fc receptors resulting in lysis of the pathogen infected cell and its eventual phagocytosis (Bonilla and Oettgen, 2010).

Antibodies are produced after stimulation of B-lymphocytes by antigens. The portion of the antigen that binds specifically with the binding site of the receptor on a lymphocyte is termed an antigenic determinant (i.e. epitope) (Janeway et al., 2001c). The antigen-specific receptors on B-lymphocytes are immunoglobulin molecules allowing interaction of epitopes sized between 5-22 aa residues (Singh et al., 2013). The size of an epitope that binds to specific T-lymphocyte is somewhat larger, since it contains an area that binds to MHC proteins of APCs and an area that binds specific receptors on the T-lymphocyte resulting in a tri-molecule complex. Therefore the size of the epitope is estimated to be 10-15 aa residues long (Singh et al., 2013).

B-lymphocytes stimulated by an antigen then differentiate into antibody secreting cells (plasma cells), to produce antibodies of different heavy chain classes or isotypes (Table 2.2). There are a total of five known antibody isotypes produced by B-lymphocytes: IgA, IgD, IgE, IgG and IgM, each possessing their own class of heavy chains (α , δ , ϵ , γ , and μ) respectively (Table 2.2) (Stavnezer et al., 2008). IgG and IgA isotypes have been divided into further subclasses (e.g. IgG1-4 and IgA1-2) that differ in heavy chains. The differing heavy chains give rise to distinct conformations in the hinge and tail regions of antibodies so that each class and subclass is unique (Janeway et al., 2001b).

Table 2.2: Main functions of human immunoglobulin antibodies (Warrington et al., 2011, Panda and Ding, 2015).

Immunoglobulin isotype	Function
IgM	First Ig expressed during B-lymphocyte development (primary response) Opsonizing antigen for destruction Complement fixation
IgG	Main Ig during secondary immune response Only antibody capable of crossing placental barrier Neutralization of toxins and viruses

	Opsonizing antigen for destruction Complement fixation
IgD	Function unclear, but appears to play a role in homeostasis
IgA	Mucosal response Protects mucosal surfaces from toxins, viruses and bacteria through direct neutralization or prevention of binding to mucosal surface
IgE	Associated with hypersensitivity and allergic reactions Plays a role in immune response to parasites

Antibody isotypes are able to enter the blood through a process of transduction and progress to the site of infection. This function allows antibodies to fight infection throughout the host's body. Based on the type of signals elicited by this response, B- and T-lymphocytes differentiate to a memory phenotype and obtain a particular effector function (Bonilla and Oettgen, 2010). These effector mechanisms are recalled whenever effector/memory lymphocytes are re-exposed to the specific epitope (Swain et al., 1991, Richter et al., 1999). Memory lymphocytes influence the immune responses to subsequent exposure to the same epitope (Benjamin, 1996a). The ease with which memory lymphocytes are triggered, even at extremely low antigen concentrations may explain why immune responses tend to be dominated by memory lymphocytes from previous infections (Rogers et al., 2000). Memory lymphocytes of a certain responsive mode may direct the differentiation of new, naïve clonotypes of lymphocytes via cytokine secretion (Paul and Seder, 1994).

The innate immune system generally induces the appropriate type of adaptive immune response to pathogens. Pre-existing memory lymphocytes can dramatically increase the chances of surviving following an infection, an example of this is seen with memory responses following vaccination (Borghans and De Boer, 2002). There are many advantages of memory lymphocytes, as they are not confined to lymphoid tissue and freely enter solid tissue, respond from anywhere and anytime where a specific epitope is encountered (Zinkernagel et al., 1997). Responses as a result of memory lymphocytes are typically faster than in primary immune responses, because memory lymphocytes are more sensitive to low concentration of antigen, have stringent requirements for co-stimulation and are primed for specific responses (Borghans and De Boer, 2002).

iii) B-lymphocyte activation

For an antigen to be immunogenic it must be exogenous to the host being exposed, must be of relatively high molecular weight and possess a certain degree of chemical complexity (Benjamin, 1996a). Processing of antigens can be classified as either T-lymphocyte independent (TI) or T-lymphocyte dependent (TD) in nature (Lesinski and Westerink, 2001, Bonilla and Oettgen, 2010).

iv) T-independent antigens

TI immune responses are associated with a less important role of T-lymphocytes in inducing B-lymphocyte production of epitope specific antibodies or their differentiation (Bonilla and Oettgen, 2010). TI immune responses are also not associated with inducing differentiation of B- or T-lymphocytes into memory cells. TI antigens are generally polysaccharide epitopes and can either be Th1 or Th2 based on their interaction with B-lymphocytes (Lesinski and Westerink, 2001). Antigens such as lipopolysaccharides are known potent B-lymphocyte mitogens. They function by non-specific, polyclonal activation of most B-lymphocytes; they do not induce memory-lymphocytes or isotype switching of antibodies. In contrast, Th2 antigens are capsular polysaccharides; they do not function as B-lymphocyte mitogens and can only activate mature B-lymphocytes (Lesinski and Westerink, 2001).

v) T-dependent antigens

Conversely, antigens eliciting TD immune responses, include peptides or proteins, and involve B-lymphocyte stimulation through Th1 lymphocytes. TD antigens are presented to T-lymphocytes by APCs in conjunction with MHC molecules (Bonilla and Oettgen, 2010). The activation of T-lymphocytes results in cytokine production and an array of immunologic reactions. Epitope recognition by Th1 lymphocytes results in their differentiation as well as presentation of epitopes and stimulation of B-lymphocytes. This consequently induces immunoglobulin production including maturation and production of high affinity antibodies of varying isotypes and differentiation into memory B- and T-lymphocyte sub-populations (Bonilla and Oettgen, 2010, Lesinski and Westerink, 2001).

vi) Immunopathology

Defects or malfunctions in either innate or adaptive immunity can occur as a result of hypersensitivity reactions, autoimmunity and immunodeficiency (Warrington et al., 2011). As part of this section, immunodeficiency will be reviewed. Immunodeficiency often refers to the immune system's inability to fight infectious disease. Immunodeficiency disorders can be classified into two forms: 1) primary congenital defect (primary immunodeficiency) or 2) acquired from a secondary source (secondary immunodeficiency), such as bacterial or viral infections, malnutrition or treatment with immunosuppressive drugs. A number of diseases can cause direct or indirect impairment of the immune system such as leukaemia and multiple myeloma. Immunodeficiency is also the hallmark of AIDS, which is caused by HIV-infection (Warrington et al., 2011).

The adaptive immune system is designed to ward off infection and allow for the maintenance of good health. However, the immune system is often challenged by organisms 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 status of HIV/AIDS pandemic

In 2014, an estimated 36.9 million individuals globally were living with HIV (UNAIDS, 2015). On average there was a 35% decrease in new infections globally since 2000, including a 58% decrease in children at the end of 2014 (UNAIDS, 2015). At the same time AIDS related deaths decreased by 42% since the peak in 2004 (UNAIDS, 2015).

Sub-Saharan Africa is a region with the highest HIV prevalence worldwide and HIV/AIDS remains a leading cause of death including in South African children (UNAIDS, 2015). It is estimated that more than 3 million children under the age of 15 years were infected with HIV worldwide, including 91% of whom live in sub-Saharan Africa, in 2007 (UNAIDS, 2015)(UNAIDS, 2013). Furthermore, in 2007 there were an estimated 1 800 new HIV-infections daily in children through mother-to-child transmission (UNAIDS, 2007, UNAIDS, 2015). Approximately 29.5% of the 1.1 million children born each year in South Africa were born to

HIV-infected women (South African Department of Health, 2005; Theron, 2007). In 2007, there were an estimated 93 000 children who were infected with HIV through peri-natal transmission and a further 21 000 who were subsequently infected by HIV through breast milk exposure (UNICEF, 2008), with an estimated 14.6% receiving adequate antiretroviral treatment for the prevention of mother-to-child transmission in 2007 (South African Department of Health, 2005; Theron, 2007; (UNICEF, 2008). The estimated mortality rate of HIV-infected children in South Africa, in the absence of antiretroviral treatment programmes, was 50% within the first two years of life (UNICEF, 2008). However, this had decreased by 18% in 2008 due to improved antiretroviral mother-to-child transmission programme (UNAIDS, 2009)(UNAIDS, 2015). Despite all progress made in reducing mother-to-child HIV transmission through peri-partum antiretroviral programmes, paediatric HIV-infection remains a public-health problem in African countries (Buchanan et al., 2014).

2.3 HIV PATHOGENESIS AND IMMUNOLOGICAL ABERRATIONS

HIV has continued to be a major health problem worldwide, owing to the intricate nature of its interaction with the immune system. HIV has remained an enigma that cleverly uses the host machinery to survive. Its ability to evade the hosts' defences at both levels, innate and adaptive, allows it to replicate and transmit from one host to another. HIV-infection leads to immune dysfunction and subsequent depletion of the immune system. In the past 3 decades there has been extensive progress on the understanding the immunopathogenesis of HIV-infection.

2.3.1 Structure of HIV

HIV-1 and HIV-2 belonging to the family *Retroviridae* within the subfamily *Lentivirinae* (Fanales-Belasio et al., 2010). Of these two, HIV-1 is more prevalent, and has established global epidemics, while HIV-2 is more prevalent in West African nations (Janeway et al., 2001a, Fanales-Belasio et al., 2010).

The structure of the HIV-1 consists of an internal core particle made up of 1200 molecules of capsid protein (p24 *gag*) surrounded by a lipid envelope (*env*) spiked with 75 trimers of a highly glycosylated protein, gp160, wrapped around a scaffold made of the matrix protein (p17 *gag*) (Cosson, 1990, Hill et al., 1996). The gp160 *env* molecules are made of two non-covalently

linked polypeptide chains. The *trans*-membrane chain, gp41, that anchors the spikes in the lipid bilayer of the envelope, maintains their trimeric structure, and plays a major role in fusion of the virus and cell membranes (Bugge et al., 1990, Eckert and Kim, 2001). The external chain, gp120, carries the important antigenic determinants of the virus and binds CD4⁺ receptor with either CXCR-4 (T-lymphocyte-tropic) or CCR-5 (macrophage-tropic) co-receptors (Kwong et al., 1998, Wyatt and Sodroski, 1998).

2.3.2 Viral entry and life cycle

Cellular tropism is defined as the ability of HIV to enter target 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 macrophages, DCs and CD4⁺ T-lymphocytes (Janeway et al., 2001a).

HIV first infects macrophages that carry the HIV to the lymph nodes, where they encounter follicular dendritic cells (FDCs). FDCs remove the HIV from the macrophages and contain the virus within the lymph nodes. The FDCs come into contact with CD4⁺ T-lymphocytes (naïve), resulting in T-lymphocyte infection. Therefore, HIV changes from macrophage (M)-tropic to CD4⁺ T-lymphocyte (T)-tropic because the T-lymphocytes have the appropriate receptor and co-receptors necessary for establishment of infection (Alcamo, 1997). HIV adsorbs to primary (CD4⁺) target cells by interaction of gp120, CD4⁺ and CC-chemokine co-receptor 5 (CCR5) or CX-chemokine co-receptor 4 (CXCR4). This results in conformational changes that expose gp41, which fuses with the cellular membrane resulting in a passage way and the nucleocapsid is deposited inside the cytoplasm of the target cell (Strauss and Strauss, 2002).

The capsid is uncoated by cellular enzymes, releasing the diploid single stranded (ss)-RNA and reverse transcriptase. Reverse transcriptase, reverse transcribes ss-RNA to double stranded-deoxyribonucleic acid (ds-DNA) that is transported to the nucleus of the infected cell. Integration occurs with the aid of the integrase enzyme, where the ds-DNA is inserted randomly into the host cell genome (to be known as provirus). After integration, the provirus is able to undergo transcription and translation. The proteins made initially are very long precursor proteins that must be spliced to size by the viral protease. Finally, mature infectious virions are released by budding to complete the life cycle (Strauss and Strauss, 2002). HIV can alternatively cause latent infection through the integrated provirus, this has been observed to occur in memory CD4⁺ T-

lymphocytes and macrophages, thus serving as important reservoirs of infection (Sierra et al., 2005).

2.2.3 Immune response following HIV-infection

Infected individuals develop several immunological responses to HIV, including neutralizing antibodies, antibody-dependent cell-mediated cytotoxicity and HIV-specific CTLs. A quarter of HIV-infected individuals develop broadly neutralizing antibodies targeting a number of conserved epitopes on the viral envelope (Euler et al., 2010). Neutralizing antibodies may play a role in the control of HIV in the blood after primary infection (Eley, 2003). In general, many viral infections are not cleared but rather controlled by an effective immune response (Eley, 2003). Both specific CD4⁺ Lymphocytes and CD8⁺ CTLs are required to limit replication during viral infections (McMichael et al., 2010).

However, during the advanced stages of infection, neutralizing antibodies do not influence the viraemia. This is probably because of successive waves of viral escape as a means of escaping immune pressure (Moore et al., 2013). Antibody-dependent cell-mediated cytotoxicity is present throughout infection and is primarily directed against gp120 (Fouda et al., 2011). Expression of several cytokines is altered during HIV-infection. In *in vitro* experiments, cytokines such as IFN- α and IFN- β suppress HIV replication, whereas transforming growth factor (TGF) - β , IL-4, IL-10, IL-13 and IFN- γ may either induce or suppress HIV expression (McMichael et al., 2010). The altered cytokine network may therefore influence viral replication (Fauci, 1996).

In the early stages of HIV disease, specific CD8⁺ CTLs restrict viral replication and limit CD4⁺ cell suppression (McMichael et al., 2010). Anti-HIV specific CD8⁺ CTLs are activated via surface expressed T-lymphocyte receptors after encountering HIV-specific antigens that are presented in association with MHC class I molecules, on the surface of target cells.

Consequently, CTLs lyse infected cells either by secreting proteases, granzyme A and B, and perforin or via Fas-mediated killing (Cocchi et al., 1995). CD8⁺ CTL further induces non-lytic inhibition of viral replication by MHC restricted release of β -chemokines including MIP-1 α , MIP-1 β and RANTES (Fauci, 1996).

2.3.4 Immunopathogenesis of HIV-infection

HIV directly infects and replicates within cells of the immune system particularly macrophages and CD4⁺ T-lymphocytes, resulting in abnormalities of both humoral and cell mediated immune responses (Resino et al., 2004, Letvin and Walker, 2003), as well as in B-lymphocytes (Resino et al., 2004, Shearer et al., 2000, Lane et al., 1983). The dysfunction of the immune response becomes apparent early on during HIV-infection, when individuals show a gradual loss of their T lymphocytic proliferative responses (Gona et al., 2006, Letvin and Walker, 2003, Palumbo et al., 1992, Borkowsky et al., 1992), progressive decline in total B-lymphocytes, with polyclonal hyperglobulinemia (Lane et al., 1983, Shearer et al., 2000), impaired reactivity to immunization (Arpadi et al., 1996, Pensiero et al., 2009) and loss of specific antibodies (Pensiero et al., 2009, al-Attar et al., 1995). In addition HIV viral replication may impair immune function through immunosuppressive effects of viral proteins and by a state of hyper activation that leads to immunosuppression and accelerated apoptosis (Letvin and Walker, 2003). The consequences of this is the loss of CD4⁺ T-lymphocytes; increased risk of susceptibility to infections or poor immune response due to defective antigen processing by macrophages and dendritic cells; defective immunological memory; failure to prime memory cells or failure of primed B-lymphocytes differentiation into memory cells; loss of immunological memory of T- and B-lymphocytes after priming i.e. clonal deletion or depletion (Resino et al., 2004, Obaro et al., 2004).

2.4 ACCESS TO ANTIRETROVIRAL THERAPY

The global estimates of ART coverage in HIV-infected children (23%) in 2009 was similar to that of HIV-infected adults (UNAIDS, 2012, UNAIDS, 2009). There has been a steady increase in access and ART treatment of HIV-infected adults and children since 2003, with a 10-fold increase in usage having been reported by 2008 (UNAIDS, 2009). It is estimated that ART coverage in HIV-infected children in South Africa increased from an estimated 7% in 2004 to 44% in 2010 (UNAIDS, 2011). The improved access in antiretroviral treatment programme, including changes to triple therapy in HIV-infected pregnant women (Goga et al., 2015), has reduced the vertical transmission of HIV to newborns of HIV-infected women to 2.5% in 2010 (Barron et al., 2013, Goga et al., 2015). In addition, improved access to ART of HIV-infected children, particularly when initiated early in life, results in improved survival among HIV-

infected children (Violari et al., 2008). ART has been shown to dramatically change the current course of HIV-infection by drastically reducing viral load and enabling the recovery of the immune system of children and adults (Resino et al., 2004, Valdez, 2002). In developed countries, where children have access to ART, 80% are still alive by the age of 6 years (WHO, 2005). Resino and colleagues reported that immune reconstitution due to ART in HIV-infected children seemed to be a consequence of a decrease in activation of naïve T-lymphocyte reconstitution (Resino et al., 2004). This immune system restoration supports normalization of immune parameters such as cytokine production, lymphoproliferative response and immunoglobulin levels in plasma (Pensieroso et al., 2009). ART treated children have shown improved numbers of CD4+ T-lymphocytes, including increased levels of both naïve and memory cells (Melvin, 1997, van Rossum, 2000).

However, additional challenges faced by HIV-infected children include the faster progression of HIV/AIDS illness; a prevailing immature immune system; greater difficulties in accessing ART programmes; and delayed initiation of ART. These factors are thought to contribute to faster progression to AIDS and death in HIV-infected children (UNAIDS, 2008). A multi-centered study in South Africa, however, indicated that early diagnosis and early initiation of ART within eight weeks of birth was able to delay HIV-related disease progression by 75% and reduce childhood mortality rates by 76% (Violari et al., 2008). This study was instrumental in the WHO revising its recommendation on the timing of initiation of ART in HIV-infected children in 2010 (WHO, 2010a). In an older group of Zambian HIV-infected children, a combination of ART and co-trimoxazole prophylaxis was associated with a six-fold decrease in mortality among HIV-infected children (Walker et al., 2007). HIV-infected children, especially in the absence of ART, rapidly develop progressive deterioration of various arms of their immune system (Moss et al., 2003). The resultant immunodeficiency in these children renders them highly susceptible to potentially fatal infections (Arrazola *et al*, 1995), increasing the need for strategies aimed at preventing infectious diseases in these children.

2.5 PREVENTION OF MOTHER-TO-CHILD TRANSMISSION (PMTCT) PROGRAMMES

Prior to implementation and routine use of ART, the rate of HIV transmission from mother-to-child ranged from 15 to 25% among children who were formula-fed and from 25 to 40% among children who were breast-fed (Luzuriaga and Mofenson, 2016). In the absence of preventative measures, an HIV-infected woman has a one in three chance of transmitting the virus to her offspring (UNAIDS, 2010). Almost half of the children who acquire HIV *in utero* will die before the age of 2 years without effective treatment (UNAIDS, 2010). In order to decrease the rate of transmission from an infected mother to her child, PMTCT programme along with increased access to ART need to be implemented (Country progress report on the declaration of commitment on HIV/AIDS 2010 report, 2010). Therefore, with the administration of ART during pregnancy and delivery to both mother and child, the risk of transmitting the virus from mother-to-child has decreased to less than 2% (UNAIDS, 2016, Massyn et al., 2015).

South Africa, along with Botswana, Mozambique, Namibia, Swaziland and Uganda, have thus far achieved above 90% coverage of ART to prevent mother to child transmission (UNAIDS, 2016). Coverage of ART for PMTCT in South Africa and Namibia was reported to be >95%, whereas other African countries such as Nigeria and Côte d'Ivoire have reported coverage of less than 30% (UNAIDS, 2016). 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, amended in 2008 to combination/dual therapy including AZT and NVP, as recommended by the WHO (DOH, 2009).

Due to the increased mortality observed in HIV-infected women and children, PMTCT and ART programmes were accelerated and modified to address this. During the course of April 2010, revisions of the PMTCT programme in keeping with the guidelines stipulated by WHO, stated that all HIV-infected women with a CD4+ cell count less than 350 cells/ μ l should be 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 therapy initiation in pregnant mothers (DOH, 2010). These changes highlight constant improvements to decrease the spread of HIV from the infected mother to child. 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).

2.6 HIV-EXPOSED UNINFECTED (HEU) CHILDREN

2.6.1 The increasing number of HEU children

Out of the 1 million children born in South Africa, approximately 300 000 of these children are exposed to HIV *in utero* (DOH, 2009). Several studies have reported increased morbidity and mortality in HEU children due to infectious agents (Newell et al., 2004, McNally et al., 2007, Filteau, 2011, Slogrove et al., 2010). Increased morbidity and mortality in HEU is believed to be multifactorial, including low-birth weight, failure to thrive, exposure to ART, inadequate clinical follow-up and reduced breastfeeding have been shown to be contributing risk factors (Filteau, 2011). Furthermore, HEU children experience abnormalities in the physiologic development of the immune system (Clerici et al., 2000). A major challenge exists in identifying the underlying mechanisms of immune dysfunction HEU children. Different pathogens interact differently with the maternal immune system; therefore, their mechanisms of interaction with the fetal immune system are different (Dauby et al., 2012). However, some mechanisms have been postulated to be common, such as the activation of adaptive immune responses by microbial epitopes either free or complexed with immunoglobulins (May et al., 2009), crossing the placenta (Malhotra et al., 1997, Pit et al., 2000, Attallah et al., 2003, Broen et al., 2007, Kuhn et al., 2002, Koumbi et al., 2010). Innate immune responses can be triggered by infection of the placenta with parasites such as plasmodium (Broen et al., 2007) and schistosoma (Kurtis et al., 2011), or by microbial products inducing inflammatory responses in the placenta or after transfer to the fetus (Lucchi et al., 2011, Diouf et al., 2004, Dauby et al., 2012).

McNally et al. and others have reported heightened susceptibility of HEU children to severe infections caused by opportunistic pathogens such as *Pneumocystis jirovecii* or *Mycobacterium tuberculosis*, and the more common early life pathogens such as cytomegalovirus and group A streptococcus, which rarely occur in HIV-unexposed children (McNally et al., 2007, Slogrove et al., 2010, Heresi et al., 2007). The results of these studies suggest that prenatal or perinatal factors that are associated with maternal HIV-infection increase the susceptibility of children to infectious diseases in different parts of the world (Dauby et al., 2012).

Furthermore, several other factors besides maternal HIV-infection have been implicated in the increased susceptibility of HEU children to infectious diseases. They include increased exposure to pathogens as a result of maternal HIV associated disease, poor socio-economic status, and absence of breastfeeding as a recommended measure to prevent MTCT and toxicity associated with antenatal antiretroviral prophylaxis (Dauby et al., 2012). In addition, the role played by alterations in passive and acquired immunity has been suggested by several studies (Miles et al., 2010, Neilsen et al., 2001, Kuhn et al., 2001, Rich et al., 1997, Chougnet et al., 2000, Farquhar et al., 2005, Jones et al., 2011, de Moraes-Pinto et al., 1996, Embree et al., 2001). HEU children have lower CD4+ T-lymphocyte counts compared to HIV-unexposed children in the first few months of life (Miles et al., 2010, Embree et al., 2001). The low CD4+ T-lymphocyte counts in HEU children has been associated with low thymic output, decreased cloning efficiency of progenitor cells, and increased serum concentrations of IL-7 (Neilsen et al., 2001). Furthermore, altered *in-vitro* T-lymphocyte activation was detected in HEU neonates with an increased production of IL-10 and decreased production of IL-2 after polyclonal stimulation (Kuhn et al., 2001, Rich et al., 1997). Decreased production of IL-12 by cord blood lymphocytes in HEU children also suggests that the function of antigen presenting cells might be altered in them. (Chougnet et al., 2000) Furthermore, HEU children have reduced transfer of maternal antibodies across the placenta compared to HIV-unexposed children (Farquhar et al., 2005, de Moraes-Pinto et al., 1996, Jones et al., 2011).

The mechanisms behind the immune dysregulation among HEU children is yet to be fully elucidated, but may involve the transfer of non-infectious HIV virion particles with immunosuppressive properties across the placenta, or the activation of the maternal immune system with transfer of soluble mediators or maternal cells across the placenta (Dauby et al., 2012). Also, although maternal ART has been highly successful in the PMTCT programme, maternal ART exposure has been shown to have negative effects on foetal and childhood development in HEU children (Noguera et al., 2004). A common side effect of ART exposure (zidovudine) in HEU children is mitochondrial dysfunction (Noguera et al., 2004), although this was not evident in another study (European-Collaborative-Study., 2005). Studies have also shown an association of *in utero* ART exposure with abnormal T-lymphocyte counts, CD8+ CTL counts, abnormal neutrophil and red blood cell counts in the first few years of life (Bunders et al.,

2005, Feiterna-Sperling et al., 2007, Pacheco et al., 2003, Le Chenadec et al., 2003). Even though the effects of ART should be carefully examined, the benefits of maternal ART far outweigh its side effects, however, the increased infectious diseases morbidity among HEU children require on-going monitoring (Thea et al., 1997, Marinda et al., 2007).

The integrity of the immune system of HEU children can be evaluated *in vivo* by use of vaccine induced responses as a model of controlled antigen exposure. Results suggest that HEU children, have antibody and T-lymphocyte responses to vaccines that are similar to those in HIV-unexposed children (Helfand et al., 2008, Mansoor et al., 2009, Jones et al., 2011, Reikie et al., 2013). Furthermore, some studies also reported that HEU children have increased antibody responses to vaccines such as pertussis, pneumococcus and *H. Influenzae* type b compared to HIV-unexposed children (Reikie et al., 2013, Jones et al., 2011, Madhi et al., 2005). This could be due to less interference from maternal-acquired antibody to the vaccine epitopes (Jones et al., 2011, Reikie et al., 2013). In contrast, other studies have reported sub-optimal responses to oral polio, tetanus, hepatitis B, and BGC vaccines (Abramczuk et al., 2011, Van Rie et al., 2006, Mazzola et al., 2011, Sanz-Ramos et al., 2013). Possibly due to exposure to ART at birth, *in utero* exposure to HIV may affect the cytokine profile that is necessary for vaccine response and lower total B-lymphocyte population in HEU children (Hesseling et al., 2015, Kakkar et al., 2014, Abramczuk et al., 2011). The discrepancy in results in HEU children needs to be further evaluated especially now in the era where ART for prevention of mother to child transmission is being scaled up.

2.7 PREVENTION OF INFECTION IN EARLY LIFE (MATERNAL ANTIBODIES)

2.7.1 Role of maternal antibodies

Neonates have a relatively incompetent immune system in relation to their ability to respond to infections. Therefore, the innate mechanisms play a key role in protecting the neonate against potential pathogens. Neonates are also endowed with maternal antibodies that are important for protection against infections in the first months of life (Siegrist, 1998). Placental antibody transfer generally occurs between 28-32 weeks gestational age (Malek, 2003). Naturally acquired antibodies have been shown to be variable in both level and rate of deterioration (Glezen, 2003, Palfi and Selbing, 1998). By the time of birth, passively acquired neonatal IgG exceeds maternal

concentrations (Saji et al., 1999, Leuridan and Van Damme, 2007). These maternal antibodies against pathogen-specific antigens disappear during the first months of life while the neonate's own immune system develops (Saji et al., 1999, Palfi and Selbing, 1998, Offit et al., 2002, Leuridan and Van Damme, 2007). The interval between the loss of protection once maternal antibodies have disappeared and protection by vaccination should be as narrow as possible for all vaccine-preventable diseases, because of the risk of early infection (Glezen, 2003). Therefore the amount of maternal antibody transferred to the child determines the length of protection, as well as response to vaccination (de Moraes-Pinto et al., 1998).

2.7.2 Mechanisms of maternal antibody transfer

Immunoglobulin G subclasses are the only antibody subclass (predominantly IgG1 and IgG3) that can cross the placenta. This transfer is an active, selective and intracellular process, which usually occurs within the third trimester (Englund, 2007). This transfer 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. 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 (Janeway et al., 2001d) (Englund, 2007). A number of factors have been described that may influence the effectiveness of maternal antibody transfer, some of which include placental abnormalities, total IgG concentration in maternal blood, vaccine-antigen type, gestational age of child, as well as time duration of maternal vaccination and delivery (Englund, 2007, van den Berg et al., 2010).

2.7.3 How maternal antibodies influence the child's immune response

At primary vaccination, most children still have high levels of maternal antibodies. Studies have raised concerns on the interference of maternal antibodies with immune response to vaccination. The extent of this effect is vaccine antigen specific (Glezen, 2003, Jones et al., 2014). For example, 25-56% decrease in antibody response to whole cell pertussis vaccine was observed in the presence of maternal antibodies (Englund et al., 1995). However in the same study this effect was not observed when acellular pertussis vaccine was utilised (Englund et al., 1995). Other

antigens that have shown the negative effects of maternal antibodies are the oral and inactivated polio vaccines given at birth. The response was further decreased in breastfed babies (Warren et al., 1964, Simoes et al., 1985). Immune response to diphtheria-toxoid was shown to be decreased in children who had antibody levels $>0.1\text{IU/ml}$ prior to vaccination, however, when a booster was given at 12 months of age there was no difference in response between children with high maternal derived antibodies and those who had low antibodies (Bjorkholm et al., 1995). Similar results were shown for Hib and tetanus toxoid vaccines (Nohynek et al., 1999). These studies suggest that passively acquired antibodies play a role in suppressing antibody responses to immunization during early childhood, but do not influence responses to booster vaccinations. No evidence exists to suggest that maternal cellular immune response can be transferred to the child.

The exact mechanism by which transferred maternal antibodies in children interfere with their immune response to vaccines is still unclear. However, there is a suggestion that the interference may be epitope specific, and mainly affects B-lymphocyte responses (Siegrist, 2003). It may be related to maternal antibody to vaccine antigen ratio at the time of immunization, which may result in epitope masking. The concept of epitope masking refers to maternal antibodies that disguise vaccine antigenic epitopes, therefore preventing binding of the child's B-lymphocytes to these epitopes; Figure 2.2. It has been suggested that to overcome the epitope masking effect, would be to increase the dose of vaccine antigen, thereby increasing the number of antigenic epitopes that will allow sufficient binding of child's B-lymphocytes (Siegrist, 2003). However, recent data from a cotton rat model of measles virus vaccination suggests that the inhibitory effect of maternally derived IgG on infant B-lymphocyte responses to vaccination is mediated through cross-linking of the inhibitory receptor Fc γ RIIB to the B-lymphocytes receptor by antibody-antigen complexes (Kim et al., 2011) and not through epitope masking as suggested by Siegrist (Siegrist, 2003); resulting in inhibition of B-lymphocyte activation and therefore antibody secretion (Kim et al., 2011). No human studies have been performed to prove this observation.

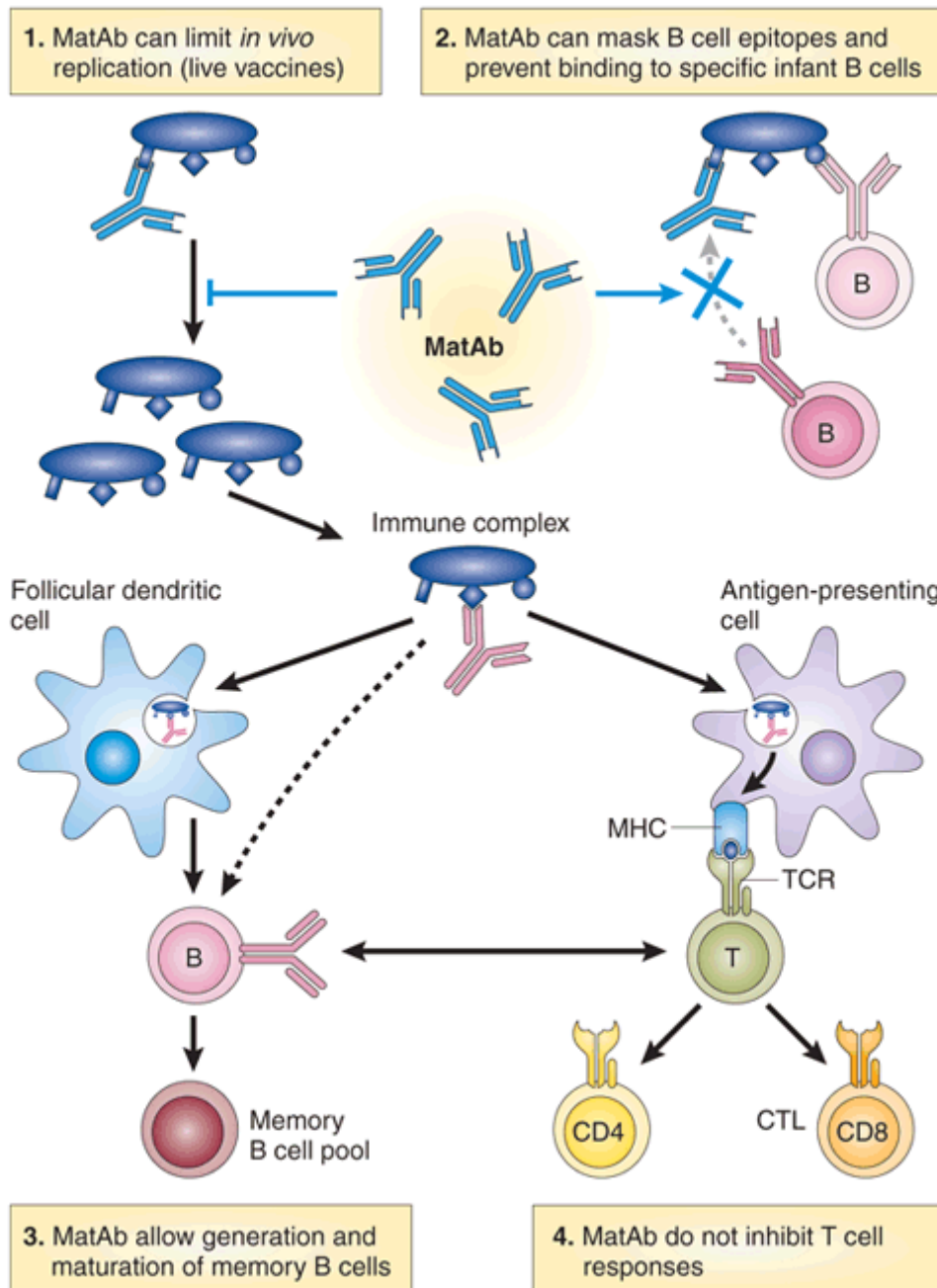


Figure 2.2: The dual effects of maternal antibodies on response to immunization by children. The presence of maternal antibodies inhibits B-lymphocyte responses to masked epitope, but not to the generation of memory B-lymphocytes. Secondly, maternal antibodies also play a role in increasing T-lymphocyte responses by enhancing antigen capture and presentation (Lambert et al., 2005).

2.7.4 HIV and its effects on maternal antibody transfer

Studies have suggested that HIV impairs the transport of IgG from an infected mother to her foetus (de Moraes-Pinto et al., 1998, Farquhar and John-Steward, 2003). Thus, a child born to an HIV-infected woman may be deprived from early protection against disease. In addition, the effective transfer on antibody is dependent on the integrity of the placenta. Diseases such as malaria and HIV have shown a negative impact on transplacental antibody transfer (Farquhar, 2004, Englund, 2007). In the case of malaria, Fc receptors impairment seems to play a role in decreased antibody transfer (Englund, 2007). On the other hand, increased levels of specific HIV antibodies present in maternal blood during the final trimester may obstruct the Fc receptors, thus inhibiting the active transport of antibodies (Farquhar, 2004).

In South Africa, significantly lower concentrations of maternal antibodies against pertussis, tetanus, Hib and pneumococcus were observed in HEU compared to HIV-unexposed children (Jones et al., 2011). In addition, HIV-infected women had significantly low levels of antibodies to PRP (Hib vaccine epitope) and pneumococcus compared to HIV-uninfected woman. However, the levels to tetanus and pertussis were similar between HIV-infected and -uninfected women (Jones et al., 2011). Similarly, a study on *Streptococcus pneumoniae* specific IgG transfer in Malawi showed a low transplacental maternal *S. pneumoniae* antibody transfer in children born to HIV-infected woman (de Moraes-Pinto et al., 1998). However the presence of IgG antibodies to tetanus toxoid and measles were not reduced (Farquhar and John-Steward, 2003, de Moraes-Pinto et al., 1998). On the contrary a study conducted in Kenya showed that maternal HIV-infection was associated with a 52% reduction of tetanus antibodies in children after adjusting for maternal vaccination. The study concluded that HIV interferes with the transfer of maternal antibodies (Cumberland et al., 2007). These observations are not unique to bacterial antibody transfer, as similar findings have been observed for antibodies targeting viral epitopes. A Zambian study showed a decrease in measles antibody transfer from HIV-infected mother to her offspring, resulting in both HIV-infected and HEU children being more susceptible to measles infection by 6 months of age (Scott et al., 2007). These results were further supported by studies conducted in Kenya and Brazil, which also showed lower measles virus antibodies in cord blood of children born to HIV-infected woman (Scott et al., 2005, de Moraes-Pinto et al., 1993). There is limited understanding on the role of HIV viral load mechanism associated with antibody transfer. Although Jones *et al* (2011) found a 23% reduction in the transplacental transfer for PRP

antibody, 40% for pertussis (PT and FHA) and 27% for tetanus antibody, there was no association between maternal CD4⁺ count or HIV viral load and placental transfer (Jones et al., 2011). In contrast, Farquhar *et al* (Farquhar *et al.*, 2005), showed a positive correlation with maternal viral load and reduced transfer of maternal antibodies.

Other studies have shown that mothers with hypergammaglobulinaemia transferred lower levels of antibodies to their children (Hartter et al., 2000). This was confirmed in a Malawian study that showed that maternal HIV-infection and hypergammaglobulinaemia were independently associated with reduced 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 (Hartter et al., 2000). They also observed that despite German mothers having half the IgG measles antibody concentration than detected in Nigerian mothers, they still transferred a higher amount of measles antibody to their foetuses (Hartter et al., 2000).

2.8 VACCINATION FOR THE PREVENTION OF DISEASE

Acquired immunity can be induced either through passive or active immunization. Passive immunization includes the transfer or administration of externally derived antibodies (polyclonal or monoclonal) with the aim of preventing infection in exposed individuals (Warrington et al., 2011). It can occur through transplacental transfer of maternal antibodies to either the developing foetus, or in colostrum and milk during breastfeeding (Horzinek and Thiry, 2009, Offit et al., 2002). However, maternally acquired antibodies have a short life-span, usually declining during the first few months of life (Offit et al., 2002, Siegrist et al., 1998, Siegrist and Lambert, 1998). More importantly, maternally acquired antibodies offer limited immunologic protection (Offit et al., 2002). Alternatively, immediate protection can be achieved by injecting antibody in the form of serum, immunoglobulin or monoclonal antibodies (Horzinek and Thiry, 2009). The latter is used when there is a high risk of infection and limited time for the body to develop its own immune response, or to reduce the symptoms of chronic or immunosuppressive diseases (Warrington et al., 2011).

Active immunization refers to administration of an antigen (attenuated pathogens, inactivated organism or toxin) so as to induce the production of antibodies against a specific target (Horzinek and Thiry, 2009, Warrington et al., 2011). The next section details the immune response to vaccines.

2.8.1 The burden of vaccine preventable diseases

On a global scale; persistence of vaccine-preventable diseases; including morbidity and mortality due to measles, diphtheria, tetanus, pertussis, hepatitis B and *H. influenzae* type b remain high, (Gentile et al., 2010) especially in low-income settings. Despite the significant progress in reducing the death among children under 5 years of age, the progress has lagged in many low-middle income countries, including due to vaccine preventable diseases (Gentile et al., 2010, Offit, 2014). Included among these are death due to diphtheria, tetanus, pertussis, hepatitis B , Hib and measles (Offit, 2014). Deaths attributable to vaccine preventable diseases among children younger than 5 years of age, varies regionally and globally ranging between 25 000 for diphtheria to 371 000 for invasive Hib disease; Table 2.3.

Table 2.3: Worldwide reported incidence, estimated worldwide incidence and prevalence, and deaths of diphtheria, tetanus, pertussis, Hepatitis B, *H. influenzae* type b disease and measles.

Disease	Worldwide incidence (cases/year)	Estimated actual worldwide incidence/prevalence	Deaths	Reference
Diphtheria	4729	Incidence: 50 000	25 000	(Offit, 2014)
Tetanus	8473	Incidence: 500 000-1 million	72 600-200 000	(Ergonul et al., 2016)
Pertussis	117 889	Incidence: 30-50 million	300 000	(Offit, 2014)
Hepatitis B	6-10 million	Prevalence: 400 million	>686 000	(WHO, 2016a)
Hib disease	3 million	Incidence: 8.13 million	371 000	(Watt et al., 2009)
Measles	244 704	Incidence: 344 000	114 900	(Offit, 2014)

Some of the most striking annual rates of infection worldwide include 138 million cases of rotavirus, 80 million cases of varicella and 40 million cases of pertussis (Offit, 2014). The annual deaths due to vaccine-preventable diseases include one million deaths from hepatitis B, 1.6 million deaths from pneumococcus, 450,000 deaths from rotavirus (Offit, 2014).

Although South Africa reportedly has a high immunization coverage (94-97% in 2015 for DTP3, Hib3, HepB3 and MV1) since the inception of the EPI in South Africa in 1995 (WHO, 2016b), outbreaks of vaccine-preventable diseases that have been observed over the last 10 years. One such disease is measles which occurred between 2008 to 2010, with many cases occurring among children exposed to maternal HIV and were too young to have been vaccinated at 9 months of age (Moss and Griffin, 2012). In 2010, more than 20 000 cases were reported in South Africa, which shows the ease with which measles virus can re-enter communities and cause large outbreaks if population immunity is not sustained (Moss and Griffin, 2012). In addition, an outbreak of pertussis was reported between 2008 and 2009 in 18 children, with 12 children being 6 months of age and had incomplete immunization. Of the 12 children younger than 6 months of age, five were HIV-infected and younger than 10 weeks, however, no deaths were reported (Hallbauer et al., 2011). Recently an outbreak of diphtheria was reported, which involved 14 cases of individuals aged 4-40 years of age and 4 deaths which occurred among children who had not been fully vaccinated (NICD, 2015).

2.8.2 History of childhood immunization

The WHO recommends immunization against a number of serious infectious diseases, including diphtheria (WHO, 2006a), tetanus (WHO, 2006b), pertussis (WHO, 2005), HepB (WHO, 2009a), invasive Hib disease (WHO, 2013b), and measles (WHO, 2009b) for all children, as well as pneumococcal disease, yellow fever, and rotavirus disease for children in some areas as part of their EPI. The EPI was established in 1974 with the main objective of expanding immunization services beyond smallpox and with an emphasis on improving vaccine coverage in children from developing countries (Galazka, 1993). The EPI at the time included vaccines against tuberculosis (BCG), polio, diphtheria, tetanus, pertussis and measles (Galazka, 1993). Since their introduction more than 3 million lives have been saved each year, which approximates to 10 000 lives a day and also protects millions from illness and permanent disability as well as averting 6 million deaths globally (Kane and Lasher, 2002, Ehreth, 2003). Recently there has been a drastic increase in the implementation of new and under-utilized vaccines providing additional prevention of premature deaths and disabilities (WHO, 2010c). These include vaccines against HepB, Hib, *S. pneumoniae* (pneumococcus), rotavirus, and rubella (WHO, 2010c).

Pre-exposure immunization of children with several antigens is the main aim of any successful immunization programme (Andre et al., 2008). Specific vaccines have also been utilized to

protect those at risk of infection such as pregnant women, cancer patients and immunocompromised individuals (Succi and Farhat, 2006). However, breakthrough infections have been observed in immunized individuals that may have resulted from primary vaccine failure; in such cases the disease is milder than in unvaccinated individuals (Schmitt et al., 1996, Vazquez et al., 2004, Ruiz-Palacios et al., 2006, Preziosi and Halloran, 2003). This observation has been confirmed on many occasions, for example in Germany people vaccinated with acellular pertussis developed whooping cough, but had a significantly shorter duration of chronic cough than unvaccinated controls (Schmitt et al., 1996), this was also observed for rotavirus and varicella infections (Ruiz-Palacios et al., 2006, Vazquez et al., 2004).

2.8.3 South African EPI (EPI-SA)

The South African EPI programme was established in 1995, and initially included BCG vaccine, oral polio vaccine, and DTwP and measles vaccines. HepB vaccine (1995) and HibCV (1999) were subsequently introduced (Baker, 2010). The success of the EPI programme in South Africa included near elimination of neonatal tetanus by 2002 and the declaration of South Africa being polio-free by 2006. More recently, the EPI vaccine repertoire has been expanded with the inclusion of pneumococcal polysaccharide protein conjugate vaccine (PCV) and rotavirus vaccine in 2009; as well as a change from wP and oral polio vaccine (OPV) to acellular pertussis (aP) and inactivated polio vaccines (IPV) since 2009 (Table 2.4). In addition, a booster dose of HibCV, as part of the pentavalent DTaP-HibCV-IPV vaccine used in the primary series of vaccination was also introduced into the immunization programme in 2009 (Baker, 2010). The EPI-SA schedule is represented in Table 2.4.

Table 2.4: Vaccination schedule for South Africa for the years 2005 and 2013

Age of child	EPI schedule for 2005	EPI schedule for 2013
At birth	OPV (0) BCG	OPV (0) BCG
6-weeks	OPV (1) DTwP/Hib (1) HepB (1)	OPV (1) DTaP-IPV/Hib (1) RV (1) HepB (1) PCV (1)
10-weeks	OPV (2) DTwP/Hib (2) HepB (2)	DTaP-IPV/Hib (2) HepB (2)
14-weeks	OPV (3) DTP/Hib (3) HepB (3)	DTaP-IPV/Hib (3) HepB (3) PCV (2)

		RV (2)
9-months	MV (1)	MV (1) PCV (3)
18-months	OPV (4) DTP (4) MV (2)	DTaP-IPV/Hib (4) MV (2)

Footnote: **(0)** : Birth dose, which doesn't count as part of primary series; **(1)** : First dose in a series; **(2)** : Second dose in a series; **(3)** : Third dose in a series; **(4)** : Fourth dose (booster); **BCG** : Bacille Calmette-Guérin (tuberculosis vaccine); **DTaP-IPV/Hib** (pentavalent) : Diphtheria, tetanus, acellular pertussis/inactivated polio vaccine and *Haemophilus influenzae* type b vaccine; **DTwP/Hib**: Diphtheria, tetanus, whole cell pertussis and *Haemophilus influenzae* type b vaccine combined; **EPI** : Expanded Programme of Immunisation; **HepB** : Hepatitis B vaccine; **MV** : Measles vaccine; **OPV** : Oral polio vaccine; **PCV** : Pneumococcal conjugated vaccine; **RV** : Rotavirus vaccine

Since the introduction of the EPI programme, as well as additional vaccines becoming available worldwide, there has been a major impact on the burden of vaccine-preventable diseases in South Africa; which could be contributing to the decline in under-5 mortality in South Africa (WHO, 2013a).

2.8.4 Immunology of vaccines

Since the success of the smallpox vaccine that was discovered by Edward Jenner in 1796, there have been dramatic reductions in disease incidence as a result of vaccination (Glezen, 2003). These include reduction in disease from viruses including polio virus, hepatitis B virus, measles, mumps and rubella viruses. In addition, control of bacterial infections including that from pertussis, tetanus, diphtheria and many more (Andre, 2003, Walker and Burton, 2008). For these vaccines and many others, protection has been achieved by mimicking infection with the pathogen and thereby establishing immunologic memory that can rapidly respond should an actual infection occur (Walker and Burton, 2008). A perfect example of mimicry is in the use of live-attenuated vaccines such as the measles vaccine, which infects the host without causing severe illness and elicits a strong and long-lasting immune response (Walker and Burton, 2008). A second example is inactivated vaccines such as inactivated-polio virus vaccine and whole cell pertussis which functions in a similar manner as the live-attenuated vaccine but does not replicate within the host. A third approach utilizes exposure of the immune system to recombinant viral proteins alone or toxoids, as in the case of the hepatitis B vaccine and diphtheria and tetanus toxoids (Walker and Burton, 2008).

To be effective and confer long-lasting immunity, a vaccine should be capable of eliciting the following: a) activation of APCs to initiate antigen processing and producing interleukins; b) activation of both B- and T-lymphocytes to give a high yield of memory cells; c) generation of Th and CTLs to several epitopes, to overcome the variation in the immune response in the population due to MHC polymorphism and d) persistence of antigen, probably on follicular dendritic cells in lymphoid tissue, where memory B-lymphocytes are recruited to form antibody secreting cells that will continue to produce antibody. Live-attenuated vaccines fulfil these criteria excellently (Siegrist, 2008). Subunit vaccines induce poor immune responses and several doses with adjuvant are required to get an adequate response. The two main functions of an adjuvant antigen are to keep the antigen at or near the injection site and to activate APCs to achieve effective antigen processing and interleukin production (Janeway et al., 2001d). The main outcome of each vaccination strategy is to induce an immunologic response that will prevent infection or failing that, minimize the severity of illness.

i) Immunology of viral vaccines

The mainstay of protection using vaccines targeting viruses is through the induction of neutralizing antibodies (Warrington et al., 2011). Pre-existing serum or mucosal antibody induced by an earlier infection or through vaccination can bind to free virus particles, prevent viral entry to host T-lymphocytes, resulting in neutralization of the pathogen, and thereby prevent establishment of infection (Walker and Burton, 2008). In the case where host T-lymphocytes become infected; antibodies can bind to such cells, trigger their elimination via host effector mechanisms, and perhaps contribute to abortion of infection. This type of immunity is known as ‘sterilizing immunity’ (Walker and Burton, 2008, Warrington et al., 2011).

Sterilizing immunity has been demonstrated in many animal models where antibodies were administered intravenously or mucosally. However, for human vaccines, antibodies are unlikely to provide sterilizing immunity and instead limit the initial burst of virus replication so that it can then be contained by the on-going immune response without substantial disease symptoms (Walker and Burton, 2008). The second line of defence is the cellular immune response, particularly CTLs. Vaccination with TD antigens generates a population of memory T-lymphocytes that can rapidly expand in the first days after infection (Walker and Burton, 2008). These expanding CTLs kill virus infected cells by recognizing foreign viral proteins that bind to developing MHC-1 molecules and are displayed on the surface, providing a signal that the cell

should be destroyed. Lysis of a virus infected cell before the assembly of mature progeny virions leads to elimination of the virus, which undergoes rapid degradation in the extracellular milieu in the absence of the protective outer envelope. CTLs also release antiviral cytokines which may act to limit the impact of progeny virions already produced (Walker and Burton, 2008).

Many live-attenuated vaccines elicit both neutralizing antibody and CTL responses, the combination of which is likely necessary for rapid elimination of the virus or successful immunologic containment, preventing the development of disease (Walker and Burton, 2008). CTL can also be induced by killed and recombinant vaccines through cross-priming when these vaccine antigens are taken up by professional antigen presenting cells (e.g. dendritic cells) (Walker and Burton, 2008).

In summary, protection against viral infections are mediated by neutralizing antibodies and cytotoxic T-lymphocytes, therefore both these arms of immune response need to be correctly balanced, (i.e. they need the correct type of cells being activated, according to tissue type and type of pathogen for an effective elimination of the pathogen and control) thus resulting in clearance of the infection and prevention of severe disease, as well as immunological memory against that pathogen (Kalinski et al., 2001).

ii) Immunology of bacterial vaccines

The acquired immune response may exert its effects either independently of certain innate defence mechanisms for example neutralization of bacterial exotoxin by antitoxic antibodies. Alternatively, they may function in concert with other components of the host immune system, for example when antibodies opsonize infectious particles or when antibodies interact with the complement pathway and an infectious microorganism resulting in lysis of the microorganism (Benjamin, 1996a).

Antitoxic immunity is predominantly associated with IgG, although IgA many also neutralize exotoxins such as diphtheria toxin (Benjamin, 1996a). Exotoxins bind firmly to their target tissue; they generally cannot be displayed by the subsequent administration of antitoxin. In those diseases mediated by exotoxins (diphtheria and tetanus) prompt administration of antitoxin is necessary to prevent attachment of additional exotoxin and the damage caused by the exotoxin

(Benjamin, 1996a). The presence of antitoxic activity in IgG means that an adequately immunized mother can transmit antitoxin to her fetus and thus confer protection to the neonate (Benjamin, 1996a). Antibacterial immune responses include lysis of bacterial cells via antibody and complement, opsonization and phagocytosis with elimination of phagocytosed bacteria by the liver, spleen and other components of the reticuloendothelial system.

Opsonization and phagocytosis of gram-positive bacteria involves the action of IgG and IgM alone or with *C3b*, which promotes phagocytosis of bacteria by polymorphonuclear cells. The alternative complement pathway may be non-specifically triggered by the lipopolysaccharide endotoxin found in the cell wall of gram-negative bacteria, or by the polysaccharide of gram-positive and gram-negative bacteria acting on *C3*. This alternative complement pathway results in the formation of chemotactic molecules *C3a* and *C5a* and the opsonin *C3b*. In addition to this, the activation of the alternative complement pathway may lead to immune adherence and bacteriolytic action of *C5-C9*. The classical lytic complement pathway operates in conjunction with IgM against gram-negative bacteria. Cell mediated immunity is active against certain bacteria characterized by their intracellular habitat e.g. *Mycobacterium tuberculosis* (Benjamin, 1996a).

2.8.5 Early life vaccine antibody responses

Vaccine induced protection relies on the production of sufficient concentration of antigen specific antibodies to allow neutralization of pathogen at mucosal or immediately after pathogen invasion (Siegrist, 2001). Vaccines that have been shown to be successful examples of antibody mediated protection at preventing both colonization and invasive disease include polysaccharide-protein conjugate *Haemophilus influenzae type b* and *Streptococcus pneumoniae* vaccines (Mulholland et al., 1997). In contrast, vaccine induced antibodies against many viral pathogens (Measles, Hepatitis B, polio and influenza, etc.) are unable to fully prevent viral infection, such that viral clearance and hence clinical disease prevention rely on both antibody-mediated-neutralization and cell mediated direct (toxicity) and indirect (cytokine release) effects (Siegrist, 2001). All childhood vaccines developed to date (with the exception of BCG) require more than one dose in order to provide adequate protection from disease when administered before 6 months of age (Siegrist, 2001, Plotkin, 2010). This can be noted by the persistent risk of pertussis or Hib in partly vaccinated children. This slow development of vaccine mediated protection in

children is directly correlated with the progressive maturation of their immune system, as well as that multiple antigen exposure through vaccination is required for inducing high-avidity antibody (Siegrist, 2001).

Immune responses to vaccination are complex and require the integration of several components of the immune system that include both B- and T-lymphocytes, which are still undergoing development in the first year of life (Siegrist, 2001, Pensiero et al., 2009, Butler et al., 2009, Jaspan et al., 2006). HIV-infection has been shown to cause detrimental effects on these components of the immune system in a time dependant manner (Pensiero et al., 2009). Pensiero and others have, however, proposed that initiation of ART in the first year of life allows the normal development of an intact T-lymphocyte repertoire (Zanchetta et al., 2008, Violari et al., 2008, Romiti et al., 2001, Palma et al., 2008, Pensiero et al., 2009), as well as the B-lymphocyte compartment and limit the deterioration of antigen specific pools of memory B-lymphocytes that could occur in the absence of ART (Pensiero et al., 2009). Although quantitative measures are frequently performed to evaluate immune recovery (example: assays of CD4⁺ T-cell number and Ig concentrations), these measures do not fully evaluate functional recovery (Pensiero et al., 2009). Understanding the degree of functional immune recovery after ART initiation may be important in guiding vaccine administration.

2.8.6 Correlates of protection

Correlates of protection in vaccine-induced immunity is important not only for research purposes, but for linking immunity with protection. These factors provide insight into the reliability of vaccine production and protection at the community level (Plotkin, 2010). According to Plotkin a correlate is “an immune response that is responsible for a statistically interrelated protection” (Plotkin, 2010). Defining protection is the first step in determining correlates of protection. The mechanism by which protection is achieved by vaccination may vary by the type of immune response required against the pathogen. In addition, the ideal way of evaluating correlates of protection involve the assessment of longitudinal studies with the end outcome being those that get the disease (Plotkin, 2010).

Most vaccines induce antibodies of which correlates of protection may relate to functionality and specific concentrations. In addition, immune memory is also a critical measure of correlates of protection (Plotkin, 2010).

2.9 SELECTED VACCINES OF THE EPI

2.9.1 Vaccination in HIV-infected children

In 2010, 50% of the close to 8 million deaths in children <5 years were due to infectious diseases (WHO, 2013a), of which approximately half were vaccine-preventable (WHO, 2013a). National immunization programmes represent major milestones in preventative medicine and public health (Levine, 2011, Clemens et al., 2010), contributing to a reduction in under 5 mortality from 12 million deaths in 1990 to 7.6 million in 2010 (WHO, 2013a). Nevertheless, an estimated 3 million children under 5 years died in 2010, due to vaccine preventable diseases (Clemens et al., 2010).

The majority of available vaccines have been developed through approaches where the efficacy has been studied in relatively healthy populations. The diminished level of protective immune response in immunocompromised people remains problematic. In addition, there are data to suggest that immunization coverage may be less in HIV-exposed children (Healy et al., 2013). A survey of over 2000 children between the ages of 12 and 23 months in South Africa found that maternal HIV status was a risk factor for decreased immunization coverage for several childhood immunizations (Ndirangu et al., 2009).

Even in those that are relatively healthy, immunization schedules are being updated continuously in accordance with scientific knowledge, epidemiology and advances in vaccine design (Menson et al., 2012) and this is also country specific (Geretti and Doyle, 2010). There are uncertainties with regards to the correct vaccination schedule among HIV-infected children (Geretti and Doyle, 2010). The status of selected single mandatory and recommended vaccines for HIV-infected children in relation to ART strategies prior to and post ART initiation compared with vaccination outcomes of healthy children, will be reviewed below.

2.9.2 Non-replicating vaccines

Non-replicating vaccines have been reportedly found to be safe in HIV-infected children, although antibody responses may be suboptimal especially in those with immunosuppression (Moss et al., 2003). The WHO has recommended that all HIV-infected children be immunized with inactivated vaccines including diphtheria, tetanus, pertussis, HibCV and hepatitis B vaccine. Immune response among HIV-infected vaccinees already on ART varies depending on the

vaccine administered (Sutcliffe and Moss, 2010), sero-protective responses range from 40-65% for DT (Zaccarelli-Filho et al., 2007), 38-65% for TT (Ching et al., 2007, Rigaud et al., 2008, Farquhar et al., 2009, Zaccarelli-Filho et al., 2007) and 1-100% for hepatitis B (Fernandes et al., 2008, Siriaksorn et al., 2006, Abzug et al., 2009, Zaccarelli-Filho et al., 2007).

2.9.2.1 Diphtheria toxoid vaccine

Infection with *Corynebacterium diphtheriae* causes symptoms of diphtheria. The pathogenic nature of *C. diphtheriae* is caused by an exotoxin, the gene for which is located in a prophage (Efstratiou et al., 2000). The toxin is a 62 kilodalton polypeptide composed of two fragments (A and B) of different functionality (Demirci, 2011). The toxin binds to receptors on the target cell via fragment B and is absorbed into the cell. Once inside the cell, fragment A blocks the synthesis of proteins irreversibly (WHO, 2006a). Two syndromes may occur from *C. diphtheriae* infection: the first is local infection and second is primary systemic toxicity. Diphtheria vaccines are based on diphtheria toxoid that induces protective antitoxin. Diphtheria toxoid is part of a triple component vaccine DTP and has been part of the WHO EPI since 1974. During 1980-2000 the total number of reported diphtheria cases has decreased by >90% (WHO, 2006a). Following primary immunization the average duration of protection afforded by the diphtheria vaccine is said to be 10 years, however, boosting of immunity is required either by exposure to wild-type strains or by booster vaccination at early school age (WHO, 2006a).

In the era before HIV-infection, responses to the diphtheria-toxoid vaccine were 100% (WHO, 2006a), while only 18-76% of HIV-infected persons develop protective antibody concentrations (Borkowsky et al., 1987, Borkowsky et al., 1992, Ryder, 1993, Zaccarelli-Filho et al., 2007); Table 2.5. HIV-infected persons develop lower geometric mean concentrations than HIV-uninfected persons after primary vaccination. Furthermore, there is greater waning of antibody concentration over time (Tejiokem et al., 2007). Between 18-71% (Table 2.5) of HIV-infected children not on ART, who developed protective levels of antibodies post primary vaccination, retained these levels 6-36 months after vaccination (Borkowsky et al., 1987, Tejiokem et al., 2007). There is limited information on the immunogenicity of the diphtheria-toxoid vaccine in sub-Saharan Africa (Table 2.5) and especially in countries with a high prevalence of HIV-infection (Tejiokem et al., 2007, Ryder, 1993). Additionally, studies have not systemically evaluated the effect of timing of ART on the primary and memory immune response in HIV-

infected children (Essajee et al., 1999, Zaccarelli-Filho et al., 2007). To date, only two studies have studied immune response to DT in HEU children in comparison with HIV-unexposed children. In both studies no differences in sero-protective threshold (>0.1 IU/ml) was observed between HEU and HIV-unexposed children (Abramczuk et al., 2011, Ryder, 1993). From these studies, it can be seen that there is limited data on response to diphtheria-toxoid vaccine in the era of high HIV-prevalence as well as increased access to ART. None of the studies conducted thus far have evaluated the effects of ART on primary vaccine response, as well as anamnestic response to booster vaccination. Furthermore, there is a paucity of data with regards to response to diphtheria-toxoid in HEU children.

Table 2.5: Comparison of antibody response to diphtheria-toxoid epitope between HIV-infected, HEU and/or HIV-unexposed children

Study setting and Reference	Study design	HIV-infected N (Age at study entry)	HEU children N (Age at study entry)	HIV-unexposed children N (Age at study entry)	Vaccination schedule and timing of blood sample	Number on ART	Initiation of ART prior to vaccination and duration on ART	CD4+ at study entry	Main findings HIV-infected only [#] or (HIV-infected vs. HIV-unexposed) [†] or (HIV-infected vs. HEU) ^{††} or (HEU vs. HIV-unexposed) ^{†††}	Summary
HIV-infected only										
1987 USA (Borkowsky et al., 1987)	NS	17 (11-90 months)	ND	ND	DTP: 3 doses Bloods: NS	ND	ND	ND	>0.02 U/ml; N/n (%): 3/17 (18%) [#] CMI, N/n (%): 8/17 (47%)	Sub-optimal response seen after primary vaccination. 8/17 had cell mediated response, of which they 5 had no evidence of antibody response to DT
1999 USA (Essajee et al., 1999)	Cohort	25 (8.9 years)	ND	ND	Previously vaccinated [†] n=15: 3 doses followed by booster doses at 4-6 years and 12 years Re-vaccination [‡] n=6: 1 dose Bloods: NS [†] and 1-2 months [‡]	25	ART was initiated post vaccination in the previously vaccinated children and were on ART for 18.8 months prior to blood sampling. ART initiated 16 months prior to booster dose in 6 children who were re-vaccinated.	2%	LPA SI>3: 0% ^{†#} LPA SI>3 N/n (%): 0/6 (0%) ^{#‡}	Sub-optimal response to vaccination, with ART not improving immunological memory responses to additional doses of DT vaccine.

HIV-infected vs. HIV-unexposed										
1993 Zaire (Ryder, 1993)	Non-randomized prospective cohort	48 (NS)	202 (NS)	440 (NS)	DTP at 6, 10, 14 weeks Bloods: 9 months	ND	ND	ND	<0.01, IU/ml, N/n (%): 14/48 (29.7%) vs. 19/440 (4.3%), p<0.001^{*†} 14/48 (29.7) vs. 3/202 (1.5%), p<0.001^{*††} ≥0.01-0.09 IU/ml, N/n (%): 29/48 (60.4%) vs. 260/440 (59%), p=1.00^{*†} 29/48 (60.4%) vs. 114/202 (56%), p=0.63^{*††} ≥0.1 IU/ml, N/n (%): 5/48 (10%) vs. 161/440 (37%), p<0.001[†]; 5/48 (10%) vs. 85/202 (42%), p<0.001^{††} GMT, IU/ml: 0.018 vs. 0.070 and 0.018 vs. 0.080 p<0.05 significant difference between HIV-infected vs. HIV-unexposed and HEU	Difference between HIV-infected vs. HEU and HIV-unexposed for DT ≥0.1 IU/ml and GMT.
2007 Brazil (Zaccarelli-Filho et al., 2007)	Observational	Good VLR: 9 (11 years) Partial VLR: 13 (7.4 years) Poor VLR: 14 (9.4 years)	ND	20 (11 years)	DTP: 2, 4, 6 months Booster 15 months Booster 15 4-6 years Bloods: NS	41	Good VLR: initiated prior to 2nd booster dose at 5.8 years of age Partial VLR: initiated prior to 2nd booster dose at 3.7 years of age Poor VLR: Initiated post 2nd booster dose at 7.3 years	Good VLR: 33% Partial VLR: 26% Poor VLR: 37%	>0.1 IU/ml: Good VLR: 65% vs. Partial VLR: 61% vs. Poor VLR: 40%, p=0.239 Mean antibody levels IU/ml, (95% CI): Good VLR: 0.26 (0.005-20.8) vs. Partial VLR: 0.156 (0.001-15.1) vs. Poor VLR: 0.36 (0.001-22.3), p=0.172	Weak response between levels of viral control compared to the HIV-unexposed control group.

							of age			
HIV-infected vs. HEU										
1992 USA (Borkowsky et al., 1992)	Prospective	33 (<4 years)	53 (<4 years)	ND	DTP: primary series 2, 4, 6 months Booster 18 months Bloods: 2 and 6 months	ND	ND	ND	>0.02 U/ml, N/n (%): 21/24 (88%) vs. 10/10 (100%), p=0.54 ^{††††} >0.02 U/ml, N/n (%): 7/13 (54%) vs. 8/8 (100%), p=0.048 ^{††††}	No differences between humoral responses in HIV-infected vs. HEU after primary doses. Attrition in the 2 nd year of life resulted in differences being observed in HIV-infected vs. HEU.
2007 Cameroon and Central Africa (Tejiokem et al., 2007)	Cross sectional	51 (25 months)	78 (25 months)	ND	DTwP: 6, 10, 14 weeks 18 months booster Bloods: 17 months (range: 3.9 to 31 months)	33	No ART was initiated prior to primary or booster dose. ART was initiated 6 months before blood sampling.	<15 to >25%	>0.1 IU/ml, N/n(%): 22/44 (50%) vs. 51/66 (77%), p=0.003 >0.1 IU/ml, N/n(%): 5/7 (71%) vs. 12/11 (92%), p=0.29 GMC IU/ml, (95% CI): 245 (166-361) vs. 312 (246-396), p=0.13 Proportion of children with CD4+<25% had low DT titers (OR: 4.1; 95% CI: 1.7-10.1, p=0.002)	Significantly reduced titers in HIV-infected vs. HEU
HEU vs. HIV-unexposed										
1993 Zaire (Ryder, 1993)	Non-randomized prospective cohort	ND	202 (NS)	440 (NS)	DTP at 6, 10, 14 weeks Bloods: 9 months	ND	ND	ND	<0.01, IU/ml N/n (%): 3/202 (1.5%) vs. 19/440 (4.3%), p=0.10 ^{††††} >0.01 IU/ml N/n (%): 114/202 (56%) vs. 260/440 (59%), p=0.55 ^{††††} >0.1 IU/ml: 85/202 (42%) vs. 161/440 (37%), p=0.19 ^{††††} GMT, IU/ml: 0.080 vs. 0.070, p=NS	No difference in sero-protective antibody titers ≥0.01 and ≥0.1 IU/ml as well as GMT between HEU vs. HIV-unexposed
2011 Brazil (Abramczuk et al., 2011)	NS	ND	19 (7.7 months)	112 (7.5 months)	DTP-Hib at 2, 4, 6 months Bloods: 16 weeks	ND	ND	ND	>0.1 IU/ml N/n(%): 18/19 (95%) vs. 110/112 (98%), p=0.38 ^{††††} GMT, IU/ml (95% CI): 0.526 (0.289-0.967) vs. 0.559 (0.465-0.67), p=0.81	No difference between sero-protective titers and GMT for HEU vs. HIV-unexposed

Footnote: HEU: HIV-exposed, uninfected; N: total number of participants; ART: antiretroviral therapy; ND: not done; LPA= lymphoproliferative assay; SI= stimulation indices; NS= not stated; [†] previously vaccinated in early childhood; [‡] re-vaccination: children who received all primary and booster doses in early childhood, and received a full series of vaccination in older age; [√] response after primary vaccination; ^{√√} response to booster vaccination; IU/ml: international

units per milliliter; IQR: interquartal range; VLR: viral load responders; GMT/C: geometric meant titer/concentration; *Calculated from data given in paper, using Fischer' exact test; DTP: diphtheria, tetanus, pertussis vaccine; DTwP: diphtheria, tetanus, whole cell pertussis vaccine; Hib: *Haemophilus influenzae* type b; CI; confidence interval.

2.9.2.2 Tetanus toxoid vaccine

Neonatal tetanus is a potentially fatal infection that is caused by *Clostridium tetani*. Maternal and neonatal tetanus, is the most common form of tetanus in developing countries, and was responsible for approximately 5% of global neonatal deaths in 2000 (WHO, 2004, Vandelaer et al., 2003). A significant reduction in maternal and neonatal associated infection has been reported following the introduction of tetanus vaccine in pregnant women (WHO, 2006b). In 2004 an estimated 290 000 individuals had died as a result of tetanus infection this included 257 000 cases of neonatal tetanus (WHO, 2004). The World Health Assembly called for the elimination of neonatal tetanus, with elimination being defined as less than 1 case per 1000 live births at district level (WHO, 2006b). 2005 was the target date for the global elimination of neonatal tetanus by all member states of WHO, United Nations Children's Fund (UNICEF) and the United Nations Population Fund (UNFPA) (Esen et al., 2007). So far 104 developing countries had achieved this goal by 2000 (WHO, 2004). Exceptional progress has been made in the 15 years since the implementation of the neonatal tetanus elimination programme, worldwide mortality from neonatal tetanus was estimated at 180 000 in 2002, which represents a 78% reduction since the late 80s (WHO, 2006b). In developed countries, maternal and neonatal tetanus are exceedingly rare (Pascual et al., 2003, Rushdy et al., 2003), however, deaths due to neonatal tetanus continue to be reported in low-income countries from South Asia and Africa (WHO, 2006b).

Tetanus-toxoid is a commonly included as part of the DTP combination vaccines. It is prepared by formaldehyde treatment of cell free tetanus toxin, which is adsorbed to an adjuvant (WHO, 2006b). It is given as a 3 dose primary series, in addition to which it is recommended that children receive a booster dose before school entry (3-5 years) and at 13-18 years of age (WHO, 2004). Tetanus-toxoid antigen is one of the most effective, safe, stable and inexpensive vaccines to be developed, and can be safely given during pregnancy and to immunosuppressed individuals (Balmer et al., 2007). The duration of protection is dependent on dose and on the age at which it is administered (Balmer et al., 2007). Antibody responses to the first dose develop slowly and consist of mainly non-neutralizing IgM and a small amount of IgG, this response is insufficient to provide protection. After the second dose, approximately 90% of people develop protective antibody levels, which however declines to <80% within a year. A third dose induces sero-protective titers in 98% of recipients, which remains high for several years (Balmer et al., 2007). The long-term protection has been observed after receiving 5-6 doses, and lasts for 20-25 years if

the primary vaccination series was administered during infancy, and booster doses are given during late childhood and adolescence (Simonsen et al., 1987, de Melker et al., 1999, Matouskova et al., 2005).

Children infected with HIV generally mount a protective response to tetanus toxoid; Table 2.6. Low levels of antibody titers against tetanus in HIV-infected individuals have been previously reported, and found to be more common amongst those with CD4+ cell counts less than 300cell/ μ l (Dieye et al., 2001, Rosenblatt et al., 2005, Lederman et al., 2003, Talesnik et al., 1998, Bonetti et al., 2004). In a study conducted in Brazil on maternal tetanus IgG transplacental transfer, it was observed that HIV-infected mothers transferred fewer antibodies than HIV-uninfected mothers (Rosenblatt et al., 2005), however, this was not evident in Zambia (de Moraes-Pinto et al., 1996). Persistence of these tetanus antibodies in HIV-infected children has been shown to be short lived as compared to HIV-uninfected control of similar age (Rosenblatt et al., 2005). They also showed that HIV-infected children with relatively well-preserved immune function who initiated ART had partial reconstitution, and responded well to re-vaccination (Rosenblatt et al., 2005).

The majority of studies conducted in HEU children, have indicated similar responses to primary TT vaccination (3 dose schedule) when compared to HIV-unexposed children; Table 2.6 (Abramczuk et al., 2011, Reikie et al., 2013, Jones et al., 2011, Garcia-Knight et al., 2015, Hesseling et al., 2016), except for one from Brazil in which GMTs were lower in HEU than HIV-unexposed children (Abramczuk et al., 2011). Furthermore, there is only a single study which evaluated booster response to TT at 18-months of age (received a 3 dose primary vaccination series 6, 10 and 14 weeks), which reported higher GMCs in HEU compared to HIV-unexposed children (Reikie et al., 2013).

Table 2.6: Comparison of vaccination response to tetanus-toxoid epitope between HIV-infected children and HEU and/or HIV-unexposed children

Study setting And Reference	Study design	HIV-infected N (Age at study entry)	HEU children N (Age at study entry)	HIV-unexposed children N (Age at study entry)	Vaccination schedule and timing of blood sample	Number on ART	Initiation of ART prior to vaccination and duration on ART	CD4+ at study entry	Main findings HIV-infected children only [#] or (HIV-infected vs. HIV-unexposed) [†] or (HIV-infected vs. HEU) ^{††} or (HEU vs. HIV-unexposed) ^{†††}	Summary
HIV_infected only										
1987 USA (Borkowsky et al., 1987)	NS	17 (11-90 months)	ND	ND	DTP: 3 doses Bloods: NS	ND	ND	ND	>0.001 U/ml; N/n (%): 9/15 (60%) ^{#v} CMI, N/n (%):8/13 (62%) ^{#v}	Sub-optimal response seen after primary vaccination.
1999 USA (Essajee et al., 1999)	Cohort	25 (8.9 years)	ND	ND	Previously vaccinated [†] n=15: 3 doses followed by booster doses at 4-6 years and 12 years Re-vaccination [‡] n=6: 1 dose Bloods: NS [†] and 1-2 months [‡]	25	ART initiated prior to booster dose in 6 children who were re-vaccinated	2%	LPA SI>3: 0% ^{†#} LPA SI>3 N/n (%): 3/6 (50%) [‡] ; 1/6 (16.6%) had an additional booster 6 weeks post re-vaccination	Sub-optimal response to vaccination even after re-vaccination and being on ART for more 16 months prior to booster dose.
2005 USA (Rosenblatt et al., 2005)	Randomized clinical trial	37 (6.1 years)	ND	ND	DTaP booster at either 16 or 36 weeks Bloods: 4, 8, 18, 32 weeks	37	ART initiated at 16 weeks or 36 weeks prior to booster dose	ND	>1:243 ^{#vv} 74% vs. 67% vs. 53% vs. 38% at 4, 8, 18 and 32 weeks after vaccination	Increase in tetanus titers after vaccination, with an increase of 27-fold over the baseline values at weeks 4 and 8. The effect on tetanus titers faded to a 9-fold and 3-fold increase over baseline values at weeks 18 and 32, respectively.

2007 USA (Ching et al., 2007)	Prospective	15 (12 years)	ND	ND	Booster TT Bloods: 6 years post primary, 2.3 months post booster	15	No ART initiated post primary, but initiated ART at booster and on ART for 2 years prior to blood sampling.	35%	>0.15IU/ml: Pre-booster:: 38% [√] 92% after booster ^{√√}	HIV-infected children had modest responses to TT post booster vaccination
2008 USA (Rigaud et al., 2008)	Randomized comparative response	Group 1: 22 (13 years) Group 2: 24 (12 years)	ND	ND	Group1: DTaP, Td or DT at 8, 16, 24 weeks of the study Group 2: 32, 40, 48 weeks in the study Bloods: 12, 28, 36, 52, 76, 100 weeks	46	Initiated ART prior to re-vaccination in both groups. Duration on ART was not stated	7% in both groups	>0.1IU/ml N/n (%): Group 1: 18/18(100% vs. Group 2: 11/20(55%), p=0.001 [#] Weeks 28 median titre (IQR) IU/ml: Group 1: 3.63 (1.99-6.19) vs. 0.11 (0.06-0.47), p<0.001 [#]	Significant differences were seen between children receiving vaccination and not receiving vaccination
2009 Kenya (Farquhar et al., 2009)	Prospective	90 (4.9 years)	ND	ND	Re-vaccination [†] Bloods: 4 weeks	90	No ART post primary ART given 8 months prior to booster dose.	6.3	>0.01IU/ml 75% ^{##}	Sero-conversion was associated with older age (p=0.03).
2009 Italy (Pensiero et al., 2009)	Cross sectional	70 (Early treatment: 6.8 years: Late control: 13.7 years Late failure: 15.8 years Treatment naïve: 11.8 years)	ND	ND	Vaccination NS Bloods: Early control: 5.3 years Late control: 7.4 years Late failure: 9.7 years Treatment naïve: 5.0 years	64	ART initiated in early and late controller and late failure. Duration on ART was stated.	Early treatment : 35% Late control: 33% Late failure: 22% Treatment naïve: 25%	Early control: 92% Late control: 38% Late failure: 25% Treatment naïve: 100%	Antibodies were higher in the early-treated compared with the late-treated patients (p= 0.02). The late control group and for the late failure group p= 0.05. Significant difference was also found between the late failure and the treatment naïve group (p= 0.01).
HIV-infected vs. HIV-unexposed										
1993 Zaire (Ryder,	Non-randomized	48 (NS)	202 (NS)	440 (NS)	DTP at 6, 10, 14 weeks	ND	ND	ND	≥0.01IU/ml, N/n (%): 46/48 (96%) vs. 418/440 (94%),	No difference between percentage with sero-protective antibody titers in

1993)	prospecti ve cohort				Bloods: 9 months				$p=1.00^{*†\vee}$ 46/48 (96%) vs. 190/202 (94%), $p=1.00^{*††\vee}$ GMT, IU/ml: 9.1 vs. 9.1[†][∨] and 9.1 vs. 7.6^{††∨} $p>0.05$ no significant difference between HIV-infected vs. HIV- unexposed and HEU	HIV-infected vs. HEU and HIV-unexposed
2007 Brazil (Zaccarelli- Filho et al., 2007)	Observat ional	Good VLR: 9 (11 years) Partial VLR: 13 (7.4 years) Poor VLR: 14 (9.4 years)	ND	20 (11 years)	Previously vaccinated: DTP: 2, 4, 6 months Booster 15 months and 4-6 years Bloods: NS	41	Good VLR: initiated prior to 2nd booster dose at 5.8 years of age Partial VLR: initiated prior to 2nd booster dose at 3.7 years of age Poor VLR: Initiated post 2nd booster dose at 7.3 years of age	Good VLR: 33% Partial VLR: 26% Poor VLR: 37%	>0.1 IU/ml: Good VLR: 71% vs. Partial VLR: 77% vs. Poor VLR: 73%, $p=0.851^{#\vee\vee}$ Mean antibody levels IU/ml, (95% CI): Good VLR: 0.26 (0.005-20.8) vs. Partial VLR: 0.156 (0.001- 15.1) vs. Poor VLR: 0.36 (0.001-22.3), $p=0.823^{#\vee\vee}$	Weak response between levels of viral control compared to the HIV- unexposed control group.
2009 Germany (Ghosh et al., 2009)	Cross sectional	48 (9.36 years)	ND	62 (6.03 years)	DTP at 2, 3, 4 and 11-14 months Booster at 5- 6 years Bloods: 7 years	9	No ART was initiated prior to either primary or booster doses. ART was initiated >12 months prior to blood sampling.	68.9%	SI: before initiation of ART 36 months after primary vaccination mean (SD), EU/ml: 0.67 (0.9) 84 months after booster mean (SD):3.3 (1.8)	All children had specific immunity to TT at an average of 7 years post vaccination. Three children had spontaneous reconstitution of tetanus antibody
HIV-infected vs. HEU										
1992 Italy	NS	20 (18-24 months)	52 (8-24 months)	ND	2 or 3 doses	ND	ND	ND	>0.1 IU/ml, N/n %; (95% CI):	Symptomatic HIV could be considered one of the

(Barbi et al., 1992)					Bloods: 9 months for HIV-infected 4 months for HEU				13/17 (76.5%) vs. 39/41(95.1%), p=0.055 ^{††} GMT, antitoxin titer $\geq$ 1:1024: 1:1737 vs. 1:5884, p<0.05 ^{††}	causes of short persistence of antibody.
2007 Cameroon and Central Africa (Tejiokem et al., 2007)	Cross sectional	51 (25 months)	78 (25 months)	ND	DTwP: 6, 10, 14 weeks Booster at 18 months Bloods: 17 months (range: 3.9 to 31 months)	33	No initiation of ART prior to either primary or booster dose. ART was initiated 6 months prior to blood sampling.	<15 to >25%	>0.1 IU/ml, N/n(%) ^{††} : 36/44 (82%) vs. 64/66 (97%), p=0.01 >0.1 IU/ml, N/n(%) ^{††} : 7/7 (100%) vs. 12/112 (100%), p=NE GMC IU/ml, (95% CI): 289 (228-367) vs. 491(396-609), p=0.001 Proportion of children with CD4+<25% had low DT titres (OR: 7.3; 95% CI: 1.3-39.9, p=0.02)	Significantly reduced titres in HIV-infected vs. HEU children Post primary vaccination Similar proportion protected post booster vaccination GMC was lower in HIV-infected than HEU
1992 USA (Borkowsky et al., 1992)	Prospective	33 (<4 years)	53 (<4 years)	ND	DTP: primary series 2, 4, 6 months Booster 18 months Bloods: 2 and 6 months	ND	ND	ND	>0.01 U/ml, N/n (%): 24/24 (100%) vs. 10/10 (100%), p=NE ^{††} >0.01 U/ml, N/n (%): 10/13 (77%) vs. 8/8 (100%), p=NE ^{††}	No differences between humoral responses in HIV-infected vs. HEU after primary and booster doses.
HEU vs. HIV-unexposed										
2010 Uganda (Elliott et al., 2010)	NS	ND	$\pm$ 129 (NS)	$\pm$ 1304 (NS)	DTwP-HepB-Hib at 6, 10, 14 weeks Bloods: NS	ND	ND	ND	Reduced IFN- γ , IL-5 and IL-13 responses to tetanus toxin in HEU children ^{†††}	Reduced response but not significant between HEU vs. HIV-unexposed
2011 Brazil (Abramczuk et al., 2011)	NS	ND	19 (7.7 months)	112 (7.5 months)	DTP-Hib at 2, 4, 6 months Bloods: 16 weeks	ND	ND	ND	>0.1 IU/ml N/n(%): 19/19 (100%) vs. 108/112 (98%), p=NE ^{†††} GMT, IU/ml (95% CI): 1.520 (1.247-1.851) vs. 2.712 (2.258-3.258),	No difference between proportion protected in HEU vs. HIV-unexposed HEU had reduced GMT to HIV-unexposed

									p=0.013 ^{√††}	
2011 South Africa (Jones et al., 2011)	Prospect ive cohort	ND	38 (<4 months)	55 (<4 months)	DTwP-Hib at 6, 10, 14 weeks Bloods: 16 weeks	ND	ND	ND	No difference after 3 doses, median concentration in infants not completed all 3 doses, mg/L (IQR): 1.86 (0.51- 2.21) vs. 0.50 (0.10- 0.93), p=0.01 ^{√†††} Fold increase (IQR): 14.0 (3.26-116.2) vs. 12 (2.8-136.35), p=0.54 ^{√†††}	Similar results between HEU and HIV-unexposed
2013 South Africa (Reikie et al., 2013)	Prospect ive cohort	ND	27 (3 months)	28 (3 months)	DTwP-Hib at 6, 10, 14 weeks Booster at 18 months Bloods: 3, 6, 12, 18, 24 months	ND	ND	ND	No differences post- primary DTP, but higher post-booster DTP in HEU infants, p<0.05 No differences in proportion protected ^{†††}	Similarities between HEU and HIV-unexposed post primary vaccination; and HEU had significantly higher titres post-booster dose
2015 Kenya (Garcia- Knight et al., 2015)	Cross sectional	ND	8 (3 months) 26 (12 months)	8 (3 months) 17 (12 months)	DTwP- HepB-Hib at 6, 10, 14 weeks Bloods: 3, 12 months	ND	ND	ND	No difference in response to TT antigen at 3 months ^{†††} At 12 months, lowered CD4+ IL-2 response and dual IL-2 TNF- α response to TT antigens in HEU children	Similar response post primary vaccination in HEU vs. HIV-unexposed Reduced responses at 12 months post primary vaccination
2016 South Africa (Hesseling et al., 2016)	NS	ND	118 (NS)	60 (NS)	DTwP-Hib at 6, 10, 14 weeks Bloods: 14, 24, 52 weeks	ND	ND	ND	GMCs and proportions protected similar between HEU and HIV-uninfected children ^{†††}	Similar between HEU and HIV-unexposed

Footnote: HEU: HIV-exposed, uninfected; N: total number of participants; ART: antiretroviral therapy; ND: not done; LPA= lymphoproliferative assay; SI= stimulation indices; NS= not stated; [†] previously vaccinated in early childhood; [‡] re-vaccination: children who received all primary and booster doses in early childhood, and received a full series of vaccination in older age; [√] response after primary vaccination; ^{√√} response to booster vaccination; IU/ml: international units per milliliter; IQR: interquartal range; VLR: viral load responders; GMT/C: geometric mean titer/concentration; *Calculated from data given in paper, using Fischer's exact test; NE: not evaluable; SD: standard deviation; DTP: diphtheria, tetanus, pertussis vaccine; DTwP: diphtheria, tetanus, whole cell pertussis vaccine; Early control= children who started on combined ART in the first year of life; Late control= children who were started on combined ART after the first year of life and in whom viral suppression was achieved; Late failure= children who started on combined ART after the first year of life and in whom viral suppression was not

achieved. DT: diphtheria toxoid; TT= tetanus toxoid; DTaP= diphtheria, tetanus, and acellular pertussis vaccine; Td= tetanus and diphtheria vaccine; Hib: *Haemophilus influenzae* type b; HepB: hepatitis B vaccine; CI; confidence interval; EU: ELISA Units.

2.9.2.3 Pertussis vaccine

Bordetella pertussis is a gram-negative pleomorphic bacillus which is the causative agent of pertussis or “whooping cough”. It is a severe, potentially life-threatening infection of the lower respiratory tract (Watanabe et al., 2006, Healy et al., 2004). *Bordetella* species has been shown to alter their phenotypes depending on the surrounding environment, and may show differing expression of virulence factors (WHO, 2010d). These factors are PT, FHA, pertactin (PRN), fimbriae (FIM) type 2 and type 3, adenylate cyclase toxin (ACT), tracheal cytotoxin (TCT), lipooligosaccharide and *B. pertussis* endotoxin (WHO, 2010d, Mills, 2001). The full spectrum of pertussis pathogenesis is not fully understood, however, FHA, PRN and FIM assist with attachment to the target cell, while PT, TCT and ACT play a role in the destruction of the host epithelial lining and evasion of the hosts’ immune defences (WHO, 2010d).

B. pertussis is transmitted from infected to susceptible persons through droplets. During the primary stages of pertussis (catarrhal phase), the infection is highly contagious, which lasts for one to two weeks (WHO, 2010d, Galazka and Robertson, 1996). Individuals in this stage, exhibit mild symptoms of upper respiratory infection with rhinorrhoea, conjunctivitis, lacrimation, mild cough, and low grade fever (Galazka and Robertson, 1996). The paroxysmal phase, which follows usually lasts for two to four weeks, however, can persist for as long as 20 weeks. This phase is characterized by a repetitive series of paroxysmal coughs during a single expiration, followed by a sudden massive inspiratory effort that produces a “whoop” as air is inhaled forcefully against a narrow glottis (Galazka and Robertson, 1996). The coughing becomes so severe that patients cannot sleep or eat, and may lead to vomiting resulting in weight loss and malnutrition, especially in children with borderline nutritional status (Galazka and Robertson, 1996). The convalescent phase is the final phase of the disease, when the paroxysmal symptoms gradually decrease in frequency and severity. Following natural infection, antibodies to PT (the only *B. pertussis* antigen) are found in 80-85% of patients. Neither type nor concentration of antibodies is correlated with immunity and thus far no protective correlate of protection has been identified for cell mediated immunity (Plotkin, 2010, WHO, 2010d). Natural infection does not confer long-term protection against pertussis (Konig, 2010).

Before the vaccine era, *B. pertussis* infection was responsible for substantial morbidity and mortality in childhood (WHO, 2010d, Healy et al., 2004). Following the scale up of vaccination during the 1950s and 1960s there was a dramatic decrease (>90%) in the incidence of pertussis in

developed countries. The pertussis vaccine (combined with diphtheria toxoid and tetanus toxoid) has been part of WHO's EPI since 1974. As of 2008, the WHO estimated that worldwide, 16 million cases of pertussis had occurred of which 95% occurred in developing countries and with approximately 687 000 deaths being averted as a result of vaccination (WHO, 2010d). Majority of clinically recognized pertussis cases occur in children between the ages of one and five years, with severe disease and death being observed in the first weeks and months of life (Archer et al., 2011). In South Africa, the incidence of pertussis has been increasing ever since improved surveillance was introduced. With 287 cases being reported in 2011, of which 67% (192/287) were children under three months of age, and 22% of these children were under 6 weeks of age (Archer et al., 2011).

After natural infection with *B. pertussis*, relatively long-lived immunity results against subsequent infection. Two types of vaccines against *B. pertussis*, the killed wP vaccine, which protects against disease (Mills, 2001), however, the reactogenicity of this vaccine and its association with mild to severe neurological complications (Donnelly et al., 2001, Geier and Geier, 2004) have been major influences in the development of new generation aP vaccines. aP vaccines may include 2-5 of the following antigens PT, FHA, PRN and FIM 2 & 3 (WHO, 2010d). Efficacy trials under controlled conditions during the 1990s have shown that aP vaccines have a considerably better safety profile than wP vaccines, and have been introduced into most EPIs of developed countries (Olin et al., 1997, Gustafsson et al., 1996, Greco et al., 1996, Trollofors et al., 1995). However, due to the cost and ease of local production wP vaccines are still widely used in developing countries (Mills, 2001).

Pertussis vaccination has dramatically decreased the incidence of whooping cough, but the organism still continues to circulate in the human population (Watanabe et al., 2006). Severe pertussis infection occurs in children under the age of five years and in children who have not been adequately vaccinated (Watanabe et al., 2006). Contrasting results are available on the presence of maternal antibodies on immune response to vaccines. Response to acellular pertussis vaccine in children with and without maternal antibodies was evident in 81.1% vs. 71.9% of children, respectively, indicating that maternal antibodies had no effects on immune response to vaccine (Saffar et al., 2007). However some studies that were similar to the latter have shown that vaccination with wP in the presence of maternal antibodies reduce the immune response,

possibly due to neutralization of vaccine antigen by maternal antibodies (Siegrist and Lambert, 1998, Englund et al., 1995).

Therefore, the schedule at which the wP vaccine is administered might influence the immune response and persistence of antibody elicited by wP (Silfverdal et al., 2007). Silfverdal and colleagues reported that giving three wP vaccine doses within or less than 5-months of age resulted in the vaccine being less effective in protection against clinical pertussis infection in childhood as compared with a schedule with a longer interval between doses (over 10 months between second and third dose) (Silfverdal et al., 2007). These studies show that the wP vaccine is highly effective at protecting against whooping cough in later in life.

Since the emergence of HIV, re-emergence of *B. pertussis* has been observed resulting in significant increases in acute and prolonged respiratory infection in children and adults with HIV-infection (Colebunders et al., 1994, Nordman et al., 1992, Doebbeling et al., 1990). The extent at which HIV-infected children are protected (by preventing the incidence of whooping cough) by pertussis-containing vaccines during early childhood has previously been determined (Abzug et al., 2007, Tejiokem et al., 2007, Tovo et al., 2000). In studies conducted prior to the wide spread use of ART, immunogenicity of the primary whole cell pertussis vaccine was found to be decreased in HIV-infected children (30.0%-75.0%) than HIV-uninfected children after booster dose (Tejiokem et al., 2007, Abzug et al., 2007). These results were correlated with CD4+ T-lymphocyte counts and comparable to HIV-exposed uninfected children (75.0%); Table 2.7. There is however no direct evidence that HIV-infected children have higher vaccine failure rates than their healthy counterparts. de Martino et al (1997), reported that HIV-infected children who sero-converted to the acellular pertussis vaccine, had lower GMTs to PT when compared to HIV-uninfected children (30% vs. 55.1%, respectively) (de Martino et al., 1997).

In contrast responses to pertussis vaccine, particularly PT and FHA epitopes, indicate that aP vaccination induced higher antibody levels post primary vaccination in HEU compared to HIV-unexposed children (Reikie et al., 2013, Jones et al., 2011, Hesseling et al., 2016), which was however similar following the booster dose of vaccine (Reikie et al., 2013, Hesseling et al., 2016).

There is limited or no data available on the immune response to whole cell pertussis especially in sub-Saharan Africa (summarized in Table 2.7). Studies suggest that ART is associated with improved responses to re-vaccination with a variety of vaccine antigens, however, the response of HIV-infected children who are on ART at the time of the primary series of pertussis vaccine has not been fully described (Melvin and Mohan, 2003, Sutcliffe and Moss, 2010). Furthermore, response to primary immunization with pertussis vaccine in HIV-infected children initiated on early ART has not been described.

Table 2.7: Comparison of vaccination response to pertussis epitopes between HIV-infected children and HEU and/or HIV-unexposed children

Study setting and Reference	Study design	HIV-infected N (Age at study entry)	HEU children N (Age at study entry)	HIV-unexposed children N (Age at study entry)	Vaccination schedule and timing of blood sample	Number on ART	Initiation of ART prior to vaccination and duration on ART	CD4+ at study entry	Main findings HIV-infected [#] (HIV-infected vs. HIV-unexposed) [†] or (HIV-infected vs. HEU) ^{††} or (HEU vs. HIV-unexposed) ^{†††}	Summary
HIV-infected only										
1997 Italy (de Martino et al., 1997)	Cohort	12 (6-107 months)	ND	ND	3 dose schedule at 8 week intervals Bloods: 0, 2, 4, 6, 8 months	ND	ND	ND	GMC EU/ml (95% CI) [‡] : 0 weeks: PT: 3.01 (0-7.9) vs. 2.1 (1.4-3.2) [†] FHA: 6.6 (2.2-10.9) vs. 7.6(4.9-11.9) [†] 2 months PT: 7.24 (0-15.04) vs. 27.6 (17.2-44.6) [†] FHA: 12.02 (7.9-10.1) vs. 82.4 (49.5-137.4) [†] 4 months PT: 13.8 (3.5-23.8) vs. 133.8 (79.4-225.5) [†] FHA: 19.9 (13.7-26.15) vs. 611.3 (439.2-1088) [†] 6 months PT: 29.5 (18.01-41) vs. 106.5 (37.8-304.4) [†] FHA: 25.7 (19.9-31.4) vs. 759.7 (439.2-1088) [†]	Antibody titre fold-rise correlated with pre-immunization CD4+ cell counts. Significant titres were still detectable 4 months after the third dose.

									8 months PT: 11.7 (2.7-20.7) vs. ND FHA:19.00 (13.6-24.21) vs. ND	
2007 USA (Abzug et al., 2007)	Cohort	92 (9.3 years) 4 strata Group1: 2 (8.9 years) Group 2: 33 (10 years) Group 3: 28 (9.3 years) Group 4: 29 (5.6 years)	ND	ND	Booster DTaP at study entry Bloods: 0, 8, 24, 72 weeks post booster vaccination	92	No ART was initiated prior to primary vaccination. ART was initiated > 3 months prior to booster dose in Group 1 and >6 months for Group 2-4	Group 1: 16% Group 2: 27% Group 3: 33% Group 4: 39%	Pre-booster antibody levels were low: GMC EU/ml, (95% CI) PT#: 4.8 (3.7-6.5) & FHA: 4.1 92.9-5.8) Post-booster GMCs rose [§] : PT: 22.3 (15.9-31.2) FHA: 77.0 (51.0-116.2)	Antibody levels fell 24 weeks after vaccination but remained higher than pre-booster levels. Predictors of response were pre-booster antibody levels, lower HIV-RNA level, and higher CD4+% at entry. .HIV-infected children may be deficient in immunologic memory from previous DTP containing vaccination and/or that immune reconstitution with ART may be incomplete for pertussis antigens.
HIV-infected vs. HEU										
2007 Cameroon and Central Africa (Tejiokem et al., 2007)	Cross sectional	51 (25 months)	78 (25 months)	ND	DTwP: 6, 10, 14 weeks Booster at 18 months Bloods: 17 months (range: 3.9 to 31 months)	33	No ART prior to primary or booster dose. ART was initiated 6 months prior to blood sampling.	<15 to >25%	Seropositive after primary vaccination [§] N/n (%) : 10/43 (23) vs. 34/66 (51.5), p=0.003 ^{††} Seropositive after booster vaccination N/n (%) [§] : 5/7 (71.4) vs. 9/12 (75), p=0.63 ^{††} GMT, (95% CI): 52.8 (41.4-67.3) vs.62.8 (52.2-75.6), p=0.28 Proportion of children with CD4+<25% had low wP titers	Significantly reduced titres in HIV-infected vs. HEU children Post primary vaccination Similar proportion protected post booster vaccination GMC were similar in HIV-infected and HEU

									(OR: 4.9; 95% CI: 1.8-13.4, p=0.002)	
HEU vs. HIV-unexposed										
2011 South Africa (Jones et al., 2011)	Prospective cohort	ND	38 (<4 months)	55 (<4 months)	DTwP-Hib at 6, 10, 14 weeks Bloods: 16 weeks	ND	ND	ND	PT & FHA median, FDA U/mL (IQR): 270.1 (84.4-355.0) vs. 91.7 (27.9-168.4), p = 0.006 ^{†‡} Fold increase (IQR): 9.51 (IQR, 2.80-24.25) vs. 2.16 (IQR, 0.41-6.84), p = 0.002 ^{†‡}	HEU had increased antibody against pertussis than HIV-unexposed
2013 South Africa (Reikie et al., 2013)	Prospective cohort	ND	27 (3 months)	28 (3 months)	DTwP-Hib at 6, 10, 14 weeks Booster at 18 months Bloods: 3, 6, 12, 18, 24 months	ND	ND	ND	PT & FHA higher concentrations post primary vaccinations (6 & 12 months age, p=<0.001), no difference at 24 months, post booster. Higher proportion of seropositive HEU infants at 6 months, P<0.001*	HEU had significantly higher titres post-primary vaccination; And similarities between HEU and HIV-unexposed post booster dose.
2013 South Africa (Hesseling et al., 2016)	NS	ND	118 (NS)	60 (NS)	DTwP-Hib at 6, 10, 14 weeks Bloods: 14, 24, 52 weeks	ND	ND	ND	PT & FHA higher at week 24 (p=0.003) ^{†††} , similar at weeks 14 and 52 Higher proportion of seropositive HEU infants at week 24 (OR: 1.80; 95% CI: 1.19-2.72, p=0.007)	HEU had higher titres post-primary vaccination; but was similar at weeks 14 and 52

Footnote: HEU: HIV-exposed, uninfected; N: total number of participants; ART: antiretroviral therapy; ND: not done; NS= not stated; [†] response after primary vaccination; [‡] response to booster vaccination; IU/ml: international units per milliliter; GMT/C: geometric mean titer/concentration; DTaP: diphtheria, tetanus, acellular

pertussis vaccine; DTwP: diphtheria, tetanus, whole cell pertussis vaccine; Hib: *Haemophilus influenzae* type b; HepB: hepatitis B vaccine; CI: confidence interval; EU: ELISA Units; IQR: interquartile range.

2.9.2.4 Hepatitis B vaccine

Of the 400 million people worldwide who have chronic hepatitis B, a disproportionate number (46 million) of those are infected with HIV (Mahoney, 1999, UNAIDS, 2007, Thio, 2003). Hepatitis B virus (HBV) is not classified as an opportunistic infection in HIV-infected persons, but due to the common modes of transmission, co-infections occur frequently (up to 10%) (Phung et al., 2014). In Africa, where hepatitis B endogenous antigen (HBeAg) positivity is low, most childhood exposure to HBV is a result of horizontal transmission rather than vertical transmission (Kiire, 1996). Thus the most practical approach in controlling early childhood transmission of HBV in Africa, while children are still protected by maternal antibodies (Ayoola, 1988), and before they are exposed to exogenous sources of HBV (Kiire, 1996), is through vaccination programmes of neonates and infants (Burnett et al., 2005). Only a few studies of long-term effects of the hepatitis B vaccine in childhood have been published in Africa (Coursaget et al., 1990, Whittle et al., 1995). In The Gambia and Senegal, the HepB vaccine has been reported to elicit protective antibodies against Hepatitis B surface antigen (HBsAg) in 85-90% of the vaccinated children (Coursaget et al., 1990, Fortuin, 1993). The Gambia is one of the first African countries to introduce HepB vaccine into its public immunization programme, with other southern African countries following thereafter. Long-term follow up studies in The Gambia and Senegal indicated that between 51-76% of vaccinated children had sero-protective HepB antibody titers 10 years following vaccination (Coursaget et al., 1990, Whittle et al., 1995). Evidence of eradication of HBV carrier state in The Gambia in children under 5 year age has already been achieved (Whittle et al., 1995). In South Africa, one study described a decline in prevalence of HBsAg in the Eastern Cape from 10.1% in unvaccinated children to 0% in vaccinated children (Schoub, 2002). In another study by Tsebe et al (2001), none of the 598 children tested were positive for HBsAg, even though some (n=6) were born to HBsAg positive mothers (Tsebe et al., 2001). Overall, 93% had sero-protective antibody levels at 23.3 months of age, which was similar to that observed in two other studies, with no breakthrough infections or chronic carriage of HBsAg (Aspinall and Kocks, 1998, Tshatsinde et al., 1996). These studies were conducted in relatively healthy HIV-uninfected children. In three other studies conducted in South Africa to determine chronic carriage in HIV-infected children, the prevalence of chronic HBV infection was 1.2-2.1% (Simani et al., 2009, Beghin et al., 2016, Amponsah-Dacosta et al., 2014).

Approximately 30% of HIV-infected patients not on ART are non-responsive to primary hepatitis B vaccination compared to 2.5% in immunocompetent individuals, with sero-protective responses occurring in 20-70% of HIV-infected children and adults (Bruguera et al., 1992, Zuccotti et al., 1994, Rey et al., 2000); Table 2.8. This lower immunogenicity of HepB vaccine seems to correlate more with lower CD4+ counts than with the mode of HIV acquisition (Thio, 2003). In such cases, re-vaccination is recommended at double the dose (Wasmuth and Rockstroth, 2004), however, Rey et al (2000) demonstrated that three additional doses of HepB separated by 1 month each increased the response rate in HIV/HBV co-infected adults to 90%. Nevertheless, this declined to only 59% having persistence of sero-protective titers one-year later (Rey et al., 2000). HIV-positive individuals, who had a primary response to HepB vaccine, are likely to lose their immunity over time (Bruguera et al., 1992). HIV-infected children and adults who respond to the HepB vaccine have a more rapid decline in antibody titer than uninfected persons, as was observed in a study by Scolfaro et al (1996), in which only 42% of children who responded to the primary three dose series had protective antibody 13-18 months post immunization (Scolfaro et al., 1996). Long-term follow up studies of HIV-negative children and adults, however, suggest that loss of antibody to HepB over time does not mean loss of immunity (Mahoney and Kane, 1999). Whether this phenomenon of retained memory response applies to HIV-positive persons is unknown (Moss et al., 2003).

HIV-1-infected children may be unprotected from HBV in spite of receiving the full three-dose immunization series, due to a less robust initial immunologic response and waning of HepB antibody titers (Simani et al., 2009, Pippi et al., 2008, Abzug et al., 2009, Beghin et al., 2016); Table 2.8). Immune reconstitution after treatment with cART does not restore HepB immunity in the majority of children, and the response to re-vaccination is variable. In several small cohorts, 1–40% of HIV-infected children with a history of receiving their primary HepB vaccine series had protective HepB antibody levels (>10 IU/ml) detected when measured after initiation of treatment with cART (Aurpibul et al., 2012, Abzug et al., 2009, Fernandes et al., 2008, Pessoa et al., 2010, Siriaksorn et al., 2006, Lao-araya et al., 2007); Table 2.8. In a study conducted by the International Maternal Pediatric Adolescent AIDS Clinical Trials group, only 24% of 204 HIV-infected cART-treated children (median age: 9-years) with a history of previous immunization with three doses of HepB vaccine had protective HepB antibody levels prior to receiving a

booster vaccine. Forty five percent of the children became seropositive 8 weeks after a booster vaccine (Abzug et al., 2009). Vaccine response was greatest in those children with higher CD4+ counts and undetectable viral loads. Lao-araya *et al.* reported on 64 HIV-infected children (median age: 10 years) on cART for a median of 31 months with a previous history of routine infant HepB immunization. Eighty-seven percent had no measurable HepB antibody prior to re-vaccination. The response rate to a repeat HBV immunization series was 17% after the first vaccine, 82.5% after the second and 92% after the third (Lao-araya et al., 2011). After 3 years, 71% of these children maintained protective HBsAg antibody levels, suggesting that HIV-infected children will benefit from a repeat HBV vaccine series after initiation of cART (Abzug et al., 2009). In study conducted in Thailand adolescents, low overall sero-protection was detected in HIV-infected adolescents on ART (18%), however after re-vaccination of 75 adolescents and stratification according to time of ART, they found that 93% of those on ART prior to re-vaccination and only 6% had anti-HepB titers ≥ 10 mIU/ml (Aurpibul et al., 2012).

Table 2.8: Comparison of vaccination response to antibody to HepB epitopes between HIV-infected children and HEU and/or HIV-unexposed children

Reference and Country	Study design	Vaccination Schedule And timing of blood sample	Study Population (n)	Age at study entry	CD4+ status at study entry	Number taking ART	Initiation of ART prior to vaccination and duration on ART	Main findings HIV-infected* (HIV-infected vs. HIV-unexposed) [†] or (HIV-infected vs. HEU) ^{††} or (HEU vs. HIV-unexposed) ^{†††}	Summary
HIV-infected only									
2006 Thailand (Sirikakorn et al., 2006)	Cross sectional	Previously vaccinated [‡] : HepB at 0, 2 and 6 months Bloods: NS	HIV-infected (75)	9.6 years	5%	75	No ART initiated prior to primary vaccination. ART was initiated 2 years prior to blood sampling.	N/n (%); ≥10 mIU/ml (%): 1/69 (1) ^{‡#}	Despite being immunized with 3 doses of HepB in early childhood and showing signs of immune recovery as a result of c ART. Most most HIV-infected children are still susceptible to HBV infection
2007 Thailand (Lao-araya et al., 2007)	Cohort	Re-vaccination [‡] : HepB: 0, 2, 6 months Bloods: 2 months after 1 st dose; 4 months after second dose and 1 month after 3 rd dose	HIV-infected: (63)	10.1 years	27.2%	63	ART initiated 31 months prior to re-vaccination.	≥10 mIU/ml ^{‡#} : 2 months after 1 st dose: 17% 4 months after 2 nd dose: 83% 1 month after 3 rd dose: 92%	Viral load below detection limit at the time of re-vaccination was associated with successful vaccine response.
2008 Tanzania (Pippi et al., 2008)	Cohort	Re-vaccination [‡] : HepB: 0, 1 and 6 months Bloods: 5 months	HIV-infected: (84)	4.7 years	40.4%	47	ART initiated 69 weeks before re-vaccination.	Sero-conversion rate was 59.5%; Sero-conversion was found in 70.8% of ART-treated and 44.4% of treatment naive children. ≥10 mIU/ml: 71% ^{‡‡}	Sero-conversion rate was higher in individuals in CDC category 1 than in CDC Categories 2 and 3. ART use and absolute CD4+ cell counts were independently associated with probability of sero-conversion and with higher antibody titer
2009 Country NS	NS	Re-vaccination [‡] :	HIV-infected:	9.1 years	34%	204	No ART was initiated prior to re-vaccination	% ≥10 mIU/ml ^{‡‡} : 8 weeks: 46%	Decreased sero-protection in HIV-infected children with

(Abzug et al., 2009)		HepB: NS Bloods: 8, 48, 96 weeks 4.6 years post booster dose	(204)				vaccination. ART was initiated > 6 months prior to booster dose	48 weeks: 38% 96 weeks: 38% 4.6 years: 25%	decreased responses to booster dose despite being on cART for more than 6 months.
2011 Thailand (Lao-araya et al., 2011)	Cohort	Re-vaccinated [‡] : HepB: 0, 2 and 6 months Bloods: 1 month and 3 years	HIV-infected: (69)	12.9 years	5.4%	69	ART was initiated prior to re-vaccination and duration was 5 years	% ≥10 mIU/ml: 1 month: 95% [‡] 3 years: 71.0% [‡]	Improved sero-protection in cART treated children; and recommend giving a 3-dose HBV vaccination to HIV-infected children with immune recovery while receiving cART.
2012 Thailand (Aurpibul et al., 2012)	Cross-sectional	Previously vaccinated [‡] : HepB: NS Bloods: 4.3 years	HIV-infected: (521)	14.8 years	685 cells/μL	521	ART-initiated post vaccination, but duration on ART was not stated.	% ≥10 mIU/ml: 18% ^{#†}	HIV-infected children showed low level of sero-protective antibody.
HIV-infected vs. HIV-unexposed									
2007 Brazil (Zaccarelli-Filho et al., 2007)	Observational	Previously vaccinated: HepB at 2, 4 and 6 months Bloods: NS.	Good VLR: (9) Partial VLR (13) Poor VLR (14) HIV-unexposed (25)	Good VLR: 10.9 years Partial VLR: 7.4 years Poor VLR: 9.4 years HIV-unexposed: 10.6 years	Good VLR: 33.0% Partial VLR: 26.4% Poor VLR: 21.6%	41	Good VLR: ART initiated post primary vaccination at 5.8 years of age. Partial VLR: ART initiated post primary vaccination at 3.7 years of age Poor VLR: ART initiated post primary vaccination at 7.3 years of age	N/n; (%) ≥10 IU/ml [‡] : Good VLR: 9/9 (100) vs. Partial VLR: 13/13 (100) vs. Poor VLR: 13/14 (93); p=NE [#]	No difference in sero-protective levels in children with good or partial or poor viral load controllers
2008 Brazil (Fernandes et al., 2008)	Cohort	Previously vaccinated [‡] : HepB: 3, 4 and 5 months Bloods: 68 months for HIV-infected 64 months for HIV-unexposed	HIV-infected: (58) HIV-unexposed: (116)	7 years	831 cells/μL	53	No ART was initiated prior to primary vaccination. ART was initiated prior to blood sampling for 27 months.	N/n (%); ≥10 mIU/ml [‡] : 14/58 (24.1) vs. 101/116 (87.1); p<0.001 ^{*†}	Difference between sero-protective rate between HIV-infected and HIV-unexposed children.

2009 South Africa (Simani et al., 2009)	Cross-sectional	HepB: 6, 10 and 14 weeks Bloods: from 4 weeks	HIV-infected: (73) HIV-unexposed: (230)	HIV-infected: 8.7 months HIV-unexposed: 11.9 months	ND	ND	No ART was initiated prior to primary vaccination. No ART was initiated on blood sampling.	≥ 10 mIU/ml N/n (%): 57/73 (78%) vs. 197/230 (86%) p=0.125 ^{††} GMT IU/ml: 289.2 vs. 370.6	There were no differences in sero-protective titers between HIV-infected and HIV-unexposed children. However hospitalized HIV-infected children had lower responses to vaccine than children attending routine immunization clinic.
2016 South Africa (Beghin et al., 2016)	Retrospective	Previously vaccinated [†] : HepB at 6, 10 and 14 weeks Bloods: NS	HIV-infected (183) HIV-unexposed (108)	HIV-infected: 9.1 years HIV-unexposed: 9.0 years	ND	ND	ND	N/n (%) ≥ 10 mIU/ml [†] : 28/183 (15.1) vs. 66/108 (61.1); p<0.001 ^{††}	HIV-infected children have reduced response to previous vaccination.
HIV-infected vs. HEU									
1992 Italy (Zuin et al., 1992)	Prospective	HepB: 3 doses Bloods: 2-4 months	HIV-infected: (18) HEU: (33)	<24 months	ND	ND	ND	N/n (%) ≥ 10 mIU/ml [†] : 414/18 (77.8) vs. 32/33 (97.0); p=0.046 ^{††}	Sero-protection was significantly higher in HIV-unexposed than in HEU children post primary vaccination.
1994 Italy (Zuccotti et al., 1994)	Longitudinal	HepB: 0,1 and 6 months Booster at 3-4 months after last dose Blood: NS	HIV-infected (5) HEU (13)	NS	ND	ND	ND	N/n (%) ≥ 10 mIU/ml [†] : 1/5 (20%) vs. 13/13 (100); p=NE ^{††}	Response of HIV-infected children to the hepatitis B vaccine is poor, even when an adult dose of the vaccine is administered at birth. A rapid fall in levels of antibody to hepatitis B virus is observed when a response is present. It is likely that immunologic damage is already present at birth.
HEU vs. HIV-uninfected									
2011 Brazil (Abramczuk et al., 2011)	NS	HepB: 0, 1 and 6 months Bloods: 1 month	HEU: (45) HIV-unexposed: (112)	HEU: 7.7 months HIV-unexposed: 7.5 months	ND	ND	ND	N/n (%); ≥ 10 mIU/ml [†] : 13/45 (28.9) vs. 65/112 (58.0); p=0.001 ^{†††}	Significant differences in sero-protective rates between HEU and HIV-unexposed children, decreased response in HEU was associated with exposure to ART for the prevention of mother to child transmission of HIV.
2013 South Africa (Reikie et al., 2013)	Prospective cohort	HepB: 6, 10, 14 weeks Bloods: 3, 6, 12, 18, 24 months	HEU: (27) HIV-unexposed: (28)	3 months	ND	ND	ND	N/n (%); ≥ 10 mIU/ml [†] : 20/27 (75%) vs. 21/28 (75%), p>0.99 ^{†††}	No significant differences were observed in response to primary vaccination.

Footnote: HEU: HIV-exposed uninfected; ND: Not done; N: total number of participants; ART: antiretroviral therapy; ND: not done ; NS= not stated; mIU= milli international unit; HepB= hepatitis B virus vaccine; [†] previously vaccinated in early childhood; [‡] re-vaccination: children who received all primary and booster doses in early childhood, and received a full series of vaccination in older age; [√] response after primary vaccination; ^{√√} response to booster vaccination; *Calculated from data given in paper, using Fischer's exact test; NE: not evaluable; VLR: viral load responders.

2.9.2.5 *Haemophilus influenzae* type b disease and Hib conjugated vaccine use in HIV-infected children.

Haemophilus influenzae type b, is a gram-negative coccobacillus which is encapsulated (Mangtani et al., 2010). In the 1930s it was discovered that some strains of *H. influenzae* possessed a polysaccharide capsule with 6 capsular serotypes (a-f) (Pittman, 1931). Invasive isolates from patients where predominantly type b (Hib) organisms which possess a PRP capsule (Kelly et al., 2004). Hib is said to be an important cause of bacterial infections, in unvaccinated individuals, which include meningitis, septicaemia, epiglottitis, and cellulitis, pneumonia and septic arthritis (Watt et al., 2009, Mangtani et al., 2010). However, Hib has also been shown to be a commensal of the nasopharynx in 3-11% of unvaccinated children (Adegbola et al., 1998, Takala et al., 1991), and only a minority of those exposed or who are carriers of the organism suffer invasive disease (Kelly et al., 2004). At the end of 2007, the World Health Organization estimated that Hib disease caused 8.1 million episodes of serious illness and 371, 000 childhood deaths yearly, of which 8100 were in HIV-infected children (WHO, 2013b, Watt et al., 2009).

Of the 2.5 million HIV-infected children under the age of 15 years, in the absence of antiretroviral therapy, one in three HIV-infected children would die before the age of one year from serious bacterial diseases, including Hib (Newell et al., 2004). Even though scale up of ART in many high HIV prevalence countries has increased, less than 30% of children requiring this have access to it (UNICEF, 2010). The use of cotrimoxazole prophylaxis in HIV-infected children has been instrumental in reducing hospitalization by 23% and childhood mortality by 43%, however, the prolonged use of this prophylaxis has some limitations (Mulenga et al., 2007). Therefore, immunization with vaccines such as HibCV remains an important approach in reducing the risk of bacterial infections in HIV-infected children (Mangtani et al., 2010).

The type b polysaccharide capsule is instrumental in invasive disease and thus is an attractive antigen to use as part of a vaccine, as invasive disease is almost exclusively restricted to type b organisms and PRP antibodies are important in natural immunity (Kelly et al., 2004). However, early observations with Hib demonstrated the limitations of plain polysaccharide as a vaccine antigen. When administered in children less than two years of age, purified PRP induces sub-optimal responses and does not provide sufficient protection against invasive disease (Parke et al., 1977, Peltola et al., 1977, Smith et al., 1973). With regards to immune memory post

vaccination, antibody levels wane quickly and follow up immunization shows no evidence of immunological priming in any age group (Kayhty et al., 1984). Polysaccharides, although recognized by B-lymphocyte receptors, cannot be presented to T-lymphocytes in conjunction with major histocompatibility complex class II molecules. B-lymphocytes therefore lack the ability to directly recruit cognate T-cell help when stimulated with polysaccharide. The lack of specific T-cell interaction in the immune response limits the immunogenicity of PRP, and the development of memory B-lymphocytes with class-switched antibody and subsequent avidity maturation cannot occur (Kelly et al., 2004). It is still unclear whether the poor antibody responses in the first 2 years of life are caused by infrequent Hib carriage at this age or immunological immaturity. The same vaccine administered later in childhood results in increased antibody responses (Peltola et al., 1977, Kayhty et al., 1984, Weinberg et al., 1987). There is some evidence for specific inhibitory mechanisms that act in infancy to delay the development of TI response (Rijkers et al., 1998). Protein conjugation has provided a solution for the lack of interaction between B-lymphocytes and T-lymphocytes when using purified polysaccharide as a vaccine during early childhood (Kelly et al., 2004).

H. influenzae type b is an important cause of invasive bacterial infections amongst children especially those with HIV-infection, in whom the infection rate is increased 6 to 8 fold when compared to HIV uninfected children (Madhi et al., 2000, Madhi et al., 2002). Despite this increased disease burden and the widespread use of HibCV in much of the developed world, there is limited data regarding the immunogenicity and efficacy of the vaccine amongst HIV-infected children (Peltola, 2000). In the absence of immunosuppression or $CD4^+ \leq 25\%$, 37-86% of HIV-infected children develop protective antibody responses to HibCV, but the geometric mean titers are significantly lower than in HIV uninfected children (Madhi et al., 2002, Peters and Sood, 1997, Rustein et al., 1996, Jeena, 2008). Furthermore, they found that waning of memory response by 15 months of age (Jeena, 2008). In addition, the bactericidal activity and avidity of anti-PRP was investigated by Madhi and colleagues (2005), they concluded that a lower percentage of HIV-infected children not on ART (51.5%) had protective antibody levels when compared to HIV-uninfected children (95.3%). Furthermore, a lower percentage of HIV-infected children had serum bactericidal antibody activity (76.1%) than in HIV-uninfected children (97.1%). The antibody concentration required for 50% bactericidal activity in HIV-infected children was much higher compared to HIV-uninfected children, and the risk of vaccine

failure was greater (Madhi et al., 2005). This observation has led to the recommendation of booster doses to be administered after 2 years of age in HIV-infected children (Mangtani et al., 2010).

A study conducted in Uganda and the USA reported higher vaccine responses to PRP epitope in HEU (75µg/ml) and HIV-unexposed children (3.3µg/ml; $p=0.002$) 48 weeks post primary vaccination (Gaensbaier et al., 2014). Furthermore, studies conducted in South Africa found similar results to the study in Uganda and the USA (Jones et al., 2011, Hesselning et al., 2016) although this was not observed by Reikie et al. either post primary and post booster vaccination (Reikie et al., 2013).

2.9.3 Live-attenuated vaccines

Although live-attenuated vaccines may cause serious complications in immunosuppressed individuals (WHO, 2010b), HIV-infected children without signs of immunosuppression can be safely immunized with live-attenuated vaccines (Sutcliffe and Moss, 2010, Melvin and Mohan, 2003), except for BCG (Hesselning et al., 2006). In some developing countries with high HIV prevalence, the advantage of routine administration of live vaccines i.e. OPV and measles vaccines far outweigh the theoretical risks of complications from these vaccines (Halsey, 2002). Additionally, vaccination at birth or early in life often results in an adequate immune response as it precedes HIV-induced immunosuppression. Therefore, the WHO and many countries recommend routine immunization of HIV-infected children with these vaccines (Sutcliffe and Moss, 2010).

2.9.3.1 Measles vaccine

The measles virus belongs to the Paramyxoviridae family, which include other human pathogens such as mumps virus, and parainfluenza virus (WHO, 2009b). Measles is a single-stranded RNA virus with a diameter of 90-140 nm, and has a pleomorphic structure and a coating which is derived from the cell membrane (WHO, 2009b). The measles virus genome encodes for 8 proteins, which include two major proteins shown to be major role players in life long immunity following infection, they are the haemagglutinin (H) and fusion (F) proteins (de Swart et al., 2005). Sequencing of the measles genome has revealed 23 different genotypes which have been used in tracking transmission (Rota et al., 2009).

In the pre-vaccine era, measles virus was highly infectious and resulted in >90% of individuals being infected by 10 years of age (WHO, 2009b). Humans are the only known reservoir of measles and transmission is through aerosolized respiratory droplets and close contact with infected fluid. The typical incubation period is 10-14 days following exposure to measles virus, and individuals may be contagious from approximately 4 days before eruption of the rash until 4 days after eruption (WHO, 2009b). Near the end of the incubation period, individuals develop prodromal symptoms of high fever, cough, coryza and conjunctivitis (WHO, 2009b). Severity and case fatality of measles is increased in children <5 years of age, overcrowded living conditions, malnutrition (especially vitamin A deficiency), and immunosuppression (such as advanced HIV-infection).

The case fatality in developing countries has been shown to be 5-10% among young children (Helfand et al., 2007). On a global scale, mortality due to measles in children under five years of age has shown trends of reduction from 544 200 to 145 700 per year between 2000 and 2013 (United Nations, 2015). This reduced mortality is a result of substantial increases in measles vaccination coverage through routine health services as well as mass immunization campaigns.

The measles vaccine is composed of a live-attenuated measles virus (Edmonston-Zagreb strain) (Arpadi et al., 1996). In four of six WHO regions, substantial progress has been made in measles prevention and control, transmission no longer occurs indigenously and importation does not result in sustained spread of the virus (Muller et al., 2007). The key reason for this success was 95% vaccination coverage through a two dose vaccination regimen (Andre et al., 2008). Increasing vaccination levels in Africa, where the coverage average was 67% in 2004, is essential for eradication of measles disease (Andre et al., 2008). Between 2001 and 2005, >360 million children in sub-Saharan Africa had been vaccinated against measles, on the African continent 126 000 deaths had been reported during the same time period compared to the previous 506 000 (Katz, 2007).

In sub-Saharan Africa, where a substantial number of measles-related deaths still occur, a major obstacle in the control of measles is the HIV epidemic (Muller et al., 2007). HIV-infection may modify the clinical manifestations of measles, thus disrupting surveillance efforts, and HIV-

infection may prolong the infectious period of measles (Helfand et al., 2005). Furthermore, HIV-infection may result in high rates of primary and secondary vaccine failure after vaccination, thus decreasing vaccine efficacy. Measles vaccine has been recommended for children, adolescents and young adults with known HIV-infection (Kaplan et al., 1992). In developing countries, the case-fatality rate attributable to measles has been between 5-10% for HIV-infected children (Wolfson et al., 2009, WHO, 2009b). It has been observed that 25-77% of HIV-infected children sero-convert to the initial dose of the vaccine (Brunell et al., 1995, Arpadi et al., 1996); (Table 2.9), in contrast 95% of HIV uninfected children sero-convert to the measles vaccine (Moss et al., 2003). The potential impact of HIV-infection on maternal antibody transfer in children born to HIV-infected mothers is unclear, but the risk of exposure to measles is reduced in early childhood in regions where measles has been successfully controlled in older children (Moss et al., 1999, Gans et al., 1998). Clinical experience with measles demonstrated that a critical maturation of the immune system occurs between 6- and 12-months, as a result of high vaccine failures observed in children less than 12-months. This vaccine failure was attributed to the persistence of maternal antibodies in mothers who had been exposed to wild-type measles virus (Gans et al., 1998). Recently most children are now born to mothers who have vaccine induced immunity to measles and can be expected to lose maternal antibodies by 9-12-months of age instead of 12-15-months (Leuridan, 2010). This might be further accelerated in children born to HIV-infected mothers due to waning of immunity in the mothers. Scott et al. reported that HIV-exposed infants had low levels of protective maternal antibodies by 6-months of age, compared to 9-12-months in children born to HIV-uninfected women (Scott et al., 2007). As a consequence of this decrease in maternal antibodies, there is also an increase in the pool of susceptible children to wild type measles virus infection, which could result in high morbidity and mortality (Scott et al., 2007).

Because the measles vaccine consists of a live-attenuated virus, it has been found that HIV-infected people, who did not respond to the primary and secondary doses of the vaccine, shed the vaccine virus for a longer period than HIV negative persons (Permar et al., 2001).

Table 2.9: Immunogenicity of measles virus vaccine in children with HIV prior to use of ART in HIV-infected children

Country and Reference	Number studied	Age group	Response to primary vaccination	Response to repeat vaccination
USA 1989 (Krasinski and Borkowsky, 1989)	8	11-41 months	25%	-

Zaire 1989 (Oxtoby et al., 1989)	37	9 months	36% of symptomatic CD4+ >25%; 77% of asymptomatic CD4+<25%	-
USA 1992 (Palumbo et al., 1992)	35	12-194 months	37%	0%
USA 1993 (Brena et al., 1993)	2	unknown	-	50%
USA 1994 (Frenkel et al., 1994)	10	22-121 months	-	20%
USA 1994 (Rudy et al., 1994)	25	6-12 months	69.20%	50%
USA 1995 (Brunell et al., 1995)	11	72-120 months	-	36%
USA 1996 (Arpadi et al., 1996)	7	31-120 months	-	14%
Uganda 1999 (Waibale et al., 1999)	50	15 months- 3.5 years	-	19%
Thailand 2000 (Thaithumyanon, 2000)	16	9 months	57%	-
Cameroon and Central Africa 2007 (Tejiokem et al., 2007)	129	18.1-35.6 months	16.0% for HIV-infected 62.3% in HIV-exposed uninfected children results correlated with CD4+ cell count	-
Zambia 2007 (Moss et al., 2007)	50	2-27 months	88%	55%

Improved immunization of children against measles has reduced measles-associated mortality globally over the past decade (Wolfson et al., 2007, WHO, 2009b). Sporadic measles-outbreaks persist in many settings, disproportionately involving children too young to be vaccinated (Machaira and Papaevangelou, 2012). Twenty-four percent of cases in a recent measles-outbreak in South Africa were aged less than 9-months (Diseases, 2010). A greater proportion of measles associated hospitalization occurs in children aged less than 9-months among HIV-infected (33%) compared to HIV-uninfected African children (23%) (Moss et al., 2002). Factors contributing to the sporadic outbreaks of measles include vaccine-coverage below the 95% threshold required to interrupt wild-type virus circulation in communities, pockets of susceptible individuals who remain unimmunized or have sub-optimal immune-responses to vaccine and reduced transplacental measles-antibody transfer to fetuses as maternal immunity is now mainly vaccine-

induced (Machaira and Papaevangelou, 2012, Leuridan et al., 2010, WHO, 2009b). Furthermore, maternal HIV-infection has been associated with lower measles-antibody titers (Farquhar et al., 2005), impaired transplacental antibody transfer to the fetus and more rapid decay of maternal-derived antibodies in HIV-infected children (Caceres et al., 2000, Farquhar et al., 2005, Kruczek, 2010, Scott et al., 2005). A recent meta-analysis identified a limited number of studies (n=1) evaluating the immunogenicity of measles-vaccine following primary vaccination in predominantly antiretroviral treatment (ART)-naïve HIV-infected children (Scott et al., 2011). The meta-analysis reported that 59% (95% confidence intervals [95%CI], 46-71%) of HIV-infected children were sero-positive after receiving standard-titer measles vaccine at 6, 9 or 12 months of age without any differences by age of vaccination. Fewer HIV-infected children (40%) remained protected compared to HIV-exposed, uninfected children 12 months post-vaccination (Scott et al., 2011). A higher proportion of older HIV-infected children (78-89%) developed sero-protective titers when vaccinated post immune-reconstitution following ART initiation (Aurpibul et al., 2007, Aurpibul et al., 2006); Table 2.10. Despite sub-optimal immune responses and higher rates of vaccine failure in HIV-infected children, this was not considered a major hurdle in measles control and elimination because of the high mortality rate in the absence of ART (Helfand et al., 2005). Improved survival of HIV-infected children on ART may, however, paradoxically impair measles control because of sub-optimal immune responses to vaccine (Scott et al., 2008). In another study, 57% of HIV-infected children and 99% in HEU children, however, when the HIV-infected children were stratified according to ART status and number of measles doses received, 85% of HIV-infected children on sustained combined ART and receiving more than 2 doses of measles vaccine had sero-protective measles antibody titers (Siberry et al., 2015). None of the studies were undertaken in the context of early-initiation of ART in HIV-infected children; Table 2.10.

Table 2.10: Studies reporting immune response to measles virus vaccination in children born to HIV-infected mothers

Reference and country	Study design	Vaccination Schedule and blood sample collection	HIV-status (n)	Age at study entry	Number taking ART	Initiation of ART prior to vaccination	CD4+ status at study entry	Main findings HIV-infected children only* or (HIV-infected vs. HIV-unexposed) [†] or (HIV-infected vs. HEU) ^{††} or (HEU vs. HIV-unexposed) ^{†††}	Summary
HIV-infected only									
2001 USA (Berkelhamer et al., 2001)	NS	Re-vaccination [‡] : MMR: Between 11 and 40 months Bloods: 1-4 months post vaccination	HIV-infected: cART (14) no cART (14)	2.2-11 years	14	ART initiated >4 months prior to re-vaccination in the cART group. No cART was initiated in the no cART group.	10-45%	N/n (%); >0.7 ELFA: 9/14 (64.3) vs. 3/14 (21.4); p=0.027 [#]	cART improves response to additional measles vaccination.
2006 Amsterdam (Bekker et al., 2006)	Prospective cohort	Previously vaccinated [‡] : MMR: NS Bloods: 48 weeks	HIV-infected (59)	4.3 years	59	ART initiated post vaccination and duration on ART was 205 weeks post vaccination.	NS	% ≥9.0 AU/ml ^{†#} : HIV-infected: 43%	Despite immune reconstitution, antibodies against MMR disappear over time in up to 40% of children with HIV- infection
2006 Thailand (Aurpibul et al., 2006)	Cross-sectional	Previously vaccinated [‡] : MMR: NS Bloods: 79.6 months	HIV-infected (93)	9.7 years	93	Children were on ART for 24 months prior to booster dose of MMR.	>15%	N/n (%); >320mIU/ml [†] : 39/93 (41.9) Mean antibody level (SD): 1389 (4147)	Low prevalence of measles protective antibody in HIV-infected children with immune recovery after HAART, despite routine immunization

									during childhood. Re-vaccination may be indicated.
2007 Thailand (Aurpibul et al., 2007)	Cross-sectional	Re-vaccination [‡] : MMR: NS Bloods: 4 and 24 weeks	HIV-infected (51)	10.2 years	51	Children were on ART for 127 weeks prior to re-vaccination	≥15%	4 weeks: N/n (%); >320 mIU/ml [#] : 46/51 (90) Mean antibody level (SD); mIU/ml: 3166 (2659) 24 weeks: N/n (%); >320 mIU/ml [#] : 39/49 (80) Mean antibody level (SD); mIU/ml: 1952 (1949)	Children re-vaccinated after immune recovery after cART respond well to MMR.
2009 Italy (Pensiero et al., 2009)	Cross-sectional	Re-vaccination [‡] : MMR: Booster Bloods: Early control: 4.2 years Late control: 4.7 years Late failure: 3.7 years Treatment naïve: 6.1	HIV-infected (70)	Early treatment: 6.8 years Late control: 13.7 years Late failure: 15.8 years Treatment naïve: 11.8 years	64	ART initiated in in early and late controller and late failure prior to booster dose. Duration on ART was not stated	Early treatment: 35% Late control: 33% Late failure: 22% Treatment naïve: 25%	Early control: mean 4.2 years; 82% protected Late control: mean 4.7 years; 39% protected. Late failure: mean 3.7 years; 40% protected. Treatment naïve: 33% ^v	Late-treated HIV-1 controllers and Late failures lose protective antibody titers against measles vaccine.
2009 Kenya (Farquhar et al., 2009)	Prospective	Re-vaccination [‡] : MMR: booster dose Bloods: 4 weeks	HIV-infected (90)	4.9 years	90	No ART post primary ART given 8 months prior to booster dose.	Median 6.3%	Among 62 children completing 6 months on cART: 17/43 (40%) without protective measles IgG sero-converted; 10/19 (53%) who were positive prior to ART lost	After 6 months of cART, more than half of previously immunized children still lacked measles antibody.

								antibodies (p=0.3). Children who lost responses had significantly lower measles antibody concentrations than those who remained measles IgG positive during follow-up (7.1 vs. 20.3 mg/mL; p=0.003).	
2016 India (Seth et al., 2016)	Prospective	Re-vaccination [†] : MMR: NS Bloods: 8-12 weeks	HIV-infected: (66)	5-18 years	66	ART initiated >6 months prior to re-vaccination.	>15%	Pre-vaccination: N/n (%): 16/66 (24.2) [#] Post-vaccination [†] : N/n (%) 62/66 (93.3)	Immune reconstitution post cART resulted in adequate responses to additional dose of measles.
HIV-infected vs. HIV-unexposed									
2007 Brazil (Zaccarelli-Filho et al., 2007)	Observational	MMR: 9 and 15 months Bloods: NS	HIV-infected: Good VLR: (9) Partial VLR: (13) Poor VLR: (14)	Good VLR: 11 years Partial VLR: 7.4 years Poor VLR: 9.4 years Controls: 10.6 years	41	Good VLR: initiated prior to 2 nd booster dose at 5.8 years of age Partial VLR: initiated prior to 2 nd booster dose at 3.7 years of age Poor VLR: Initiated post 2 nd booster dose at 7.3 years of age	Good VLR: 33.0% Partial VLR: 26.4% Poor VLR: 21.6%	N/n (%) >0.12 IU/ml [#] : Good VLR: 4/9 (43) vs. Partial VLR: 6/13 (44) vs. Poor VLR: 6/14 (45); p=0.99* Good VLR: 0.10 (0.06–0.33) vs. Partial VLR: 0.09 (0.02–0.22) vs. Poor VLR: 0.09 (0.01–0.55); p>0.99	Mean measles, plasma antibody levels were similarly low for all three groups of HIV-infected children. No differences were observed when assessing the percentage of children with protective antibody levels
2007 Zambia	Prospective cohort	MV: 9 months	HIV-infected (50)	HIV-infected: 9.0	ND	No ART was initiated prior to	ND	6 months: N/n (%); >120	Despite good primary

(Moss, 2007)		Bloods: 6 after vaccination	HIV-unexposed (98) HEU (211)	months HIV-unexposed: 9.1 months HEU: 9.1 months		vaccination		mIU/ml [†] : 44/50 (88) vs. 92/98 (94) vs. 198/211 (94); p=0.3 GMC (95% CI) mIU/ml: 504 (349-727) vs. 484 (399-588) vs. 555 (486-634)	antibody responses to measles vaccine, there was rapid waning of antibody.
HIV-infected vs. HEU									
2007 Cameroon and central Africa (Tejiokem et al., 2007)	Cross sectional	MMR at 18 months Bloods: 12.8 months	HIV-infected (51) HEU (78)	18-36 months	33	No initiation of ART prior to either primary or booster dose. ART was initiated 6 months prior to blood sampling	<15 to >25%	N/n (%); >335 mIU/ml ^{iv} : 8/50 (16.0) vs. 48/77 (62.3); p<0.001 ^{††} GMC (95% CI); mIU/ml: 1091.5 (576.1-2067.8) vs. 943.2 (782.7-1136.6); p=0.56 ^{††}	Antibody concentration after a single dose of the measles vaccine was substantially lower than expected, particularly low in HIV-infected children with low CD4+ T-lymphocyte counts.
2015 USA (Siberry et al., 2015)	Prospective cohort	MMR: Previously vaccinated [†] Bloods: 9.2 years after primary vaccination	HIV-infected (428) HEU (221)	13.9 years	347	No ART was initiated prior to vaccination; in those on ART duration was not stated.	34%	N/n (%); mIU/ml [†] : HIV-infected: 57% [†] HEU: 99% ^{††}	High percentage of HIV-infected children lack serologic evidence of immunity to measles vaccine, despite full immunization and current cART.
HEU vs. HIV-unexposed									
2013 South Africa (Reikie et al., 2013)	Prospective cohort	MV: 9 and 18 months Bloods: 12, 18, 24 months	HEU (27) HIV-unexposed (28)	3 months	ND	ND	ND	No differences post primary or booster measles vaccine between HEU and HIV-unexposed children	Similar rates of sero-protection were observed between HEU and HIV-unexposed children.

Footnote: HEU: HIV-exposed, uninfected; ND: not done; Early control= children who started on HAART in the first year of life; Late control= children who were started on HAART after the first year of life and in whom viral suppression was achieved; Late failure= children who started on HAART after the first year of life and in whom viral suppression was not achieved. EIA= enzyme immunoassay; IU= international units; † previously vaccinated in early childhood; ‡ re-vaccination: children who received all primary and booster doses in early childhood, and received a full series of vaccination in older age; √ response after primary vaccination; √√ response to booster vaccination; mIU= milli international unit; MMR= measles, mumps, and rubella; MV: measles vaccine; ELFA: enzyme linked fluorescent immunoassay; AU= arbitrary unit; VLR: viral load responder in children receiving ART.

2.10 ASSESSMENT OF ANTIBODY RESPONSE TO VACCINES

ELISA has been seen for decades as the most widely used assay for vaccine antibody serology, which is both specific and sensitive, and is ideal for testing many samples against a single analyte (Edwards et al., 1995, Lal et al., 2005, Farquhar et al., 2009, Maede et al., 1995) However, evaluation of IgG responses following routine immunization, entails measurement of multiple analytes, and screening by ELISA has become time consuming, costly and requires large sample volumes (Pickering et al., 2002a). Studies have demonstrated the ability of ELISA to accurately determine vaccine responses (Fu et al., 2010), however, with increased number of combined antigens (Fletcher et al., 2004) the use of ELISA has become increasingly unsuitable for purposes of studying immunogenicity and efficacy of vaccines in large numbers of samples (Pickering et al., 2002a). Therefore new methods which can rapidly and reliably determine specific IgG levels are required. Microsphere-based flow cytometric assays allow the simultaneous measurement of many analytes from a single sample (Ling et al., 2007). Studies have shown the ability to effectively multiplex a range of biological analytes such as cytokines (de Jager et al., 2003), bacterial pathogen nucleic acid (Dunbar et al., 2003) as well as antigen and antibodies to specific pathogens (van Gageldonk et al., 2008, Pickering et al., 2002a, Pickering et al., 2002b). Multiplexing allows high sample throughput and use of very small sample volumes, whilst still retaining the advantages of a conventional ELISA (Fu et al., 2010). Microsphere-based technology permits transfer of the simple ELISA format into a bead format in which the fluorescent bead is used as the solid support for the target antigen (Lal et al., 2005). Since ELISA kits are expensive, and are only available in a monoplex layout, we developed a multiplex method for the simultaneous detection of antibodies to five antigens, DT, TT, PT, FHA and HBsAg. This assay uses Luminex multiple analyte profiling technology (Luminex, Austin, TX), which is a flow cytometric system that allows a single sample to be tested simultaneously for several different analytes (Pickering et al., 2002b).

The Luminex platform has several advantages when compared to the conventional ELISA: the ability to multiplex, high sensitivity and specificity, has higher throughput, has a greater dynamic range on smaller sample volume and reduced time and less labour intensive (van Gageldonk et al., 2008). With all these advantages in mind, we aimed to determine whether the Luminex system was comparable to ELISA assays for measuring select vaccine responses.

2.11. CHAPTER SUMMARY

The WHO has recommended that all HIV-infected children should receive routine childhood vaccination, with the exception of BCG and measles vaccine in severely immunosuppressed children. HIV-infection impairs humoral and cell mediated immune functions early in childhood (Resino et al., 2004, Letvin and Walker, 2003). Immune dysfunction in HIV-infected individuals include loss of T-lymphocytic proliferative responses and hyper-activation of T-lymphocytes leading to accelerated apoptosis (Letvin and Walker, 2003, Palumbo et al., 1992, Borkowsky et al., 1992), decline in B-lymphocyte number and function (Lane et al., 1983, Shearer et al., 2000), and impaired priming for immunological memory and/or loss of anamnestic responses (Pensieroso et al., 2009, al-Attar et al., 1995). Early initiation of combination ART in HIV-infected children prior to immunological or clinical deterioration is associated with improved survival (Violari et al., 2008). Control of HIV replication limits and reverses immunosuppression in HIV-infected individuals (Resino et al., 2004, Valdez, 2002, Melvin and Mohan, 2003), improving immune responses to vaccines (Simani et al., 2013, Madhi et al., 2010, Obaro et al., 2004).

Due to effective ART regimens for preventing mother-to-child HIV-transmission, the majority (>97%) of children born to HIV-infected women are HIV-uninfected (Chuachoowong et al., 2000, Connor et al., 1994, Kind, 1998, Simonds et al., 1998). HEU children may, however, have increased risk of morbidity and mortality than children born to HIV-uninfected mothers (i.e. HIV-unexposed) (Newell et al., 2004, Koyanagi et al., 2011). This may be due to perturbations of the immune systems of HEU children due to *in-utero* HIV virion particle exposure and/or ART-exposure (Hygino et al., 2008, Faye et al., 2007, Bunders et al., 2005, Clerici et al., 2000, Gesner et al., 1994, Neilsen et al., 2001), affecting lymphocyte differentiation and function (Hygino et al., 2008, Faye et al., 2007, Feiterna-Sperling et al., 2007, Borges-Almeida et al., 2011, Bunders et al., 2005, Clerici et al., 2000). There are conflicting data on vaccine-related immune responses of HEU compared to HIV-unexposed children. These include variable responses to BCG vaccine (Van Rie et al., 2006, Mahomed et al., 2006), tetanus-toxoid, pertussis, HibCV, HBV and PCV (Jones et al., 2011, Abramczuk et al., 2011, Madhi et al., 2010). Differences in immune responses to some vaccines in HEU compared to HIV-unexposed children is attributed to lower maternal-derived vaccine-epitope specific antibody in HEU children, possibly reducing interference in immune responses to the homotypic vaccines (Jones et al., 2011, Abramczuk et al., 2011).

Memory induced by vaccination is crucial to long-term protection, particularly for diseases with long incubation periods such as hepatitis B (Plotkin, 2010). The establishment of long-term serologic memory is dependent on the generation and maintenance of antigen-specific memory CD4⁺ T-lymphocytes and memory B-lymphocytes (Cagigi et al., 2012). These lymphocytes are capable of re-circulating between the peripheral circulation and the lymphoid tissues, upon antigen encounter through vaccination or natural infection, B-lymphocytes in the presence of cognate help from CD4⁺ T-lymphocytes, are able to differentiate into plasma cells with the production of high-affinity antibodies in the periphery (Cagigi et al., 2012).

HIV-infection is associated with aberrations in all major lymphocyte populations, including B-lymphocytes which are the cornerstone of humoral immune responses (Letvin and Walker, 2003, Shearer et al., 2000, Pensiero et al., 2009, Gona et al., 2006, Palma et al., 2008, Moir and Fauci, 2009, Moir et al., 2004, Titanji et al., 2005, Titanji et al., 2006). In HIV-infected individuals T-lymphocyte and B-lymphocyte dysfunction may result in lower rates of sero-conversion post-vaccination, quicker decline of antibody levels, and impaired generation or accelerated loss of antigen-specific memory B-lymphocytes (Ghosh et al., 2009, Moir and Fauci, 2008). Extensive progress has been made in improving the lives of children born to HIV-infected mothers, through access to ART (Miller and Rathore, 2012). Successful HIV viral suppression through ART treatment has been shown to restore CD4⁺ T-lymphocytes and to normalize the percentage of B-lymphocytes when therapy is available during primary infection (Faye et al., 2004, Moir et al., 2008). Regarding the impact of ART on the immune responses to vaccines in vertically HIV-infected children, available data has shown that initiation of ART as early as possible induced better immune responses compared to when ART was deferred until there was immunological or clinical deterioration (Cagigi et al., 2012, Pensiero et al., 2009, Madhi et al., 2010). Early initiation of ART has been associated with relatively normal development of the T-lymphocyte lineage, and with preservation of high numbers of functional memory B-lymphocytes in HIV-infected individuals (Palma et al., 2008, Pensiero et al., 2009, Zanchetta et al., 2008, Romiti et al., 2001). In some studies, however, despite B-lymphocyte function being improved following ART initiation, memory responses remained low at least over a year after viral control was attained (Iwajomo et al., 2015, Moir et al., 2008, Moir et al., 2010, Lederman et al., 1998, Soriano-Sarabia et al., 2005, Bekker et al., 2006).

Therefore, the data shows that the problem with children born from HIV-infected mothers (infected or uninfected) is that they are at an increased risk of severe, recurrent, or unusual infections by vaccine preventable infectious agents. Even though routine immunization programmes have been the most practical and safe approach in controlling childhood infections in HIV-infected children, their responses are sub-optimal, and results in HIV-infected children on ART and those who are HEU are conflicting. In the absence of ART, progressive immunodeficiency and disease is an inevitable consequence. A review of studies conducted in HIV-infected children indicates a paucity of studies that evaluate the effects of ART on the immune response to childhood vaccines, especially in sub-Saharan Africa. A large number of studies have shown the effects of HIV-infection or risk of being born to an HIV-infected mother on the immune response to vaccines, however, a limited number have assessed the effects of HIV-infection and ART on the immune response to vaccines prior to and post vaccination. Of the limited number of studies conducted on the effects of HIV and ART, these effects were assessed in either HIV-infected adolescents and/or adults; including evaluation of the effects of ART after primary or re-vaccination.

CHAPTER 3: STUDY OBJECTIVES

The primary objectives of this study were to:

- (i) Design a microbead multiplex assay on the Bio-Plex 200 instrument that can qualitatively and quantitatively detect antibody responses to diphtheria-toxoid (DT) vaccine, tetanus-toxoid (TT) vaccine, whole cell pertussis (wP) vaccine (DTwP vaccine), and recombinant hepatitis B vaccine (HepB). To compare Luminex and ELISA assays for the detection and quantification of antibodies to DT vaccine, TT vaccine, wP vaccine and HepB vaccine. Validate the Luminex method for the evaluation of immunological responses to DT vaccine, TT vaccine, wP vaccine and HepB vaccine (Results in Chapter 5).
- (ii) Evaluate sero-conversion and sero-protection rates after primary series against combined DT, TT, wP vaccine, as well as HepB in HIV-infected children that are receiving versus those not receiving ART and compare this to HIV-unexposed children (Results in Chapter 6).
- (iii) Evaluate sero-conversion and sero-protection rates after primary series against combined DT, TT, wP vaccine, as well as HepB vaccine in HEU children following the primary series of vaccines and compare this to HIV-unexposed children (Results in Chapter 6).
- (iv) Evaluate sero-conversion and sero-protection rates after a booster-dose against combined DT, TT, and wP vaccine, in HIV-infected children that are receiving versus those not receiving ART and compare this to HIV-unexposed children. In addition persistence of HepB vaccine antibodies was compared between the same children (Results in Chapter 7).
- (v) Evaluate sero-conversion and sero-protection rates after a booster-dose against combined DT, TT, and wP vaccine, in HEU children and compare this to HIV-unexposed children. In addition persistence of HepB vaccine antibodies was compared between the same children (Results in Chapter 7).

- (vi) Evaluate sero-conversion and sero-protection rates to a primary dose and following a booster dose of measles vaccine in HIV-infected children that with a CD4+ percentage ≥ 25 randomized to either early or deferred initiation of ART compared to HIV-unexposed children (Results in Chapter 8).
- (vii) Evaluate sero-conversion and sero-protection rates to a primary dose and following a booster-dose of measles vaccine in HEU children compared to HIV-unexposed children (Results in Chapter 8).

The secondary objectives of this study were to:

- (viii) Determine effects of ART on responses rate to HepB and DTwP vaccines by comparing ART-Def children and ART-Immed children and HIV-infected children with CD4+ <25% receiving early ART (CD4+<25%), pre-vaccination and post-primary vaccination (Results in Chapter 6).
- (ix) Determine effects of ART on response to DTwP vaccine by comparing ART-Def children and ART-Immed children, pre-booster, post-booster (Results in Chapter 7).
- (x) Determine effects of ART on persistence of antibody to HepB and DTwP vaccine by comparing ART-Def children and ART-Immed children at 15-18 months and 24 months of age (Results in Chapter 7).
- (xi) Determine effects of antiretroviral therapy on responses rate to measles vaccine by comparing ART-Def children with ART-Immed and CD4+<25% children, pre-vaccination and post-vaccination (Results in Chapter 8).

CHAPTER 4: METHODS

4.1 Ethical clearance and approval

Permission to conduct the study was sought and obtained from the Human Research Ethics Committee (HREC), at the University of the Witwatersrand (M080966) and the faculty of Health Sciences Higher Degrees Committee. This study was performed retrospectively on samples that were obtained as part of Comprehensive International Programme for Research on AIDS in South Africa (CIPRA-SA Project 4) HREC: 040704. All participants were given a unique study identification (ID) number at the beginning of the study. All laboratory specimens, evaluation forms, reports and other records were identified only by the study ID number to maintain subject confidentiality. All records were stored in a secure place, and all computer entry and networking programmes were carried out with coded numbers only.

4.2 Study design and site

This was a retrospective analysis of a prospective cohort on the immunogenicity of vaccines within the EPI-SA in HIV-infected, HEU and HIV-unexposed, uninfected children (HIV-unexposed) as the control group. These children were predominantly from the public sector either born or receiving care at the Chris Hani-Baragwanath Hospital in Johannesburg, and at Tygerberg Hospital in Stellenbosch, South Africa. HIV-uninfected children were recruited from the maternity wards of the respective study sites by a trained health worker in HIV counselling. The mothers must have tested HIV negative based on their participation in the PMCT programme that was conducted at each study site. HEU children were recruited from a cohort of children originally identified to participate in the “Children with HIV Early Antiretroviral (CHER)” study, but the child tested HIV negative at 4 weeks of age by polymerase chain reaction (PCR). HIV-exposed infected children were identified to be HIV-infected within the CHER study and had to have a baseline CD4+ T-lymphocyte count available. These children were randomized to either early or delayed antiretroviral treatment groups, detailed information on the groups of children are discussed in section 4.3. The vaccination schedule was based on the WHO and South African Department of Health guidelines, however, at 64-76 weeks of age children from all groups were either randomized, in equal proportions, to either receive a PncCV or HibCV booster dose of the vaccines by means of stratified permuted block randomization. Quantitative antibody responses to DTwP-HibCV, HepB and live-attenuated measles virus vaccines among HIV-uninfected (exposed and non-exposed) and HIV-infected children stratified by ART status were measured.

4.3 Description of study population

The study was conducted on archived serum samples from 578 children in whom the effect of HIV exposure and timing of ART initiation was evaluated on the quantitative and qualitative antibody responses to PncCV as the primary objective (Madhi et al., 2010). Five groups of children, aged at 6-12-weeks, were enrolled from April 2005 to June 2006. This included HIV-unexposed children born to HIV-uninfected mothers; and HEU children born to HIV-infected mothers; Figure 4.1. In addition, peri-natally HIV-infected children with a CD4+ T-lymphocyte percentage $\geq 25\%$ at enrolment were randomized to initiate ART when clinically and/or immunologically indicated (ART-Def) as per then WHO recommendations, or immediately upon enrolment (ART-Immed) (Violari et al., 2008). ART-Immed children were subsequently randomized to either interrupt ART at 12- (ART-12m) or 24-months of age (ART-24m) if immunologically stable and virologically suppressed, with ART being re-initiated if clinically and/or immunologically indicate; Figure 4.1. Furthermore, a convenience sample of HIV-infected children with CD4+ < 25% initiated on ART at enrolment (CD4+ < 24%) were included. HIV-infected children were co-enrolled from the CHER Study as described (Violari et al., 2008).

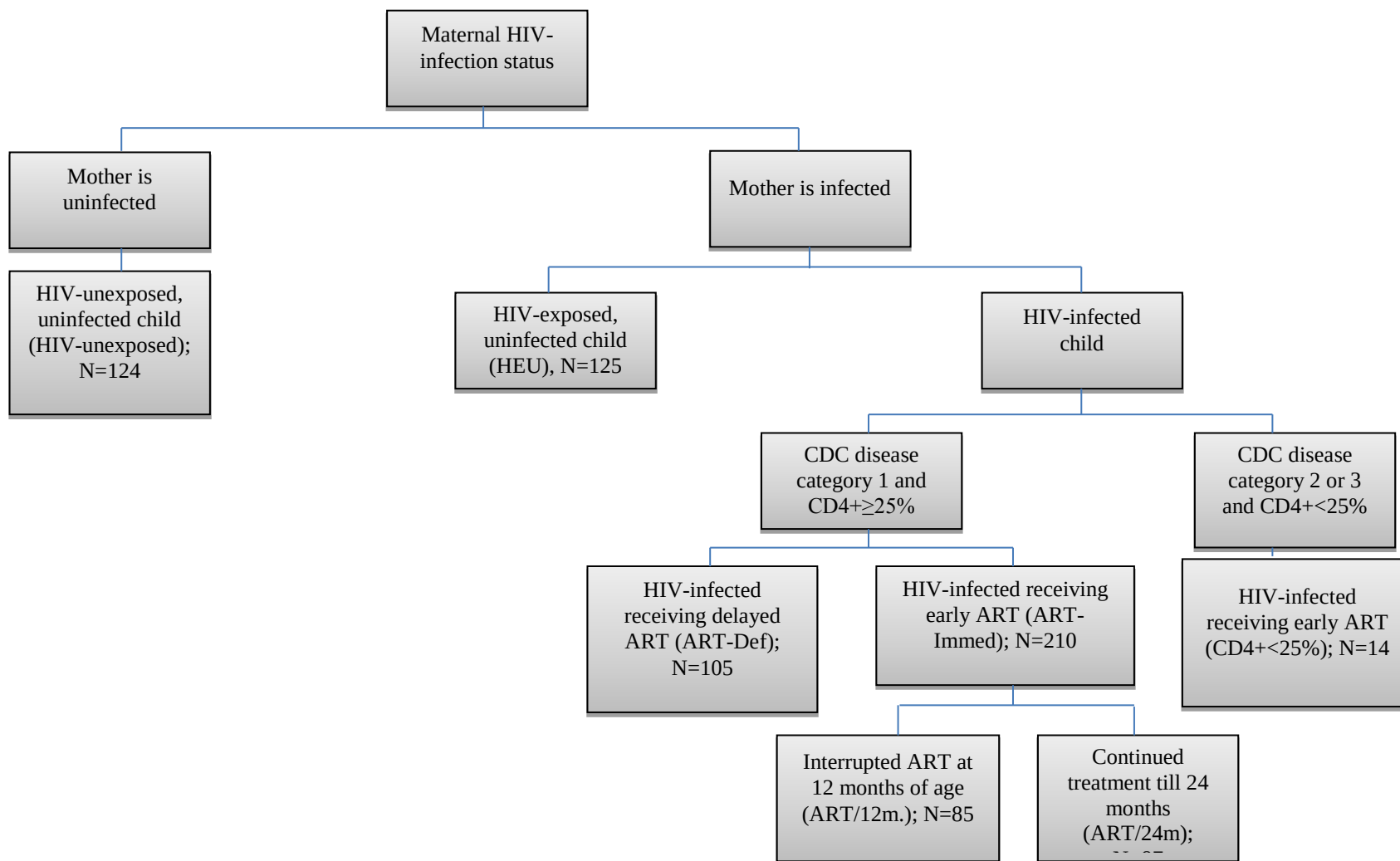


Figure 4.1: Flow diagram of all recruited study participants. N=total number recruited, however, there may have been missing samples at later visits.

4.3.1 Inclusion criteria

Recruited children had to be between the age of four and ten weeks and have a birth weight of at least 2000 grams. The parent or legal guardian of the child had to have indicated that they would stay in the study area for the study duration. Documentation of the mothers HIV status at 24 weeks gestation had to be available, and breastfeeding was permitted. Inclusion criteria for HIV-infected children were that the child had to be co-enrolled in the CHER study, HIV status had to have been confirmed by a positive DNA or RNA PCR at birth and a second one at four to ten weeks of age. Children born to HIV-infected mothers were allowed into the study if they were ART naïve except for receipt of ART within the PMCT programme. Allowed medications were antipyretics for auxiliary temperatures $\geq 38^{\circ}\text{C}$ after vaccination. HIV-infected children were allowed to receive ART as part of the study as well as trimethoprim-sulfamethoxazole prophylaxis as per the South African guidelines ((NDoHSA), 2004).

4.3.2 Exclusion criteria

Children were excluded from the study if they had received any blood products, any vaccine with the exception of BCG and OPV, prior to study entry. Children, who had received immunosuppressant agents for more than 2 weeks and were unable to tolerate oral medication, were excluded from the study. Presence of any major congenital anomalies that were life threatening. Children with acute, moderate to severe intercurrent illness or fever $\geq 38^{\circ}\text{C}$ within 72 hours before vaccination that resulted in hospitalization, were excluded from the study. The presence of Grade 2 allergic reaction and any Grade 3 clinical toxicity; the presence of any Grade 3 or 4 clinical or laboratory toxicity; were excluded from the study. Parent or guardian unable or unwilling to attend scheduled study visits. The use of ART other than the regimens described within the study, systemic cytotoxic chemotherapy and interleukin or other immune modulators. Insufficient sample volume or children with more than two visits not attended.

4.4 Antiretroviral therapy administered

At Chris-Hani Baragwanath Hospital, mothers who were identified as HIV-infected received a single dose of nevirapine at the time of delivery; this was also give to their child at the time of delivery to prevent mother-to-child transmission. While at Tygerberg Hospital the regimen consisted of zidovudine given to the HIV-infected mother from 34 weeks gestation and to the neonates for seven days and a single-dose nevirapine to both at the time of delivery. ART received

during the study was as follows: first-line antiretroviral was zidovudine at a dose of 240mg per square meter of body-surface area twice daily; lamivudine at a dose of 4mg per kilogram of body weight twice daily and lipinavir (300mg)-ritonavir (75mg) given twice daily up-to 6 months of age and thereafter at 230mg and 57.5mg respectively. The second-line regimen consisted of didanosine, abacavir and nevirapine (Violari et al., 2008).

4.5 Study vaccine regimens, administration and duration

Study subjects had five immunization visits where they received routinely administered childhood vaccines, as recommended in the EPI-SA, which included combined DTwP-HibCV, HepB and live-attenuated measles virus vaccine. PncCV was administered along with study vaccines and immunological responses were measured within the CIPRA-SA project 4 study. Table 4.1 below, illustrates the timing and administration of EPI vaccines being studied (in bold). DTwP-HibCV (CombAct-Hib®, Aventis, France) and HepB (Heberbiovac HB®; HeberBiotec S.A., Cuba) vaccines were administered intramuscularly into the right or left anterolateral external aspect of the upper thigh, respectively following the standard three dose schedule of 6, 10, 14 weeks of age (with a 4 to 6 week interval between doses) and a booster dose of DTwP at 67.8 weeks of age. Children were randomized at 67.8 weeks of age to either receive HibCV or PncCV booster dose, as part of the CIPRA-SA project 4. Live-attenuated measles vaccine (Schwarz strain, Rouvax®; Aventis, France) was administered intramuscularly into the right anterolateral external aspect of the upper thigh following a two dose schedule at 39.5 weeks and 67.8 weeks of age.

Table 4.1: EPI vaccine regimen

Immunization	Dose 1	Dose 2	Dose 3	1° Measles	2° Measles
Age in Weeks	6-8	10-12	14-16	38-40	66-68
DTwP-HibCV (CombAct-Hib®)^a	X	X	X		X ^b
Trivalent oral polio vaccine (OPV) (POLIORAL-trivalEnt®)	X	X	X		
HepB (Heberbiovac HB®)^c	X	X	X		
PncCV (Prenar®)	X	X	X		X
Measles vaccine (Rouvax®)^d				X	X

Footnote: ^aDTwP-HibCV was administered as a combination vaccine, CombAct-Hib® (diphtheria toxoid+ tetanus toxoid + whole cell pertussis+ HibCV); ^bChildren were randomized to either receive a booster dose of HibCV or PncCV; ^cThe hepatitis B vaccine was administered as a monovalent vaccine, Heberbiovac HB®; ^dMeasles vaccine (Rouvax®) was administered as a monovalent vaccine.

4.6 Specimen collection

Clotted blood samples were collected immediately prior to the first DTwP-HibCV/HepB doses (Time-point 1), one-month following the third dose (Time-point 2), prior to first live-attenuated measles vaccine dose (Time-point 3), prior to booster-doses of DTwP-HibCV or PnCV and live-attenuated measles vaccine (Time-point 4), two-weeks following booster doses (Time-point 5) and at two years of age (Time-point 6); at the Respiratory and Meningeal Pathogens Research Unit (RMPRU) and Tygerberg Hospital; Table 4.2. Serum was archived at -70°C until processed at the RMPRU, Johannesburg, South Africa.

Table 4.2: Specimen collection schedule

Time-point	1 ^a			2 ^b	3 ^c	4 ^d	5 ^e	6 ^f
Age in weeks	6-8	10-12	14-16	18-20	38-40	66-68	67-70	109-112
Serum for DTwP-HibCV and HepB antibodies	X			X	X	X	X	X
Serum for measles antibodies	X			X		X	X	X

Footnote: ^a1= Enrollment time-point and first dose of DTwP-HibCV and hepatitis B vaccines as well as pre-vaccination for measles vaccine; ^b2= immunogenicity visit which was four weeks post third dose of vaccines; ^c3=first dose of measles vaccination and 28 weeks post third dose of vaccination against DTwP-HibCV and Hepatitis b vaccine; ^d4= Booster-dose of DTwP and monovalent HibCV as well as second measles vaccine dose, no hepatitis b vaccine booster was given; ^e5=two weeks post-booster doses of study vaccines; ^f6= two year follow-up visit.

4.7 MEASUREMENT OF QUANTITATIVE AND FUNCTIONAL ANTIBODY RESPONSES

Archived serum samples were used to measure the quantitative immune response (total IgG antibodies) to study vaccine antigens (DT, TT, HepB); response to whole cell pertussis vaccine was measured using PT and FHA as surrogate markers for *B. pertussis*, these antigens have been shown to be good representatives of the whole cell response (Plotkin, 2010). Immune responses were measured at study Time-points 1, 2, 3, 4, 5 and 6 (Table 4.2), with an in-house multiplex fluorescent microbead immunoassay. Anti-PRP for HibCV was detected by standard in-house ELISA (Time-points 1-6) and a bactericidal assay to measure antibody functionality at study time-point 2. Immune response to live-attenuated measles vaccine (IgG antibodies) were measured at

time-points 1, 2, 4, 5 and 6, with a commercially available enzyme immunoassay and tested according to the manufacturers' instructions. All assays were conducted at RMPRU. The assays used are briefly described in sections 4.7.1 to 4.11. However, a detailed description on the preparation of reagents and detailed standards of operations (SOPs) are presented in Appendix 5.

4.7.1 Quantification of antibodies to DT, TT, PT, FHA and HepB using commercially available assays as gold standard assays for comparison with the Luminex assay

4.7.1.1 Material

Diphtheria toxoid (Batch: D52-2; Statens Serum Institute, Copenhagen, Denmark) from *Corynebacterium diphtheriae* and tetanus toxoid (Batch: T177-1; Statens Serum Institute, Copenhagen, Denmark) from *Clostridium tetani*. Pertussis toxin (Batch: 181209A1; List Biological Laboratories, INC, USA) and Filamentous haemagglutinin (Batch: 17040B1; List Biological Laboratories, INC, USA). Hepatitis B surface antigen (Engerix B B59, Batch: AHBVBPA 097; Glaxo-Smith Kline, Belgium). R-phycoerythrin (*R-PE*) conjugated goat anti-human IgG (gamma-chain specific) (Jackson ImmunoResearch Laboratories Inc., Westgrove, Pa). Carboxylated microsphere beads (Bio-Rad Laboratories, Hercules, CA). *N*-hydroxy-sulphosuccinimide, Sulpho-NHS (Cat # 56485; Sigma-Aldrich, Germany). 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl, EDC, (Cat # 77149; Thermo Scientific, Rockford IL, USA). 96 well round bottom plates Costar (Cat # 9017; Costar USA). AcroPrep multi-well filter-plates, 1.2 µm (Pall Corporation, USA). Anti-diphtheria toxoid IgG enzyme immunoassay MK014 (Lot # 260929; The Binding Site Ltd, U.K.) and VaccZymeTM Anti-tetanus toxoid IgG enzyme immunoassay MK010 (Lot # 265507; The Binding Site Ltd, U.K.). SERION ELISA classic *Bordetella pertussis* IgG ESR120G (Lot # SKY.CX; Institut Virion/Serion GmbH, Germany). ImmunoLISATM HBsAb (Lot # 40261000; Organics Ltd. Part of the Inverness Medical Innovations Group, Israel).

4.7.1.2 Measurement of antibody to DT by quantitative ELISA

Quantification of DT IgG antibodies was achieved by the use of Diphtheria toxoid IgG EIA (Binding site Ltd, UK), and tested according to the manufacturers' instructions. Briefly, 96 well microtiter plates were supplied pre-adsorbed with purified DT antigen derived from *C. diphtheriae*. The reference sera (National Institute of Biological Standards and Control (NIBSC London, UK) diphtheria antitoxin reference preparation 00/496), controls and diluted unknown samples (1:100) were added in duplicate to each well and antibodies recognizing the DT antigen were bound during

the first incubation. Optical densities were determined at 450 nm on a microplate reader (Labsystems Multiskan RC; USA). DT-specific IgG antibody levels (IU/ml) were calculated from the log/linear scale of the plotted reference sera graph with a range of 0.004-3 IU/ml. Assays with concentrations between 0.1 and 0.2 IU/ml were considered as low positives and 0.6 and 1.0 IU/ml were considered as high positives. Samples for which the concentration was below the detection limit (0.004 IU/ml) were assigned a concentration of half the detection limit of the assay (i.e. 0.002 IU/ml) when calculating GMC.

4.7.1.3 Measurement of antibody to TT by quantitative ELISA

Quantitative TT IgG antibodies were measured with VaccZyme™ Anti-Tetanus Toxoid IgG EIA (Binding site Ltd, UK), and tested according to the manufacturers' instructions. Briefly, 96 well microtiter plates were pre-coated with purified TT antigen derived from the toxin of *C. tetani*. The reference sera (NIBSC tetanus antitoxin reference preparation 76/589), controls and diluted unknown samples (1:100) were added in duplicate to each well and antibodies recognizing the TT antigen were bound during the first incubation. Optical densities were determined at 450 nm on a microplate reader (Labsystems Multiskan RC; USA). TT-specific IgG antibody levels (IU/ml) were calculated from the log/linear scale of the plotted reference sera graph with a range of 0.01-7 IU/ml. Assays with concentrations between 0.15 and 0.35 IU/ml were considered as low positives and 1.05 and 1.75 IU/ml were considered as high positives. Samples for which the concentration was below the detection limit (0.009 IU/ml) were assigned a concentration of half the detection limit of the assay (i.e. 0.005 mIU/ml) when calculating GMC.

4.7.1.4 Measurement of antibody to PT and FHA by quantitative ELISA

Measurement of IgG antibodies against PT and FHA was undertaken with the Serion ELISA (Institut Virion Serion GmbH, Germany) and tested according to the manufacturers' instructions. Briefly, 96 well microtiter plates were adsorbed with PT and FHA from *B. pertussis* and this constituted the solid phase. Reference serum (human sera in PBS with protein) and unknown samples (1:100) and controls were added to the wells in duplicate and any antibodies specific to the antigens present were bound to the solid phase. The optical density was read at 405 nm on a microplate reader (Labsystems Multiskan RC; USA). The mean optical density value of the standard serum should have been within the validity range (OD 0.48-1.63), which was given on the lot specific quality control certificate (Lot number: SKY.CX) of the kit (after subtraction of the substrate blank).

Concentration of samples was calculated using the SERION *evaluate*-Software, which operates by inputting the 4-parameters and the reference value of the standard serum and antibody activity were calculated online. The following criteria were used to interpret the results, IgG>30 FDA-U/ml were considered as positive; <20 FDA-U/ml was negative and 20 to 30 FDA-U/ml was indeterminate.

4.7.1.5 Measurement of antibody to HepB by quantitative ELISA

The quantification of HepB antibodies was done by the use of ImmunoLISA™ HBs Ab EIA (Organics Ltd Inverness Medical Innovations Group, Israel), according to the manufactures' instructions. The 96 well microtiter plates were pre-coated with highly purified heat inactivated HBsAg from subtypes "ad" and "ay". Reference sera (NIBSC WHO anti-HBsAg 1st reference preparation 1977, lot 17-2-77), controls and unknown samples (undiluted) were added in duplicate to each of the pre-coated wells and incubated to allow antigen antibody interaction. Optical density was measured at 450nm with a microplate reader (Labsystems Multiskan RC; USA). The amount of antibody was quantitated by means of a standard curve calibrated against the WHO reference preparation. A positive sample was interpreted as a sample with HepB antibody concentration ≥ 10 mIU/ml. Samples for which the concentration was below the detection limit (10 mIU/ml) were assigned a concentration of half the detection limit of the assay (i.e. 5 mIU/ml) when calculating GMC.

4.7.1.6 Quantitative measurement of antibodies against DTwP and HepB vaccines using Luminex assay

Luminex microspheres are colour-coded microbeads of approximately 50nm in size and are available in 100 distinct colour sets. Each bead set was coated with a different antigen, specific to a particular bioassay (which will be detailed in section *Quantitative antibody assay for the measurement of DT, TT, PT, FHA and HepB antibody responses* below). By combining multiple bead sets in a single reaction, the capture and detection of antibodies against multiple antigens can be made simultaneously from a single sample. Bound antibody is detected by the addition of rabbit anti-human IgG antibody which has been conjugated to R-phycoerythrin (R-PE), a fluorescent protein. The beads are read on a dual-laser flow-based detection instrument, such as the Luminex 200™ or Bio-Plex 200™ analyzer. One laser classifies the bead colour and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of the bound analyte.

i) Coupling of DT, TT, PT, FHA and HBsAg antigens to carboxylated microspheres

The first step to this assay was the coupling of purified DT, TT, PT, FHA and HBsAg to activated microspheres, and this was achieved by using a two step carbodiimide reaction (Staros et al., 1986, van Gageldonk et al., 2008). Briefly, the protein antigens were linked to the microspheres by an EDC/Sulpho-NHS reaction, in which the COOH groups of the microspheres was dehydrated with EDC, and in the presence of NHS, modifies the COOH to form an amine-reactive ester (Staros et al., 1986). The NHS-ester-containing molecule can then be reacted spontaneously with a primary amine-containing molecule e.g. amino group found on the N-terminal of the protein and certain amino acids. While the carboxyl-containing molecule can be made to react directly with the amine-containing molecule by the addition of EDC, the reaction is much more efficient with Sulpho-NHS present because a stable intermediate is created and incubated in the dark at room temperature (Staros et al., 1986). The final bead ratio for each antigen was 25µg/6.25x10⁶ for DT and TT antigens; 5µg/6.25x10⁶ for PT and FHA; and 14µg/6.25x10⁶ for HepB.

ii) Reference, controls and serum samples

An in-house standard sera consisted of pooled adult sera, which was calibrated against the International standards (NIBSC code 00/496 (diphtheria antitoxin), 76/589 (tetanus antitoxin), 07/164 (anti-Hepatitis B surface antigen immunoglobulin) and 06/140 (Pertussis antiserum (human) 1st International Standard) and used as an in-house reference sera for determining concentrations of natural and vaccine induced antibodies to all study antigens. The in-house positive control was derived from pooled human plasma (Hebagam® IM, National Bioproduct Institute, Pinetown South Africa), each unit was individually tested and found negative for HBsAg, anti-HCV and anti-HIV-1/-2. The negative control was assay buffer. For assay validation and optimization purposes (first phase of the study), unknown samples were collected from 25 HIV-uninfected children who were participants of the “Durability study” (HREC number: 031013) which include children between 4 – 7 years of age (described in detail in section 4.8). (For the second phase of the study (determining passive and vaccine induced immune responses to DT, TT, PT, FHA and HBsAg), serum samples were collected as described in section 4.6.

iii) Multiplex immunoassay (MIA) for quantitation of DT, TT, PT, FHA and HepB specific antibodies

This was the second phase of the study; we assessed serum samples described in section 4.6. A standard curve was prepared from seven 4-fold dilutions (1:50 to 1: 204800) of the reference serum

in phosphate buffered saline with 10% Foetal Bovine Serum (PBS-F). Controls and unknown sera were diluted in PBS-F at 1:100. Unknown serum samples that were above the detection limit of the assay were further diluted until they were within the dynamic range of the assay. Reference, controls and unknown sera, were mixed with the coupled microsphere beads in a 96-well AcroPrep multi-well filter-plates 1.2 μm (5000 microspheres/region/well) and incubated for one hour at room temperature with shaking. Microspheres were collected by vacuum filtration and washed with PBS-T. *R*-phycoerythrin-conjugated anti-human IgG was added across all wells and incubated for 30 min, in a shaker, at room temperature. Following incubation, the plates were washed and the beads were re-suspended in PBS-T buffer and transferred into a 96-well round-bottom plate, and read with a Luminex platform Bio-Plex 200 instrument (Bio-Rad, Hercules CA, USA) for quantification of IgG antibodies. Antibody concentrations in either IU/ml for DT, -TT, -PT and -FHA and mIU/ml for HepB were determined relative to the standard curve, with a five parameter logistic log model with the BioPlex software (van Gageldonk et al., 2008, Pickering et al., 2002a).

4.8 VALIDATION OF LUMINEX ASSAY

4.8.1 Calibration curve and its fitting algorithm

The dose response curve expressed in terms of response y (fluorescent intensity (FIs) on a linear scale) versus the dose x (IU on a log scale) commonly display a pronounced sigmoidal shape. The five parameter logistic function fits these data with a high degree of accuracy (van Gageldonk et al., 2008, Cambron et al., 2009, Plikaytis et al., 1991). An unweighted five-parameter logistic function was used to fit the standard curve for all multiplex reference sera. In order to estimate the accuracy of the standard curves, R^2 , the percentage of the relative error (RE) and percentage recovery (percentage backfit dose error) were determined for each standard point. The backfit dose is the recalculated concentration of each standard obtained by extrapolating its response (FIs) through the fitted curve. The RE of each standard point is as follows: $\%RE = [(recalculated\ value - assigned\ value)/assigned\ value] \times 100\%$. To evaluate the overall quality of the fitting of the standard curve, standard curves collected during the development phase of the assay were assessed for R^2 and RE.

4.8.2 Comparison of Luminex assay with ELISA

The Luminex assay was compared to a monoplex ELISAs for quantification of IgG antibodies against DT, TT, PT, FHA and HepB. The same reference sera, controls and sera ($n=25$) were used for comparison of antibodies to all five antigens. Twenty five serum samples from children aged

between 4-7 years were tested with both assay systems. Samples that fell outside of the upper detection limit for the assays were repeated at a higher dilution. Serum antibody concentrations to the protein antigens were determined by both methods and were subjected to linear regression analysis and the correlation coefficients were also calculated and designated R^2 .

4.8.3 Competitive inhibition studies of Luminex assay

To determine the specificity of the Luminex method and reported as percent change in concentration in response to the addition of individual antigens to the serum, inhibition studies were performed as described previously (van Gageldonk et al., 2008, Pickering et al., 2002a) with slight modifications. A serum with known high concentrations of IgG antibodies directed against all five antigens was diluted 1:1000 times. Four aliquots of each serum dilution were prepared. Each aliquot was incubated with an equal volume of one of the five purified antigens in a concentration of 30 $\mu\text{g/ml}$ for DT, TT, PT, FHA and 14 $\mu\text{g/ml}$ for HepB for 1 hour at room temperature or overnight at 4°C. A 1:1000 dilution of the serum was used as a control (non-absorbed). Homologous and heterologous inhibition of the antibody concentration compared to the control was calculated as a percentage inhibition.

4.8.4 Sensitivity of Luminex and ELISA serum IgG

The lower limit of quantitation (LLOQ) for the Luminex assay was determined from the recorded blank values from independent runs ($n=16$) for each antigen. This value was given as the equivalent antibody concentration of the reference at the point where the fluorescence intensity was equal to the blank plus three standard deviations. For the ELISAs the manufacturers' results were reported as indicated in the package insert of the kit.

4.8.5 Assay Reproducibility

The reproducibility of the assay was determined by calculation of inter and intra assay variation. Intra-assay variation within a plate was determined by testing an independent duplicate of different samples. For each analyte the coefficient of variation (%CV) for each duplicate result per sample was calculated and averaged. Intra-assay variation (plate to plate variation) was done by testing a serum panel in dependent dilutions between different plates on the same day. The % CV between each of these results for each analyte was calculated and averaged. Inter-assay variation (variation from assay to assay) was assessed by testing a serum panel in two separate assay runs. The %CV between each of these results for each analyte was calculated and averaged. The reproducibility of

the results generated from separate batches of conjugated microsphere beads was determined with considerable time intervals. A serum panel was tested with different batches of conjugated microsphere beads. The results obtained with the different batches were compared by calculating the correlation of coefficient (*R*).

4.9. MEASUREMENT OF *HAEMOPHILUS INFLUENZAE* TYPE B ANTI-PRP ANTIBODIES WITH AN IN-HOUSE ELISA

4.9.1 Principle of the Method

PRP antibodies were measured with a standardized in-house enzyme immunoassay (Kurikka et al., 1995). Briefly, Hib antigen (HboHA; Wyeth Vaccines distributor NIBSC, UK) at a final concentration of 1 mg/ml was pre-coated onto 96 well microtiter plates, which act as the solid phase. Hib specific antibodies in the reference serum (anti-*H.influenzae* type b capsular polysaccharide serum, lot 1983 (NIBSC)), sample, and low and high control sera were bound to the antigens on the surface of the well, all were added in duplicate. The antibody-antigen complex was detected by adding anti-human IgG (γ -chain specific) alkaline phosphatase antibody (Sigma, USA) conjugate. Substrate (4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma, USA)) was added to the antigen-antibody conjugate complex and measured colometrically at 405 nm. Sero-protective antibody titre was defined as ≥ 0.15 $\mu\text{g/ml}$, samples for which the OD value was < 0.1 were arbitrarily assigned a titre of half the detection limit of the assay (i.e. 0.06 $\mu\text{g/ml}$) to provide an unbiased calculation of GMTs.

4.10 MEASUREMENT OF SERUM BACTERICIDAL ACTIVITY SPECIFIC FOR *HAEMOPHILUS INFLUENZAE* TYPE B

4.10.1 Principle of Assay

This assay differs from the quantitative ELISA that was performed for *H. influenzae* type b, in that it measures the functional binding of PRP antibodies to viable bacteria (*H. influenzae* Type b, strain GB3292), and their ability to illicit bacterial killing in the presence of complement. This protocol was based on the original method by Schlesinger et al (1992) and later modified by Romero-Steiner et al (2001). In this assay, serum samples are serially diluted in a microtiter plate and a known concentration of live bacteria are added to each well. An active source of complement is added to induce killing of opsonized bacteria. After incubating for an hour; the number of viable bacteria in

each well was determined by plating out each serum dilution onto agar plates. Functional killing titers were expressed as the reciprocal of the dilution at which the serum was able to illicit 50% bacterial killing. For statistical purposes, samples that were found to be below the detection limits were given an arbitrary value of half the detection limit.

4.11 MEASUREMENT OF MEASLES VIRUS ANTIBODY TITRES

4.11.1 Principle of assay

Measurement of quantitative levels of measles-specific IgG antibody titres for all serum specimens were determined by the Enzygnost (Dade Behring, Marburg, Germany) anti-measles-virus/IgG enzyme immunoassay (sensitivity =99.6%; specificity =100%), as described previously (Ovsyannikova et al, 2004). Briefly, serum samples were added in duplicate to a microtitre plate, which contained two parallel wells coated with a test whole measles virus antigen or control antigen derived from non-infected cells. Optical densities were determined at 450 nm and corrected at 650 nm on a microplate reader (Labsystems Multiskan RC; USA). The difference in mean absorbance between the viral test antigen and the control antigen for each sample was calculated and multiplied by a correction factor (determined by dividing a kit specific nominal value by the mean of the reference standard) to give the corrected change in absorbance (ΔA). Measles-specific IgG antibody levels (mIU/ml) were calculated from the antilog of the following formula: $\log_{10} = \alpha * \Delta A^\beta$ (where α and β are lot-dependent constants). Assays with titers <150 mIU/ml were considered as negative (equivalent to <0.1 optical density [OD]), 150–329 mIU/ml as equivocal (equivalent to 0.1-0.2 OD) and ≥ 330 mIU/ml as sero-protective (equivalent to >0.2 OD) per manufacturers criteria. For this study, sero-positivity was defined as antibody titers ≥ 150 mIU/ml. Samples for which the OD value was <0.1 were assigned a titer of half the detection limit of the assay (i.e. 75 mIU/ml) when calculating GMT. The coefficient of variation for this assay in our laboratory was 8.9%.

4.12 Statistical analysis of antibody kinetics

Statistical analyses were performed using GraphPad Prism 5a (GraphPad Software Inc, La Jolla, CA, USA), R (R Foundation for Statistical Computing, Vienna, Austria) and STATA statistical package (STATA Corporation, College Station, TX, USA). The thresholds for correlates of sero-protection were defined as antibody concentrations greater or equal to 0.01 IU/ml and 0.1 IU/ml for DT, 0.1 IU/ml for TT, 10 mIU/ml for HepB, ≥ 0.15 $\mu\text{g/ml}$ for PRP (i.e. Hib epitope) and 230 mIU/ml for measles (Plotkin, 2010). Surrogate of immune response to pertussis was defined as ≥ 4 -

fold increase in antibody concentration from pre- to post-vaccination for PT and FHA (Plotkin, 2010). Alternate antibody threshold levels evaluated included ≥ 100 mIU/ml for HepB and ≥ 1.0 $\mu\text{g/ml}$ for PRP, which may be a predictor for long-term protection (Plotkin, 2010). Pre- and post-vaccination GMCs/GMTs and proportions with sero-protective levels or surrogates of antibody responses were compared using analysis of covariance or multivariate logistic regression considering gender, age, race (black African or mixed ancestry), CD4+ T-lymphocyte percentage, study center, birth weight, mother-to-child HIV-prevention antiretroviral regimen and pre-vaccination antibody concentrations. Pre- and post-booster vaccination GMCs/GMTs and proportions with sero-protective levels or surrogates of antibody responses were compared using analysis of covariance or multivariate logistic regression considering gender, age, race (black African or mixed ancestry), CD4+ T-lymphocyte percentage, study center, and post-primary vaccination antibody concentrations. Additional factors that may be associated with response to vaccination included breastfeeding, exposure to PMTCT and time since vaccination were evaluated by linear regression.

To minimize additional confounders, the analysis was limited to children who received all three vaccine doses and for who blood for immunogenicity assays were collected within protocol-specified window periods. A paired *t*-test was used in the comparison of pre- and post-vaccination CD4+ percentages. Any missing data for children in all the groups was excluded from all comparison analysis. For all the tests, a two-sided *p*-value of ≤ 0.05 was considered statistically significant.

CHAPTER 5 RESULTS: VALIDATION AND OPTIMIZATION OF LUMINEX ASSAY

5.1 Results

5.1.1 Calibration curve and its fitting algorithm

The results for calibration curve and its fitting algorithms represented by Figure 5.1 and Table 5.1 which summarize R^2 , %RE and % recovery results. R^2 was ≥ 0.99 for the study reference tested for all antibodies with %RE being less than 20% for the seven-step dilution series, which indicates high variability at the lower end of the plot. FHA showed high variability and decreased fit. Current literature and guidelines recommend an absolute value of RE of $\leq 20\%$ and an r^2 of >0.99 (Braggio et al, 1996; US FDA, 2001), which was observed for all the tested antibodies. Percentage recovery was set to be between 80 to 130%, from the data in Table 5.1 our reference sera performed very well with this regard.

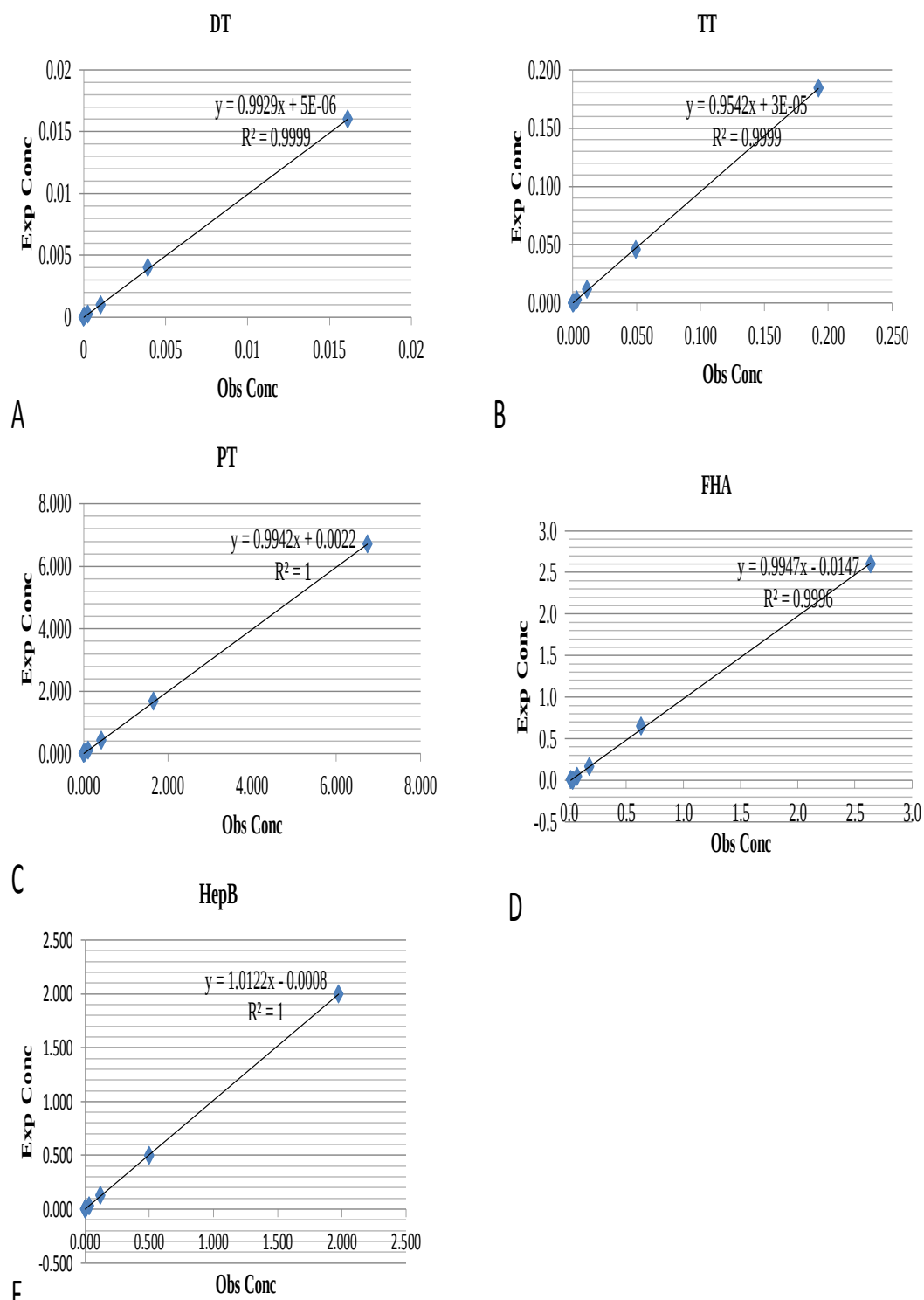


Figure 5.1: Fitting algorithm of standard curves for observed versus expected concentration in a seven step 4-fold dilution of the reference sera.

Table 5.1: Data on percentage recovery and relative error for each standard point

Antigen	Dilution	Expected concentration (IU/ml)	Mean obs. Conc. (IU/ml)	CV (%)	%Recovery	% RE
DT	50	0.016	0.016	0.86	100.82	0.82
	200	0.004	0.004	2.84	98.06	-1.94
	800	0.001	0.001	8.50	103.83	3.83
	3200	0.00025	0.000268	11.90	107.17	7.17
	12800	0.00006	0.000065	10.99	107.50	7.50
	51200	0.00002	0.000022	23.26	110.00	10.00
	204800	0	0.000000	0	100	0
TT	50	0.184	0.193	17.74	104.65	4.65
	200	0.046	0.049	28.13	107.20	7.20
	800	0.0115	0.0111	15.68	96.37	-3.63
	3200	0.00288	0.00295	6.34	102.38	2.38
	12800	0.00072	0.00072	7.79	99.47	-0.53
	51200	0.00018	0.00019	12.89	105.56	5.56
	204800	0.00004	4.23077E-05	19.67	105.77	5.77
PT	50	6.7	6.7	2.21	100.63	2.21
	200	1.68	1.66	4.08	99.05	4.08
	800	0.42	0.42	6.52	100.98	6.52
	3200	0.10	0.11	13.56	105.44	13.56
	12800	0.03	0.03	12.57	102.33	12.57
	51200	0.01	0.01	18.21	95.73	18.21
	204800	0.002	0.002	27.47	93.84	27.47
FHA	50	2.6	2.6374	1.55	101.44	1.44
	200	0.65	0.6291	3.48	96.78	-3.22
	800	0.1625	0.1767	15.81	108.72	8.72
	3200	0.04063	0.0689	54.55	169.59	69.59
	12800	0.01016	0.0145	52.47	142.83	42.83

	51200	0.00254	0.0288	226.08	1133.13	1033.13
	204800	0.00063	0.0331	191.17	5254.95	5154.95
HepB	50	2	1.975	12.80	98.77	-1.23
	200	0.5	0.500	5.71	100.06	0.06
	800	0.125	0.121	7.00	96.86	-3.14
	3200	0.03125	0.0328	5.08	105.06	5.06
	12800	0.00781	0.0077	6.97	98.44	-1.56
	51200	0.00195	0.0020	12.75	103.79	3.79
	204800	0.00049	0.00047	25.18	94.98	-5.02

Footnote: DT: diphtheria-toxoid; TT: tetanus-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; HepB: hepatitis B; %RE: percentage relative error.

5.2 Comparison of Luminex assay with ELISA

Twenty five serum samples were assessed for antibody concentrations to the study protein antigens using both methods and were subjected to linear regression analysis and the correlation coefficients were determined as shown in Figure 5.2. The correlation coefficients for DT, TT, PT, FHA and HepB were 0.90, 0.93, 0.97, 0.89 and 0.72 respectively.

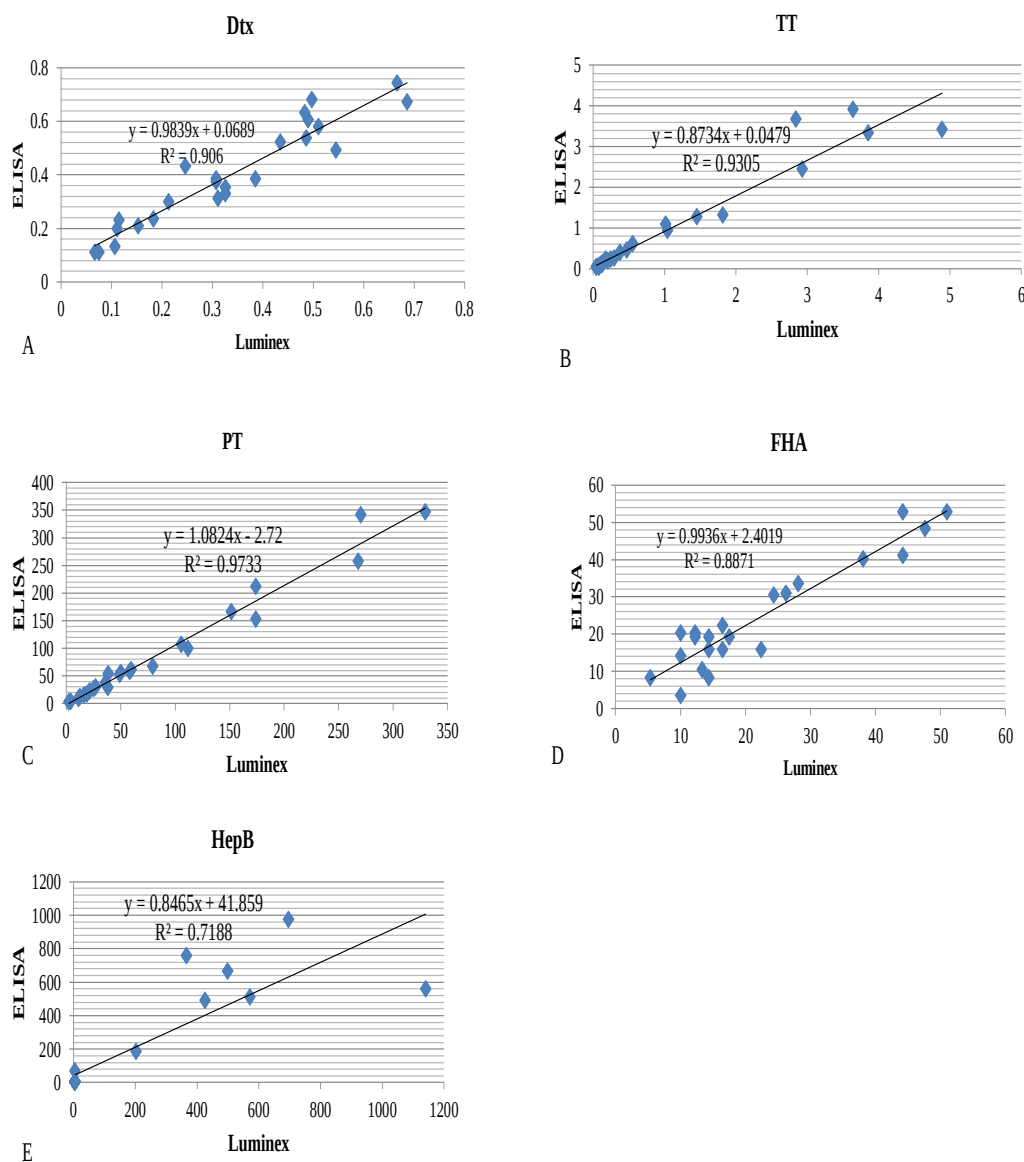


Figure 5.2: Comparison of the IgG concentrations against DT, TT, PT, FHA and HepB determined by Luminex assay and monoplex ELISA. Sera from 25 patients were quantitated by both Luminex and ELISA methods. Antibody concentrations for all five protein antigens were reported in either IU/ml (DT, TT, PT and FHA) or mIU/ml (HepB).

The results were further analysed by determining frequencies of agreement with respect to a cut-off of the sero-protective concentration for each antigen; Table 5.2. There was <8% non agreement observed for DT, TT, and HepB between ELISA and Luminex.

Table 5.2: Frequency table of agreement between the Luminex assay and ELISA (antibody concentration classified as above or below sero-protective titers for DT (≥ 0.1 IU/ml), TT (≥ 0.1 IU/ml) and HepB (≥ 10 mIU/ml). While PT was classified by antibody concentration ≥ 20 IU/ml and FHA was classified by antibody concentration ≥ 2 IU/ml).

Antibody	Luminex and ELISA $\geq$ sero-protection	Luminex and ELISA $<$ sero-protection	Luminex $\leq$ sero-protection and ELISA $>$ sero-protection	Luminex $>$ sero-protection and ELISA $\leq$ sero-protection
DT	23 (92%)	0 (0%)	0 (0%)	2 (8%)
TT	18 (72%)	5 (20%)	2 (8%)	0 (0%)
PT	20 (80%)	5 (20%)	0 (0%)	0 (0%)
FHA	25 (100%)	0 (0%)	0 (0%)	0 (0%)
HepB	9 (36%)	14 (56%)	0 (0%)	2 (8%)

Footnote: DT: diphtheria-toxoid; TT: tetanus-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; HepB: hepatitis B; ELISA: enzyme immunoassay.

We then studied the difference between ELISA and Luminex methods for the detection of antibodies; Figure 4.3. A difference of 0.022 [95% CI: -0.40-0.44] for antibody to DT; TT was 0.09 [95% CI: -0.67-0.84]; PT was -4.32 [95% CI: -39.93-31.29]; FHA was 4.44 [95% CI: -89.23-98.10] and HepB was 10.31 [95% CI: -57.70-78.31] between ELISA and Luminex methods by Bland-Altman regression test.

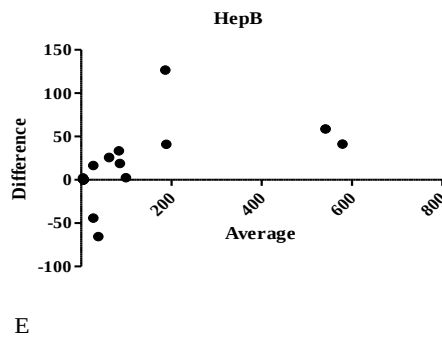
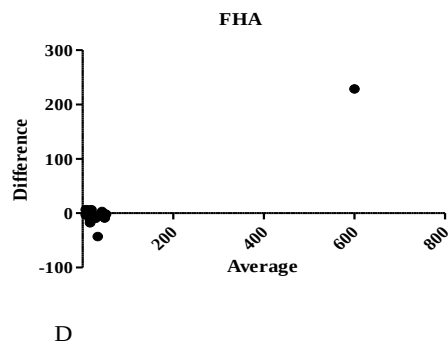
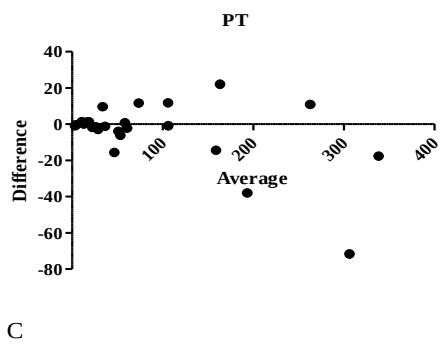
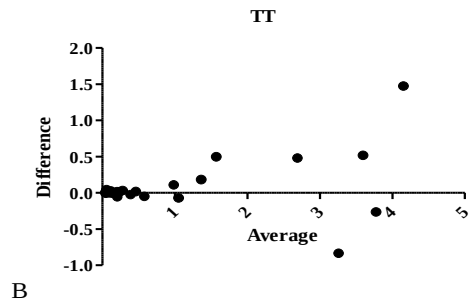
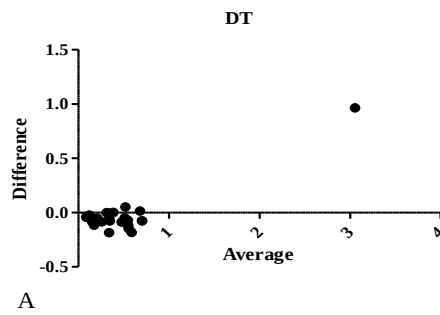


Figure 5.3: Difference between Luminex assay and ELISA using the Bland-Altman plot. The closer the difference is to zero the more alike the methods are; DT: diphtheria-toxoid; TT: tetanus-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; HepB: hepatitis B

5.3 Competitive inhibition studies of Luminex assay

The addition of homologous antigen to the serum at saturated concentrations of 30 µg/ml for DT, TT, PT, FHA and 14 µg/ml for HepB, resulted in inhibition of >90% of signal compared to the control sample for all tested antigens with the Luminex assay; Figure 5.4.

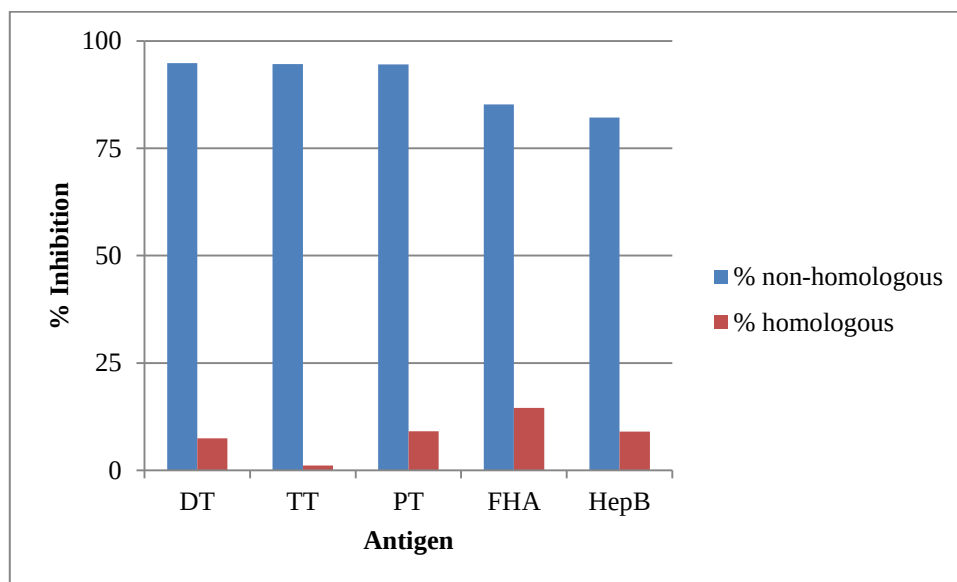


Figure 5.4: Specificity assay: Percentage inhibition on addition to homologous antigen inhibitor. DT=93%, TT= 99%, PT=91%, FHA=85% and HepB= 91% for homologous inhibition per antigen.

Non-heterologous antigen was added to the samples, inhibition was <15% for all other antigens except HepB and FHA which resulted in 16.88% non-homologous inhibition; Figure 5.4.

5.4 Sensitivity of Luminex and ELISA serum IgG

For the ELISAs the manufacturer results were reported as indicated in the package insert of the kit. The calculated LLOQ and the fold difference between assays are shown in Table 5.3. The Multiplex assays were 4-, 9-fold more sensitive than ELISA for DT and TT respectively. While a decreased sensitivity of 0.25-fold was observed for PT, FHA and HepB. No upper limit of detection was determined since all samples above the measurable range were re-diluted to fall within the measurable range of the standard curve of the reference serum.

Table 5.3: Lower limit of quantitation of antigen specific IgG antibodies against DT, TT, PT, FHA and HepB antigens determined by ELISA and Multiplex methods, and the –fold difference in sensitivity of Multiplex over ELISA.

Antigen	ELISA (*LLOQ)	Multiplex (*LLOQ)	**Fold difference between assays
DT	0.004 IU/ml	0.001 IU/ml	4
TT	0.0093 IU/ml	0.001IU/ml	9
HepB	2.5 mIU/ml	10.00 mIU/ml	0.25
PT	†5 FDA-U	19.823 IU/ml	0.25
FHA		1.657 IU/ml	3

Footnote: *LLOQ: lower limit of quantification. **Fold difference between assays was calculated as ELISA LLOQ/ Multiplex LLOQ, † PT and FHA in the ELISA were coated together in a single well on the plate therefore the results could not be differentiated for each antigen.

5.5 Linearity and range of assay

Each antigen was conjugated to an individual microsphere bead region. To assess the conjugation and to determine the range of fluorescence generated each microsphere bead region (monoplex) and all five microsphere bead regions (pentaplex) were assayed with seven 4-fold dilutions of the reference serum. The MFI generated from the pentaplex assay were similar for all five microsphere bead sets and demonstrated linearity over the seven 4-fold dilution range; Figure 5.5.

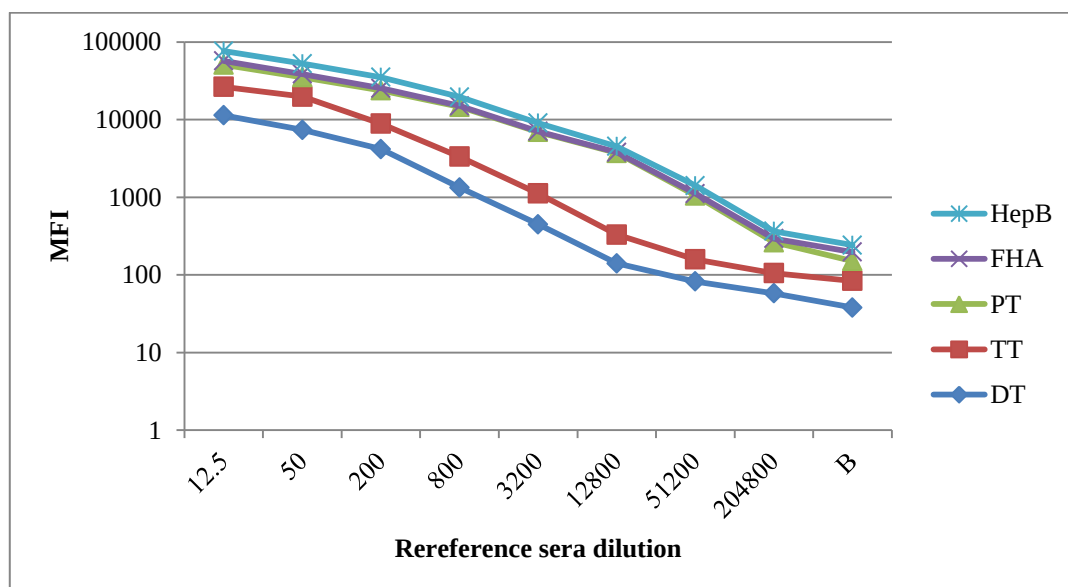
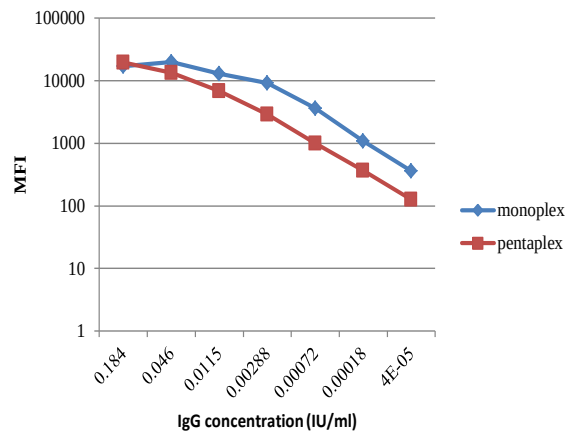
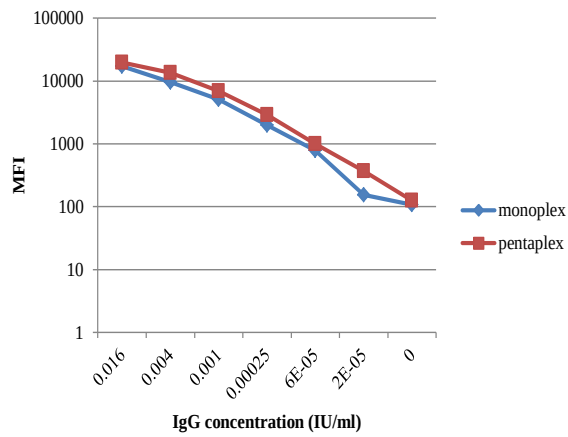


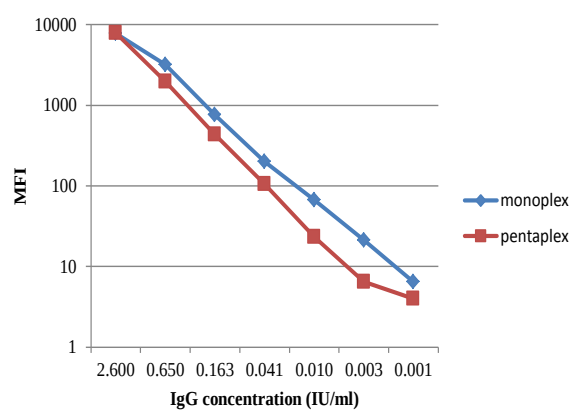
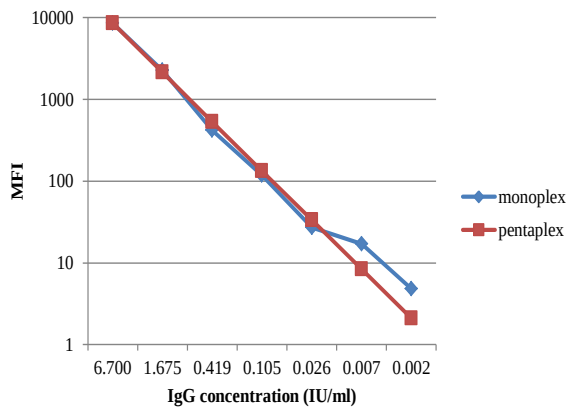
Figure 5.5: MFI generated from the pentaplex assay with in-house standard reference sera titration.

The possibility of interference between the different microsphere bead regions was determined by comparing the MFIs generated from the monoplex assay with those obtained with the pentaplex assay. The pentaplex was performed by combining all microsphere bead regions. Figure 5.6 shows the MFIs obtained from all microsphere bead regions run individually and in a pentaplex assay. MFIs generated by the pentaplex were comparable to that generated by the monoplex, for all combinations of microsphere bead regions.



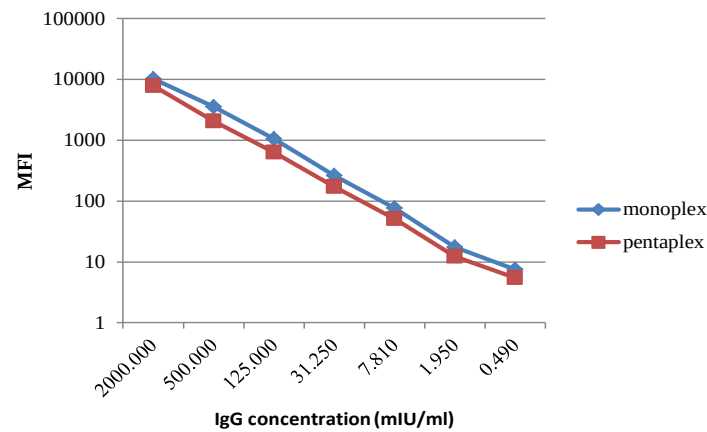
A

B



C

D



E

Figure 5.6: Comparison of MFI generated from monoplex and pentaplex assays for (A) DT, (B) TT, (C) PT, (D) FHA and (E) HepB.

5.6 Multiplex assay reproducibility

The mean percentage CV obtained from duplicates of 5 samples within a plate ranged from 7.7% for DT to 22.7% for TT (Table 5.4), indicating the variation within a plate was acceptable. Inter-assay variation was determined by comparison of antibody concentrations obtained from 5 samples run in three separate assays. The mean percentage CV was similar to that obtained for intra-assay variation.

Table 5.4: Intra- and inter assay variation.

Intra-assay variation (Within Assay)				
	DT	TT	HepB	PT
Day 1	2.10	17.91	405.97	1355.24
	2.20	23.02	464.87	1254.92
	2.60	18.98	551.80	1507.75
	2.50	21.59	537.69	1514.39
	2.41	22.53	502.16	1421.25
Day 2	2.36	33.18	661.78	1053.97
	2.85	27.28	490.65	1052.52
	2.52	37.40	635.01	1071.26
	2.59	25.34	576.72	1249.58
	2.59	36.97	849.83	1340.41
Day 3	2.67	26.59	513.33	1385.15
	2.57	23.59	540.32	1361.66
	2.68	25.74	519.92	1432.53
	2.61	25.13	602.70	1246.27
	2.38	22.19	516.48	1455.76
Mean	2.51	25.83	557.95	1313.51
SD	0.19	5.86	103.37	155.89
CV %	7.69	22.67	18.53	11.87
Inter-assay variation (Between assays)				
	DT	TT	HepB	PT
Day 1 mean	2.36	20.81	492.50	1410.71
Day 2 mean	2.58	32.03	642.80	1153.55
Day 3 mean	2.58	24.65	538.55	1376.27
Mean	2.51	25.83	557.95	1313.51
SD	0.13	5.71	77.01	139.60
CV %	5.13	22.09	13.80	10.63

5.7 Concluding comments

We describe for the first time the determination of anti-HBs with a multiplex platform. We also show that a single reference serum can be used to verify all the test antibodies. Commercially

available antigens with high purity were utilized to ensure antigen availability for future testing. The major advantage of this multiplex assay was the need for small sample volumes (5µl) compared to ELISAs which ranged from 10µl to 100µl of sample. In addition, reduction of assaying time with the multiplex assay, that required 2.5 hours to test 38 samples for all five vaccine antigens simultaneously; instead of the 10.5 hours required for ELISAs. Studies have demonstrated the ability of ELISAs to accurately determine vaccine responses (Fu et al., 2010), but with advances in the vaccinology fields of new vaccines with increased number of combined antigens the use of ELISA has become increasingly unsuitable (Pickering et al., 2002a). The optimal bead conjugation concentration and procedure was found to be similar to those reported for elsewhere.(van Gageldonk et al., 2008) MFIs generated from all five conjugated microsphere bead regions showed linearity over a seven step 4-fold reference serum dilution range, the consequence of this increase in range was the resultant increase in accuracy, a reduction in the number of sample dilutions required and the ability to detect much lower antibody levels. MFI comparisons between monoplex and pentaplex assays allowed for determination of interference between the microsphere bead regions. Multiplexing did not affect specificity and sensitivity of the assay when compared to monoplexing. The multiplex assay was found to be reproducible, with minimal variability inter- and intra assay, and correlated well with commercially available ELISAs.

CHAPTER 6 RESULTS: EFFECT OF HIV-1 EXPOSURE AND ANTIRETROVIRAL TREATMENT STRATEGIES IN HIV-INFECTED CHILDREN ON IMMUNOGENICITY OF VACCINES DURING INFANCY

In this chapter, results on the effects of HIV exposure in HIV-uninfected and timing of ART initiation in HIV-infected children on the kinetics of antibodies to epitopes of diphtheria-toxoid, tetanus-toxoid, whole cell pertussis(i.e. DTwP-HibCV) and monovalent HBV prior to and one-month following the primary three-dose series of vaccines were evaluated (The published paper is attached in Appendix 2). In addition, independent factors that may be associated with response to vaccination will be presented.

6.1 Results

6.1.1 Study population and demographic information

Briefly, four groups of children aged 6-12 weeks were enrolled at two South African Centers (Chris Hani-Baragwanath Hospital in Soweto, Johannesburg and Tygerberg Children's Hospital, Stellenbosch) from April 2005 to June 2006. HIV-unexposed children born to HIV-uninfected mothers (confirmed by a non-reactive HIV ELISA at enrolment); and HEU children in whom HIV-PCR was non-reactive at enrolment. HIV-infected children participating in the "Children with HIV Early Antiretroviral (CHER)" study were co-enrolled (Violari et al., 2008). The latter were aged less than 12 weeks with CD4+ % $\geq 25\%$ and randomized to deferred ART until immunological and/or clinically indicated (ART-Def) or immediately started on ART upon confirmation of their HIV-infection status (ART-Immed). Additionally, a convenience sample of HIV-infected children from CHER with CD4+T-lymphocyte $< 25\%$ (CD4+ $< 25\%$) was recruited. The first-line ART regimen in CHER included zidovudine, lamivudine and lopinavir-ritonavir. Following an interim analysis of the CHER study in which HIV disease progression to AIDS or death was greater in ART-Def compared to ART-Immed children, the Data Safety and Monitoring Board recommended halting recruitment to the ART-Def group and that all children in this group be evaluated whether they required ART to be initiated (Violari et al., 2008). Participants were scheduled to receive three doses of combined DTwP-HibCV (CombAct-Hib®, Sanofi Pastuer, France) and monovalent recombinant HBV (HerberBiotec S.A., Cuba) at approximately 6, 10 and 14 weeks of age with a 4-6 week interval between doses. Both, DTwP-HibCV and HBV were administered at separate sites in the left arm, PCV (Prevnar-7®; Wyeth Vaccines and Pediatrics, Philadelphia, US) in the right arm and trivalent oral polio vaccine (POLIORAL-trivalEnt®, Novartis Vaccines and Diagnostics, Srl, Italy) orally. Values for unknown test sera were generated from a standard curve of MFI against

expected IgG concentration for the in-house reference sera and converted to either IU/ml or mIU/ml. The LLOQ were 0.001 IU/ml for antibody to diphtheria-toxoid and tetanus-toxoid, 20 IU/ml for antibody to pertussis toxoid, 2 IU/ml for FHA and 10 mIU/ml for HepB. These values were regarded as sero-positive thresholds. Samples with a concentration below the LLOQ were assigned a concentration of half the LLOQ when calculating GMC. The thresholds for correlates of sero-protection were defined as antibody concentrations greater or equal to 0.01IU/ml and 0.1 IU/ml for DT, 0.1 IU/ml for TT, 10 mIU/ml for HepB (Plotkin, 2010). Surrogate of immune response to pertussis was defined as ≥ 4 -fold increase in antibody concentration from pre- to post-vaccination for PT and FHA.(Plotkin, 2010) Alternate antibody threshold level evaluated was ≥ 100 mIU/ml for HepB, which may be a predictor for long-term protection (Plotkin, 2010).

Samples from 493 (85.3%) of 578 children in the parent-study (CIPRA-4) were available as shown in Table 6.1. The main reasons for unavailability of samples post-vaccination were the high mortality rate in ART-Def children as reported (Violari et al., 2008) and time-points undertaken beyond the protocol-defined window-period; Figure 6.1. The mean age at pre-vaccination ($\pm$ SD) was 7.3 (SD 1.3) weeks and 19.5 (SD 1.3) weeks at post-vaccination being similar between groups; Table 1. Forty six percent were male and 90.7% were of black-African descent; Table 1. All children in the ART-Immed and CD4+<25% groups were initiated on ART within four days prior to the first DTwP-HibCV/HBV dose. Thirteen (16.9%) ART-Def children were initiated on ART at a median of 40 days following the first vaccine-doses (Madhi et al., 2010). The mean CD4+-lymphocyte percentages were 35.1% in ART-Immed, 37.5% in ART-Def and 22.0% in CD4+<25% group pre-vaccination; and 40.7% ($p<0.001$), 30.1% ($p<0.001$) and 27.5% ($p=0.06$) one-month post third-dose in the respective groups; Table 6.1.

Table 6.1: Demographic and baseline features of participants included in the analysis evaluating the immunogenicity of a three doses primary series of diphtheria toxoid-tetanus toxoid- whole cell pertussis and *Haemophilus influenzae* type-b conjugate vaccine and hepatitis B vaccine.

Variable	Total	HIV-unexposed ^a	HEU ^b	ART-Immed ^c	ART-Def ^d	CD4+<25% ^e
Total subjects enrolled	578	124	125	210	105	14
Number included in analysis	493	114	120	172	77	10
Male sex (%)	227 (46.1)	61 (53.5)	65 (54.2)	68 (39.5)	29 (37.7)	4 (40.0)
Total Race: Black African (%)	447 (90.7)	86 (75.4)	112 (93.3)	164 (95.3)	76 (98.7)	9 (90.0)
Mixed ancestry (%)	46 (9.3)	28 (24.6)	8 (6.7)	8 (4.7)	1 (1.3)	1 (10.0)
Mean age (Standard deviation; SD) at time of vaccine administration in weeks						
Dose-1 (SD) ^f	7.3 (1.3)	6.7 (1.3)	7.4 (1.0)	7.5 (1.2)	7.3 (1.2)	8.6 (1.6)
Dose-2	11.4 (1.3)	11.1 (1.2)	11.4 (1.1)	11.5 (1.4)	11.4 (1.4)	12.7 (1.8)
Dose-3	15.4 (1.3)	15.2 (1.3)	15.5 (1.2)	15.5 (1.4)	15.4 (1.3)	16.6 (1.7)
Post dose-3 (SD) ^g	19.5 (1.3)	19.2 (1.2)	19.5 (1.0)	19.6 (1.3)	19.6 (1.5)	20.6 (1.6)
9 months of age (SD)	39.3 (1.1)	38.8 (0.8)	39.2 (0.8)	39.4 (1.1)	39.9 (1.2)	40.5 (1.3)
Pre-vaccination median HIV-1 Viral load copies/ml (range)	Not done			>750000 (394500 to >750000)	>750000 (448000 to >750000)	>750000 (>750000 to >750000)
Pre-vaccination median CD4+ lymphocyte Count (IQR) ^h				2070 (1572 to 2070)	2083 (1654 to 2083)	1568 (1194 to 1568)
Pre-vaccination mean CD4+ lymphocyte % (SD)				35.1 (8.3)	37.5(9.2)	22(6.0)
One-month post dose-3 median CD4+ lymphocyte count at (IQR) ⁱ				2230 (1713 to 2230)	1872 (1112 to 1872)	1827 (990 to 1827)
One-month post dose-3 mean CD4+ % (SD) ⁱ				40.7 (9.4)	30.1 (9.0)	27.49(8.7)
Six-months post dose-3 median CD4+ lymphocyte count at (IQR)				2201 (1604 to 2751)	1587 (1202 to 2253.5)	1772.5 (1284 to 1981)
Six-months post dose-3 mean CD4+ % (SD)				40.0 (9.3)	31.4 (7.9)	32.4 (6.1)

Median days (range) to ART initiation relative to 1st vaccine		0 (-4 to 0)	40 (7.0 to 71.0) ^j	0 days

Footnote: ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers. ^bHEU: HIV-uninfected children born to HIV-infected mothers. ^cART-Immed: HIV-infected children with CD4+ $\geq$ 25% at enrolment randomized to initiate antiretroviral treatment (ART) immediately. ^dART-Def: HIV-infected children with CD4+ $\geq$ 25% at enrolment randomized to deferred anti-retroviral treatment (ART). ^eCD4+<25%: HIV-infected children with CD4+ cell count <25% started on ART at enrolment. ^fBlood draw was undertaken just prior to and on the same day of DTwP-HibCV and hepatitis B vaccination where applicable (Time-point 1). ^gIncluded subjects who received all three doses within protocol-defined window periods and available immunogenicity data within 3-6 weeks after 3rd dose of diphtheria toxoid- tetanus toxoid- whole cell pertussis- *Haemophilus influenzae* type b conjugate vaccine (DTwP-HibCV) and hepatitis B (Time-point 2). ^hIQR: interquartile range. ⁱInvolve 171, 77 and 10 observations in ART-Immed, ART-Def and CD4+<25% children, respectively. ^j13 (16.8%) of 77 ART-Def children had been initiated on ART at the time of the immunogenicity analysis.

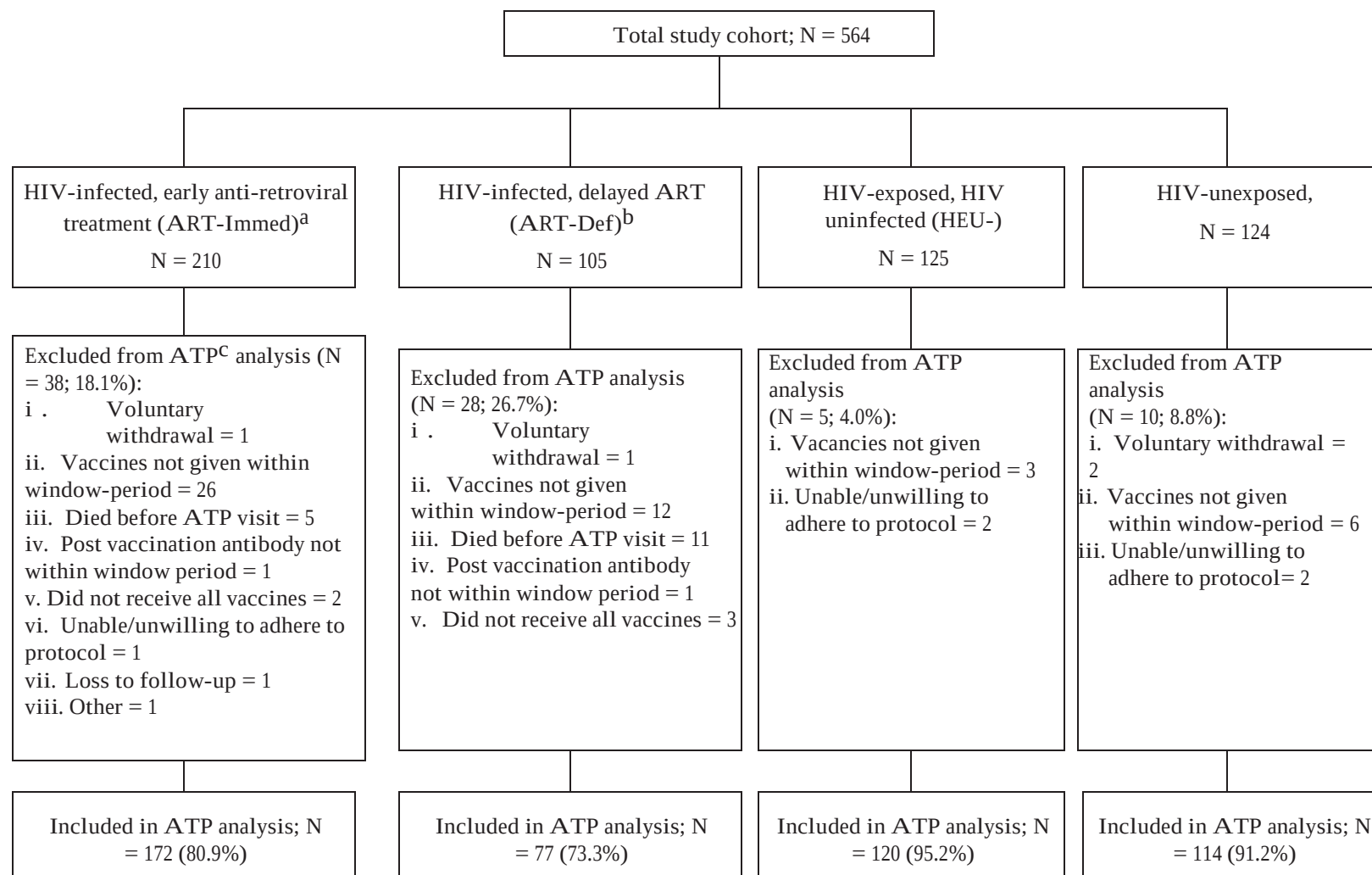


Figure 6.1: Stratification of study population and reasons for children being excluded from analysis.

^a ART-Immed: HIV-infected children with $CD4^{+}\% \geq 25\%$ who were initiated on treatment upon immediately upon enrolment. ^b ART-Def: HIV-infected children with $CD4^{+}\% \geq 25\%$ who were initiated on treatment when clinically and/or immunologically indicated. ^c According to protocol, i.e. received all vaccines within window-period and also pre-vaccination and post-vaccination blood available per defined window-period.

Note. In addition, a convenience sample of 10 HIV-infected children who had CD4⁺ cell count <25% at enrolment were also included as a fifth descriptive group.

6.1.2 Antibody responses in HIV-unexposed compared to HIV-infected children

Compared to HIV-unexposed, ART-Immed and ART-Def children had lower antibody GMCs to TT ($p < 0.001$ for both), HepB ($p = 0.01$ for ART-Immed; $p = 0.002$ for ART-Def) and PT ($p < 0.001$ for both); Table 6.2. The proportion of children with antibodies above the sero-protective threshold were generally similar between HIV-unexposed and the HIV-infected groups, except for HepB which was lower in ART-Def (39.5% vs. 49.6% in HIV-unexposed, $p = 0.03$); Table 6.2.

Table 6.2: Antibody concentrations and proportion of children with sero-protective antibody levels prior to first dose of diphtheria toxoid-tetanus toxoid- whole cell pertussis- *Haemophilus influenzae* type b conjugate vaccine (DTPw-HibCV) and Hepatitis-B vaccines

Antibody	Measure	HIV-unexposed ^a n=114	HEU ^b n=120	p-value ^c	ART-Immed ^d n=172	p-value ^e	p-value ^f	ART-Def ^g n=77	p-value ^h	p-value ⁱ	p-value ^j	CD4+<25 % ^k n=10
TT^m	GMC (95% CI) ^l	0.421 (0.309-0.573)	0.219 (0.181-0.265)	0.001	0.086 (0.071-0.105)	<0.001	<0.001	0.074 (0.054-0.103)	<0.001	<0.001	0.34	0.100 (0.065-0.155)
	≥0.01IU/ml N/n ^m (%; 95%CI)	110/113 (97.4; 92.5-99.1)	117/118 (99.2; 95.4-100)	0.07	164/168 (97.6; 94.0-99.1)	0.12	0.41	72/76 (94.8; 87.5-97.9)	0.48	0.15	0.45	10 (100; 72.3-100)
DT^o	GMC (95% CI)	0.078 (0.069-0.088)	0.136 (0.115-0.161)	<0.001	0.085 (0.074-0.097)	0.26	<0.001	0.076 (0.064-0.089)	0.08	<0.001	0.54	0.127 (0.088-0.184)
	≥0.1 IU/ml N/n (%; 95%CI)	33/113 (29.2; 21.6-38.2)	72/118 (61.0; 52.0-69.3)	<0.001	67/168 (39.9; 32.8-47.4)	0.86	<0.001	26 /76 (34.2; 24.5-45.4)	0.45	<0.001	0.76	8 (80.0; 49.0-94.3)
HepB^p	GMC (95% CI)	7.74 (5.25-11.42)	29.77 (21.05-42.11)	<0.001	5.81 (4.12-8.17)	0.01	<0.001	4.26 (2.70-6.73)	0.002	<0.001	0.38	396.98 (152.6-1032.9)
	≥10 mIU/ml N/n (%; 95%CI)	56/113 (49.6; 41.0-59.0)	95/118 (80.5; 72.5-86.7)	<0.001	77/168 (45.8; 38.9-53.4)	0.11	<0.001	30/76 (39.5; 29.3-50.7)	0.03	<0.001	0.47	10 (100; 72.3-100)
FHA^q	GMC IU/ml (95% CI)	20.09 (17.91-22.55)	35.08 (29.65-41.50)	<0.001	22.88 (20.30-25.79)	0.52	<0.001	20.83 (17.99-24.12)	0.23	<0.001	0.63	30.17 (21.39-42.54)
PT^r	GMC IU/ml (95% CI)	40.65 (33.83-48.84)	31.70 (27.67-36.30)	0.06	17.67 (15.57-20.04)	<0.001	<0.001	16.29 (13.63-19.46)	<0.001	<0.001	0.53	26.76 (16.56-43.26)

Footnote: ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers. ^cp-value comparing HEU to HIV-unexposed children; ^dART-Immed: HIV-infected children with CD4+ ≥25% at enrolment randomized to initiate antiretroviral treatment (ART) immediately; ^ep-value comparing ART-Immed with HIV-unexposed children; ^fp-value comparing ART-Immed with HEU children; ^gART-Def: HIV-infected children with CD4+ ≥25% at enrolment randomized to deferred ART; ^hp-value comparing ART-Def with HIV-unexposed children; ⁱp-value comparing ART-Def with HEU children; ^jp-value comparing ART-Def with ART-Immed children; ^kCD4+<25%: HIV-infected children with CD4+ cell count <25% started on ART at enrolment; ^lGMC (95% CI): geometric mean concentration with 95% confidence interval in parenthesis; ^mn: Total number of samples available for calculating percent response. ^oTT=Tetanus-toxoid. ^pDT=diphtheria-toxoid. ^pHepB= antibody to Hepatitis B. ^qFHA=Filamentous haemagglutinin. ^rPT= pertussis toxin.

Compared to HIV-unexposed, lower post-vaccination GMCs were observed in the ART-Immed and ART-Def groups for TT ($p<0.001$ for both) and HepB ($p<0.001$ for both); Table 6.3. Also, lower GMCs were observed in ART-Immed children to DT ($p=0.001$) and FHA ($p<0.001$), as well as for PT in ART-Def children ($p<0.001$) compared to HIV-unexposed children, Table 6.3.

In HIV-unexposed, despite higher post-vaccination antibody GMCs to some epitopes, the proportion with sero-protective levels of antibody were similar for TT (82.7% to 100%), DT at ≥ 0.01 IU/ml (100%) and HepB ($>84\%$) compared to ART-Immed and ART-Def children; Table 6.3. Also, a similar proportion in each group had antibody above ≥ 100 mIU/ml for HepB ($>84\%$) and DT ≥ 0.1 IU/ml (76.9% to 100%); Table 6.3. Sero-conversion rates associated with pertussis vaccination, however, were higher in HIV-unexposed (38.2%) for FHA compared to ART-Immed children (20.6%, $p=0.008$), and lower for PT in HIV-unexposed (39.1%) compared to ART-Def (51.4%, $p=0.02$) children, Table 6.3.

Antibody to HepB was lower in ART-Def (601.99 mIU/ml) compared to HIV-unexposed (1571.17 mIU/ml; $p<0.001$); Table 6.4. Similar proportions between HIV-unexposed and HIV-infected children were observed for all study vaccine epitopes 4 months post vaccination.

Table 6.3: Antibody concentrations and proportion of children with sero-protective antibody levels following primary series of three doses of diphtheria toxoid- tetanus toxoid- whole cell pertussis- *Haemophilus influenzae* type b conjugate vaccine and hepatitis B vaccines.

Antibody	Measure	HIV-unexposed ^a n= 114	HEU ^b n= 120	p-value ^c	ART-Immed ^d n= 169	p-value ^e	p-value ^f	ART-Def ^g n= 75	p-value ^h	p-value ⁱ	p-value ^j	CD4+<2 5% ^k n=9
TT ^o	GMC (95% CI) ^l	0.952 (0.849-1.068)	0.762 (0.679-0.854)	0.11	0.405 (0.343-0.478)	<0.001	<0.001	0.323 (0.228-0.457)	<0.001	<0.001	0.24	0.470 (0.128-1.722)
	≥0.1 IU/ml N/n ^m (%; 95%CI)	111/111 (100; 96.7-100)	116/118 (98.3; 94.0-99.5)	NE ⁿ	156 (92.3; 87.3-95.5)	NE	0.41	62 (82.7; 72.6-89.6)	NE	0.02	0.03	8 (88.9; 56.5-98.0)
DT ^p	GMC (95% CI)	0.272 (0.250-0.296)	0.372 (0.333-0.415)	0.14	0.200 (0.173-0.231)	0.001	<0.001	0.233 (0.194-0.281)	0.25	0.03	0.10	0.172 (0.060-0.492)
	≥0.01 IU/ml N/n (%; 95%CI)	111/111 (100; 96.7-100)	118/118 (100; 96.9-100)	NE	169 (100; 97.8-100)	NE	NE	75 (100; 95.2-100)	NE	NE	NE	8 (88.9; 56.5-98.0)
	≥0.1 IU/ml N/n (%; 95%CI)	111/111 (100; 96.7-100)	117/118 (99.2; 95.4-100)	NE	130 (76.9; 70.0-82.6)	NE	<0.001	60 (80.0; 69.6-87.5)	NE	0.001	0.65	8 (88.9; 56.5-98.0)
HepB ^q	GMC (95% CI)	2521.03 (2249.84-2824.92)	2019.28 (1740.28-2343.00)	0.04	924.87 (778.28-1099.07)	<0.001	<0.001	536.60 (372.97-772.02)	<0.001	<0.001	0.02	6545.81 (1256.29-34106.64)
	≥10 mIU/ml N/n (%; 95%CI)	111/111 (100;	117/118 (99.2;	NE	167 (98.8;	NE	0.67	73 (97.3;	NE	0.540	0.52	9 (100.0;

		96.7-100)	95.4-100)		95.8-99.7)			90.8-99.3)				70.1-100.0)
	≥100 mIU/ml N/n (%; 95%CI)	111/111 (100; 96.7-100)	117/118 (99.2; 95.4-100)	NE	164 (97.0; 93.3-98.7)	NE	0.47	63 (84.0; 74.1-90.6)	NE	<0.001	0.02	8 (88.9; 56.5- 98.0)
FHA ^f	GMC (95% CI)	61.24 (53.65- 69.91)	72.23 (62.59- 83.36)	0.54	40.48 (34.09-48.08)	<0.001	<0.001	51.05 (40.39- 64.51)	0.23	0.14	0.02	39.26 (14.39- 107.06)
	4-fold increase N/n (%; 95%CI)	42/110 (38.2; 29.7-47.5)	25/116 (21.6; 15.1-29.9)	0.53	34/165 (20.6; 15.1-27.4)	0.008	0.060	19/74 (25.7; 17.1-36.7)	0.13	0.37	0.52	1 (11.1; 2.0-43.5)
PT ^s	GMC (95% CI)	134.34 (111.97- 161.17)	261.30 (221.56- 308.18)	<0.001	138.11 (114.47- 166.64)	0.44	<0.001	72.55 (52.81- 99.66)	<0.001	<0.001	0.06	76.05 (20.38- 283.78)
	4-fold increase N/n (%; 95%CI)	43/110 (39.1; 30.5-48.4)	89/116 (76.7; 68.3-83.5)	<0.001	117/165 (70.9; 63.6-77.3)	0.56	<0.001	38/74 (51.4; 40.2—62.4)	0.02	<0.001	0.14	5 (55.6; 26.7- 81.1)

Footnote: ^a HIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^b HEU: HIV-uninfected children born to HIV-infected mothers; ^c p-value comparing HEU with HIV-unexposed children; ^d ART-Immed: HIV-infected children with CD4+ ≥25% at enrolment randomized to initiate ART immediately; ^e p-value comparing ART-Immed with HIV-unexposed children; ^f p-value comparing ART-Immed with HEU children; ^g ART-Def : HIV- infected children with CD4+ ≥25% at enrolment randomized to deferred ART; ^h p-value comparing ART-Def with HIV-unexposed children; ⁱ p-value comparing ART-Def with HEU children; ^j p-value comparing ART-Def to ART-Immed children; ^k CD4+<25%: HIV-infected children with CD4+ cell count <25% started on ART at enrolment ; ^l GMC (95% CI): geometric mean concentration with 95% confidence interval in parenthesis; ^m n: Total number of samples available for calculating percent response; ⁿ NE: not evaluable p-value is not calculable through logistic regression due to group having 100%. ^o TT=Tetanus toxoid. ^p DT=diphtheria toxoid. ^q HepB= Hepatitis B surface antigen. ^r FHA=Filamentous haemagglutinin. ^s PT= pertussis toxoid.

Table 6.4: Antibody concentrations and proportion of children with sero-protective antibody levels six months following primary series of three doses of diphtheria toxoid- tetanus toxoid- whole cell pertussis- *Haemophilus influenzae* type b conjugate vaccine and hepatitis B vaccines (9-months of age).

Antibody	Measure	HIV-unexposed ^a n= 106	HEU ^b n= 112	p-value ^c	ART-Immed ^d n= 163	p-value ^e	p-value ^f	ART-Def ^g n= 59	p-value ^h	p-value ⁱ	p-value ^j	CD4+<25% ^k n=8
TT^p	GMC (95% CI) ^l	0.251 (0.216-0.293)	0.228 (0.194-0.268)	0.58	0.148 (0.123-0.179)	0.66	0.23	0.158 (0.108-0.230)	0.37	0.41	0.59	0.223 (0.124-0.399)
	Fold-change ^m	-1.9 (1.7-2.2)	-1.8 (1.5-2.2)	0.59	-2.0 (1.7-2.4)	0.94	0.50	-1.5 (1.0-2.1)	0.11	0.31	0.38	-4.5 (0.7-26.5)
	≥0.1 IU/ml N/n (%; 95%CI)	103/103 (100; 96.4-100)	110/111 (99.1; 95.1-99.8)	NE ^o	144/146 (98.6; 95.1-99.6)	NE	0.72	52/52 (100; 93.1-100)	NE	NE	NE	8/8 (100; 67.6-100)
DT^q	GMC (95% CI)	0.097 (0.084-0.112)	0.073 (0.053-0.099)	0.58	0.095 (0.084-0.107)	0.66	0.23	0.118 (0.094-0.147)	0.37	0.41	0.59	0.101 (0.077-0.133)
	Fold-change	-3.5 (3.1-4.0)	-1.2 (0.8-1.9)	<0.001	-3.8 (3.2-4.5)	0.90	<0.001	-3.3 (2.5-4.2)	0.69	<0.001	0.48	-7.1 (1.5-34.3)
	≥0.01 IU/ml N/n (%; 95%CI)	103/103 (100; 96.4-100)	102/111 (91.9; 85.3-95.7)	NE	146/146 (100; 97.4-100)	NE	NE	52/52 (100; 93.1-100)	NE	NE	NE	8/8 (100; 67.6-100)
	≥0.1 IU/ml N/n (%; 95%CI)	103/103 (100;96.4-100)	101/111 (91.0; 84.2-95.0)	NE	146/146 (100;97.4-100)	NE	NE	52/52 (100; 93.1-100)	NE	NE	NE	8/8 (100; 67.6-100)
HepB^r	GMC (95% CI)	1571.17 (1302.0-1896.0)	1422.49 (1087.14-1861.28)	0.09	1099.36 (955.89-1264.30)	0.83	0.84	601.99 (430.88-841.04)	<0.001	0.03	0.001	10941 (5829.67 - 20533.85)
	Fold-change	-1.2 (1.0-1.4)	-0.7 (0.5-1.0)	0.007	-1.3 (1.0-1.5)	0.60	0.001	-1.1 (.8-1.5)	0.97	0.04	0.31	-2.2 (0.3-18.2)
	≥10 mIU/ml N/n (%; 95%CI)	103/103 (100; 96.4-100)	111/111 (100; 96.7-100)	NE	146/146 (100; 97.4-100)	NE	NE	52/52 (100; 93.1-100)	NE	NE	NE	8/8 (100; 67.6-100)
	≥100 mIU/ml N/n (%; 95%CI)	103/103 (100; 96.4-100)	104/111 (93.7; 87.6-96.9)	NE	144/146 (98.6; 95.1-99.6)	NE	0.05	47/52 (90.4; 79.4-95.8)	NE	0.74	0.05	8/8 (100; 67.6-100)

FHA^s	GMC (95% CI)	41.26 (35.23-48.31)	45.23 (38.88-52.62)	0.04	31.02 (27.41-35.12)	0.55	0.03	33.49 (27.22-41.20)	0.01	0.07	0.78	31.48 (14.56-68.05)
	Fold-change	-3.1 (2.4-4.1)	-3.2 (2.6-4.0)	0.55	-5.6 (4.6-6.8)	0.002	<0.001	-4.7 (3.4-6.5)	0.09	0.01	0.31	-7.6 (1.6-35.2)
PT^t	GMC (95% CI)	55.16 (46.81-65.01)	78.85 (67.17-92.55)	0.37	59.56 (50.34-70.47)	0.41	0.81	51.97 (38.20-70.71)	0.70	0.77	0.80	46.48 (16.56-130.44)
	Fold-change	-1.8 (1.5-2.2)	-1.4 (1.2-1.7)	0.04	-1.9 (1.5-2.3)	0.79	0.05	-2.1 (1.4-3.0)	0.54	0.02	0.95	-3.8 (0.7-19.8)

Footnote: ^a HIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^b HEU: HIV-uninfected children born to HIV-infected mothers; ^c p-value comparing HEU with HIV-unexposed children; ^d ART-Immed: HIV-infected children with CD4+ $\geq$ 25% at enrolment randomized to initiate ART immediately; ^e p-value comparing ART-Immed with HIV-unexposed children; ^f p-value comparing ART-Immed with HEU children; ^g ART-Def : HIV- infected children with CD4+ $\geq$ 25% at enrolment randomized to deferred ART; ^h p-value comparing ART-Def with HIV-unexposed children; ⁱ p-value comparing ART-Def with HEU children; ^j p-value comparing ART-Def to ART-Immed children; ^k CD4+<25%: HIV-infected children with CD4+ cell count <25% started on ART at enrolment ; ^l GMC (95% CI): geometric mean concentration with 95% confidence interval in parenthesis; ^m Fold-change: Fold-change in GMC between post-vaccination and 9 months of age; ⁿ n: Total number of samples available for calculating percent response; ^o NE: evaluable. p-value is not calculable through logistic regression due to group having 100%. ^p TT=Tetanus toxoid. ^q DT=diphtheria toxoid. ^r HepB= Hepatitis B surface antigen. ^s FHA=Filamentous haemagglutinin. ^t PT= pertussis toxoid.

6.1.3 Antibody responses in HIV-unexposed compared to HEU children

Prior to vaccination, HEU only had lower antibody GMCs to TT ($p=0.001$) than HIV-unexposed children; but higher GMCs for DT, HepB and FHA ($p<0.001$ for all); Table 6.2. HEU were also more likely than HIV-unexposed children to have sero-protective DT (61.0% vs. 29.2%, $p<0.001$) and HepB (80.5% vs. 49.6%, $p<0.001$) antibody concentrations at pre-vaccination; Table 6.2.

Antibody GMCs were similar post-vaccination between HIV-unexposed and HEU for TT, DT and FHA. Antibody GMCs in HIV-unexposed were, however, slightly higher for HepB (2521.0 vs. 2019.3 mIU/ml; $p=0.041$) and lower for PT (134.3 vs. 261.3 IU/ml, $p<0.001$) than in HEU; Table 6.3. A high proportion ($>99\%$) of HEU and HIV-unexposed children developed sero-protective levels to TT, DT and HepB post-vaccination, which did not differ by group. Similarly, $>99\%$ of HIV-unexposed and HEU children had antibody levels ≥ 100 mIU/ml to HepB post-vaccination. The proportion of HIV-unexposed compared to HEU children with sero-conversion (i.e. ≥ 4 fold increase between pre-vaccination and post-vaccination) to pertussis antigens was similar for FHA (38.2% vs. 21.6%; $p=0.53$), however, against PT this proportion was lower in HIV-unexposed children (39.1% vs. 76.7%; $p>0.001$); Table 6.3.

Similar GMCS were observed between HIV-unexposed and HEU, for TT, DT, HepB and PT; Table 6.4. Antibody to FHA was slightly higher in HEU (45.23 IU/ml) than in HIV-unexposed (41.26 IU/ml; $p=0.04$). Over 90% of study children had antibody levels above the protective threshold for all study epitope; Table 6.4.

6.1.4 Antibody responses in HEU compared to HIV-infected children

Pre-vaccination antibody GMCs were lower in ART-Immed and ART-Def children than HEU children for all epitopes ($p<0.001$ for all comparisons); Table 6.2. HEU children were also more likely to have sero-protective antibody concentrations against DT (61.0%) and HepB (80.5%) compared to ART-Immed (39.9%, and 45.8%, respectively) and ART-Def (34.2%, and 39.5%, respectively); $p<0.001$ for all comparisons, Table 6.2.

Post-vaccination antibody GMCs were higher in HEU than ART-Immed or ART-Def children to TT, DT, HepB and PT; Table 6.3. The proportion of children with sero-protective antibody levels

were similar in HEU and ART-Immed children, although the latter were less likely to have antibody threshold ≥ 0.1 IU/ml for DT (99.2% vs. 76.9%; $p < 0.001$). In contrast, lower proportions of ART-Immed children had sero-protective antibody levels post-vaccination compared to HEU children to TT (82.7% vs. 98.3%; $p = 0.02$), DT at the ≥ 0.1 IU/ml threshold (80.0% vs. 99.2%; $p = 0.001$), HepB at ≥ 100 mIU/ml threshold (84.0% vs. 99.2%; $p = 0.02$) and were also less likely to demonstrate sero-conversion to PT (51.4% vs. 76.7%; $p < 0.001$); Table 6.3.

Significant differences were observed when comparing HEU and ART-Def children for the HepB epitope (1422 vs. 601.99 mIU/ml; $p = 0.03$ respectively); Table 6.4. However, the proportion of children with sero-protective thresholds for the vaccine epitopes was similar between HEU and HIV-infected children.

6.1.5 Antibody responses in antiretroviral treatment-Immed compared to antiretroviral treatment-Def. children

There were no differences between ART-Immed and ART-Def groups prior to vaccination in either GMCs or the proportion of children with sero-protective antibody levels for any vaccine epitope; Table 6.2.

Post-immunization immune GMCs against most epitopes were similar, but higher in ART-Immed for HepB ($p = 0.017$) and lower for FHA ($p = 0.02$) than ART-Def children; Table 6.3. A similar proportion of ART-Immed and ART-Def children developed antibody concentrations above the sero-protective threshold for most vaccines, except for more ART-Immed children with TT antibody ≥ 0.1 IU/ml (92.3% vs. 82.7%; $p = 0.03$) and HepB antibody ≥ 100 mIU/ml (97.0% vs. 84.0%; $p = 0.02$); Table 6.3. The proportion of CD4+ < 25% group with sero-protective levels of antibody and/or with sero-conversion to PT and FHA was similar to ART-Immed children.

No significant differences were observed in the persistence of vaccine induced antibodies six months post primary vaccination, when comparing ART-Immed to ART-Def children; Table 6.4.

6.2 Correlation of pre-vaccination response with post-vaccination response in study participants

There was correlation between pre- and post-vaccination antibody levels for TT ($r=-0.35$; $p<0.001$) and PT ($r=0.18$; $p<0.001$) in HIV-unexposed children. Children born to HIV-infected mothers showed correlation for FHA between pre- and post primary vaccination antibody concentrations, except for $CD4+<25\%$; Table 6.5. In addition to this, ART-Immed children had correlation for DT ($r=0.49$; $p<0.001$) and HepB ($r=0.45$; $p<0.001$) epitopes; Table 6.5. No correlation was observed for post vaccination antibody level and $CD4+$ percent; Table 6.6.

Table 6.5: Correlation of pre-vaccination (Time-point 1) and post-vaccination (Time-point 2) antibody levels.

Group (N)	TT		DT		PT		FHA		HepB	
	r^* (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value
HIV-unexposed (104)	-0.35 (-0.51 to - 0.17)	<0.001	0.18 (-0.02 to 0.37)	0.06	-0.49 (- 0.63 to -0.33)	<0.001	-0.0003 (-0.20 to 0.20)	1.00	0.0003 (-0.20 to 0.20)	1.00
HEU (110)	-0.25 (-0.43 to - 0.06)	0.01	0.30 (0.11 to 0.46)	0.002	-0.15 (- 0.34 to 0.04)	0.11	0.27 (0.08 to 0.44)	0.004	-0.09 (-0.27 to 0.11)	0.37
ART-Def (60)	-0.22 (-0.46 to 0.04)	0.08	0.66 (0.48 to 0.78)	<0.001	0.03 (- 0.23 to 0.29)	0.83	0.63 (0.45 to 0.77)	<0.001	0.24 (- 0.02 to 0.47)	0.06
ART-Immed (147)	0.04 (-0.13 to 0.20)	0.66	0.49 (0.36 to 0.61)	<0.001	0.004 (-0.16 to 0.17)	0.96	0.53 (0.40 to 0.64)	<0.001	0.45 (0.28 to 0.55)	<0.001
CD4+<25% (8)	-0.04 (-0.73 to 0.68)	0.92	-0.18 (-0.78 to 0.06)	0.68	-0.23 (- 0.81 to 0.56)	0.58	0.19 (- 0.59 to 0.79)	0.65	-0.36 (-0.85 to 0.47)	0.39

Footnote: TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemolglutanin; HepB: hepatitis B vaccine; r^* = Spearman's correlation coefficient; HIV-unexposed: HIV-unexposed uninfected; HEU: HIV-exposed uninfected; ART-Def: HIV-infected on deferred ART; ART-Immed: HIV-infected on immediate ART; $CD4+<25\%$: HIV-infected on immediate ART with $CD4+ \leq 25\%$.

Table 6.6: Correlation of CD4+% with response to primary vaccination (Time-point 2)

Group (N)	TT		DT		PT		FHA		HepB	
	r* (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value
ART-Def (60)	-0.08 (-0.33 to 0.19)	0.56	-0.13 (-0.38 to 0.14)	0.32	0.18 (-0.09 to 0.42)	0.18	-0.11 (-0.36 to 0.16)	0.42	0.13 (-0.14 to 0.38)	0.32
ART-Immed (150)	-0.01 (-0.17 to 0.16)	0.92	-0.02 (-0.19 to 0.14)	0.80	0.15 (-0.02 to 0.31)	0.07	-0.04 (-0.21 to 0.12)	0.59	-0.02 (-0.19 to 0.14)	0.76
CD4+<25 % (8)	0.13 (-0.63 to 0.76)	0.76	0.18 (-0.60 to 0.79)	0.66	0.56 (-0.24 to 0.91)	0.15	-0.01 (-0.71 to 0.70)	0.97	-0.13 (-0.76 to 0.63)	0.76

Footnote: TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemolglutinin; HepB: hepatitis B vaccine; r*= Spearman's correlation coefficient; ART-Def: HIV-infected on deferred ART; ART-Immed: HIV-infected on immediate ART; CD4+<25%: HIV-infected on immediate ART with CD4+ % <25%.

6.3 Factors associated with response to vaccination at following primary vaccination for all study children

In multiple linear regression model for factors associated with magnitude of specific antibody response to vaccination, breastfeeding, study center, baseline antibody titer and combined AZT/NVP for the prevention of mother to child transmission in HIV-exposed children, birth weight and CD4+% were found to be associated with response and were also found to be vaccine epitope specific; Table 6.7.

Table 6.7: Independent variables and their association to response to primary vaccination at Time-point 2 for DTwP and HepB vaccines

	TT			DT			PT			FHA			HepB		
Independent variables	b	SE	P	b	SE	P	b	SE	P	b	SE	P	b	SE	P
Gender	-0.08	0.09	0.42	-0.08	0.09	0.42	-0.08	0.12	0.52	-0.13	0.08	0.08	-0.18	0.11	0.09
Age	0.003	0.04	0.94	0.003	0.04	0.94	0.05	0.05	0.34	-0.02	0.03	0.56	-0.01	0.04	0.86
Birth-weight	0.0002	0.0001	0.12	0.002	0.0001	0.12	0.0001	0.0001	0.55	0.0001	0.0001	0.16	0.0002	0.0001	0.04
Race	0.27	0.18	0.13	0.27	0.18	0.13	0.01	0.23	0.96	-0.21	0.15	0.16	-0.29	0.20	0.16
Breastfeeding	0.35	0.11	0.002	0.35	0.11	0.002	-0.04	0.14	0.76	0.16	0.09	0.07	0.59	0.12	<0.001
Study center	-1.19	0.11	0.002	-1.19	0.12	0.002	0.08	0.15	0.60	-0.86	0.09	<0.001	-0.42	0.13	0.001
Time since last dose	-0.16	0.12	0.17	-0.16	0.12	0.17	-0.14	0.16	0.37	0.06	0.10	0.55	-0.02	0.14	0.91
Time-point 1 antibody	0.15	0.04	<0.001	0.15	0.04	<0.001	-0.003	0.07	0.97	0.42	0.51	<0.001	0.15	0.02	<0.001
R ²			0.25			0.25			0.006			0.36			0.18
Born to HIV-infected mother	TT			DT			PT			FHA			HepB		
Independent variables	b	SE	P	b	SE	P	b	SE	P	b	SE	P	b	SE	P
NVP	-0.24	0.16	0.14	-0.24	0.16	0.14	-0.17	0.20	0.41	-0.24	0.15	0.12	-0.15	0.20	0.46
AZT	0.33	0.41	0.43	0.33	0.42	0.43	0.63	0.52	0.23	0.13	0.39	0.75	-0.28	0.51	0.59
Combined AZT/NVP	0.77	0.17	<0.001	0.77	0.17	<0.001	-0.13	0.21	0.55	0.73	0.16	<0.001	0.27	0.21	0.20
CD4+%	0.002	0.01	0.80	0.002	0.01	0.80	0.03	0.01	<0.001	-0.01	0.01	0.18	0.01	0.01	0.19
R ²			0.16			0.16			0.10			0.17			0.02

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed). TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; HepB: hepatitis B; MV: measles virus vaccine; NVP: Nevirapine; AZT: Zidovudine.

6.4 Factors associated with immune response to three dose schedules of DTwP and HepB vaccines stratified by maternal HIV status

Being born to an HIV-infected mother had a negative association with antibody levels for TT ($b=-0.59$; SE, 0.19; $p=0.002$), DT ($b=-0.59$; SE, 0.19; $p=0.002$) and HepB ($b=-0.53$; SE, 0.21; $p=0.01$). Children from Soweto were less likely to have antibody to TT, DT, FHA and HepB; Table 6.8. Pre-vaccination antibody levels had a positive association with presence of post vaccination antibody levels for TT ($b=0.13$; SE, 0.04; $p<0.001$), DT ($b=0.13$; SE, 0.04; $p<0.001$), FHA ($b=0.42$; SE, 0.05; $p<0.001$) and HepB ($b=0.15$; SE, 0.02; $p<0.001$); Table 6.8.

Table 6.8: Independent variables and their association to response to primary vaccination at Time-point 2 for DTwP and HepB vaccines by maternal exposure to HIV in their children

All children	TT			DT			PT			FHA			HepB		
Independent variables	b	SE	P	b	SE	P	b	SE	P	b	SE	P	b	SE	P
Gender	-0.09	0.09	0.34	-0.09	0.09	0.34	-0.07	0.12	0.54	-0.13	0.08	0.08	-0.20	0.11	0.06
Age	0.02	0.04	0.69	0.02	0.04	0.69	0.05	0.05	0.36	-0.02	0.03	0.59	0.005	0.04	0.92
Birth-weight	0.0001	0.0001	0.25	0.0001	0.0001	0.25	0.0001	0.0001	0.53	0.0001	0.0001	0.18	0.0001	0.0001	0.11
Race	0.33	0.18	0.06	0.33	0.18	0.06	0.002	0.23	0.99	-0.20	0.15	0.18	-0.22	0.21	0.29
Breastfeeding	-0.05	0.17	0.75	-0.05	0.17	0.75	0.01	0.22	0.96	0.13	0.14	0.37	0.22	0.19	0.25
Study center	-1.15	0.12	<0.001	-1.15	0.12	<0.001	0.08	0.15	0.60	-0.86	0.09	<0.001	-0.41	0.13	0.002
Time since last dose	-0.16	0.12	0.18	-0.16	0.12	0.18	-0.14	0.16	0.37	0.06	0.10	0.55	-0.02	0.14	0.90
Time-point 1 antibody	0.13	0.04	<0.001	0.13	0.04	<0.001	0.001	0.07	0.98	0.42	0.05	<0.001	0.15	0.02	<0.001
HIV status	-0.59	0.19	0.002	-0.59	0.19	0.002	0.08	0.25	0.75	-0.04	0.16	0.77	-0.53	0.21	0.01
R²			0.26			0.26			0.01			0.36			0.20

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed); Status (0 unexposed, 1 HIV-exposed). TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; HepB: hepatitis B; MV: measles virus vaccine; NVP: Nevirapine; AZT: Zidovudine.

6.5 Factors associated with immune response to three dose schedules of DTwP and HepB vaccines, as well as after booster dose of DTwP in children born to HIV-infected woman

HIV-infection was associated with less robust immune response to primary vaccination for all vaccine epitopes, Table 6.9. In addition to this, children from Stellenbosch were more likely to have antibody to TT ($b=-1.26$; SE, 0.40; $p=0.002$), DT ($b=-1.26$; SE, 0.40; $p=0.002$), FHA ($b=-0.67$; SE, 0.34; $p=0.05$) and HepB ($b=-1.04$; SE, 0.47; $p=0.03$), Table 6.9. Interestingly female gender was associated with less robust immune response to HepB vaccination ($b=-0.32$; SE, 0.13; $p=0.02$), this was not seen for any of the other vaccine epitopes; Table 6.9. PMTCT did not have an effect on response to primary vaccination, for all children who were exposed to either AZT/NVP or a combination of both for a mean of 8 days (± 5.7).

Table 6.9: Independent variables and their association to response to primary vaccination at Time-point 2 for DTwP and HepB vaccines in HEU and HIV-infected children

Independent variables	TT			DT			PT			FHA			HepB		
	b	SE	P	b	SE	P	b	SE	P	b	SE	P	b	SE	P
Gender	-0.20	0.11	0.07	-0.20	0.11	0.07	-0.24	0.14	0.09	-0.15	0.09	0.12	-0.32	0.13	0.02
Age	-0.001	0.04	0.98	-0.001	0.04	0.98	0.03	0.05	0.63	-0.02	0.04	0.58	-0.0	0.05	0.82
Birth-weight	0.00002	0.0001	0.87	0.00002	0.0001	0.87	-0.00003	0.0001	0.82	0.00005	0.0001	0.66	0.0002	0.0001	0.27
Race	0.29	0.26	0.26	0.29	0.26	0.26	0.0004	0.32	0.99	0.09	0.21	0.68	-0.08	0.30	0.78
Breastfeeding	-0.01	0.18	0.95	-0.01	0.18	0.95	0.04	0.23	0.85	0.16	0.15	0.30	0.26	0.22	0.24
Study center	-1.26	0.40	0.002	-1.26	0.40	0.002	-0.16	0.50	0.74	-0.67	0.34	0.05	-1.04	0.47	0.03
Time since last dose	-0.04	0.14	0.75	-0.04	0.14	0.75	-0.08	0.17	0.63	0.09	0.12	0.43	0.06	0.16	0.71
Time-point 1 antibody	0.09	0.05	0.07	0.09	0.05	0.07	0.004	0.09	0.38	0.40	0.06	<0.001	0.14	0.03	<0.001
NVP	-0.21	0.15	0.17	-0.21	0.15	0.17	-0.17	0.20	0.38	-0.24	0.13	0.06	-0.10	0.18	0.59
AZT	0.45	0.44	0.31	0.45	0.44	0.31	0.98	0.55	0.08	0.36	0.37	0.32	0.04	0.52	0.93
Combined AZT/NVP	-0.27	0.41	0.51	-0.27	0.41	0.51	-0.26	0.52	0.61	0.04	0.34	0.92	-0.68	0.48	0.16
HIV status	-0.70	0.13	<0.001	-0.70	0.13	<0.001	-0.96	0.16	<0.001	-0.48	0.10	<0.001	-0.64	0.15	<0.001
R²			0.29			0.29			0.13			0.39			0.21

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed); Status (0 unexposed, 1 HIV-exposed). TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; HepB: hepatitis B; MV: measles virus vaccine; NVP: Nevirapine; AZT: Zidovudine.

6.6 Factors associated with immune response to three dose schedules of DTwP and HepB vaccines, in HIV-infected children stratified by ART status

Initiation of ART in the first year of life was associated with a more robust response to HepB only ($b=0.68$; SE, 0.18; $p<0.001$). The inverse was seen with FHA for ART-Immed children ($b=-0.27$; SE, 0.13; $p=0.03$); Table 6.10. Being from Stellenbosch was associated with more robust immune response to all vaccine epitopes, except PT which showed there is no association between which region the children received vaccination; Table 6.10. CD4+ percent was only associated with immune response to the PT epitope ($b=0.03$; SE, 0.01; $p=0.003$). Pre-vaccination antibody concentration was associated with FHA ($b=0.53$; SE, 0.09; $p<0.001$) and HepB ($b=0.15$; SE, 0.04; $p<0.001$) epitopes only.

Table 6.10: Independent variables and their association to response to primary vaccination at Time-point 2 for DTwP and HepB vaccines in ART-Def and ART-Immed children

Independent variables	TT			DT			PT			FHA			HepB		
	b	SE	P	b	SE	P	b	SE	P	b	SE	P	b	SE	P
Gender	-0.23	0.16	0.14	-0.23	0.16	0.14	-0.01	0.19	0.95	-0.14	0.12	0.26	-0.32	0.17	0.06
Age	-0.05	0.06	0.42	-0.05	0.06	0.42	-0.04	0.07	0.62	-0.08	0.05	0.11	-0.10	0.07	0.14
Birth-weight	0.0001	0.002	0.68	0.0001	0.002	0.68	-	0.00004	0.0002	0.86	0.0001	0.0001	0.28	0.0002	0.19
Race	0.43	0.38	0.27	0.43	0.38	0.27	0.18	0.46	0.70	0.01	0.29	0.98	0.06	0.42	0.88
Breastfeeding	0.02	0.25	0.93	0.02	0.25	0.93	0.23	0.29	0.43	0.10	0.19	0.60	0.43	0.27	0.12
Study center	-1.51	0.19	<0.001	-1.51	0.19	<0.001	-0.39	0.23	0.08	-1.16	0.14	<0.001	-0.76	0.20	<0.001
Time since last dose	-0.07	0.19	0.72	-0.07	0.19	0.72	-0.06	0.22	0.79	0.09	0.14	0.52	0.10	0.20	0.59
Time-point 1 antibody	0.10	0.06	0.11	0.10	0.06	0.11	-0.03	0.12	0.78	0.53	0.09	<0.001	0.15	0.04	<0.001
CD4+%	0.002	0.01	0.80	0.002	0.01	0.80	0.03	0.01	0.003	-	0.01	0.96	-0.004	0.01	0.68
ART status	0.22	0.16	0.19	0.22	0.16	0.19	0.35	0.20	0.07	-0.27	0.13	0.03	0.68	0.18	<0.001
R²			0.28			0.28			0.10			0.47			

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed); ART status (0 ART-Def, 1 ART-Immed). TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; HepB: hepatitis B.

6.7 Sensitivity Analysis

Because of the imbalance of race in the population among the different groups, a sensitivity analysis was performed restricting the analysis to black African participants. P-values were similar to those when including mixed race participants suggesting that any differences can be attributed to HIV exposure and not race (data not shown).

6.8 Concluding comments

HEU children were not at increased susceptibility to most of the studied vaccine-preventable diseases by 6-weeks of age, suggesting similar acquisition of antibody from their mothers in our study. Furthermore, the immune responses in HEU were similar to HIV-unexposed children post-vaccination. Although HIV-infected children had lower GMCs post-vaccination, including to TT and HepB irrespective of timing of ART initiation, the proportion with sero-protective antibody concentrations post-vaccination were similar and timing of ART initiation generally did not affect immune responses between HIV-infected groups. The results indicate that vaccination of HEU and HIV-infected children initiated on early-ART with DTwP/HBV would provide similar immunity to the targeted vaccine-preventable disease compared with HIV-unexposed children. Short-term persistence of vaccine induced antibodies 4 months post vaccination, showed that over 90% of children maintained sero-protective antibody concentrations. However, persistence of sero-protective antibody concentrations (>4 months) and anamnestic responses to the studied vaccines among HEU and HIV-infected children initiated on early-ART needs to be investigated.

CHAPTER 7 RESULTS: ANTIBODY PERSISTENCE AND BOOSTER RESPONSE TO DIPHTHERIA-TETANUS-PERTUSSIS AND HEPATITIS B VACCINATION AMONG HIV-UNINFECTED AND HIV-INFECTED CHILDREN INITIATED ON EARLY ANTIRETROVIRAL THERAPY

Anamnestic immune response induced by vaccination is crucial for long-term protection against disease (Plotkin, 2010). HIV-infection is associated with aberrations in all major lymphocyte populations, including B-lymphocytes which are the cornerstone of humoral immune responses. (Letvin and Walker, 2003, Shearer et al., 2000, Pensiero et al., 2009, Gona et al., 2006, Palma et al., 2008, Moir and Fauci, 2009, Moir et al., 2004, Titanji et al., 2005, Titanji et al., 2006) In HIV-infected individuals T-lymphocyte and B-lymphocyte dysfunction may result in lower rates of sero-conversion post-vaccination, quicker waning of antibody levels, and impaired generation or accelerated loss of antigen-specific memory B-lymphocytes. (Ghosh et al., 2009, Moir and Fauci, 2008) This might be mitigated through advances made in the antiretroviral therapy (ART) of HIV-infected children. (Miller and Rathore, 2012) Early initiation of ART has been associated with relatively normal development of the T-lymphocyte lineage, and with preservation of high numbers of functional memory B-lymphocytes in HIV-infected individuals. (Palma et al., 2008, Pensiero et al., 2009, Zanchetta et al., 2008, Romiti et al., 2001) In some studies, however, despite B-lymphocyte function being improved following ART initiation, memory responses remained low at least over a year after viral control was attained. (Iwajomo et al., 2015, Moir et al., 2008, Moir et al., 2010, Lederman et al., 1998, Soriano-Sarabia et al., 2005, Bekker et al., 2006) We and others have previously reported that early initiation of ART induced better immune responses to vaccines compared to when ART was deferred until there was immunological or clinical deterioration (Simani et al., 2014, Cagigi et al., 2012, Pensiero et al., 2009, Madhi et al., 2010).

Furthermore, immunological aberrations have also been reported in HIV-uninfected children born to HIV-infected women. This could be due to *in-utero* HIV virion particle exposure and/or ART-exposure (Hygino et al., 2008, Faye et al., 2007, Bunders et al., 2005, Clerici et al., 2000, Gesner et al., 1994, Neilsen et al., 2001), affecting lymphocyte differentiation and function (Hygino et al., 2008, Faye et al., 2007, Feiterna-Sperling et al., 2007, Borges-Almeida et al., 2011, Bunders et al., 2005, Clerici et al., 2000), which might affect immune responses to vaccines, including anamnestic responses and persistence of antibody responses.

In this chapter, results on antibody persistence and responses to a booster dose of with or without *H. influenzae* type-b conjugate vaccine (DTwP-HibCV) in HIV-unexposed, HEU and HIV-infected children with CD4+ $\geq 25\%$ previously randomized to initiate ART within 4-10 weeks of age (ART-Immed) or when clinically or immunologically indicated per then World Health Organization criteria (ART-Def). We also compared the persistence of antibody to HepB vaccine up to 24-months of age (The published paper is under review).

7.1 Results

7.1.1 Study population and demographic information

This study was conducted on archived serum samples from children in whom the effects of HIV-exposure and timing of ART initiation on immune responses to 7-valent pneumococcal conjugate vaccine (PCV7) had been evaluated (Madhi et al., 2010). Briefly children aged 6-12 weeks were enrolled in Soweto (Gauteng) and Stellenbosch (Western Cape) in South Africa from April 2005 to June 2006. Participants included HIV-infected children with CD4+ T-lymphocyte cells $\geq 25\%$ randomized to initiate ART immediately (ART-Immed); or when clinically (CDC Stage C or investigator-selected severe Stage B) and/or immunologically indicated as per then WHO recommendations for ART treatment (ART-Def). All HIV-infected children were co-enrolled in the Children with HIV Early Antiretroviral (CHER) study, in which the first line ART regimen used was zidovudine, lamivudine and lopinavir/ritonavir (Violari et al., 2008). Children in the ART-Immed group were randomized to interrupt ART if clinically and immunologically stable at either 12- (ART/12m) or 24-months (ART/24m) of age (Simani et al., 2013, Violari et al., 2008). Furthermore, children born to HIV-infected mothers who tested negative for HIV (HEU) or born to HIV-uninfected mothers (HIV-unexposed) were also enrolled. Details of verification of the HIV-infection status of the children and mothers have been described, including repeat testing of the HEU to confirm their HIV-uninfected status (Violari et al., 2008).

Participants within each group were randomized to receive either a booster-dose of HibCV or PCV7 (Prevnar®, Wyeth Vaccines, NJ, USA) at 15-18-months of age. The immune responses to the booster dose of PCV7 and HibCV will be reported separately. Those children randomized to receive HibCV, received a DTwP-HibCV (CombAct-Hib®, Sanofi Pasteur, Marcy L'Etoile,

France) dose, whereas the others received DTwP (Sanofi Pasteur, Marcy L'Etoile, France) at 15-18-months of age. No booster vaccine was scheduled for HBV.

Antibodies to DT, TT, HepB, PT and FHA were measured by an in-house Luminex-multiplex immunoassay as previously described (Simani et al., 2014). Antibody persistence and booster response were described for established putative thresholds of sero-protective levels for diphtheria (≥ 0.1 IU/ml), tetanus (≥ 0.1 IU/ml) and HepB (≥ 10 mIU/ml) (Plotkin, 2010). Immune responses to pertussis vaccine were evaluated by measuring sero-conversion rates (≥ 4 -fold increase from pre-booster compared to post-booster antibody concentration) for PT and FHA. Other predefined antibody thresholds were ≥ 0.01 IU/ml and ≥ 1.0 IU/ml (threshold correlated with long-term protection) for tetanus and diphtheria and ≥ 100 mIU/ml for HepB (Madhi et al., 2013b).

Of the 483 children who completed the primary series vaccination (Simani et al., 2014), 465 (96.3%) returned for booster-vaccination at 15-18 months of age; Table 1. The reasons for exclusion from analyses at the different study-visits are summarized in Figure 1. The mean age ($\pm$ standard deviation) at the 3 study-visits was 15.1 months (± 0.5) at the time of booster, 15.4 months (± 0.6) at the post-booster visit and 24.9 months (± 0.7) at last study-visit. Forty-seven percent of children were male and 90% of black-African descent; Table 1. All children in the ART-Def group were initiated on ART at 6.4 months (± 3.9) of age. In the early ART group, 81 children interrupted treatment at 12 months of age (ART/12m) and 90 continued on ART until their second birthday (ART/24m) (Simani et al., 2013). The mean CD4⁺ cell percentages were similar between the ART-Immed compared to the ART-Def groups pre-booster (31.1% vs. 32.3%, respectively) and at 24 months of age (34.3% vs. 36.3%, respectively); Table 1.

Table 7.1: Demographic and baseline information of participants who had received three doses of diphtheria toxoid-tetanus toxoid-whole cell pertussis-*Haemophilus influenzae* type b conjugate vaccine and hepatitis B vaccines and where available for follow-up.

Variable	Total	HIV-unexposed ^a	HEU ^b	ART-Immed ^c	ART-Def ^d
Number vaccinated	465	110	115	171	69
Male, n (%)	218 (46.9)	59 (53.6)	63 (54.8)	71 (41.5)	25 (36.2)
Black race, n (%)	420 (90.3)	81 (73.6)	108 (93.9)	163(95.3)	68 (98.6)
Coloured race, n (%)	45 (9.7)	29 (26.4)	7 (6.1)	8(4.7)	1 (1.5)
Soweto study center, n (%)	312 (67.1)	71 (64.6)	80 (69.6)	116 (67.8)	45 (65.2)
Stellenbosch study center, n (%)	153 (32.9)	39 (35.5)	35 (30.4)	55 (32.2)	24 (34.8)
Mean time in months between last dose in primary series and booster dose (±SD)	12.0 (0.6)	11.9 (0.4)	11.8 (0.6)	12.0 (0.4)	12.2 (0.8)
Mean age in months (±SD)					
Pre-booster ^e	15.1 (0.5)	14.9 (0.4)	14.9 (0.6)	15.2 (0.4) [*]	15.4 (0.6) ^{+√}
Post-booster ^f	15.4 (0.6)	15.2 (0.4) ^{††}	15.4 (0.6) ^{**}	15.4 (0.5) ⁺	15.6 (0.7) ^{√√}
24 months	24.9 (0.7)	24.8(0.5) ⁺⁺	24.9 (0.6)	24.9 (0.7)	24.7 (0.9) [°]
Age in months at ART initiation [±SD]				0	6.4 (3.9)
Booster median CD4+ T-lymphocyte count (IQR) ^g				1592 (1138 to 2166); n=171	1611 (1357 to 2272); n=69
Booster mean CD4+ T-lymphocyte % (±SD)				31.1 (8.7); n=171	32.3 (7.1); n=69
24 months of age median CD4+ T-lymphocyte count (IQR)				1404 (982 to 1765); n=107	1826 (1272 to 20921828); n=43
24 months of age mean CD4+ T-lymphocyte % (±SD)				34.3 (7.5); n=107	36.3 (7.1); n=43

Footnote: ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ^cART-Immed: HIV-infected children with CD4+ % ≥25% at enrolment who were initiated on antiretroviral therapy (ART) at enrolment; ^dART-Def: HIV-infected children with CD4+ % ≥25% at enrolment randomized to deferred ART; ^eBlood draw was undertaken just prior to and on the same day of DTP booster vaccination. ^fChildren who received booster dose within protocol-defined window periods and available immunogenicity data within 2-4 weeks after booster. ^gIQR: interquartile range.

^{*}p-value<0.001 when comparing HIV-unexposed and HEU to ART-Immed; [†]p-value<0.001 when comparing HIV-unexposed and HEU to ART-Def; [√]p-value=0.048 when comparing ART-Immed to ART-Def; ^{**}p-value=0.001 when comparing HIV-unexposed to HEU; ^{††}p-value<0.001 when comparing ART-Immed and ART-Def to HIV-unexposed; ^{√√}p-value=0.03 when comparing HEU to ART-Def; ⁺p-value=0.02 when comparing ART-Immed to ART-Def; ⁺⁺p-value=0.03 when comparing HEU to HIV-unexposed; [°]p-value=0.03 when comparing HEU to ART-Def

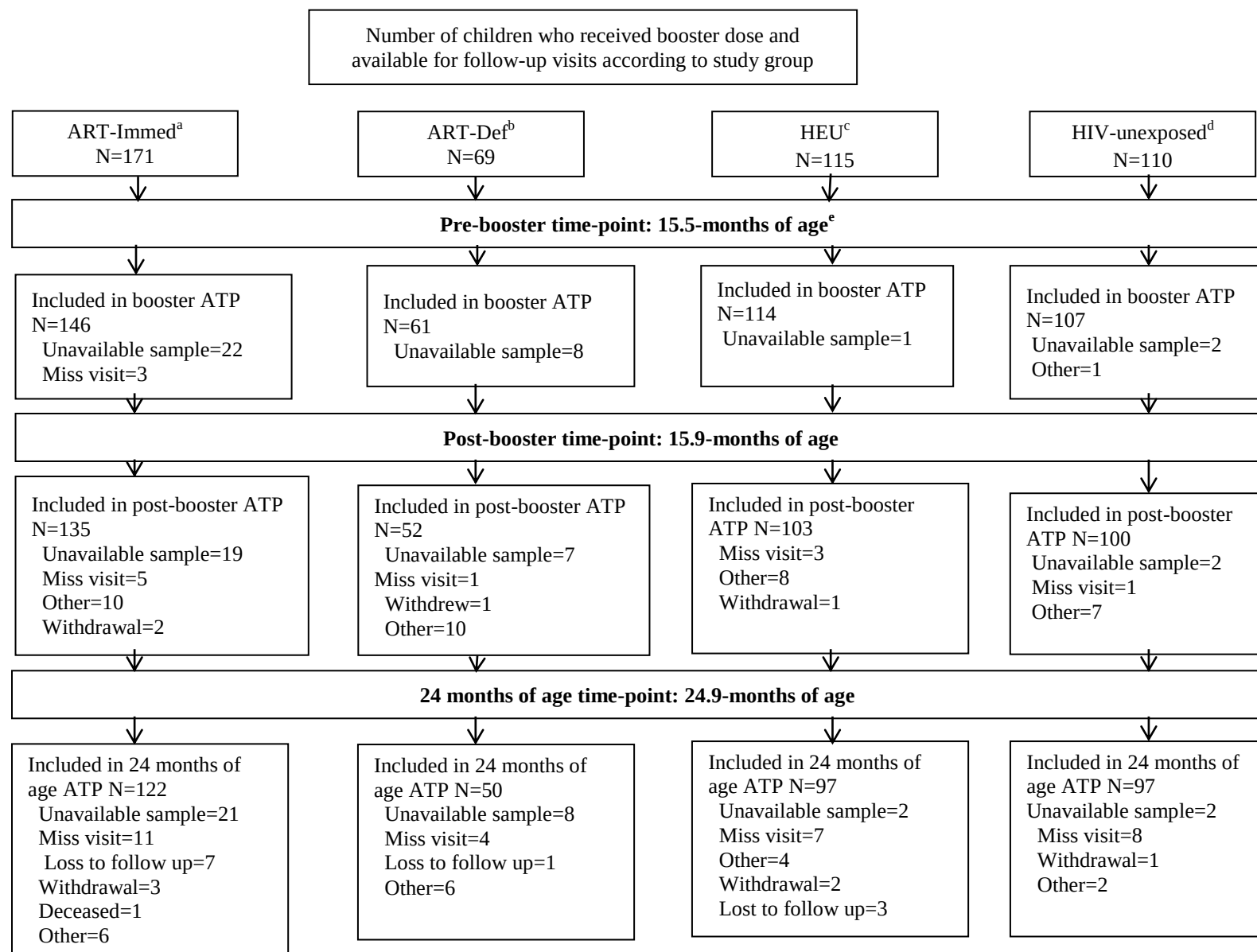


Figure 7.1: Reasons for exclusion from analyses at the different study time-points.

^aART-Immed: HIV-infected children with CD4+ $\geq$ 25% at enrolment who were initiated on antiretroviral therapy (ART) at enrolment; ^bART-Def: HIV-infected children with CD4+ $\geq$ 25% at enrolment randomized to deferred ART; ^cHEU: HIV-uninfected children born to HIV-infected mothers; ^dHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^ePre-booster: 15.5 months of age, all children received a booster dose of DTP and no booster dose of hepatitis-B vaccine was administered.

Explanatory notes: ATP: analysis time-point; Unavailable sample: sample unavailable for further testing; Lost to follow-up: due to relocation or the children stopped the study without reason; Withdrew: parent or guardian withdrew informed consent; Missed visit: children missed a schedule visit; Other: Unable/unwilling to adhere to study protocol; number of participants who left the study (excluded, missed visit, lost to follow-up, withdrawals, deceased) are represented as cumulative pre- and post-vaccination.

7.1.2 Pre- and post-booster antibody kinetics to tetanus-toxoid

Prior to the booster dose, TT IgG antibody GMC were lower among the ART-Immed group (0.08 IU/ml; $p<0.001$) and similarly so in the ART-Def group (0.11 IU/ml; $p=0.09$) compared to HIV-unexposed children (0.13 IU/ml); Table 7.2. Nevertheless, $\geq 99\%$ of children in all groups had antibody levels ≥ 0.01 IU/ml to TT pre-booster dose; Table 7.2. Compared to HIV-unexposed children (79%), the ART-Immed (49%; $p<0.001$) and ART-Def (43%; $p<0.001$) were, however, less likely to have TT antibody levels ≥ 0.1 IU/ml.

Following the booster dose of TT, IgG GMC were lower among the ART-Immed (6.6 IU/ml; $p<0.001$) and ART-Def groups (4.4 IU/ml; $p<0.001$) compared to HIV-unexposed children (17.9 IU/ml). Furthermore, the ART-Def group had lower TT GMC post-booster compared to the ART-Immed group ($p=0.01$); Table 7.2. Also, the fold-increase in GMC were lower in the ART-Def group (39.8) compared to either the HIV-unexposed (135.6; $p<0.001$) or the ART-Immed group (87.9; $p=0.001$). There was an association with reduced antibody response to booster dose of TT in children born to HIV-infected mothers (unstandardized coefficient [b] = -0.59; SE, 0.11; $p<0.001$) and older age ($b=-0.21$; SE, 0.08; $p=0.01$); however, there was a positive association between time lapsed pre- and post-booster, which was associated with a more robust response ($b=0.39$; SE, 0.06; $p<0.001$); Table 7.3.

One hundred percent of HIV-infected and HIV-unexposed children, however, had antibody levels ≥ 0.1 IU/ml to TT post-boost. The majority of children in each group also had antibody concentrations ≥ 1.0 IU/ml, although this too was lower in the ART-Def group (85%) than HIV-unexposed (100%; $p<0.001$) and ART-Immed groups (96%; $p=0.01$).

At 24 months of age, the TT antibody GMC were lower among the ART-Immed (0.79 IU/ml; $p<0.001$) and ART-Def groups (0.23 IU/ml; $p<0.001$) compared to HIV-unexposed children (1.7 IU/ml); Table 7.2. There was greater fold decline in TT antibody between the post-booster visit and at 24 months of age in the ART-Def group (-21.3) when compared to HIV-unexposed (-10.1; $P<0.001$) and ART-Immed (-9.7; $p=0.002$) groups; Table 7.2. Although $\geq 96\%$ of children in all groups retained TT antibody levels ≥ 0.01 IU/ml, the percentage of ART-Def children (72%) with TT antibody levels ≥ 0.1 IU/ml was lower than HIV-unexposed children (100%; $p<0.001$) or the

ART-Immed group (100%; $p<0.001$). Furthermore, TT antibody levels ≥ 1.0 IU/ml were less prevalent in the ART-Immed (36%; $p<0.001$) and ART-Def groups (14%; $p<0.001$), compared to HIV-unexposed children (76%); Table 7.2.

Comparing the HEU to HIV-unexposed children, in general the TT antibody kinetics were similar pre-boost and in response to a booster dose of vaccine. The TT IgG GMC were, however, lower in HEU at 24 months of age (1.7 vs. 1.3 IU/ml; $p=0.01$), as were the percentage of HEU children with TT IgG ≥ 1.0 IU/ml (66% vs. 76%; $p=0.05$). However, no differences were observed in the fold decrease of antibody between the post-boost and 24 month of age time-point between these two groups; Table 7.2.

Table 7.2: Percentage of children with sero-protective antibody levels and geometric mean concentrations to Tetanus-toxoid at pre-booster, post-booster and at 24 months of age

Threshold	Time-point	HIV-unexposed ^a N=107	HEU ^b N=114	p-value ^c	ART-Immed ^d N=146	p-value ^e	ART-Def ^f N=61	p-value ^g	p-value ^h
GMC	Pre-Booster	0.13 (0.12-0.15)	0.13 (0.12-0.15)	0.47	0.08 (0.07-0.09)	<0.001	0.11 (0.08-0.15)	0.09	0.07
	Post-booster	17.9 (15.8-20.4)	15.4 (13.3-17.9)	0.72	6.6 (5.7-7.6)	<0.001	4.4 (3.2-6.1)	<0.001	0.01
	Fold-change ⁱ	135.6 (114.1-161.0)	116.6 (97.5-139.3)	0.72	87.9 (71.7-107.7)	0.12	39.8 (23.0-68.8)	<0.001	0.001
	24 months	1.7 (1.5-1.9)	1.3 (1.1-1.5)	0.01	0.79 (0.69-0.90)	<0.001	0.23 (0.15-0.35)	<0.001	<0.001
	Fold-change ^j	-10.1 (8.7-11.8)	-11.9 (9.8-14.5)	0.08	-9.7 (8.0-11.7)	0.74	-21.3 (13.8-32.7)	<0.001	0.002
≥0.01	Pre-booster: n/N; % (95% CI)	107/107; 100 (97-100)	114/114; 100 (97-100)	NE ^k	145/146; 99 (96-100)	0.39	61/61; 100 (94-100)	NE	0.52
	Post-booster: n/N; % (95% CI)	100/100; 100 (96-100)	103/103; 100 (96-100)	NE	135/135; 100 (97-100)	NE	52/52; 100 (93-100)	NE	NE
	24 months: n/N; % (95% CI)	97/97; 100 (96-100)	97/97; 100 (96-100)	NE	122/122; 100 (97-100)	NE	48/50; 96 (87-99)	0.05	0.03
≥0.1	Pre-booster: n/N; % (95% CI)	85/107; 79 (71-86)	90/114; 79 (71-85)	0.63	72/146; 49 (41-57)	<0.001	26/61; 43 (31-55)	<0.001	0.38
	Post-booster: n/N; % (95% CI)	100/100; 100 (96-100)	103/103; 100 (96-100)	NE	135/135; 100 (97-100)	NE	52/52; 100 (93-100)	NE	NE
	24 months: n/N; % (95% CI)	97/97; 100 (96-100)	95/97; 98 (93-99)	0.16	122/122; 100 (97-100)	NE	36/50; 72 (58-83)	<0.001	<0.001
≥1	Pre-booster: n/N; %	0/107; 0	1/114; 0.9 (0.2-5)	0.33	1/146; 0.7 (0.1-4)	0.39	1/61; 2 (0.3-9)	0.18	0.47

	(95% CI)								
	Post-booster: n/N; % (95% CI)	100/100; 100 (96-100)	103/103; 100 (96-100)	NE	130/135; 96 (92-98)	<i>0.05</i>	44/52; 85 (73-92)	<i><0.001</i>	0.01
	24 months: n/N; % (95% CI)	74/97; 76 (67-84)	64/97; 66 (56-75)	0.05	44/122; 36 (28-45)	<i><0.001</i>	7/50; 14 (7-26)	<i><0.001</i>	0.01

Footnote: Percentage of participants with concentration above threshold (95% confidence interval) and geometric mean concentration (95% confidence interval); Units for geometric mean concentration (GMC) are IU/ml. ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ^cp-value: HIV-unexposed vs. HEU; ^dART-Immed: HIV-infected children with CD4+ $\geq 25\%$ at enrolment who were initiated on ART at enrolment; ^ep-value: HIV-unexposed vs. ART-Immed; ^fART-Def: HIV-infected children with CD4+ $\geq 25\%$ at enrolment randomized to deferred ART; ^gp-value: HIV-unexposed vs. ART-Def; ^hp-value: ART-Immed vs. ART-Def; ⁱFold-change: Fold-change in GMC between pre-booster and post-booster; ^jFold-change: Fold-change in GMC between post-booster and 24 months of age; ^kNE: not evaluable;

Explanatory notes:

Percentage participants: p-values were either calculated by logistic regression and adjusted for age, gender, race, study center, pre-booster antibody concentration (for post-booster and 24 months time-point) and CD4+ cell percentage in HIV-infected children; or by Pearson's chi-square test for values in italics.

Geometric mean concentration: All p-values were calculated by linear regression and adjusted for age, gender, race, study center, pre-booster antibody concentration (for post-booster and 24 months time-point) and CD4+ cell percentage in HIV-infected children.

Table 7.3: Independent variables and their association with response to booster vaccination for DTwP-HibCV and DTwP vaccines by HIV status

Independent variables	TT			DT			PT			FHA		
	b	SE	P	b	SE	P	b	SE	P	b	SE	P
Gender	0.11	0.09	0.22	0.09	0.06	0.16	-0.04	0.10	0.69	-0.003	0.08	0.97
Age	-0.21	0.08	0.01	-0.15	0.06	0.01	-0.25	0.09	0.005	-0.09	0.07	0.23
Race	-0.40	0.17	0.02	-0.40	0.12	0.001	-0.49	0.18	0.008	-0.49	0.15	0.001
Study center	0.09	0.12	0.44	0.01	0.08	0.95	0.57	0.12	<0.001	-0.04	0.10	0.67
Time since vaccination	0.39	0.06	<0.001	0.26	0.04	<0.001	0.48	0.07	<0.001	0.34	0.06	<0.001
Time-point 4 antibody	0.03	0.06	0.62	-0.07	0.03	0.03	0.05	0.07	0.49	-0.005	0.05	0.93
HIV status	-0.59	0.11	<0.001	0.01	0.08	0.92	-0.26	0.12	0.03	0.27	0.10	0.005
R²			0.23			0.16			0.22			0.14

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Status (0 unexposed, 1 HIV-exposed). TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemoglutinin.

7.1.3 Pre- and post-booster antibody kinetics to diphtheria-toxoid

Prior to receiving the booster dose of DT, IgG antibody GMCs were similar in HIV-unexposed children (0.08 IU/ml); compared to either the ART-Immed (0.10 IU/ml; $p=0.22$) or ART-Def (0.12 IU/ml; $p=0.05$) group; Table 7.4. The percentage of children with pre-booster DT antibody ≥ 0.1 IU/ml was, however, higher among the ART-Immed (54%; $p=0.001$) and ART-Def groups (57%; $p=0.02$) compared to HIV-unexposed children (38%); Table 7.4.

Post-booster of DT, the fold increase in IgG GMC was lower in ART-Def children (17.9) than HIV-unexposed (49.6; $p=0.001$) or ART-Immed group (30.4; $p=0.001$). Consequently, the IgG GMC post-booster were lower in the ART-Def (2.3 IU/ml) compared to HIV-unexposed (3.9 IU/ml; $p=.003$) or ART-Immed children (3.0 IU/ml; $p<.001$). Younger age ($b=-0.15$; SE, 0.06; $p=0.01$), coloured race ($b=-0.40$; SE, 0.12; $p=0.001$) and low pre-booster antibody level ($b=-0.07$; SE, 0.03; $p=0.03$) were negatively associated with immune response to the DT booster, but not so for HIV-infection ($b=0.01$; SE, 0.08; $p=0.92$); Table 7.3. Furthermore, shorter time since last time-point was associated with a more robust immune response ($b=0.26$; SE, 0.04; $p<0.001$).

One hundred percent of the HIV-infected and HIV-unexposed groups had antibody levels ≥ 0.1 IU/ml post-booster. Furthermore, $\geq 93\%$ of children in each group had DT antibody levels ≥ 1 IU/ml, except for the ART-Def group (85%) which was lower compared to HIV-unexposed children (99%; $p=0.02$); Table 7.4.

At 24 months of age, compared to HIV-unexposed children (0.68 IU/ml), GMC were lower in ART-Immed (0.46) IU/ml; $p<0.001$) and ART-Def (0.20 IU/ml; $p<0.001$) groups; Table 7.4. Furthermore, antibody decay (fold-decline in GMC) was more pronounced in ART-Def group (-11.4) compared to HIV-unexposed (-5.5; $p=0.001$) and ART-Immed children (-6.8; $p=0.008$). Nevertheless, 100% of HIV-infected and HIV-unexposed children maintained DT antibody levels ≥ 0.01 IU/ml, although a lower percentage of ART-Def children (80%) had DT antibody levels ≥ 0.1 IU/ml compared to HIV-unexposed (99%; $p=0.02$) or ART-Immed children (99.2%; $p=0.004$). Furthermore, a lower percentage of children in the ART-Immed (13%, $p=0.001$) and

ART-Def groups (2%; $p=0.003$) had DT antibody levels ≥ 1.0 IU/ml compared to HIV-unexposed children (32%).

Comparing HEU to HIV-unexposed children, the pre-boost DT IgG GMC was similar (0.07 vs. 0.08 IU/ml, respectively). Although HEU children were less likely to have DT IgG ≥ 0.01 IU/ml (92% vs. 100%; $p=0.003$), a greater percentage had titres ≥ 0.1 IU/ml (54% vs. 38%; $p=0.03$). Post-boost, HEU children had a higher fold increase in DT GMC (68.4 vs. 49.6; $p=0.004$), with resultant higher GMCs as well (4.6 vs. 3.9 IU/ml, respectively; $p=.002$). There was, however, no difference in the percentage of children between these two groups for the different analysed DT IgG thresholds, including >97% having a threshold of ≥ 1.0 IU/ml. Similarly, the GMC at 24 months of age and percentage with antibody threshold above the correlate of protection were similar between these two groups, however, antibody decay was greater in HEU (-7.7) compared to HIV-unexposed (-5.5) children, Table 7.4.

Table 7.4: Percentage of children with sero-protective antibody levels and geometric mean concentration to diphtheria-toxoid at pre-booster, post-booster and at 24 months of age

Threshold	Time-point	HIV-unexposed ^a N=107	HEU ^b N=114	p-value ^c	ART-Immed ^d N=146	p-value ^e	ART-Def ^f N=61	p-value ^g	p-value ^h
GMC	Pre-Booster	0.08 (0.07-0.09)	0.07 (0.05-0.09)	0.07	0.10 (0.09-0.11)	0.22	0.12 (0.10-0.15)	0.05	0.05
	Post-booster	3.9 (3.5-4.3)	4.6 (4.1-5.2)	0.002	3.0 (2.7-3.3)	0.15	2.3 (1.8-3.0)	0.003	0.04
	Fold-change ⁱ	49.6 (41.4-59.4)	68.4 (48.9-95.8)	0.004	30.4 (25.7-36.0)	0.08	17.9 (12.2-26.2)	0.001	0.001
	24 months	0.68 (0.60-0.77)	0.61 (0.53-0.70)	0.37	0.46 (0.41-0.52)	<0.001	0.20 (0.15-0.27)	<0.001	<0.001
	Fold-change ^j	-5.5 (4.8-6.4)	-7.7 (6.5-9.1)	0.004	-6.8 (5.8-8.0)	0.08	-11.4 (8.3-15.7)	0.001	0.008
≥0.01	Pre-booster: n/N; % (95% CI)	107/107; 100 (97-100)	105/114; 92. (86-96)	0.003	146/146; 100 (97-100)	NE ^k	61/61; 100 (94-100)	NE	NE
	Post-booster: n/N; % (95% CI)	100/100; 100 (96-100)	103/103; 100 (96-100)	NE	135/135; 100 (97-100)	NE	52/52; 100 (93-100)	NE	NE
	24 months: n/N; % (95% CI)	97/97; 100 (96-100)	97/97; 100 (96-100)	NE	122/122; 100 (97-100)	NE	50/50; 100 (93-100)	NE	NE
≥0.1	Pre-booster: n/N; % (95% CI)	41/107; 38 (30-48)	61/114; 54 (44-62)	0.03	79/146; 54 (46-62)	0.001	35/61; 57 (45-69)	0.02	0.95
	Post-booster: n/N; % (95% CI)	100/100; 100 (96-100)	103/103; 100 (96-100)	NE	135/135; 100 (97-100)	NE	52/52; 100 (93-100)	NE	NE
	24 months: n/N; % (95% CI)	96/97; 99 (94-100)	95/97; 98 (93-99)	0.49	121/122; 99 (96-100)	0.76	40/50; 80 (67-89)	0.02	0.004
≥1	Pre-booster: n/N; % (95% CI)	0/107; 0	0/114; 0	NE	0/146; 0	NE	0/61; 0	NE	NE
	Post-booster: n/N; %	99/100; 99 (95-100)	100/103; 97.1 (92-99)	0.45	125/135; 93 (87-96)	0.08	44/52; 85 (73-92)	0.02	0.14

	(95% CI)								
	24 months: n/N; % (95% CI)	31/97; 32 (24-42)	26/97; 27 (19-36)	0.40	16/122; 13 (8-20)	0.001	1/50; 2 (0.3-1.0)	0.003	0.03

Footnote: Percentage of participants with concentration above threshold (95% confidence interval) and Geometric mean concentration (95% confidence interval); Units for geometric mean concentration (GMC) are IU/ml. ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ^cp-value: HIV-unexposed vs. HEU; ^dART-Immed: HIV-infected children with CD4+ $\geq 25\%$ at enrolment who were initiated on ART at enrolment; ^ep-value: HIV-unexposed vs. ART-Immed; ^fART-Def: HIV-infected children with CD4+ $\geq 25\%$ at enrolment randomized to deferred ART; ^gp-value: HIV-unexposed vs. ART-Def; ^hp-value: ART-Immed vs. ART-Def; ⁱFold-change: Fold-change in GMC between pre-booster and post-booster; ^jFold-change: Fold-change in GMC between post-booster and 24 months of age; ^kNE: not evaluable.

Explanatory notes:

Percentage participants: p-values were either calculated by logistic regression and adjusted for age, gender, race, study center, pre-booster antibody concentration (for post-booster and 24 months time-points) and CD4+ cell percentage in HIV-infected children; or by Pearson's chi-square test for values in italics.

Geometric mean concentration: All p-values were calculated by linear regression and adjusted for age, gender, race, study center, pre-booster antibody concentration (for post-booster and 24 months time-points) and CD4+ cell percentage in HIV-infected children.

7.1.4 Pre- and post-booster antibody kinetics to pertussis antigens (PT and FHA)

Comparing the HIV-unexposed to HIV-infected children, prior to wP booster dose of vaccine, FHA IgG GMC were higher in the ART-Immed (34.0 IU/ml; $p=0.001$) group, and a similar trend observed for the ART-Def group (31.9 IU/ml; $p=0.09$), compared to HIV-unexposed children (24.6 IU/ml), Pertussis toxoid IgG antibody GMCs were, however, lower in both the ART-Immed (31.8 IU/ml; $p=0.004$) and ART-Def groups (27.2 IU/ml; $p=0.004$) compared to HIV-unexposed children (38.3 IU/ml) ; Table 7.5.

Post-booster dose of wP, the fold change in GMC to FHA was similar between the HIV-infected and HIV-unexposed children; however, ART-Immed (30.1; $p=0.01$) and ART-Def groups (27.9; $p=.04$) had lower fold increase to PT than HIV-unexposed children (56.1). Also, whilst there was no difference in FHA antibody GMC between the HIV-infected and HIV-unexposed groups post-booster, PT antibody GMC were lower in the ART-Immed (951.5 IU/ml; $p<0.001$) and ART-Def groups (767.6 IU/ml; $p<0.001$) compared to HIV-unexposed children (2168.8 IU/ml). There was a positive association between children enrolled at Chris Hani Baragwanath Hospital ($b=0.57$; SE, 0.12; $p<0.001$) and time since last time-point ($b=0.48$; SE, 0.07; $p<0.001$) and immune resulting to the booster wP dose. In contrast, Age ($b=-0.25$; SE, 0.09; $p=0.005$) and Race (-0.49 ; SE, 0.18; $p=0.008$) were negatively association with response to the booster dose; Table 7.3. There were, however, no difference in the sero-conversion rates between either HIV-infected groups compared to HIV-unexposed children for either FHA ($>88\%$) or PT ($\geq 90\%$); Table 7.5.

At 24 months of age, the ART-Def group showed greater waning of antibody, as evident by the fold decrease between the post-booster and 24 month antibody concentrations, for both FHA (-3.6 fold) and PT (-12 fold) compared to HIV-unexposed (-2.6; $p=0.04$ and -7.7; $p=0.01$, respectively) or ART-Immed children (-2.1; $p=0.03$ and -4.0; $p<0.001$, respectively); Table 7.5. This was associated with the ART-Def group having lower GMC to both FHA and PT compared to HIV-unexposed and ART-Immed groups ($p\leq 0.02$ for all observations) at 24 months of age; Table 7.5. In contrast, there was no difference in the fold decline of FHA antibody concentrations between the ART-Immed and HIV-unexposed groups, for whom GMCs were similar at 24 months of age; Table 4. HIV-unexposed group (-7.7; $p=0.001$) however had a greater decline in

PT GMC compared to ART-Immed children (-4.0; $p=0.001$) between the post-boost and 24 months of age.

Comparing the HEU to HIV-unexposed children, although the pre-booster FHA GMC was higher in HEU children (34.5 vs. 24.6 IU/ml; $p=0.01$), this did not differ for PT. The post-booster fold increase in GMC was also greater in HEU than HIV-unexposed children to both FHA (23.1 vs. 18.7 fold; $p=0.01$) and PT (79.7 vs. 56.1 fold; $p=0.001$). This was associated with higher GMCs in HEU compared to HIV-unexposed children to both FHA (806.2 vs. 467.6 IU/ml; $p<0.001$) and PT (3138.2 vs. 2168.8 IU/ml; $p<0.001$). There was, however, no difference in sero-conversion rates for either FHA or PT between these two groups. Although HEU showed a greater fold decrease in GMC between the post-booster and 24 month of age visits compared to HIV-unexposed group for FHA (-5.8 vs. -2.6; $p<0.001$) and similarly so PT (-9.6 vs. -7.7; $p=0.06$), there were no difference in GMCs between the two groups at 24 months of age, Table 7.5.

Table 7.5: Geometric mean concentration at pre-booster, post-booster and at 24-months of age for pertussis antigens and percentage of children who achieved a 4-fold increase in antibody levels

Antibody	Threshold	Time-point	HIV-unexposed ^a N=107	HEU ^b N=114	p-value ^c	ART-Immed ^d N=146	p-value ^e	ART-Def ^f N=61	p-value ^g	P-value ^h
FHA	GMC	Pre-booster	24.6 (20.9-28.9)	34.5 (30.1-39.6)	0.01	34.0 (30.3-38.1)	0.001	31.9 (26.9-38.0)	0.09	0.23
		Post-booster	467.6 (395.8-552.4)	806.2 (703.2-924.2)	<0.001	426.0 (371.6-488.3)	0.71	402.2 (324.9-497.9)	0.78	0.50
		Fold-change ⁱ	18.7 (14.8-23.6)	23.1 (19.0-28.1)	0.01	13.0 (10.8-15.8)	0.22	12.3 (9.1-16.6)	0.36	>0.99
		24 months	170.3 (151.2-191.8)	140.9 (120.1-165.4)	0.14	198.4 (169.7-232.0)	0.26	103.9 (70.3-153.7)	0.02	0.001
		Fold-change ^j	-2.6 (2.1-3.2)	-5.8 (4.8-7.0)	<0.001	-2.1 (1.7-2.6)	0.40	-3.6 (2.3-5.6)	0.04	0.03
	≥4 fold increase ^k : n/N; % (95% CI)	Post-booster	91/100; 91 (84-95)	100/103; 97 (92-99)	0.06	116/131; 89 (82-93)	0.75	45/50; 90 (79-96)	0.99	0.69
PT	GMC	Pre-booster	38.3 (33.9-43.3)	39.5 (35.3-44.3)	0.64	31.8 (28.7-35.2)	0.004	27.2 (21.6-34.2)	0.004	0.38
		Post-booster	2168.8 (1856.7-2533.3)	3138.2 (2650.6-3715.5)	<0.001	951.5 (802.7-1127.8)	<0.001	767.6 (594.8-990.7)	<0.001	0.19
		Fold-change	56.1 (45.6-69.0)	79.7 (65.7-96.7)	0.001	30.1 (24.6-36.8)	0.01	27.9 (18.3-42.5)	0.04	0.89
		24 months	268.4 (228.3-315.5)	348.6 (288.6-421.2)	0.09	254.6 (225.3-287.7)	0.54	67.2 (47.9-94.3)	<0.001	<0.001
		Fold-change	-7.7 (6.1-9.7)	-9.6 (7.6-12.2)	0.06	-4.0 (3.3-4.9)	0.001	-12.0 (8.6-16.7)	0.01	<0.001
	≥4 fold increase: n/N; % (95% CI)	Post-booster	99/100; 99 (95-100)	102/103; 99 (95-100)	0.88	126/131; 96 (91-98)	0.31	45/50; 90 (79-96)	0.10	0.19

Footnote: Percentage of participants who achieved a 4-fold increase in antibody levels from pre- to post-booster (95% confidence interval) and geometric mean concentration (95% confidence interval); Units for geometric mean concentration (GMC) are IU/ml. ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ^cp value: HIV-unexposed vs. HEU; ^dART-Immed: HIV-infected children with CD4+ ≥25% at enrolment who were initiated on ART at enrolment; ^ep value: HIV-unexposed vs. ART-Immed; ^fART-Def: HIV-infected children with CD4+ ≥25% at enrolment randomized to deferred ART; ^gp value: HIV-unexposed vs. ART-Def; ^hp value: ART-Immed vs. ART-Def; ⁱFold-change: Fold-change in GMC between pre-booster and post-booster; ^jFold-change: Fold-change in GMC between post-booster and 24 months of age; ^k≥4 fold increase: sero-conversion rate, children showing 4 fold increase in antibody concentration from pre-booster to post-booster dose. Explanatory notes: ≥4 fold increase: p-values were calculated by logistic regression and adjusted for age, gender, race, study center, pre-booster antibody concentration (for

post-booster time-point) and CD4+ cell percentage in HIV-infected children; Geometric mean concentration: All p-values were calculated by linear regression and adjusted for age, gender, race, study center, pre-booster antibody concentration (for post-booster and 24 months time-points) and CD4+ cell percentage in HIV-infected children.

7.1.5 Persistence and antibody kinetics to hepatitis B

No booster dose of HepB vaccine was scheduled in this study; hence we only measured persistence of antibody to HepB post-primary vaccination. At approximately 15 months of age, 100% of children retained HepB sero-protective antibody levels ≥ 10 mIU/ml and 94% to 100% had levels ≥ 100 mIU/ml; Table 7.6. HepB GMC were, however, significantly lower in ART-Immed (769.4 mIU/ml; $p=0.001$) and ART-Def groups (700.2 mIU/ml; $p=0.002$) compared to HIV-unexposed children (1198.5 mIU/ml); and similarly so in HEU children (880.1 mIU/ml; $p=0.02$); Table 7.6. All groups showed a decrease in HepB GMC antibody between the post primary series GMC at 5 months of age compared to 15 months of age, except for the ART-Def group in whom there was a 0.8-fold increase, which was significant compared to HIV-unexposed group (-2.1; $p<0.001$). Also, the fold decrease in GMC was lower in the ART-Immed group (-1.2; $p<0.001$) than in HIV-unexposed children.

At 24 months of age, the HepB antibody GMCs were lower in the ART-Immed (242.0 mIU/ml, $p=0.001$) and ART-Def (54.8 mIU/ml; $p<0.001$) groups compared to HIV-unexposed children (396.1 mIU/ml). Also, ART-Def children were less likely to maintain HepB antibody levels ≥ 10 mIU/ml (72%) or ≥ 100 mIU/ml (50%) compared to HIV-unexposed or ART-Immed groups ($\geq 92\%$ -100%; $P<.001$ for all comparisons); Table 7.6. The decay of antibody was also more evident in ART-Def children with -13.3 fold change from the 15 months compared to 24 months of age, which was significant compared to HIV-unexposed and ART-Immed groups (-3.0 fold change; $p<0.001$ for all comparisons); Table 7.6.

Comparing the HIV-unexposed to HEU children, although there was no difference in fold decrease of GMC between 5 and 15 months of age, HEU had lower GMC (880.1 vs. 1198.5; $p=0.02$) and were less likely to have titres >100 mIU/ml (94% vs. 100%; $p=0.01$). These differences were, however, no longer evident by 24 months of age; Table 7.6.

Table 7.6: Percentage of children with sero-protective antibody levels and geometric mean concentration to hepatitis B at 15-18 months and 24 months of age

Threshold	Age	HIV-unexposed ^a N=107	HEU ^b N=114	p-value ^c	ART-Immed ^d N=146	p-value ^e	ART-Def ^f N=61	p-value ^g	p-value ^h
GMC	5 months ⁱ	2521.0 (2249.8-2824.9)	2019.3 (1740.3-2343.0)	0.04	924.9 (778.3-1099.1)	<0.001	536.6 (373.0-772.0)	<0.001	0.02
	15-18 months	1198.5 (1033.9-1389.4)	880.1 (719.2-1077.0)	0.02	769.4 (654.4-904.7)	0.001	700.2 (587.3-834.7)	0.002	0.55
	Fold-change ^j	-2.1 (1.8-2.5)	-2.3 (1.8-3.0)	0.78	-1.2 (0.9-1.5)	<0.001	0.8 (0.50-1.3)	<0.001	0.13
	24 months	396.1 (332.5-471.8)	311.9 (264.8-367.4)	0.11	242.0 (209.9-279.1)	0.001	54.8 (32.2-93.0)	<0.001	<0.001
	Fold-change ^j	-3.0 (2.5-3.7)	-3.0 (2.3-4.0)	0.97	-3.0 (2.4-3.7)	0.94	-13.3 (7.6-23.2)	<0.001	<0.001
≥10	15-18 months: n/N; % (95% CI)	107/107; 100 (97-100)	114/144; 100 (97-100)	NE ^k	146/146; 100 (97-100)	NE	61/61; 100 (94-100)	NE	NE
	24 months: n/N; % (95% CI)	97/97; 100 (96-100)	97/97; 100 (96-100)	NE	122/122; 100 (97-100)	NE	36/50; 72.0 (58-83)	<0.001	<0.001
≥100	15-18 months: n/N; % (95% CI)	107/107; 100 (97-100)	107/114; 94 (88-97)	0.01	142/146; 97 (93-99)	0.08	61/61; 100 (94-100)	NE	0.19
	24 months: n/N; % (95% CI)	93/97; 96 (90-98)	89/97; 92 (85-96)	0.17	112/122; 92 (86-96)	0.28	25/50; 50 (37-63)	<0.001	<0.001

Footnote: Percentage of participants with concentration above threshold and geometric mean concentration (95% confidence interval); Units for geometric mean concentration (GMC) are mIU/ml. ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ^cp-value: HIV-unexposed vs. HEU; ^dART-Immed: HIV-infected children with CD4+ ≥25% at enrolment who were initiated on ART at enrolment; ^ep-value: HIV-unexposed vs. ART-Immed; ^fART-Def: HIV-infected children with CD4+ ≥25% at enrolment randomized to deferred ART; ^gp-value: HIV-unexposed vs. ART-Def; ^hp-value: ART-Immed vs. ART-Def; ⁱ5 months: results of primary vaccination and seen in table 6.3; ^jFold-change: Fold-change in GMC between 5 months and 15-18 months of age; ^kNE: not evaluable. Explanatory notes: Percentage participants: p-values were either calculated by logistic regression and adjusted for age, gender, race, study center, pre-booster antibody concentration (for 24 months of age time-point) and CD4+ cell percentage in HIV-infected children or by Pearson's chi-square test for values in italics; Geometric mean concentration: All p-values were calculated by linear regression and adjusted for age, gender, race, study center, pre-booster antibody concentration (for 24 months of age time-point) and CD4+ cell percentage in HIV-infected children.

7.2 Correlation of post-primary vaccination response with post-booster response in study participants.

There was a correlation between antibody level at time-point 2 (after 3 doses of primary vaccination) and antibody levels at Time-point 5 (2-weeks post-booster vaccination) in HEU children for DT ($r=0.23$; $p=0.02$) epitope; Table 7.7.

Table 7.7: Correlation of primary vaccination and post-booster antibody levels in study participants

Group (N)	TT		DT		PT		FHA	
	r* (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value	r (95% CI)	p-value
HIV-unexposed (100)	-0.06 (-0.26 to 0.14)	0.55	-0.01 (-0.21 to 0.19)	0.93	-0.04 (-0.24 to 0.17)	0.72	0.01 (-0.19 to 0.21)	0.92
HEU (103)	0.18 (-0.02 to 0.36)	0.08	0.23 (0.04 to 0.41)	0.02	0.13 (-0.07 to 0.32)	0.18	0.10 (-0.10 to 0.29)	0.32
ART-Def (50)	-0.23 (-0.49 to 0.06)	0.10	-0.20 (-0.46 to 0.10)	0.17	-0.24 (-0.49 to 0.05)	0.09	-0.09 (-0.37 to 0.20)	0.52
ART-Immed (131)	-0.12 (-0.29 to 0.06)	0.19	-0.11 (-0.28 to 0.07)	0.21	0.05 (-0.12 to 0.23)	0.55	-0.12 (-0.29 to 0.06)	0.17

Footnote: TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin; r*: Spearman's correlation coefficient; CI: confidence interval; HIV-unexposed: HIV-exposed uninfected; HEU: HIV-exposed uninfected; ART-Def: HIV-infected on deferred ART; ART-Immed: HIV-infected on immediate ART.

7.3 Factors associated with response to booster vaccine in all children irrespective of HIV status

Factors associated with booster responses in study children included race which showed a negative association for all study epitopes, and time since last visit, showed a positive association with all vaccine epitopes; Table 7.8. Age was negatively associated with response to TT ($b=-0.30$; SE, 0.08; $p<0.001$); DT ($b=-0.14$; SE, 0.06; $p=0.01$) and PT ($b=-0.29$; SE, 0.08; $p=0.001$); Table 7.8. Hepatitis B was not included in this analysis as no booster dose was administered at this time point.

Table 7.8: Independent variables and their association to response to booster vaccination at Time-point 5 for DTwP

Independent variables	TT			DT			PT			FHA		
	b	SE	P	b	SE	P	b	SE	P	b	SE	P
Gender	0.14	0.09	0.14	0.09	0.06	0.16	-0.02	0.10	0.81	-0.02	0.08	0.81
Age	-0.30	0.08	<0.001	-0.14	0.06	0.01	-0.29	0.08	0.001	-0.04	0.07	0.57
Race	-0.72	0.17	<0.001	-0.39	0.12	0.001	-0.62	0.18	<0.001	-0.37	0.15	0.01
Study center	0.20	0.12	0.09	0.005	0.08	0.95	0.59	0.11	<0.001	-0.06	0.10	0.52
Time since last dose	0.39	0.06	<0.001	0.26	0.04	<0.001	0.48	0.07	<0.001	0.34	0.06	<0.001
Time-point 4 antibody	0.09	0.06	0.12	-0.07	0.03	0.03	0.07	0.07	0.34	0.01	0.05	0.80
R ²			0.18						0.21			0.12

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto). TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin.

7.4 Factors associated with immune response to booster dose of DTwP in children born to HIV-infected woman

Similar to primary vaccination, HIV-infection was associated with having decreased response to booster vaccination, Table 7.9. In addition to HIV-infection, time since last time-point was strongly associated with robust responses in HEU children for all vaccine epitopes.

Table 7.9: Independent variables and their association to response to booster vaccination at Time-point 5 for DTwP vaccines in HEU and HIV-infected children

Independent variables	TT			DT			PT			FHA		
	b	SE	P	b	SE	P	b	SE	P	b	SE	P
Gender	0.04	0.10	0.74	0.08	0.08	0.33	-0.14	0.10	0.16	-0.10	0.09	0.27
Age	-0.14	0.09	0.10	-0.12	0.07	0.08	-0.17	0.08	0.05	-0.04	0.07	0.57
Race	-0.17	0.25	0.49	-0.22	0.18	0.24	-0.07	0.23	0.78	-0.10	0.21	0.65
Study center	0.22	0.13	0.10	0.21	0.09	0.03	0.78	0.11	<0.001	0.14	0.10	0.17
Time since last time-point	0.17	0.08	0.02	0.14	0.06	0.01	0.21	0.07	0.004	0.20	0.06	0.002
Time-point 4 antibody	-0.02	0.07	0.74	-0.04	0.04	0.32	-0.02	0.07	0.82	-0.01	0.06	0.92
HIV status	-0.85	0.12	<0.001	-0.39	0.09	<0.001	-1.12	0.11	<0.001	-0.54	0.10	<0.001

R²			0.24			0.18			0.43			0.19

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Status (0 exposed, uninfected, 1 HIV-infected). TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin.

7.5 Factors associated with immune response to booster dose of DTwP in HIV-infected children stratified by ART status

Initiation of ART within the first year of life was associated with optimal responses to TT (b=0.40; SE, 0.13; p=0.002, DT (b=0.22; SE, 0.10; p=0.02) and PT (b=0.25; SE, 0.11; p=0.03) vaccine epitopes; Table 7.10. Children from Soweto were more likely to respond to the booster dose of DTwP than children from Stellenbosch for TT, DT and PT epitopes.

Table 7.10: Independent variables and their association to response to booster vaccination at Time-point 5 for DTwP vaccines in ART-Def and ART-Immed children

Independent variables	TT			DT			PT			FHA		
	b	SE	P	b	SE	P	b	SE	P	b	SE	P
Gender	-0.04	0.13	0.74	0.03	0.10	0.78	-0.16	0.12	0.17	-0.05	0.11	0.63
Age	0.04	0.11	0.75	0.05	0.08	0.55	-0.03	0.10	0.76	0.04	0.10	0.69
Race	-0.11	0.32	0.73	-0.04	0.24	0.86	0.22	0.28	0.42	0.09	0.27	0.74
Study center	0.78	0.17	<0.001	0.46	0.13	<0.001	1.19	0.13	<0.001	0.35	0.14	0.01
Time since last time-point	0.03	0.08	0.76	0.06	0.06	0.32	0.09	0.07	0.21	0.16	0.07	0.03
Time-point 4 antibody	0.04	0.08	0.66	-0.07	0.07	0.35	-0.06	0.08	0.43	-0.03	0.08	0.68
CD4+ % *	0.01	0.01	0.52	-0.001	0.006	0.93	0.005	0.007	0.43	-0.002	0.007	0.78
ART status	0.40	0.13	0.002	0.22	0.10	0.02	0.25	0.11	0.03	0.07	0.11	0.52
R ²			0.18			0.16			0.39			

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); ART status (0 ART-Def, 1 ART-Immed). TT: tetanus-toxoid; DT: diphtheria-toxoid; PT: pertussis toxin; FHA: filamentous haemagglutinin. *= CD4+ percent used in analysis was from CD4+ percent results from Time-point 4 as no CD4+ percent was measured for Time-point 5.

7.6 Concluding comments

Protecting children born to HIV-infected mothers against vaccine-preventable diseases is important now that ART has improved life expectancy and general well being of these children. The results show that duration of sero-protection is shorter in HIV-infected children, especially those not initiated on ART prior to receiving their primary series of vaccines, if subsequently initiated on ART and establishing normal CD4+ counts. With the current guidelines a large proportion of these children would have lost sero-protective antibody levels before the proposed booster dose schedules for the study vaccines. Therefore, this study shows that children on deferred ART require boosting for all the studied vaccine epitopes and continued monitoring of tetanus, diphtheria, pertussis and hepatitis B specific antibodies concentrations can guide the need for further booster doses to these and other vaccines.

CHAPTER 8 RESULTS: EFFECT OF IN-UTERO HIV EXPOSURE AND ANTIRETROVIRAL TREATMENT STRATEGIES ON MEASLES SUSCEPTIBILITY AND IMMUNOGENICITY OF MEASLES-VACCINE

The aim of this chapter was to 1) evaluate the effect of in-utero HIV-exposure and timing of ART initiation to measles susceptibility prior to vaccination; (2). Examine the association of HIV-exposure and different ART strategies on the kinetics of measles antibody following the primary and booster doses of measles vaccine.

8.1 Results

8.1.1 Study population and demographic information

Samples were available for 528 (91.3%) of 578 children enrolled into the study from the initial time-point. The number of samples available at each time-point in the various groups is detailed in Figure 8.1. Of the 176 ART-Immed children with samples available at the time of the booster-dose, 87 had been assigned to discontinue ART at age 12-months (ART/12m) and 89 were scheduled to interrupt ART at age 24-months (ART/24m). Forty-eight percent (n=251) of children were male and 90.9% (n=480) black-African descent. The mean age of children at each study time-points, at the time of primary-measles vaccination and booster-dose was similar between groups, Table 8.1. In addition to other childhood vaccines administered as described in chapter 6 and 7, children received the Schwarz-strain measles vaccine (Rouvax®; Aventis, France) at age 38-42 weeks and a booster dose at age 64-76 weeks. Measles antibody titers were analysed twice pre-vaccination at study enrolment (Time-point 1: age 6-12 weeks) and approximately 20-weeks of age (Time-point 2). Furthermore, measles-antibody was measured prior to (Time-point 4) and two weeks after the booster-dose (Time-point 5), as well as at approximately 24 months of age (Time-point 6). No blood was planned *a priori* immediately prior to the first dose of measles-vaccine or anytime soon thereafter. Samples were analysed for measles specific IgG antibodies with a commercial indirect ELISA kit (Enzygnost, Dade Behring, Marburg, Germany) and results calibrated against the international reference preparation of measles antibody. Antibody titer levels were calculated using the α -method and presented in milli-International Units per millilitre (mIU/ml) per manufacturer recommendation. Assays with titers <150mIU/ml were considered as negative (equivalent to <0.1 optical density [OD]), 150–329mIU/ml as equivocal (equivalent to 0.1-0.2 OD) and ≥ 330 mIU/ml as sero-protective (equivalent to >0.2 OD) per manufacturers criteria. For this study, sero-positivity was defined as antibody titers ≥ 150 mIU/ml. Samples for

which the OD value was <0.1 were assigned a titer of half the detection limit of the assay (i.e. 75 mIU/ml) when calculating GMTs. Due to the limited number of subjects in CD4+ $<25\%$, statistical comparisons were not undertaken between this and other Groups.

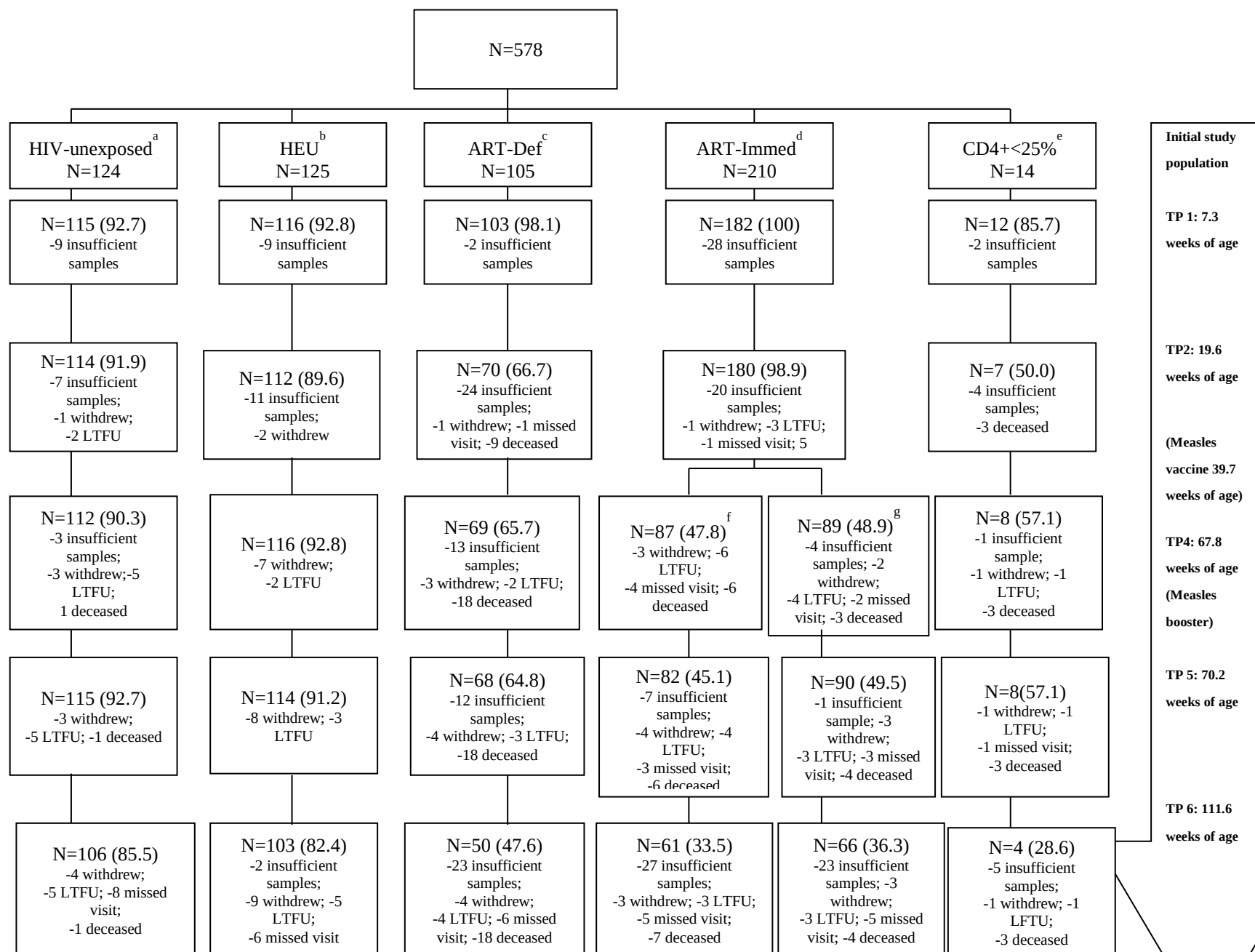


Figure 8.1: Subject follow-up during the duration of the study.

Footnote: ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ^cART-Def: HIV-infected children on deferred ART; ^dART-Immed: HIV-infected children on immediate ART; ^eCD4+<25%: HIV-infected children with CD4+<25% on immediate ART; ^fART/12m: HIV-infected children who interrupted ART at 12 months of age; ^gART/24m: HIV-infected children who continued ART till 24 months of age. Insufficient sample: sample unavailable for further testing; LTFU: loss to follow-up due to relocation or the child stopped the study without reason; Withdrawal: parent or guardian withdrew informed consent; Missed visit: children missed a schedule visit; number of participants who left the study (LTFU, Withdrawals, and Deceased) are represented as cumulative at each visit. Number in parenthesis is percentages of total enrolled.

Table 8.1: Demographic information for study groups at separate study time-points

Characteristics	HIV-unexposed ^a	HEU ^b	ART-Def ^c	ART-Immed ^d		CD4+<25% ^e	Total
Time-point 1: Enrolment	N=115	N=116	N=103	N=182		N=12	N=528
Mean age, weeks $\pm$ SD	6.9 $\pm$ 1.0	7.3 $\pm$ 1.0	7.3 $\pm$ 1.1	7.4 $\pm$ 1.3		8.4 $\pm$ 1.5	7.3 $\pm$ 1.2
Mean CD4+ cell % $\pm$ SD	NA	NA	36.5 $\pm$ 1.1	35.4 $\pm$ 0.6		22.0 $\pm$ 1.9	NA
Median CD4+ x10 ⁶ cells/ml (IQR)	NA	NA	2303 (2090-2516)	2304 (2142-2468)		1617 (1208-2027)	NA
Time-point 2: Pre measles vaccination	N=114	N=112	N=70	N=180		N=7	N=483
Mean age, weeks $\pm$ SD	19.3 $\pm$ 1.7	19.5 $\pm$ 1.1	19.7 $\pm$ 1.7	19.7 $\pm$ 1.6		20.7 $\pm$ 1.5	19.6 $\pm$ 1.6
Mean CD4+ cell % $\pm$ SD	NA	NA	29.9 $\pm$ 1.0 ^f	41.6 $\pm$ 0.7 ^f		31.5 $\pm$ 2.1	NA
Median CD4+ x10 ⁶ cells/ml (IQR)	NA	NA	1805 (1586-2025) ^f	2346 (2202-2491) ^f		2065 (1412-2718)	NA
1st Measles vaccine	N=115	N=116	N=75	N=182		N=10	497
Mean age, weeks $\pm$ SD	38.9 $\pm$ 1.0	39.4 $\pm$ 1.6	40.8 $\pm$ 3.9	39.8 $\pm$ 2.0		41.5 $\pm$ 2.9	39.7 $\pm$ 2.3
Mean CD4+ cell % $\pm$ SD	NA	NA	31.0 $\pm$ 0.9 ^f	39.1 $\pm$ 0.6 ^f		30.6 $\pm$ 2.7	NA
				ART/12m ^g	ART/24m ^h		
Time-point 4: Booster	N=112	N=116	N=69	N=87	N=89	N=8	N=481
Mean age, weeks $\pm$ SD	67.2 $\pm$ 5.0	67.1 $\pm$ 2.2	69.3 $\pm$ 7.7	68.0 $\pm$ 1.9	67.8 $\pm$ 2.1	67.9 $\pm$ 1.5	67.8 $\pm$ 4.4
Median CD4+ x10 ⁶ cells/ml (IQR)	NA	NA	1823 (1657-1987) ⁱ	1364 (1212-1517) ⁱ	1770 (1597-1942) ⁱ	1434 (872-1995)	NA
Time-point 5: Post-booster	N=115	N=114	N=68	N=82	N=90	N=8	N=477
Mean age, weeks $\pm$ SD	69.3 $\pm$ 5.4)	69.4 $\pm$ 2.8	72.1 $\pm$ 6.8 ⁱ	69.7 $\pm$ 2.2 ⁱ	69.3 $\pm$ 2.2 ⁱ	69.4 $\pm$ 1.5	70.2 $\pm$ 5.4
Mean CD4+ cell % $\pm$ SD	NA	NA	34.5 $\pm$ 0.9	27.8 $\pm$ 0.9	36.2 $\pm$ 0.9	29.4 $\pm$ 3.4	NA
Time-point 6: 24 months of age	N=115	N=114	N=68	N=82	N=90	N=8	N=477
Mean age, weeks $\pm$ SD	69.3 $\pm$ 5.4	69.4 $\pm$ 2.8	72.1 $\pm$ 6.8 ⁱ	69.7 $\pm$ 2.2 ⁱ	69.3 $\pm$ 2.2 ⁱ	69.4 $\pm$ 1.5	70.2 $\pm$ 5.4

Footnote: ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers ; ^cART-Def: HIV-infected children on deferred ART; ^dART-Immed: HIV-infected children on immediate ART; ^eCD4+<25%: HIV-infected children with CD4+%

<25% on immediate ART; ^f $p \leq 0.001$ comparing ART-Def vs. ART-Immed; ^gART/12m: HIV-infected children who interrupted ART at 12 months of age; ^hART/24m: HIV-infected children who continued ART till 24 months of age; ⁱ $p \leq 0.001$ comparing ART-Def vs. ART/12m to and ART?12m vs. ART/24m. N= total number of children with available sample; SD= standard deviation; NA= not available; IQR=interquatile range (25-75th Centile).

The CD4+ lymphocyte percentage at enrolment and subsequent time-points in HIV-infected children is summarized in Table 8.1. At a mean age of 19.6 weeks, CD4+% were higher in ART-Immed (41.6%) compared to ART-Def (29.9%; $p<0.001$) and CD4+<25% (31.5%; $p=0.003$) children; Table 8.1. Similarly, CD4+% remained higher at the time of the first vaccine-dose in ART-Immed (39.1%) compared to ART-Def (31.0%; $p<0.001$) and CD4+<25% (30.6%; $p=0.01$). ART/12m had lower CD4+% (27.8%) when measured two weeks post-booster dose compared to ART-Def (34.5%; $p<0.001$) and ART/24m (36.2%; $p<0.001$), Table 8.1. The median age of ART initiation in ART-Def was 25.0 (18.4-40.0) weeks, and 65.3% (49/75) in this group had been initiated on ART by the time they received the first measles-vaccine and 68 (98.6%) of the remaining 69 had been initiated on ART by the time of their booster dose of measles-vaccine. Also, 67 (77.0%) of 87 ART/12m children had been re-initiated on ART by the time of their booster vaccine-dose.

8.1.2 Pre-vaccination measles antibody kinetics

The proportion of HIV-unexposed children who were sero-positive (78.3%) or had sero-protective titers (65.2%) was similar to HEU children, but higher than in ART-Def (56.3% and 37.9%, respectively) and ART-Immed (59.3% and 41.8%); Table 2. Also, GMTs were higher in HIV-unexposed (963 mIU/ml) compared to ART-Def (657 mIU/ml, $p=0.04$; Table 8.2) at enrollment. By 19.6-weeks of age, the proportion of children with sero-positive and sero-protective titers among HIV-unexposed had declined to 28.9% and 16.7%, respectively; both higher than in ART-Def (5.7% and 0%,) and ART-Immed (16.1% and 7.2%;); Table 8.2. There was no difference in the fold-decrease of antibody titers in HIV-unexposed (0.49) compared to HEU (0.39, $p=0.21$), ART-Def (0.31, $p=0.97$) and ART-Immed (0.42, $p=0.96$) between enrollment and 19.6 weeks of age, Table 8.2.

Table 8.2: Geometric mean titers and proportion of children with sero-positive and sero-protective levels of measles antibody prior to measles vaccination

Time-point	Measure	HIV-unexposed ^a	HEU ^b	p-value ^c	ART-Def ^d	ART-Immed ^e	CD4+<25% ^f	p-value ^g	p-value ^h	p-value ⁱ
Time-point 1 (Mean age: 7.3-weeks)		N=115	N=116		N=103	N=182	N=12			
	GMT (95% CI) ^j	963 (759-1223)	935 (737-1186)	0.86	657 (497-869)	731 (596-896)	938 (43-20623)	0.04	0.08	0.54
	N (%) ≥150mIU/ml ^k	90 (78.3)	80 (69.0)	0.11	57 (55.3)	108 (59.3)	2 (16.7)	0.001	0.001	0.62
	N (%) ≥330mIU/ml ^l	75 (65.2)	64 (55.2)	0.12	39 (37.9)	76 (41.8)	2 (16.7)	0.0002	<0.0001	0.52
Time-point 2 (Mean age 19.6-weeks)		N=114	N=112		N=70	N=180	N=7			
	GMT (95% CI)	461 (332-639)	369 (287-476)	0.28	202 (126-324)	309 (249-385)	0 (0)	0.09	0.05	0.16
	N (%) ≥150mIU/ml	33 (28.9)	31 (27.7)	0.83	4 (5.7)	29 (16.1)	0 (0.0)	<0.0001	0.01	0.01
	N (%) ≥330mIU/ml	19 (16.7)	16 (13.8)	0.62	0 (0.0)	13 (7.2)	0 (0.0)	<0.0003	0.01	0.02
	GMT fold change ^m	0.49	0.39	0.21	0.31	0.42	1.0	0.97	0.96	0.94

Footnote: ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ^cp-value: HIV-unexposed vs. HEU; ^dART-Def: HIV-infected children on deferred ART; ^eART-Immed: HIV-infected children on immediate ART; ^fCD4+<25%: HIV-infected children with CD4+% <25% on immediate ART; ^gp-value: HIV-unexposed vs. ART-Def; ^hp-value: HIV-unexposed vs. ART-Immed; ⁱp-value: ART-Def vs. ART-Immed; ^jGMT (95% CI) = geometric mean titer with 95% confidence interval in parenthesis; ^k≥150mIU/ml= proportion sero-positive for measles antibody; ^l≥330mIU/ml= proportion with sero-protective titers; ^mFold change in GMT from time-point1 to time-point 2.

8.1.3 Kinetics of measles antibody post-primary and booster doses of measles vaccine

The first measles-vaccine was administered at 39.7 (S.D. $\pm$ 2.3) weeks of age. Twenty-eight weeks thereafter (Time-point 4), the proportion of HIV-unexposed children with sero-positive (93.8%) or sero-protective titers (91.1%) was similar to other Groups, except for a lower proportion of ART/12m (79.3%; $p < 0.001$) having sero-protective titers; Table 8.3. This occurred despite 77.0% (67 of 87) ART/12m children already having been re-initiated on ART at this stage. ART/12m were also less likely to have sero-protective titers compared to ART-Def (95.7%; $p = 0.003$) and ART/24m (92.1%; $p = 0.02$; Table 8.3); and also had lower GMTs compared to each of the other groups at Time-point 4; Table 8.3.

The booster-dose was given at 67.8 (S.D. $\pm$ 4.4) weeks of age, after which the proportion of children with sero-protective titers was $\geq 90.2\%$ and did not differ between Groups when evaluated 1.9 weeks (S.D. 0.1) later; Table 8.3. The GMTs post-booster were higher in HIV-unexposed (3124 mIU/ml) compared to HEU (2532 mIU/ml, $p = 0.02$) and ART/12m (1435 mIU/ml, $p < 0.001$); Table 8.3. GMTs in ART/12m also remained lower compared to ART-Def (3254 mIU/ml, $p < 0.001$) and ART/24m (2740 mIU/ml, $p = 0.002$) post-booster dose; Table 8.3. The proportion of CD4+<25% children with sero-protective titers was 100% post-primary and post-booster vaccine.

Persistence of antibody was evaluated 41.4 (S.D. $\pm$ 0.3) weeks following the booster-dose, at approximately 111 weeks of age, HIV-unexposed were more likely to have sero-protective titers (94.3%) compared to HEU (79.6%; $p = 0.002$) and ART/12m (80.3%; $p = 0.010$), Table 8.3.

Although 75.4% (46/61) of ART/12m children had been re-initiated on ART by this time-point, they remained less likely to have sero-protective titers compared to ART-Def (96.0%; $p = 0.013$) and similarly to ART/24m (86.4%; $p = 0.360$). At the same time-point, GMTs were higher in HIV-unexposed (2248 mIU/ml) compared to HEU (1773 mIU/ml, $p = 0.004$) and ART/12m (1156 mIU/ml, $p < 0.001$), Table 8.3. ART/12m also had lower GMTs compared to ART-Def (2577 mIU/ml, $p < 0.001$) and ART/24m (1924 mIU/ml, $p = 0.009$).

Table 8.3: Measles-antibody titers following primary and booster-vaccine stratified by HIV-exposure and HIV-infection status

Measure	HIV-unexposed ^a	HEU ^b	p-value ^c	ART-Def ^d	p-value ^e	ART/12m ^f	p-value ^g	p-value ^h	ART/24m ⁱ	p-value ^j	p-value ^k	p-value ^l	CD4+<25% ^m
Time-point 4	N=112	N=116		N=69		N=87			N=89				N=8
GMT (95% CI) ⁿ	2212 (1888-2591)	3009 (2653-3412)	0.008	2253 (1782-2847)	0.89	1044 (849-1283)	<0.001	<0.001	2224 (1822-2717)	0.96	0.94	<0.001	2566 (1188-5542)
N (%) ≥150mIU/ml ^o	105 (93.8)	111 (95.7)	0.512	68 (98.6)	0.13	80 (91.9)	0.62	0.06	85 (95.5)	0.59	0.28	0.33	8 (100.0)
N (%) ≥330mIU/ml ^p	102 (91.1)	110 (94.8)	0.267	66 (95.7)	0.25	69 (79.3)	<0.001	0.003	82 (92.1)	0.12	0.37	0.02	8 (100.0)
Time-point 5	N=115	N=114		N=68		N=82			N=90				N=8
GMT (95% CI)	3124 (2763-3533)	2532 (2134-3005)	0.015	3254 (2656-3988)	0.72	1435 (1185-1740)	<0.001	<0.001	2740 (2334-3217)	0.19	0.18	0.002	2480 (1175-5233)
N (%) ≥150mIU/ml	112 (97.4)	110 (96.5)	0.692	67 (98.5)	0.61	79 (96.3)	0.67	0.41	87 (96.7)	0.76	0.46	0.91	8 (100.0)
N (%) ≥330mIU/ml	111 (96.5)	104 (91.2)	0.095	66 (97.1)	0.84	74 (90.2)	0.07	0.10	87 (96.7)	0.96	0.89	0.09	8 (100.0)
Fold-change ^q	1.4	0.95	0.0003	1.44	0.70	1.37	0.97	0.76	1.23	0.07	0.25	0.14	0.96
Time-point 6	N=106	N=103		N=50		N=61			N=66				N=4
GMT (95% CI)	2248 (1888-2676)	1773 (1459-2157)	0.004	2577 (2005-3311)	0.379	1156 (898-1488)	<0.001	<0.001	1924 (1448-2557)	0.33	0.14	0.01	1230 (253-5990)
N (%) ≥150mIU/ml	105 (99.1)	93 (90.3)	0.005	49 (98.0)	0.584	57 (93.4)	0.04	0.25	64 (96.9)	0.31	0.73	0.35	4 (100)
N (%) ≥330mIU/ml	100 (94.3)	82 (79.6)	0.002	48 (96.0)	0.079	49 (80.3)	0.01	0.01	57 (86.4)	0.07	0.01	0.36	4 (100)
Fold-change ^q	0.72	0.70	0.81	0.79	0.482	0.80	0.64	0.85	0.70	0.29	0.84	0.67	0.50

Footnote: ^aHIV-unexposed: HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ^cp-value: HIV-unexposed vs. HEU; ^dART-Def: HIV-infected children on deferred ART; ^ep-value: HIV-unexposed vs. ART-Def; ^fART/12m: HIV-infected children who interrupted ART at 12 months of

age; ^ep-value: HIV-unexposed vs. ART/12m; ^hp-value: ART-Def vs. ART/12m; ⁱART/24m: HIV-infected children who continued ART till 24 months of age; ^jp-value: HIV-unexposed vs. ART/24m; ^kp-value: ART-Def vs. ART/24m; ^lp-value: ART/12m vs. ART/24m; ^mCD4+<25%: HIV-infected children with CD4+% <25% on immediate ART; ⁿGMT (95% CI)= geometric mean titer with 95% confidence interval in parenthesis; ^o≥150mIU/ml= proportion sero-positive for measles antibody; ^p≥330mIU/ml= proportion with sero-protective titers; ^qFold change in GMT.

8.2 Correlation of post-primary vaccination response with post-booster response in study participants

There was no correlation between pre-vaccination measles antibody titer with post-vaccination titers at time-point 4 in HIV-infected and HEU children; Table 8.4. However, post-booster dose HIV-infected children and not HEU children showed a correlation between pre-booster and post-booster antibody titers; Table 8.4. There was no correlation between CD4+ percent and measles antibody titer in ART/12m ($r=0.04$; $p=0.77$), ART/24m ($r=0.03$; $p=0.76$) and CD4+<25% ($r=0.13$; $p=0.76$) children; Table 8.5.

Table 8.4: correlation of pre- and post vaccination antibody titers for measles vaccine

Group (N)	Time-point 2 vs. Time-point 4		Group (N)	Time-point 4 vs. Time-point 5	
	r^* (95% CI)	p-value		r (95% CI)	p-value
HIV-unexposed (104)	-0.27 (-0.45 to -0.08)	0.005	HIV-unexposed (99)	0.43 (0.25 to 0.59)	<0.001
HEU (111)	-0.06 (-0.25 to 0.13)	0.54	HEU (101)	0.13 (-0.07 to 0.32)	0.19
ART-Def (56)	-0.005 (-0.28 to 0.27)	0.97	ART-Def (45)	0.84 (0.71 to 0.91)	<0.001
ART/12m (71)	0.10 (-0.14 to 0.34)	0.39	ART/12m (66)	0.61 (0.43 to 0.75)	<0.001
ART/24m (78)	-0.29 (-0.48 to -0.06)	0.01	ART/24m (72)	0.62 (0.45 to 0.75)	<0.001
CD4+<25% (5)	0.16 (-0.84 to 0.91)	0.80	CD4+<25% (8)	0.34 (-0.48 to 0.84)	0.41

Footnote: r^* = Spearman's correlation coefficient; HIV-uninfected children born to HIV-uninfected mothers; ^bHEU: HIV-uninfected children born to HIV-infected mothers; ART-Def: HIV-infected children on deferred ART; ART/12m: HIV-infected children who interrupted ART at 12 months of age; ART/24m: HIV-infected children who continued ART till 24 months of age; CD4+<25%: HIV-infected children with CD4+% <25% on immediate ART.

Table 8.5: Correlation of CD4+ percent with response to primary vaccination (Time-point 4)

Group (N)	MV1	
	r^* (95% CI)	p-value
ART-Def (58)	0.37 (0.11 to 0.58)	0.005
ART/12m (72)	0.04 (-0.20 to 0.27)	0.77
ART/24m (78)	0.03 (-0.20 to 0.26)	0.76
CD4+<25% (8)	0.13 (-0.63 to 0.76)	0.76

Footnote: r*= Spearman's' correlation coefficient; ART-Def: HIV-infected children on deferred ART; ART/12m: HIV-infected children who interrupted ART at 12 months of age; ART/24m: HIV-infected children who continued ART till 24 months of age; CD4+<25%: HIV-infected children with CD4+% <25% on immediate ART.

8.3 Factors associated with response to the two doses of measles virus vaccine administered in the study

Pre-vaccination antibody titers (Time-point 2) were associated with a response to primary (unstandardized coefficient [b] =-0.14; SE, 0.06; p=0.02) and booster dose (b=0.35; SE, 0.04; p<0.001) of the measles vaccine; Table 8.6.

Table 8.6: Independent variables and their association to response to primary vaccination at Time-point 4 (post-primary) and Time-point 5 (post-booster) for measles virus vaccine.

Independent variables	MV1			MV2		
	b	SE	P	b	SE	P
Gender	0.13	0.12	0.26	0.04	0.10	0.66
Age	0.02	0.05	0.76	-0.002	0.02	0.93
Race	-0.05	0.22	0.80	-0.02	0.18	0.91
Study center	-0.11	0.14	0.43	-0.18	0.12	0.12
Time since last dose	-0.06	0.06	0.29	-0.01	0.07	0.83
Pre-vaccination antibody	-0.14	0.06	0.02	0.35	0.04	<0.001
R ²			0.03			0.17

Footnote: b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed). MV1: first dose of measles virus vaccine; MV2 booster dose of measles virus vaccine.

8.4 Factors associated with immune response to live-attenuated measles virus vaccine in children stratified by maternal HIV status

Maternal HIV-infection was not associated with response to the first measles virus vaccine dose (b=-0.002; SE, 0.25; p=0.99); Table 8.7. Pre-vaccination antibody titer had a negative association with response to measles virus vaccine response in all children (b=-0.13; SE, 0.06; p=0.02. None of the other factors were associated with response to vaccine in children according to maternal HIV status; Table 8.7.

Table 8.7: Independent variables and their association to response to the first dose of measles virus vaccine by maternal exposure to HIV in their children

Independent variables	MV1		
	b	SE	P
Gender	0.14	0.12	0.25
Age	0.005	0.05	0.92
Birth-weight	0.00002	0.0001	0.89
Race	-0.08	0.23	0.72
Breastfeeding	-0.06	0.22	0.77
Study center	-0.12	0.15	0.43
Time since last dose	-0.05	0.06	0.42
Pre-vaccination titer (Time-point 2)	-0.13	0.06	0.02
HIV status	-0.002	0.25	0.99
R ²			0.03

Footnote: MV1: first dosed of measles virus vaccine. b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed); HIV status (0 unexposed, 1 HIV-exposed). NVP: Neverapine; AZT: Zidovudine.

HIV-unexposed children were less likely to have a robust response to measles virus vaccine (b=-0.48; SE, 0.12; p<0.001) post-booster, as well as children from Stellenbosch (b=-0.23; SE, 0.12; p=0.05). Pre-booster antibody titers were also found to be associated with a more robust response to measles vaccine in HIV-infected children (b=0.35; SE, 0.04; p<0.001); Table 8.8.

Table 8.8: Independent variables and their association to response to booster vaccination at Time-point 5 for measles virus vaccines by maternal exposure to HIV in children

Independent variables	MV2		
	b	SE	P
Gender	0.01	0.09	0.92
Age	0.02	0.02	0.41
Race	0.22	0.18	0.23
Study center	-0.23	0.12	0.05
Time since vaccination	-0.02	0.07	0.77
Pre-booster antibody (Time-point 4)	0.35	0.04	<0.001
HIV status	-0.48	0.12	<0.001
R ²			0.20

Footnote: MV2: Booster dose of measles virus vaccine. b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed); HIV status (0 unexposed, 1 HIV-exposed). NVP: Nevirapine; AZT: Zidovudine.

8.5 Factors associated with immune response to first dose of live-attenuated measles virus vaccine as well as after booster dose in children born to HIV-infected woman

HIV-infection was associated with a reduced response to the first dose of measles vaccine (b=-0.66; SE, 0.14; p<0.001) when comparing children born to HIV-infected mothers; Table 8.9. Exposure to NVP (b=-0.25; SE, 0.18; p=0.16), AZT (b=-0.92; SE, 0.49; p=0.06) or combination therapy (b=0.14; SE, 0.46; p=0.76) for PMTCT was not associated with a response to first measles virus vaccine response; Table 8.9.

Table 8.9: Independent variables and their association to response to primary vaccination at Time-point 4 for measles virus vaccine in HEU and HIV-infected children

Independent variables	MV1		
	b	SE	P
Gender	0.02	0.13	0.89
Age	0.003	0.04	0.95
Birth-weight	-0.0002	0.001	0.18
Race	0.26	0.31	0.39
Breastfeeding	0.02	0.21	0.93
Study center	0.18	0.21	0.93
Time since last dose	-0.16	0.17	0.34
Pre-vaccination titer (Time-point 2)	-0.09	0.07	0.19
NVP	-0.25	0.18	0.16
AZT	-0.92	0.49	0.06
Combined AZT/NVP	0.14	0.46	0.76
HIV status	-0.66	0.14	<0.001
R ²			0.10

Footnote: MV1: first dosed of measles virus vaccine. b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed); HIV status (0 exposed, 1 HIV-infected). NVP: Nevirapine; AZT: Zidovudine.

Post-booster; HIV-infection (b=0.57; SE, 0.13; p<0.001) and pre-booster antibody titer (b=0.43; SE, 0.05; p<0.001) were associated with response to the booster dose of live-attenuated measles virus vaccine in children; Table 8.10.

Table 8.10: Independent variables and their association to response to booster vaccination at Time-point 5 for measles virus vaccine in HEU and HIV-infected children

Independent variables	MV2		
	b	SE	P
Gender	0.06	0.11	0.57
Age	0.01	0.03	0.62
Race	0.04	0.26	0.88
Study center	-0.37	0.13	0.005
Time since last vaccination	0.16	0.08	0.06
Pre-booster antibody (Time-point 4)	0.43	0.05	<0.001
HIV status	0.57	0.13	<0.001
R ²			0.24

Footnote: MV2: Booster dose of measles virus vaccine. b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); HIV status (0 exposed, 1 HIV-infected).

8.6 Factors associated with immune response to the first dose of measles vaccine in HIV-infected children stratified by ART status

Post-primary vaccination CD4+ percent was associated with increased response to live-attenuated measles virus vaccine in ART-Immed children (b=0.03; SE, 0.01; p=0.01); Table 8.11.

Table 8.11: Independent variables and their association to response to the first dose of measles virus vaccine in ART-Def and ART-Immed children

Independent variables	MV1		
	b	SE	P
Gender	0.10	0.17	0.53
Age	-0.01	0.05	0.87
Birth-weight	-0.0002	0.0001	0.17
Race	0.26	0.42	0.54
Breastfeeding	-0.09	0.25	0.71
Study center	-0.32	0.19	0.09
Time since last dose	-0.21	0.21	0.32
Pre-vaccination titer (Time-point 2)	-0.14	0.09	0.13
CD4+ %	0.03	0.01	0.01

ART status	-0.17	0.17	0.34
R²			

Footnote: MV1: first dose of measles vaccine. b: unstandardized coefficient; SE: standard error; P: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); Breastfeeding (0 not breastfed, 1 breastfed); ART status (0 ART-Def, 1 ART-Immed).

Response to booster dose was associated with time since last vaccination (b=0.15; SE, 0.07; p=0.04) and pre-booster antibody titer (b=0.54; SE, 0.05; p<0.001) in ART-Immed children; Table 8.12.

Table 8.12: Independent variables and their association to response to booster vaccination at Time-point 5 for measles virus vaccine in ART-Def and ART-Immed children

All children	MV2		
Independent variables	b	SE	P
Gender	0.02	0.11	0.88
Age	0.04	0.03	0.16
Race	0.13	0.27	0.63
Study center	-0.03	0.14	0.82
Time since last vaccination	0.15	0.07	0.04
Pre-booster antibody (Time-point 4)	0.54	0.05	<0.001
CD4+%	0.005	0.007	0.44
ART status	-0.22	0.12	0.06
R²			0.45

Footnote: MV2: Booster dose of measles virus vaccine. b: unstandardized coefficient; SE: standard error; p: p-value. Independent variables were entered simultaneously into the model and were defined as: Gender (0 Female, 1 male); Race (0 coloured, 1 Black); study center (0 Stellenbosch, 1 Soweto); ART status (0 ART-Def, 1 ART-Immed).

8.7 Concluding comments

These results indicate that persistence of maternal measles-antibody is not an impediment to measles-vaccination by 6-months of age, possibly with the Edmonston-Zagreb vaccine-strain, irrespective of the child's HIV-infection status (Scott et al., 2011). Reasonably good responses have been observed in ART-naïve HIV-infected children when immunized with an Edmonston-Zagreb vaccine-strain as early as 6 months, followed by a booster-dose at 12 months of age (Chandwani et al., 2011). Sustainability of protection and need for further doses of measles

vaccine in HIV-infected children in whom ART has been interrupted and in HEU remains to be explored, as does the issue of sustainability of protection in HIV-infected children who are maintained on ART.

CHAPTER 9: DISCUSSION

9.1 Validation and optimization of Luminex assay

In the first phase of the study, validation and optimization of a Luminex multiplex assay for the simultaneous determination of serum IgG antibodies against diphtheria-toxoid, tetanus-toxoid, *B. pertussis* and hepatitis B epitopes in a single serum sample was undertaken, in order to evaluate IgG levels following routine immunization. Studies have demonstrated the ability of ELISAs to accurately determine vaccine responses (Fu et al., 2010), but with advances in the vaccinology fields of new vaccines with increased number of combined epitopes, the use of ELISA has become increasingly unsuitable (Pickering et al., 2002a).

The major advantage of this multiplex assay, particularly when using paediatric samples, was the need for small sample volumes (5µl) compared to ELISAs which ranged from 10µl to 100µl of sample. Multiplexing allows for considerable savings in terms of materials and labour compared to single plex ELISA. In addition the reduced assaying time with the multiplex assay, that required 2.5 hours to test 38 samples for all five vaccine antigens simultaneously; instead of the 10.5 hours required by all the ELISAs for the five study epitopes. Furthermore, the Luminex assays are approximately 50-100 folds more sensitive than conventional ELISA. Studies have been published on the multiplexing of epitopes against TT, DT, PT and FHA as either duplex, tetraplex and pentaplexes (van Gageldonk et al., 2008) , but the current study describes for the first time the determination of antibody to HepB with a multiplex system. The HepB antigen has been a problem with multiplexing assays because of its highly hydrophobic nature, and requires special attention when coupling it to the microbead. Correlation of the HepB antigen between ELISA and Luminex was $R^2=0.72$, reason being that antibody levels were higher with ELISA than with Luminex multiplex assay. Despite the low R^2 we still found over 90% agreement between the two assays as seen in results in Table 5.2 and Figure 5.3. Correlation quantifies the degree to which two variables are related. But a high correlation does not automatically imply that there is good agreement between the two methods (Giavarina, 2015). The correlation coefficient and regression technique are sometimes inadequate and can be misleading when assessing agreement, because they evaluate only the linear association of two sets of observations (Giavarina, 2015). The R^2 , named the coefficient of determination, only tells us the proportion of variance that the two variables have in common. This study also showed that a single reference serum can be used to test all the test antigens. To ensure antigen availability for future testing

commercially available antigens with high purity were utilized. The optimal bead conjugation concentration and procedure was found to be similar to those reported for elsewhere (van Gageldonk et al., 2008). MFIs generated from all five conjugated microsphere bead regions showed linearity over a seven step 4-fold reference serum dilution range, the consequence of this increase in range was the resultant increase in accuracy, a reduction in the number of sample dilutions required and the ability to detect much lower antibody levels (Lal et al., 2005). MFI comparisons between monoplex and pentaplex assays allowed for determination of interference between the microsphere bead regions. This was important as there was always a risk of cross-reactivity within a multiplexed assay than with a monoplex (Lal et al., 2005). For this study multiplexing did not seem to affect the specificity and sensitivity of the assay when compared to monoplexing. Even though heterologous and homologous inhibition was slightly lower than that reported previously (>95%) (Lal et al., 2005, van Gageldonk et al., 2008) it was still above 90% for all epitopes tested.

The multiplex assay was found to be reproducible, with minimal variability inter- and intra assay. Good correlation was obtained when comparing IgG concentrations generated from the multiplex and ELISA and this was true for all epitopes. The correlation was further tested using the sero-protective cut-offs for all epitopes, which showed an 8% discrepancy for TT, DT and HepB vaccine epitopes. The limitation of this multiplex method with the chosen epitopes is the short half-life of the coupling reaction, which was found to be a maximum of two weeks post coupling. The short half-life was also observed in a study by van Gageldonk and colleagues, especially with coupling of *B. pertussis* epitopes (van Gageldonk et al., 2008). Therefore it cannot be stored for use at a later date as are the commercially available assay that can be bought well in advance of use.

9.2 Effects of HIV and treatment of HIV on response to childhood vaccines

In the second phase of the study, the effects of HIV exposure and timing of ART initiation in HIV-infected children on the kinetics of antibodies to five EPI vaccines pre- and post vaccination as well as response to booster dose vaccination for DTwP and measles. This is the first and most-comprehensive study evaluating immune responses to routine vaccines in HIV-infected children receiving early ART, irrespective of CD4+ count/percent according to current WHO

recommendations (WHO, 2010a). As the introduction of ART to treat HIV-infected children is increasing, especially in developing countries, there is a longer life expectancy and reduction in the number of individuals progressing to HIV disease (Tejiokem et al., 2007). Studies evaluating the immunogenicity of booster DTP vaccination as well as the persistence of antibodies to the HepB vaccine in HIV-infected children who initiated early ART in accordance to WHO guidelines are relatively few, and the proportion of children in these studies are limited (WHO, 2010a).

9.2.1 Immune response to DTwP and HepB vaccines in HEU children

Data in the current study on the relative immune responses to DTwP/HBV in HEU compared with HIV-unexposed children differed from other reports. *In-utero* HIV-exposure was not consistently associated with lower GMCs prior to immunization in HEU compared to HIV-unexposed children as proposed by others (Montoya et al., 2007, Miyamoto et al., 2010, Jones et al., 2011, Abramczuk et al., 2011, Scott et al., 2007). Although lower GMCs to TT in HEU children pre-vaccination were identified, the converse was true for antibody GMCs to DT, HepB and FHA. This could be due to regional differences in maternal antibody concentrations to different vaccine-epitopes and variations in factors which may influence transplacental antibody transfer, including differences in maternal HIV-viral load, total IgG antibody concentrations and placental function sufficiency in different populations (Jones et al., 2011, Abramczuk et al., 2011, Montoya et al., 2007, Miyamoto et al., 2010, Scott et al., 2007). These factors may influence HEU children' susceptibility to vaccine-preventable diseases during early childhood and prior to vaccination, and close monitoring of the HEU group of children needs to take place.

Comparing HEU and HIV-unexposed children, HEU had a trend toward lower pre-vaccination PT GMCs but higher post-vaccination levels, and conversely had higher pre-vaccination HepB antibody GMCs, but lower post-vaccination levels to HepB. This corroborates that higher epitope-specific antibody levels pre-vaccination may interfere with the immunogenicity to homotypic epitopes. The higher pre-immunization HepB antibody GMCs in HEU in our study has been attributed to higher hepatitis B virus infection prevalence in HIV-infected than HIV-uninfected women (Reikie et al., 2013). However, we also observed higher antibody levels to HepB and other vaccine-epitope GMCs in HEU compared to both ART-Immed and ART-Def

children. Our study did not determine HIV-infection status at birth and since only 10% of HIV-exposed children were breast-fed, it is likely that the majority of HIV-infection in children of our cohort occurred either *in utero* or the immediate peri-partum period. Therefore, differences in pre-vaccination GMCs to most epitopes between HEU and either ART-Immed or ART-Def children, suggests an association between factors related to acquiring maternal-derived vaccine-related antibodies and the risk of the child being HIV-infected. Placental abnormalities can affect transplacental antibody transfer. For example, placentas from HIV-infected women from the Tygerberg site showed a higher percentage of villitis and infarcts than from HIV-uninfected controls, especially in women with CD4+ counts below 200 cells/ μ l (Vermaak et al., 2012). In-utero HEU children have increased incidence of infectious diseases, hospitalization and death compared to HIV-unexposed (Koyanagi et al., 2011, Kuhn et al., 2005, McNally et al., 2007, Slogrove et al., 2013, Kourtis et al., 2013, Slogrove et al., 2016). The mechanisms accounting for this increased vulnerability are not clear but a number of factors have been suggested including socioeconomic factors, acquisition of different pathogens from the HIV-infected mothers and possible immunological impairment either due to ART or HIV-exposure during development (Abramczuk et al., 2011, Dauby et al., 2012).

Another notable finding from this study, is that despite the heightened susceptibility to morbidity and mortality in HEU compared to HIV-unexposed children during early infancy (Simani et al., 2014), the memory responses to vaccine epitopes were generally similar if not occasionally more robust (for DT, PT and FHA) compared to HIV-unexposed children. In this study a similar proportion of HEU and HIV-unexposed children maintained sero-protective levels to TT, DT and HepB and had comparable PT and FHA GMCs 12-months post-primary vaccination series. These results are similar to those observed in another South African study, where HEU children had similar antibody levels to HIV-unexposed at 12-months to TT, DT and HepB (Reikie et al., 2013). Anamnestic responses were also similar between HEU and HIV-unexposed, although HEU had higher FHA and PT GMC compared to HIV-unexposed children, the clinical significance of which is unclear in the absence of correlates of protection against pertussis. These results suggest that HEU mount good memory responses to booster-dose of DTP.

9.2.2 Immune response to DTwP and HepB vaccines in HIV-infected children on ART or not on ART

The findings show that early ART in HIV-infected children is associated with similar quantitative and qualitative immune responses to vaccines as in HIV-unexposed children. Although the immune response in children among whom ART was deferred was generally similar to the ART-Immed group, the ART-Def children had lower proportion with HepB antibody ≥ 100 mIU/ml.

Differences observed between ART-Immed and ART-Def children may have been masked by survivor bias in the ART-Def group especially in the post-vaccination comparison. It is plausible that vaccine immune responses were even poorer among ART-Def children who died compared to surviving children. A recent review on the association of ART on vaccine immune responses in HIV-infected children, included studies in older children re-vaccinated between 28-weeks and 5.3-years after ART initiation (Sutcliffe and Moss, 2010). We report a higher proportion of children with sero-protective antibody concentrations than in previous studies, undertaken prior to early ART initiation in children becoming policy, where the proportion of HIV-infected children without severe immunosuppression developing sero-protective antibody levels varied between 37% and 86% with GMCs generally much lower than in HIV- uninfected children (Madhi et al., 2002, Peters and Sood, 1997, Rustein et al., 1996).

Timing of when ART was initiated during early infancy and in relation to receipt of the primary series of childhood vaccines had an on-going effect on the immune responses to booster dose of some of those vaccines; and persistence of protection against disease in children. Notably, even though all the children in the ART-Def group in our study had been initiated on ART during their first year of life and had similar CD4+ counts compared to the ART-Immed group at the time of the booster dose of vaccines, in general they had a more attenuated booster response to most vaccine epitopes compared to HIV-unexposed children (for TT, DT and PT) as well as compared to ART-Immed children (for TT and DT). In contrast, memory responses and persistence of antibody responses were generally similar, or differences less marked than observed for the ART-Def group, between HIV-infected children who had been initiated on ART prior to receiving their primary series of vaccines (ART-Immed), compared to HIV-unexposed children. Furthermore, interruption of ART at 12-months of age in the ART-Immed group did not negatively impact on their booster responses or persistence of antibodies to the evaluated vaccine epitopes. Also, our study noted greater reduction in the percentage of ART-Def children with HepB antibody titres

$\geq 10\text{IU/ml}$ at two years of age compared to it being 100% in other groups, however, it remains unclear whether this is indicative for booster doses of HepB vaccine in these children as underlying memory responses might persist even in the absence of detectable HepB antibody (Mahoney and Kane, 1999).

Studies evaluating the immunogenicity of booster DTP vaccination as well as the persistence of antibodies to the HepB vaccine in HIV-infected children who initiated early ART in accordance to WHO guidelines are relatively few, and the proportion of children in these studies have been limited (WHO, 2010a). The duration of sero-protection was shorter in ART-Def than in ART-Immed and HIV-uninfected children, as assessed by quantitative antibody response to TT, DT, HepB and PT. In general in previous studies, a high percentage of HIV-infected children on ART ($\pm 90\%$) were able to maintain serologic response to TT for up to one year post-vaccination (Zaccarelli-Filho et al., 2007, Rigaud et al., 2008, Melvin and Mohan, 2003). A recent meta-analysis found that approximately 75% of HIV-infected children would maintain an immune response of $\geq 0.1\text{IU/ml}$ to tetanus-toxoid vaccine 2 to 5-years post-vaccination (Kerneis et al., 2014). It has also been shown that children after ART initiation (similar to our ART-Def group), have a reduced capacity to mount sero-protective responses to toxoid antigens, especially during the first year of ART (Rigaud et al., 2008). In a small study pre- and post-booster responses to tetanus in complete versus incomplete ART responders (by suppression of HIV viral load) resulted in modest humoral and cellular responses in both groups, with short antibody persistence and antibody concentrations decaying rapidly within one year (Rosenblatt et al., 2005). Our results show that, despite reconstitution of the CD4⁺ T-lymphocyte pool over the first year of ART in ART-Def group, additional booster doses might be necessary to maintain sero-protective immunity to TT, as evident by only 72% of the ART-Def group maintaining titers $\geq 0.1\text{IU/ml}$ at 24 months of age compared to $>98\%$ in the other three groups.

Diphtheria toxoid is a weaker antigen than TT, and its immunity decays more rapidly in healthy children (Menson et al., 2012). In our study, in addition to an inferior anamnestic response to DT in the ART-Def group, these children were less likely to maintain sero-protective titers of $\geq 0.1\text{IU/ml}$ at 24 months of age (80%) than the other groups ($>98\%$). Nevertheless, these responses to DT even in the ART-Def group was higher than previously reported in children on

ART, where only 40-65% of children with viral suppression achieved sero-protective antibody levels (Zaccarelli-Filho et al., 2007).

Interpretation of immune responses to pertussis vaccination is challenging, as correlates of protection have not been established (Taranger et al., 2000, Mallet et al., 2004, Pichichero et al., 2001, Cherry et al., 1998, Storsaeter et al., 1998, Plotkin, 2010). Nonetheless, immune responses to PT and FHA were high in our study-groups, with ≥ 4 -fold increase from pre-booster to post-booster vaccination observed for each pertussis antigen in $>87\%$ of all children. The high sero-conversion rates seen in our HIV-infected children were similar to previous studies on booster responses to DTP in relatively healthy HIV-uninfected children (Lin et al., 2007). Although our HIV-infected children had a significant increase in antibody concentration to PT and FHA post-booster, the GMCs were generally lower than in HIV-uninfected children especially for PT. A cohort study of children on stable ART from the USA who received acellular pertussis vaccine reported low antibody responses to a booster-dose and shorter duration of protective immunity when compared to HIV-uninfected children, despite a minimum of four doses of acellular pertussis vaccine during their primary series (Abzug et al., 2007).

In our study persistence of antibodies to HepB surface antigen 12 months post-primary vaccination were high in all HIV-infected groups. However, GMC in HIV-infected children were substantially decreased when compared to HIV-unexposed children. Sero-protective antibody levels at 25-months of age had decreased from 100% to 72% in ART-Def children for concentrations $\geq 10\text{mIU/ml}$, with antibody levels $\geq 100\text{mIU/ml}$ only being found in 50% of these children. In contrast the ART-Immed group had similar antibody levels when compared to HIV-uninfected children, with 92% of children still having sero-protective antibody levels $\geq 100\text{mIU/ml}$ at 25-months of age. Previous studies on the persistence of antibodies following HepB vaccination in HIV-infected children and adults the follow-up times range from 12 to 115-months post-primary vaccination (Cruciani et al., 2009, Abzug et al., 2009, Fernandes et al., 2008, Lao-araya et al., 2011, Rey et al., 2000, Scolfaro et al., 1996, Siriakson et al., 2006, Cooper et al., 2008, Kaech et al., 2012, Pessoa et al., 2010, Powis et al., 2012). As expected, the percentage of participants with sero-protective antibody levels decrease over time after three doses of HepB vaccine, 71% of primary responders maintained sero-protective antibody levels

(10mIU/ml) at one year of age (Cruciani et al., 2009), 33%–61% at year 2 (Cruciani et al., 2009, Cooper et al., 2008), and 40% at year 5 (Cooper et al., 2008). Due to the high prevalence of chronic hepatitis B in HIV-infected individuals (Phung et al., 2014), it is suggested that HIV-infected individuals which show signs of immunosuppression should be tested annually and re-vaccinated if antibody to HepB falls below the sero-protective threshold of 10 mIU/ml (Sokal et al., 2013, Spearman et al., 2013). Furthermore studies have shown that the standard immunization schedule was sub-optimal in immunosuppressed children and repeated or increased doses of vaccine after immune recovery should be considered (Lao-arya et al., 2011, Sokal et al., 2013, Wilkins et al., 2013).

9.2.3 Immune response to measles virus vaccine in HIV-infected and HEU children

High susceptibility to measles infection as early as seven-weeks of age in the majority of HIV-infected children and in >83% of all groups by 20-weeks of age was found in this study. Also, interruption of ART in HIV-infected children (ART/12m) was associated with them being less likely to have sero-protective levels 28-weeks following the primary-dose of measles-vaccine and less likely to maintain sero-protective titers 9.5-months after the booster-dose. Furthermore, there was also waning of immunity in HEU children 9.5-months post-booster indicated by a lower proportion with sero-protective titers and lower GMTs compared to HIV-unexposed. This despite, the proportion of HEU with sero-protective titers being similar compared to HIV-unexposed children following the initial and immediately following the booster-dose.

This high susceptibility to measles in children under six-months is corroborated by other African studies in HIV-infected (85-91%) and HEU children (Lepage et al., 1992, Scott et al., 2007), and more recently among healthy children in developed countries (Machaira and Papaevangelou, 2012). In developed countries, the increased susceptibility to measles infection among young children has been attributed to decrease in passive-immunity acquired from transplacental maternal measles-antibody transfer, because of lower measles antibody levels in pregnant women who acquired immunity from vaccination rather than by natural infection (Leuridan and Van Damme, 2007). Additional factors which may contribute to the increased immunological-susceptibility to measles infection in children born to HIV-infected mothers compared to HIV-unexposed children, include lower measles antibody titers and impaired trans-placental transfer

of antibody in HIV-infected mothers (Kruczek, 2010). Also, there may be greater decay of antibody in HIV-infected children (de Moraes-Pinto et al., 1996). The observation that GMTs and proportion of children with sero-protective titers were similar between HIV-unexposed and HEU children suggests that factors other than solely maternal HIV-positivity contributed to increased susceptibility to measles at an earlier age in HIV-infected children. Possible reasons include that mothers' of peri-partum HIV-infected children had higher HIV viral-load, which has been associated with increased perinatal HIV transmission and inversely associated with efficiency of trans-placental transfer of measles antibody (Farquhar et al., 2005, Thea et al., 1997, Garcia et al., 1999).

The WHO has recommended that in settings of high HIV-prevalence, children should receive two doses of live-attenuated measles virus vaccine at 6- and 9-months of age (WHO, 2009b). This recommendation has, however, not been widely implemented: for example in South Africa the initial dose is still scheduled for 9-months of age. Reasons for this include the inherent low immunogenicity of measles vaccine in younger children, coupled with interference in the immune response by prevailing maternal derived measles antibody (John et al., 2004, Helfand et al., 2008, Gans et al., 2001). Based on our study, the low prevalence of maternal-derived antibody in young children (<30% at 20 weeks of age) supports immunization at 6-months of age. A recent report from Guinea-Bissau indicated that vaccination as early as 4.5-months of age using the Edmonston-Zagreb measles-vaccine strain induced sero-protective antibody levels in 77% of children, as well as being associated with 94% efficacy against measles infection (Martins et al., 2008).

A drawback that was found was that immune response following the first dose of live-attenuated measles virus vaccine was only evaluated eight months after vaccination, as earlier evaluation had not been planned *a priori* in the parent-study. Consequently, although we were unable to undertake a direct comparison of immune responses in the overall ART-Immed compared to other groups, ART/24m may be representative of the initial ART-Immed cohort at least until 24 months of age. Persistence of antibody levels up until prior to the booster-dose was similar in HIV-unexposed compared to ART/24m, as well as the surviving children in ART-Def. This indicated that the timing of ART did not affect immune responses to live-attenuated measles virus vaccine in those HIV-infected children with CD4+>25% at Time-point 1. However, a

previous reported showed a higher early-childhood mortality rate in ART-Def compared to ART-Immed children (Violari et al., 2008). Additionally, 100% of CD4+<25% children also had persistence of sero-protective titers eight months post-primary dose. The good immune responses in HIV-infected children on ART corroborate reports that initiation of ART in the first year of life allows the normal development of T- and B-lymphocyte immunity (Palma et al., 2008, Romiti et al., 2001, Pensieroso et al., 2009).

Although the proportion of ART/12m with sero-protective titers two-weeks following the booster dose of vaccine was similar (>90%) to the other groups, their GMTs were lower compared to other groups immediately post-booster dose. Furthermore, greater waning of immunity was observed in ART/12m following the initial and after the booster-dose, despite them being carefully monitored for clinical and immunological disease progression following ART interruption at 12-months of age, resulting in ART being re-initiated in 77% of them at the time of the booster-dose. CD4+ cell counts and percentage were however lower in ART/12m compared to ART-Def and ART/24m immediately following the booster-dose. This indicated that interruption of ART in ART/12m resulted in waning of immunity, including possible decline in anamnestic responses to measles. This may be explained by the deterioration of antigen specific pools of memory B-lymphocytes in the absence of ART (Pensieroso et al., 2009). The proportion of ART/12m who had sero-protective titers at approximately two-years of age was, however, higher compared to ART-naïve HIV-infected children who had been vaccinated at 6- and 9-months of age and assessed at 20-months of age (41%) as reported (Fowlkes et al., 2011).

In exploring the effect of HIV-exposure on persistence of measles antibody following vaccination, we observed that GMTs were higher in HEU compared to HIV-unexposed children prior to the booster-dose. Lower pre-vaccination antibody levels in HEU children have been previously reported to be associated with more robust immune responses to some vaccines during early-childhood.(Jones et al., 2011) This was, however, unlikely to be the reason for the higher GMTs in HEU in our study as pre-vaccination measles antibody levels were similar to that of HIV-unexposed children. The clinical relevance of the higher GMTs in HEU is unclear as the proportion of children with sero-protective titers was similar between the two groups. The higher GMTs in HEU children prior to the booster-dose is also difficult to explain in the context of lower GMTs being observed two-weeks thereafter in this group compared to pre-booster levels,

as well as relative to HIV-unexposed children post-booster. Lower GMTs persisted in HEU compared to HIV-unexposed until 9.5-months post-booster, at which time HEU were also less likely to be sero-positive or have sero-protective titers. The underlying immune aberration which may cause waning of immunity in HEU, as well as the need for further booster doses of vaccine in these children remain to be explored.

9.3 Limitations of the study

This study had some limitations, which included only having a convenience sample of HIV-infected children with CD4+ T-lymphocyte <25% at screening (i.e. CD4+<25%). Data from CHER showed that by 7-weeks of age, 20% of HIV-infected children already have severe CD4+ T-lymphocyte depletion (Violari et al., 2008). Nevertheless, the observations in this group were similar to those of HIV-infected children on ART (i.e. ART/24m), suggesting that CD4+ percentage categorization at enrolment did not have an effect of immune responses to all vaccine epitopes in children who had been initiated on ART upon confirmation of being HIV-infected. In addition, we were unable to distinguish *in utero* from intra-partum infection and placentas were not collected in our study. Also, despite having used whole cell pertussis vaccine, we elected to measure antibody to pertussis toxoid and FHA rather than using the whole-cell pertussis ELISA assay, as this was more practical for inclusion in the multiplex Luminex assay. The pertussis toxoid and FHA antibody responses, although providing a relative measure of immunogenicity of the whole cell pertussis vaccine between groups in our study, is not recommended as a measure of immunogenicity of whole cell pertussis vaccine. Furthermore, our study involved measuring measles antibody by ELISA as a surrogate of protection, which have been shown to miss low antibody level responses (Ratnam et al., 1995), rather than the plaque reduction neutralization assay which correlates more strongly with protection (Cohen, 2008). We may therefore have over-estimated the protection against measles infection, particularly among HIV-infected children in whom poorer IgG avidity may affect neutralizing activity which has, however, only been observed in the absence of ART (Nair et al., 2009). The problem that arises with multiple comparisons when using the standard alpha (α) of 0.05 to determine statistical significance is that hundreds of p-values are presented and just by chance 5% will be statistically significant (Sinclair et al., 2013). This may have accounted for the unexpected associations presented for response to vaccines especially study center.

9.4 Concluding comments

The results show that HIV-infected children on early ART require primary and booster doses at the same schedule and timing as HIV-uninfected children for tetanus, hepatitis B, pertussis and live-attenuated measles virus vaccines. This implies that early ART allowed for the development of memory B- and memory T-lymphocyte compartments thus allowing for relatively normal responses to occur in HIV-infected children (Cotton et al., 2013, Cotugno et al., 2012). On the other hand children treated later than one year after diagnosis, show similar responses to vaccines as those seen in ART naïve persons (Sutcliffe and Moss, 2010). This suggests that the development and reconstitution of the B-lymphocyte compartments is altered in the deferred treatment group (Cotugno et al., 2012). ART-Def children will require earlier booster dose schedules of diphtheria, tetanus and pertussis vaccine (depends on the countries formulation) than the specified booster dose schedule at 5-years of age. ART has been shown to partially restore memory T-lymphocytes for vaccine antigens to which children had been primed with prior to initiating treatment (Pensiero et al., 2009).

In conclusion, the first phase has shown that the multiplex assay described here was found to be rapid, specific, sensitive and reproducible. This assay has the potential to become a viable alternative to ELISAs for all the stated epitopes and allows the simplification of surveillance and vaccine efficacy studies. The second phase shows that HEU children were not at increased susceptibility to most of the studied vaccine-preventable diseases by 6-weeks of age, suggesting similar acquisition of antibody from their mothers in our study. Furthermore, the immune responses in HEU were similar to HIV-unexposed children post-vaccination. Our study indicates that persistence of maternal measles-antibody no longer appears to be an impediment to measles-vaccination by 6-months of age, possibly with the Edmonston-Zagreb vaccine-strain, irrespective of the child's HIV-infection status (Scott et al., 2011). Although HIV-infected children had lower GMCs post-vaccination, including to TT and HepB irrespective of timing of ART initiation, the proportion with sero-protective antibody concentrations post-vaccination were similar and timing of ART initiation generally did not affect immune responses between HIV-infected groups. The results indicate that vaccination of HEU and HIV-infected children initiated on early-ART confers with DTwP/HBV would provide similar immunity to the targeted vaccine-preventable disease compared with HIV-unexposed children. Post-booster vaccination, duration of sero-protection was shorter in HIV-infected children, especially those on deferred

ART treatment, for all vaccine epitopes. With the current guidelines a large proportion of these children would have lost sero-protective antibody levels before the proposed booster dose schedules for the study vaccines. Therefore, continued monitoring of tetanus, diphtheria, pertussis, hepatitis B and measles specific antibody concentrations can guide the need for earlier boosting or re-vaccination in HIV-exposed children (infected or uninfected).

CHAPTER 10: REFERENCES

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CHAPTER 11: APPENDICES

Appendix 1: [PDF] Simani, O.E., Adrian, P.V., Violari, A., Kuwanda, L., Ottembe, K., Nunes, M., Cotton, M.F. & Madhi, S.A. Effect of in-utero HIV exposure and antiretroviral treatment strategies on measles susceptibility and immunogenicity of measles vaccine. *AIDS* 2013; 27: 1583-1591.

Appendix 2: [PDF] Simani, O.E., Izu, A., Violari, A., Cotton, M.F., van Niekerk, N., Adrian, P.V. & Madhi, S.A. Effect of HIV-1 exposure and antiretroviral treatment strategies in HIV-infected children on immunogenicity of vaccines during infancy. *AIDS* 2014; 28(4): 531-541.

Appendix 3: Other trials referred to in the study

Appendix 4: Detailed standard of operations procedures

Appendix 5: Certificate of approval granted by the University of Witwatersrand Human Research Ethics Committee on the 26th September 2008 (HREC number: M080966)

Appendix 1:

Effect of HIV-1 exposure and antiretroviral treatment strategies in HIV-infected children on immunogenicity of vaccines during infancy

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Introduction: We studied the effect of maternal HIV-exposure and timing of antiretroviral treatment (ART) in HIV-infected infants on antibody responses to combined diphtheria-toxoid–tetanus-toxoid–whole cell pertussis and *Haemophilus influenzae* type b conjugate vaccine (HibCV) and monovalent hepatitis B vaccine (HBV).

Methods: HIV-uninfected infants born to HIV-infected (HEU) or HIV-uninfected (HUU) mothers were enrolled in parallel with HIV-infected children with CD4⁺ ≥25%, who were randomized to initiate ART immediately upon confirmation of HIV-infection (ART-Immed) or when clinically and/or immunologically indicated (ART-Def). Infants received three doses of diphtheria-toxoid–tetanus-toxoid –wP-HibC/HBV at 7.3, 11.4 and 15.4 weeks of age. Antibody to diphtheria-toxoid, tetanus-toxoid, pertussis toxin, filamentous hemagglutinin (FHA) and hepatitis B surface antigen (HBsAg) were measured by Luminex multiplex-immunoassay and polyribosyl-ribitol phosphate (PRP) antibodies by standard ELISA and bactericidal assay.

Results: Prevaccination antibody geometric mean concentrations (GMCs) were higher in HUU than HEU infants for tetanus-toxoid, but lower for HBsAg, diphtheria-toxoid and FHA. Postvaccination GMCs and proportion with seroprotective antibody levels or sero-conversion rates were similar between HUU and HEU infants for all vaccines. Postvaccination GMCs were higher in HUU for tetanus-toxoid, diphtheria-toxoid, HBsAg and FHA than ART-Immed infants; and for tetanus-toxoid, HBsAg and pertussis-toxoid than ART-Def infants. Nevertheless, there was no difference in proportion of HUU and HIV-infected infants who developed sero-protective vaccine-specific antibody levels postvaccination. The timing of ART initiation generally did not affect immune responses to vaccines between HIV-infected groups.

Conclusion: Vaccination with DTwP-HibCV/HBV of HEU and HIV-infected infants initiated on early-ART confers similar immunity compared with HUU children.

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Keywords: diphtheria vaccine, *Haemophilus influenzae* type b conjugate vaccine, hepatitis B vaccine, HIV-exposed uninfected, HIV-infected, pertussis vaccine, tetanus vaccine

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Introduction

HIV-infection impairs humoral and cell-mediated immune functions early in infancy [1,2]. Immune dysfunction in HIV-infected individuals include loss of T-lymphocytic proliferative responses and hyper-activation of T-lymphocytes leading to accelerated apoptosis [2–4], decline in B-lymphocyte number and function [5,6], and impaired priming for immunological memory and/or loss of anamnestic responses [7,8]. Early initiation of combination antiretroviral treatment (ART) in HIV-infected infants prior to immunological or clinical deterioration is associated with improved survival [9]. Control of HIV replication limits and reverses immunosuppression in HIV-infected individuals [1,10,11], improving immune responses to vaccines [12–14].

Due to effective ART regimens for preventing mother-to-child HIV-transmission, the majority (>97%) of infants born to HIV-infected women are HIV-uninfected (HEU) [15–18]. HEU children may, however, have increased risk of morbidity and mortality than infants born to HIV-uninfected mothers (i.e. HUU) [19,20]. This may be due to perturbations of the immune systems of HEU infants due to in-utero HIV virion particle exposure and/or ART-exposure [21–26], affecting lymphocyte differentiation and function [21–24,27,28]. There are conflicting data on vaccine-related immune responses of HEU compared with HUU children. These include variable responses to BCG vaccine [29,30], tetanus toxoid, pertussis, *Haemophilus influenzae* type b-conjugate (HibCV), hepatitis B vaccine (HBV) and pneumococcal conjugate vaccines (PCV) [13,31,32]. Differences in immune responses to some vaccines in HEU compared with HUU children is attributed to lower maternal-derived vaccine-epitope-specific antibody in HEU children, possibly reducing interference in immune responses to the homotypic vaccines [31,32].

The aim of this study was to evaluate the effect of HIV exposure in HIV-uninfected and timing of ART initiation in HIV-infected children on the kinetics of antibodies to epitopes of diphtheria-toxoid, tetanus-toxoid, whole cell pertussis-HibCV (i.e. DTwP-HibCV) and monovalent HBV prior to and 1-month following the primary three-dose series of vaccines. The HUU group was the referent group for comparison of immune responses in relation to other groups.

Methods

Study population

The study was conducted on archived serum samples from a study investigating the effects of HIV exposure and timing of ART initiation in HIV-infected infants on immune responses to seven-valent PCV [13,14]. Briefly,

four groups of infants aged 6–12 weeks were enrolled at two South African Centers (Chris Hani-Baragwanath Hospital in Soweto, Johannesburg and Tygerberg Children's Hospital, Stellenbosch) from April 2005 to June 2006 (See on-line supplementary material for Study participant selection criteria, <http://links.lww.com/QAD/A443>). HUU infants born to HIV-uninfected mothers (confirmed by a nonreactive HIV ELISA at enrolment); and HEU infants in whom HIV-PCR was nonreactive at enrolment. HIV-infected infants participating in the 'Children with HIV Early Antiretroviral (CHER)' study were co-enrolled [9]. The latter were aged less than 12 weeks with CD4% $\geq 25\%$ and randomized to deferred ART until immunological and/or clinically indicated (ART-Def) or immediately started on ART upon confirmation of their HIV-infection status (ART-Immed). Additionally, a convenience sample of HIV-infected infants from CHER with CD4% $< 25\%$ (ART-CD4<25%) was recruited. The first-line ART regimen in CHER included zidovudine, lamivudine and lopinavir-ritonavir. Following an interim analysis of the CHER study in which HIV-disease progression to AIDS or death was greater in ART-Def compared with ART-Immed infants, the Data Safety and Monitoring Board recommended halting recruitment to the ART-Def group and that all infants in this group be evaluated whether they required ART to be initiated [9].

Participants were scheduled to receive three doses of combined DTwP-HibCV (CombAct-Hib, Sanofi Pasteur, France) and monovalent recombinant HBV (HerberBiotec S.A., Cuba) at approximately 6, 10 and 14 weeks of age with a 4–6 week interval between doses. Both, DTwP-HibCV and HBV were administered at separate sites in the left arm, PCV (Prenar-7; Wyeth Vaccines and Pediatrics, Philadelphia, Pennsylvania, USA) in the right arm and trivalent oral polio vaccine (POLIORAL-trivalent, Novartis Vaccines and Diagnostics, Srl, Italy) orally.

Sample collection

Clotted blood samples were collected immediately prior to the first DTwP-Hib/HSV dose and 1-month following the third dose. Blood samples were stored in a cooler box and transported within 4 h to the site laboratory. This included the Respiratory and Meningeal Pathogens Research Unit (RMPRU), which is based at Chris Hani-Baragwanath Hospital for the Johannesburg site. The blood samples were then centrifuged and aliquots of serum were stored at -70°C . Samples from Stellenbosch were subsequently couriered on dry ice to RMPRU, where it was also archived at -70°C until testing was done at RMPRU. Antibody to diphtheria-toxoid, tetanus-toxoid, pertussis toxoid, filamentous hemagglutinin (FHA) and hepatitis B surface antigen (HBsAg) were measured by an in-house Luminex-multiplex immunoassay and polyribosyl-ribitol phosphate (PRP for HibCV) antibodies by standard ELISA

and bactericidal assay. (See on-line as supplementary material for Luminex assay methods and validation results, <http://links.lww.com/QAD/A443>).

Data were acquired in real-time using Bio-plex Manager 5.0 (Bio-Rad Laboratories, Hemel Hempstead, UK) software. Values for unknown test sera were generated from a standard curve of median fluorescent intensity (MFI) against expected IgG concentration for the in-house reference sera and converted to either IU/ml or mIU/ml. The limits of quantification (LOQ) were 0.001 IU/ml for anti-diphtheria-toxoid and anti-tetanus-toxoid, 20 IU/ml for anti-pertussis toxoid, 2 IU/ml for anti-FHA and 10 mIU/ml for anti-HBsAg, these values were regarded as sero-positive thresholds. Samples with a titer below the LOQ were assigned a titer of half the LOQ when calculating geometric mean concentrations (GMC). The specificity of the assay was more than 92% for all tested antibodies and the coefficient of variation for intra-assay and inter-assay ranged between 5 and 22% for individual assays.

Polyribosyl-ribitol phosphate IgG antibodies were measured by standardized in-house enzyme immunoassay [33], with samples below detection limit assigned a concentration half the detection limit (i.e. 0.06 µg/ml) when calculating GMCs. Polyribosyl-ribitol phosphate conjugated to human serum albumin (HbO-HA) was purchased from the National Institute for Biological Standards and Control (NIBSC, London, UK). Antibody activity was measured by bactericidal assay as described previously [34,35]. Titers were given as the reciprocal serum dilution for 50% killing activity. The lower limit of detection for the assay was a titer of 8, with samples below the threshold assigned an arbitrary value of 4.

Serological correlates of protection

The antibody thresholds used as serological correlates for protection were defined as antibody concentrations greater or equal to 0.01 and 0.1 IU/ml for diphtheria-toxoid, 0.1 IU/ml for tetanus-toxoid, 10 mIU/ml for HBsAg and 0.15 µg/ml for PRP (i.e. Hib epitope) [36]. The surrogate for measuring immune responsiveness to pertussis was defined as at least four-fold increase in antibody titer from prevaccination to postvaccination for pertussis toxoid and FHA [36]. Alternate antibody threshold levels evaluated included ≥ 100 mIU/ml for HBsAg and ≥ 1.0 µg/ml for PRP, which may be a predictor for long-term protection [36].

Statistical analyses

Descriptive statistics were reported for demographic and baseline features of participants included in this study. Paired *t*-tests were used in the comparison of pre and postvaccination CD4⁺ percentages. Prevaccination and postvaccination GMCs and proportions with sero-protective levels or surrogates of antibody responses were compared using analysis of covariance and multivariate

logistic regression, respectively, considering sex, age, race (black African or mixed ancestry), CD4⁺ lymphocyte percentage, study center, birth weight, mother-to-child HIV-prevention antiretroviral regimen and prevaccination antibody concentrations. To minimize additional confounders, the analysis was limited to children who received all three-vaccine doses and had both prevaccination and postvaccination bloods for immunogenicity assays collected within protocol-specified window periods. For all the tests, a two-sided *P*-value ≤ 0.05 was considered statistically significant.

Statistical analyses were performed using GraphPad Prism 5a (GraphPad Software Inc, La Jolla, California, USA), R (R Foundation for Statistical Computing, Vienna, Austria) and STATA statistical package (STATA Corporation, College Station, Texas, USA).

Ethics

This sub-study was approved by the Human Research Ethics Committee (HREC) of the University of the Witwatersrand (M080966). The study from which samples were obtained was approved by HREC, the Ethics Committee of Stellenbosch University, Medicine Control Council (South Africa) and the Division of AIDS of the National Institute for Health (NIH) and was registered under Clinical Trials number (NCT00099658).

Results

Samples from 493 (85.3%) of 578 children in the parent-study were available as shown in Table 1. The main reasons for unavailability of samples postvaccination were the high mortality rate in ART-Def infants as reported [9] and visits undertaken beyond the protocol-defined window-period; Fig. 1. The mean age at prevaccination ($\pm$ SD) was 7.3 (SD 1.3) weeks and 19.5 (SD 1.3) weeks at postvaccination being similar between groups; Table 1. Forty six % were male and 90.7% were of black-African descent; Table 1. All infants in the ART-Immed and ART-CD4 $\geq 25\%$ groups were initiated on ART within 4 days prior to the first DTwP-HibC/HBV dose. Thirteen (16.9%) ART-Def infants were initiated on ART at a median of 40 days following the first vaccine-doses [13]. The mean CD4⁺-lymphocyte percentages were 35.1% in ART-Immed, 37.5% in ART-Def and 22.0% in ART-CD4 $\geq 25\%$ group prevaccination; and 40.7% ($P < 0.001$), 30.1% ($P < 0.001$) and 27.5% ($P = 0.06$) 1-month post third-dose in the respective groups; Table 1.

Antibody responses in HUU compared to HIV-infected infants

Prevaccination

Compared to HUU, ART-Immed and ART-Def infants had lower antibody GMCs to tetanus-toxoid ($P < 0.001$

Table 1. Demographic and baseline features of participants included in the analysis evaluating the immunogenicity of a three doses primary series of diphtheria toxoid-tetanus toxoid- whole cell pertussis and *Haemophilus influenzae* type-b conjugate vaccine and hepatitis B vaccine.

Variable	Total	HUU ^a	HEU ^b	ART-Immed ^c	ART-Def ^d	ART-CD4<25% ^e
Total patients enrolled	578	124	125	210	105	14
Number included in analysis	493	114	120	172	77	10
Male sex (%)	227 (46.1)	61 (53.5)	65 (54.2)	68 (39.5)	29 (37.7)	4 (40.0)
Total race: Black African (%)	447 (90.7)	86 (75.4)	112 (93.3)	164 (95.3)	76 (98.7)	9 (90.0)
Mixed ancestry (%)	46 (9.3)	28 (24.6)	8 (6.7)	8 (4.7)	1 (1.3)	1 (10.0)
Mean age (SD) at time of vaccine administration in weeks						
Dose-1 (SD) ^f	7.3 (1.3)	6.7 (1.3)	7.4 (1.0)	7.5 (1.2)	7.3 (1.2)	8.6 (1.6)
Dose-2	11.4 (1.3)	11.1 (1.2)	11.4 (1.1)	11.5 (1.4)	11.4 (1.4)	12.7 (1.8)
Dose-3	15.4 (1.3)	15.2 (1.3)	15.5 (1.2)	15.5 (1.4)	15.4 (1.3)	16.6 (1.7)
Post dose-3 (SD) ^g	19.5 (1.3)	19.2 (1.2)	19.5 (1.0)	19.6 (1.3)	19.6 (1.5)	20.6 (1.6)
Prevaccination median HIV-1 Viral load copies/ml (range)				>750000 (394500 to >750000)	>750000 (448000 to >750000)	>750000 (>750000 to >750000)
Prevaccination median CD4 ⁺ lymphocyte count (IQR) ^h				2070 (1572 to 2070)	2083 (1654 to 2083)	1568 (1194 to 1568)
Prevaccination mean CD4 ⁺ lymphocyte % (SD)	Not done			35.1 (8.3)	37.5(9.2)	22(6.0)
One-month post dose-3 median CD4 ⁺ lymphocyte count at (IQR) ⁱ				2230 (1713 to 2230)	1872 (1112 to 1872)	1827 (990 to 1827)
One-month post dose-3 mean CD4 ⁺ % (SD) ^j				40.7 (9.4)	30.1 (9.0)	27.49(8.7)
Median days (range) to ART initiation relative to 1st vaccine				0 (-4 to 0)	40 (7.0 to 71.0) ^k	0 days

^aHUU: HIV-uninfected infants born to HIV-uninfected mothers.^bHEU: HIV-uninfected infants born to HIV-infected mothers.^cART-Immed: HIV-infected infants with CD4⁺ ≥25% at enrolment randomized to initiate antiretroviral treatment (ART) immediately.^dART-Def: HIV-infected infants with CD4⁺ ≥25% at enrolment randomized to deferred antiretroviral treatment (ART).^eART-CD4<25%: HIV-infected infants with CD4⁺ cell count <25% started on ART at enrolment.^fBlood draw was undertaken just prior to and on the same day of DTwP-HibCV and hepatitis B vaccination where applicable.^gInclude subjects who received all three doses within protocol-defined window periods and available immunogenicity data within 3–6 weeks after 3rd dose of diphtheria toxoid-tetanus toxoid- whole cell pertussis- *Haemophilus influenzae* type b conjugate vaccine (DTwP-Hib) and hepatitis B.^hIQR: interquartile range.ⁱInvolve 171, 77 and 10 observations in ART-Immed, ART-Def and ART-CD4<25% children, respectively.^j13 (16.8%) of 77 ART-Def children had been initiated on ART at the time of the immunogenicity analysis.

for both), HBsAg ($P=0.01$ for ART-Immed; $P=0.002$ for ART-Def) and pertussis toxoid ($P<0.001$ for both); Table 2. The proportion of infants with antibodies above the sero-protective threshold were generally similar between HUU and the HIV-infected groups, except for HBV which was lower in ART-Def (39.5% vs. 49.6% in HUU, $P=0.03$); Table 2.

Postvaccination

Compared to HUU, lower postvaccination GMCs were observed in the ART-Immed and ART-Def groups for tetanus-toxoid ($P<0.001$ for both) and HBsAg ($P<0.001$ for both); Table 3. Also, lower GMCs were observed in ART-Immed infants to diphtheria-toxoid ($P=0.001$) and FHA ($P<0.001$), as well as for pertussis toxoid in ART-Def children ($P<0.001$) compared to HUU infants, Table 3.

In HUU, despite higher postvaccination antibody GMCs to some epitopes, the proportion with sero-protective levels of antibody were similar for tetanus-toxoid (82.7% to 100%), diphtheria-toxoid at ≥ 0.01 IU/ml (100%), PRP ($>96\%$) and HBsAg ($>84\%$) compared to ART-Immed

and ART-Def infants; Table 3. Also, a similar proportion in each group had antibody above ≥ 1.0 µg/ml for PRP ($>88\%$), HBsAg ≥ 100 mIU/ml ($>84\%$) and diphtheria-toxoid ≥ 0.1 IU/ml (76.9 to 100%); Table 3. Sero-conversion rates associated with pertussis vaccination, however, were higher in HUU (38.2%) for FHA compared to ART-Immed infants (20.6%, $P=0.008$), and lower for pertussis toxoid in HUU (39.1%) compared with ART-Def (51.4%, $P=0.02$) infants, Table 3.

Despite no differences in PRP antibody concentrations, the SBA geometric mean titers (GMTs) were higher for HUU than ART-Def infants ($P<0.001$) and more HUU infants had SBA titers ≥ 8 (90.3 vs. 71.1%; $P<0.001$). There was no difference in SBA GMTs or proportion with titers ≥ 8 in HUU compared with ART-Immed infants; Table 3.

Antibody responses in HUU compared with HEU infants

Prevaccination

Prior to vaccination, HEU only had lower antibody GMCs to tetanus-toxoid ($P=0.001$) than HUU infants;

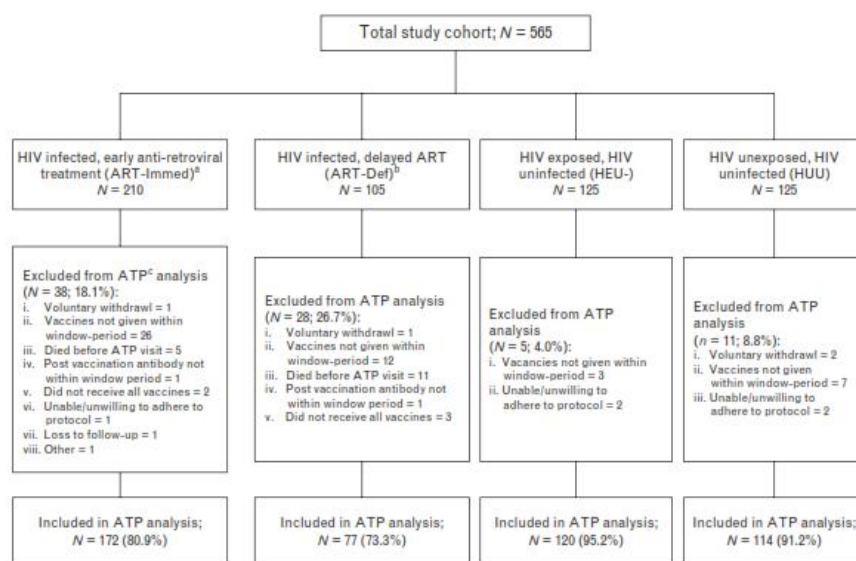


Fig. 1. Stratification of study population and reasons for children being excluded from analysis. ^aART-Immed: HIV-infected children with CD4⁺% $\geq 25\%$ who were initiated on treatment upon immediately upon enrolment. ^bART-Def: HIV-infected children with CD4⁺% $\geq 25\%$ who were initiated on treatment when clinically and/or immunologically indicated. ^cAccording to protocol, i.e. received all vaccines within window-period and also prevaccination and postvaccination blood available per defined window period. Note. In addition, a convenience sample of 10 HIV-infected children who had CD4⁺ cell count $< 25\%$ at enrolment were also included as a fifth descriptive group.

but higher GMCs for diphtheria-toxoid, HBsAg and FHA ($P < 0.001$ for all); Table 2. HEU were also more likely than HUU infants to have sero-protective diphtheria-toxoid (61.0 vs. 29.2%, $P < 0.001$) and HBsAg (80.5 vs. 49.6%, $P < 0.001$) antibody concentrations at prevaccination; Table 2.

Postvaccination

Antibody GMCs were similar postvaccination between HUU and HEU for tetanus-toxoid, diphtheria-toxoid, PRP and FHA. Antibody GMCs in HUU were, however, slightly higher for HBsAg (2521.0 vs. 2019.3 mIU/ml; $P = 0.041$) and lower for pertussis-toxoid (134.3 vs. 261.3 IU/ml, $P < 0.001$) than in HEU; Table 3.

A high proportion ($> 99\%$) of HEU and HUU children developed sero-protective levels to tetanus-toxoid, diphtheria-toxoid, HBV and PRP postvaccination, which did not differ by group. Similarly, more than 99% of HUU and HEU infants had antibody levels ≥ 100 mIU/ml to HBsAg and more than 88% had ≥ 1.0 $\mu\text{g}/\text{ml}$ to PRP postvaccination. The proportion of HUU compared with HEU children with sero-conversion (i.e. at least four-fold increase between prevaccination and postvaccination) to pertussis antigens was similar for FHA (38.2 vs. 21.6%; $P = 0.53$), however, against

pertussis toxoid this proportion was lower in HUU infants (39.1 vs. 76.7%; $P > 0.001$). There was no difference in SBA GMTs or proportion with SBA titers ≥ 8 between HUU (90.3%) and HEU (94.1%) infants; Table 3.

Antibody responses in HEU compared to HIV-infected infants

Prevaccination

Prevaccination antibody GMCs were lower in ART-Immed and ART-Def children than HEU infants for all epitopes ($P < 0.001$ for all comparisons), except for PRP; Table 2. HEU infants were also more likely to have sero-protective antibody concentrations against diphtheria-toxoid (61.0%) and HBV (80.5%) compared with ART-Immed (39.9, and 45.8%, respectively) and ART-Def (34.2, and 39.5%, respectively); $P < 0.001$ for all comparisons, Table 2.

Postvaccination

Postvaccination antibody GMCs were higher in HEU than ART-Immed or ART-Def infants to tetanus-toxoid, diphtheria-toxoid, PRP, HBsAg and PT; Table 3. The proportion of children with sero-protective antibody levels were similar in HEU and ART-Immed infants, although the latter were less likely to have anti-diphtheria-toxoid antibody ≥ 0.1 IU/ml for diphtheria-toxoid

Table 2. Antibody concentrations and proportion of children with sero-protective antibody levels prior to first dose of diphtheria toxoid-tetanus toxoid-whole cell pertussis-Haemophilus influenzae type b conjugate vaccine (DTPw-Hib) and hepatitis-B vaccines.

Antigen	Measure	HU ^a n = 114	HEU ^b n = 120	ART-Innmod ^c n = 172	P-value ^d	ART-Der ^e n = 77	P-value ^f	P-value ^g	P-value ^h	ART-CD4<25% ⁱ n = 10
TT ^j	GMC (95% CI) ≥0.01 IU/ml N/n ^m (%, 95% CI)	0.421 (0.309–0.573) 110/113 (97.4) 92.5–99.1	0.219 (0.181–0.265) 117/118 (99.2) 95.4–100	0.086 (0.071–0.105) 164/168 (97.6) 94.0–99.1	0.001 0.07	0.074 (0.054–0.103) 72/76 (94.8) 87.5–97.9	<0.001 0.41	<0.001 0.15	<0.001 0.48	0.100 (0.065–0.135) 10/100 (72.3–100)
DT ^k	GMC (95% CI) ≥0.1 IU/ml N/n (%, 95% CI)	0.078 (0.069–0.088) 33/113 (29.2) 21.6–38.2	0.136 (0.115–0.161) 72/118 (61.0) 52.0–69.3	0.085 (0.074–0.097) 67/168 (39.9) 32.8–47.4	<0.001 0.001	0.076 (0.064–0.089) 26/76 (34.2) 24.5–45.4	<0.001 0.90	<0.001 0.26	<0.001 0.08	0.127 (0.080–0.184) 8/80 (0; 49.0–94.3)
PRP ^l	GMC (95% CI) ≥0.15 µg/ml N (%, 95% CI)	0.284 (0.219–0.369) 75/65 (85.8) 56.7–73.9	0.250 (0.198–0.314) 80/66 (77.5) 57.8–74.5	0.345 (0.279–0.426) 120/69 (8) 62.5–76.1	0.20 0.51	0.306 (0.222–0.423) 51/66 (2) 35.1–75.8	0.75 0.77	0.28 0.82	0.79 0.37	0.459 (0.235–0.894) 9/90 (0; 59.6–98.2)
HibAg ^h	GMC (95% CI) ≥10 mIU/ml N/n (%, 95% CI)	7.74 (5.25–11.42) 56/113 (49.6) 41.0–59.0	29.27 (21.05–42.11) 95/118 (80.5) 72.5–86.7	5.81 (4.12–8.17) 77/164 (45.8) 38.9–59.4	<0.001 0.001	4.26 (2.70–6.73) 30/76 (39.5) 29.3–50.7	<0.001 0.12	<0.001 0.02	0.002 0.03	396.98 (152.6–1032.9) 10/100 (72.3–100)
HIA ⁱ	GMC IU/ml (95% CI)	20.09 (17.91–22.55) 40/65 (61.5) 48.6–64.4	35.08 (29.65–41.50) 31/70 (44.3) 27.67–36.30	22.88 (20.30–25.79) 17/67 (25.4) 15.57–20.04	<0.001 0.06	20.83 (17.99–24.12) 16/29 (55.2) 13.63–19.46	<0.001 0.52	<0.001 0.23	<0.001 0.23	30.17 (21.39–42.54)
PT ^j	GMC IU/ml (95% CI)	40.65 (33.83–48.84)	31.70 (27.67–36.30)	17.67 (15.57–20.04)	0.06	16.29 (13.63–19.46)	<0.001	<0.001	<0.001	26.76 (16.56–43.26)

^aHU: HIV-uninfected infants born to HIV-uninfected mothers.^bHEU: HIV-uninfected infants born to HIV-infected mothers.^cART-Innmod: HIV-infected infants with CD4⁺ ≥25% at enrollment randomized to initiate antiretroviral treatment (ART) immediately.^dP-value comparing HEU to HU infants.^eP-value comparing ART-Innmod with HU infants.^fP-value comparing ART-Innmod with HEU infants.^gART-Der: HIV-infected infants with CD4⁺ ≥25% at enrollment randomized to deferred ART.^hP-value comparing ART-Der with HU infants.ⁱP-value comparing ART-Der with HEU infants.^jART-CD4<25%: HIV-infected infants with CD4⁺ cell count <25% started on ART at enrollment.^kGMC (95% CI): geometric mean concentration with 95% confidence interval in parentheses.^ln: Total number of samples available for calculating percentage response.^mTT = tetanus toxoid.ⁿPRP = diphtheria toxoid.^oHibAg = polyribosyl-diphtheria phosphate.^pHIA = hepatitis B surface antigen.^qPT = pertussis toxin.

Table 3. Antibody concentrations and proportion of children with sero-protective antibody levels following primary series of three doses of diphtheria toxoid-tetanus toxoid-whole cell pertussis-Haemophilus influenzae type b conjugate vaccine and hepatitis B vaccines.

Antigen	Measure	HEU ^a n = 114	HEU ^b n = 120	ART-Imm ^c n = 169	ART-Def ^d n = 75	ART-CD4<25% ^e n = 9
TT ^f	GMC (95% CI) ^g ≥0.1 IU/ml N/n ^h (% 95%CI) ⁱ	0.952 (0.849–1.068) 111/111 (100) 96.7–100	0.762 (0.679–0.858) 116/118 (98.3; 94.0–99.5) NE ^j	0.405 (0.343–0.478) 156 (92.3; 87.3–95.5) NE	0.323 (0.228–0.457) 62 (82.7; 72.6–89.6) NE	0.470 (0.128–1.722) 8 (88.9; 56.5–98.0) NE
DT ^f	GMC (95% CI) ^g ≥0.01 IU/ml N/n ^h (% 95% CI) ⁱ	0.272 (0.250–0.296) 111/111 (100) 96.7–100	0.372 (0.333–0.415) 118/118 (100) 96.9–100	0.200 (0.173–0.231) 169 (100; 97.8–100) NE	0.233 (0.194–0.281) 75 (100; 95.2–100) NE	0.172 (0.060–0.492) 8 (88.9; 56.5–98.0) NE
PRP ^f	GMC (95% CI) ^g ≥0.1 IU/ml N/n ^h (% 95% CI) ⁱ	7.24 (5.47–9.60) 111/111 (100) 96.7–100	10.2 (7.95–13.12) 117/118 (99.2) 95.4–100	6.96 (5.62–8.61) 167 (98.8; 95.8–99.7) NE	7.21 (4.94–10.53) 73 (97.3; 90.8–99.3) NE	4.25 (1.23–14.66) 9 (100.0; 70.1–100.0) NE
SBA-PRP ^f	GMC (95% CI) ^g ≥1.0 µg/ml N/n ^h (% 95% CI) ⁱ	101 (88.6; 81.5–93.2) 311/362 (222.36–435.13) 102 (90.3; 83.4–94.5)	112 (93.3; 87.4–96.6) 373.86 (282.38–494.97) 112 (94.1; 88.4–97.1)	0.39 0.96 0.52	0.35 0.93 0.26	0.48 0.85 0.02
HBsAg ^f	GMC (95% CI) ^g ≥10 mIU/ml N/n ^h (% 95% CI) ⁱ	2521.03 (2249.84–2824.92) 111/111 (100) 96.7–100	2019.28 (1740.28–2343.00) 117/118 (99.2) 95.4–100	924.87 (778.28–1099.07) 167 (98.8; 95.8–99.7) NE	536.60 (372.97–772.02) 73 (97.3; 90.8–99.3) NE	6545.81 (1256.29–34106.64) 9 (100.0; 70.1–100.0) NE
FHA ^f	GMC (95% CI) ^g 4-fold increase N/n ^h (% 95% CI) ⁱ	61.24 (53.65–69.91) 42/110 (38.2) 29.7–47.5	72.22 (62.39–83.36) 25/116 (21.6) 15.1–27.4	0.54 0.53 0.44	0.00 0.00 0.44	0.23 0.13 0.06
PT ^f	GMC (95% CI) ^g 4-fold increase N/n ^h (% 95% CI) ⁱ	134.34 (111.97–161.17) 43/110 (39.1) 30.5–48.4	261.30 (221.56–308.18) 89/116 (76.7) 68.3–83.5	0.00 0.00 0.56	0.00 0.00 0.56	0.02 0.02 0.14

^aHEU: HIV-uninfected infants born to HIV-uninfected mothers.

^bHEU: HIV-uninfected infants born to HIV-infected mothers.

^cART-Imm: HIV-infected infants with CD4⁺ ≥25% at enrollment randomized to initiate ART immediately.

^dART-Def: HIV-infected infants with CD4⁺ <25% at enrollment randomized to initiate ART immediately.

^eART-CD4<25%: HIV-infected infants with CD4⁺ <25% at enrollment randomized to deferred ART.

^fPRP: diphtheria toxoid.

^gGMC: geometric mean concentration with 95% confidence interval in parentheses.

^hN/n: total number of samples available for calculating percentage response.

ⁱNE: evaluable. P-value is not calculable through logistic regression due to group having 100%.

^jTT: tetanus toxoid.

^kPRP: diphtheria toxoid.

^lSBA-PRP: serum bactericidal assay.

^mHBsAg: hepatitis B surface antigen.

ⁿFHA: Filamentous hemagglutinin.

^oPT: pertussis toxin.

(99.2 vs. 76.9%; $P < 0.001$). In contrast, lower proportions of ART-Immed infants had sero-protective antibody levels postvaccination compared to HEU infants to tetanus-toxoid (82.7 vs. 98.3%; $P = 0.02$), diphtheria-toxoid at the ≥ 0.1 IU/ml threshold (80.0 vs. 99.2%; $P = 0.001$), HBV at ≥ 100 mIU/ml threshold (84.0 vs. 99.2%; $P = 0.02$) and were also less likely to demonstrate sero-conversion to pertussis toxin (51.4 vs. 76.7%; $P < 0.001$); Table 3. HEU also had higher SBA GMTs compared with either ART-Immed ($P = 0.03$) or ART-Def ($P < 0.001$) groups and were also more likely (94.1%) to have SBA titers ≥ 8 compared with ART-Immed (87.8%, $P = 0.06$) and ART-Def infants (71.1%, $P = 0.02$).

Antibody responses in antiretroviral treatment-Immed compared to antiretroviral treatment-Def infants

Prevaccination

There were no differences between ART-Immed and ART-Def groups prior to vaccination in either GMCs or the proportion of children with sero-protective antibody levels for any vaccine; Table 2

Postvaccination

Postimmunization immune GMCs against most epitopes were similar, but higher in ART-Immed for HBV ($P = 0.017$) and lower for FHA ($P = 0.02$) than ART-Def infants; Table 3. A similar proportion of ART-Immed and ART-Def infants developed antibody concentrations above the sero-protective threshold for most vaccines, except for more ART-Immed infants with tetanus-toxoid antibody ≥ 0.1 IU/ml (92.3 vs. 82.7%; $P = 0.03$) and HBsAg antibody ≥ 100 mIU/ml (97.0 vs. 84.0%; $P = 0.02$). Serum bactericidal assay GMTs trended to be higher in ART-Immed children, among whom it was more likely to be ≥ 8 (87.8%) compared with ART-Def group (71.1%; $P = 0.02$; Table 3).

The proportion of ART-CD4 $< 25\%$ group with sero-protective levels of antibody and/or with sero-conversion to pertussis-toxoid and FHA was similar to ART-Immed infants.

Sensitivity analysis

Because of the imbalance of race in the population among the different groups, a sensitivity analysis was performed restricting the analysis to black African participants. P -values were similar to those when including mixed race participants suggesting that any differences can be attributed to HIV exposure and not race (data not shown).

Discussion

This is the first and most-comprehensive study evaluating immune responses to routine vaccines in HIV-infected

infants receiving early ART, irrespective of CD4⁺ count/percentage according to current WHO recommendations [37]. Our findings show that early ART in HIV-infected infants, is associated with similar quantitative and qualitative immune responses to vaccines as in HUU infants. Although the immune responses in infants among whom ART was deferred was generally similar to the ART-Immed group, the ART-Def infants had lower proportion with anti-HBsAg antibody ≥ 100 mIU/ml and was also associated with lower levels of functional antibody to HibCV. A similar observation was observed on opsonophagocytic activity assay following PCV vaccination in ART-Def compared to ART-Immed groups [13]. Therefore, only measuring quantitative antibody responses could over-estimate protection induced by some vaccines among HIV-infected infants not yet on ART.

Differences observed between ART-Immed and ART-Def infants may have been masked by survivor bias in the ART-Def group especially in the postvaccination comparison. It is plausible that vaccine immune responses were even poorer among ART-Def infants who died compared to surviving children. A recent review on the association of ART on vaccine immune responses in HIV-infected children, included studies in older children re-vaccinated between 28 weeks and 5.3 years after ART initiation [38]. We report a higher proportion of children with sero-protective antibody concentrations than in previous studies, undertaken prior to early ART initiation in infants becoming policy, where the proportion of HIV-infected children without severe immunosuppression developing sero-protective antibody levels varied between 37 and 86% with GMCs generally much lower than in HIV-uninfected infants [39–41].

Our data on the relative immune responses to DTwP-HibCV/HBV in HEU compared with HUU infants differed from other reports. In-utero HIV-exposure was not consistently associated with lower GMCs prior to immunization in HEU compared with HUU infants as proposed by others [31,32,42–44]. Although we also identified lower GMCs to tetanus-toxoid in HEU infants prevaccination, the converse was true for antibody GMCs to diphtheria-toxoid, HBsAg and FHA. This could be due to regional differences in maternal antibody concentrations to different vaccine-epitopes and variations in factors which may influence transplacental antibody transfer, including differences in maternal HIV-viral load, total IgG antibody concentrations and placental function sufficiency in different populations [31,32,42–44]. These factors may influence HEU infants' susceptibility to vaccine-preventable diseases during early infancy and prior to vaccination.

Comparing HEU and HUU infants, HEU had a trend toward lower prevaccination anti-pertussis toxoid GMCs but higher postvaccination levels, and conversely had

higher prevaccination HBsAg antibody GMCs, but lower postvaccination levels to HBsAg. This corroborates that higher epitope-specific antibody levels prevaccination may interfere with the immunogenicity to homotypic epitopes. The higher preimmunization HBsAg antibody GMCs in HEU in our study has been attributed to higher hepatitis B virus infection prevalence in HIV-infected than HIV-uninfected women [45]. However, we also observed higher antibody levels to HBsAg and other vaccine-epitope GMCs (except PRP) in HEU compared with both ART-Immed and ART-Def infants. Our study did not determine HIV-infection status at birth and since only 10% of HIV-exposed infants were breast-fed, it is likely that the majority of HIV-infection in infants of our cohort occurred either *in utero* or the immediate peripartum period. Therefore, differences in prevaccination GMCs to most epitopes between HEU and either ART-Immed or ART-Def infants, suggests an association between factors related to acquiring maternal-derived vaccine-related antibodies and the risk of the infant being HIV-infected. Placental abnormalities can affect transplacental antibody transfer. For example, placentas from HIV-infected women from the Tygerberg site showed a higher percentage of villitis and infarcts than from HIV-uninfected controls, especially in women with CD4⁺ cell counts below 200 cells/ μ l [46].

Our study has some limitations, including that we were unable to distinguish *in utero* from intra-partum infection and placentas were not collected in our study. Also, we recruited only a small number of HIV-infected infants in whom with baseline CD4⁺ lymphocyte <25%. Data from CHER showed that by 7 weeks of age, 20% of HIV-infected infants already have severe CD4⁺-lymphocyte depletion. The trends in immune responses in the ART-CD4<25% group included in our study was, however, similar to that of ART-Immed infants. Also, despite having used whole cell pertussis vaccine, we elected to measure antibody to pertussis toxoid and FHA rather than using the whole-cell pertussis ELISA assay, as this was more practical for inclusion in the multiplex Luminex assay. The pertussis toxoid and FHA antibody responses, although providing a relative measure of immunogenicity of the whole cell pertussis vaccine between groups in our study, is not recommended as a measure of immunogenicity of whole cell pertussis vaccine.

In conclusion, HEU infants were not at increased susceptibility to most of the studied vaccine-preventable diseases by 6-weeks of age, suggesting similar acquisition of antibody from their mothers in our study. Furthermore, the immune responses in HEU were similar to HUU infants postvaccination. Although HIV-infected children had lower GMCs postvaccination, including to tetanus-toxoid and HBsAg irrespective of timing of ART initiation, the proportion with sero-protective antibody concentrations postvaccination were similar and timing of

ART initiation generally did not affect immune responses between the HIV-infected groups. Our study indicates that vaccination of HEU and HIV-infected infants initiated on early-ART confers with DTwP-HibCV/HBV would provide similar immunity to the targeted vaccine-preventable disease compared with HUU infants. The persistence of sero-protective antibody concentrations and anamnestic responses to the studied vaccines among HEU and HIV-infected children initiated on early ART needs to be investigated.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary material: Study participant selection criteria:

Study design and site

This was a retrospective analysis of a prospective cohort on the immunogenicity of vaccines within the Expanded Program of Immunization in South Africa (EPI-SA) in HIV infected, HIV exposed uninfected and HIV unexposed uninfected children as the control group. These children were predominantly from the public sector either born or receiving care at the Chris Hani-Baragwanath Hospital in Johannesburg, and at Tygerberg Hospital in Stellenbosch, South Africa. HIV-uninfected children were recruited from the maternity wards of the respective study sites by a trained health worker in HIV counselling. The mothers must have tested HIV negative based on their participation in the mother to child transmission programme that was conducted at each study site. HIV exposed uninfected children were recruited from a cohort of children originally identified to participate in the “Children with HIV Early Antiretroviral (CHER)” study, but the child tested HIV negative at 4 weeks of age by polymerase chain reaction (PCR). HIV-exposed infected children were identified to be HIV-infected within the CHER study and had to have a baseline CD4+ T-lymphocyte count available. These children were randomized to either early or delayed antiretroviral treatment groups. The vaccination schedule was based on the WHO and South African Department of Health guidelines. Quantitative antibody responses to DTwP-Hib-CV and Hepatitis B vaccines among HIV- uninfected (exposed and non-exposed) and HIV-infected children stratified by antiretroviral therapy status were measured.

Study population, randomization, inclusion and exclusion criteria

The study was conducted on archived serum samples from 578 children in whom the effect of HIV exposure and timing of ART initiation was evaluated on the quantitative and qualitative

antibody responses to pneumococcal conjugate vaccine as the primary objective. [1] Five groups of children, aged at 6-12 weeks, were enrolled from April 2005 to June 2006. This included HIV-uninfected children born to HIV-uninfected mothers (HUU; N=124); and HIV-exposed uninfected children born to HIV-infected mothers (HEU; N=125). In addition, perinatal HIV-infected children with a CD4+T-lymphocyte cell percentage $\geq 25\%$ at baseline were randomized to initiate ART when clinically and/or immunologically indicated (ART-Def; N=105) as per then WHO recommendations, or immediately upon enrolment (ART-Immed; N=210). [2] Furthermore, a convenience sample of HIV-infected children with CD4+ $<25\%$ initiated on ART at enrolment (ART-CD4 $<25\%$; N=14) were included. HIV-infected children were co-enrolled from the “Children with HIV Early Antiretroviral (CHER)” Study as described. [2] Children were included in the study had to be between four and ten weeks of age and have a birth weight of at least 2000 grams. The parent or legal guardian of the child had to have indicated that they would stay in the study area for the study duration. Documentation of the mothers HIV status at 24 weeks had to be available, and breastfeeding was permitted. Inclusion criteria for HIV-infected children were that the child had to be co-enrolled in the CHER study, HIV status had to have been confirmed by a positive DNA or RNA polymerase chain reaction and a second one at four to ten weeks of age. Children born to HIV-infected mothers were allowed into the study if they were ART naïve except for receipt of ART to prevent mother to child transmission. Allowed medications were antipyretics for auxiliary temperatures $\geq 38^{\circ}\text{C}$ after vaccination. HIV-infected children were allowed to receive ART as part of the study as well as trimethoprim-sulfamethoxazole prophylaxis as per the South African guidelines. Children were excluded from the study if they had received any blood products, any vaccine with the exception of BCG and oral polio vaccine, prior to study entry. Children who had received immunosuppressant agents for more than 2 weeks and had were unable to tolerate oral

medication. Presence of any major congenital anomalies that were life threatening. Children with acute, moderate to severe intercurrent illness or fever $\geq 38^{\circ}\text{C}$ within 72 hours before vaccination that resulted in hospitalization, were excluded from the study. The presence of Grade 2 allergic reaction and any Grade 3 clinical toxicity; the presence of any Grade 3 or 4 clinical or laboratory toxicity; were excluded from the study. Parent or guardian unable or unwilling to attend scheduled study visits. The use of ART other than the regimens described within the study, systemic cytotoxic chemotherapy and interleukin or other immune modulators.

Antiretroviral therapy administered:

At Chris-Hani Baragwanath Hospital, mothers who were identified as HIV positive received a single dose of nevirapine at the time of delivery, this was also give to their child at the time of delivery to prevent mother-to-child transmission. While at Tygerberg Hospital the regimen consisted of zidovudine given to the HIV positive mother from 34 weeks gestation and to the neonates for 7 days and a single-dose nevirapine to both at the time of delivery. ART received during the study was as follows: first-line antiretroviral was zidovudine at a dose of 240mg per square meter of body-surface area twice daily; lamivudine at a dose of 4mg per kilogram of body weight twice daily and lipinavir (300mg)-ritonavir (75mg) given twice daily up-to 6 months of age and thereafter at 230mg and 57.5mg respectively.

Supplementary material: Luminex methods and validation results:

Methods:

We had validated the antibody measures of the Luminex assays for DT, TT, PT, FHA and HBsAg in reference to commercially available individual ELISA assays in a separate group of 25 children enrolled in a previous study which involved 4 to 7 year old children who had documented DTwP-Hib and HBV vaccination during infancy.[3] Antibodies to DT and TT were measured with the anti-diphtheria toxoid and VaccZymeTM anti-tetanus toxoid IgG EIA (MK014 and MK010, The Binding Site Ltd, Birmingham, England), with concentrations of $\geq 0.004\text{IU/ml}$ and $\geq 0.0093\text{IU/ml}$ regarded as positive, respectively.

Antibodies to PT and FHA were measured with the Serion enzyme-linked immunosorbant assay classic kit (ESR120G, Serion Immundiagnostica GmbH, Würzburg, Germany), with a titer of $\geq 30\text{FDA-U/ml}$ being considered as sero-positive. Antibodies to HBsAg were measured using ImmunoLISATM HBsAb (Organics Ltd. Part of the Inverness Medical Innovations Group, Israel), with titers $\geq 10\text{mIU/ml}$ considered as sero-positive. All reagents including controls were supplied with the commercial kits and ELISA assays were performed according to the manufacturers' instructions.

Antigens used in the multiplex Luminex assay were sourced as follows: diphtheria- toxoid (DT; batch number D52-2) and TT (batch number T177-1) from Statens Serum Institute, Denmark, pertussis toxin (PT; batch number 181209A1) and filamentous hemagglutinin (FHA; batch number: 17040B1) from List Biological Laboratories, California, USA and hepatitis B surface antigen (HBsAg, Engerix B B59, Batch number AHBVBPA 097) from GlaxoSmithKline, Rixensstadt, Belgium. The multiplex anti-human IgG antibody assays were performed similarly as described,[4] but modified by exclusion of anti- polyribosyl-ribitol phosphate (PRP) and inclusion of HBsAg, FHA and PT. Serum samples, reference standards and controls were diluted in phosphate buffered saline with 10% fetal bovine serum and

0.05% sodium azide (PBS-FN) and added to filter plates that were pre-blocked with the same buffer. An in-house reference standard was used for all assays, that consisted of pooled human serum that had been calibrated against International standards (NIBSC code 00/496 (diphtheria antitoxin), 76/589 (tetanus antitoxin), 07/164 (anti-HBsAg immunoglobulin) and 06/140 (Pertussis antiserum (human) 1st IS). Serum antibody values were either assigned in IU/ml for DT, TT, PT, FHA and mIU/ml for HBsAg.

The in-house reference serum was diluted in seven 4-fold dilutions (1/50–1/204800). The R^2 obtained was ≥ 0.99 for the study reference tested for antibodies against DT, TT, PT FHA and HBsAg with percentage of relative error (%RE) being less than 20% for the seven step dilution series. Percentage recovery was set to be between 80 to 130% and all antibodies tested fell well within the set criteria (range: 96-105%). For quality control purposes positive and negative controls were included in duplicate in all tests.

Statistical analysis: Correlation between ELISA and multiplex assay was measured with Pearson's correlation coefficients (R^2) and *kappa* statistic used to compare the proportion of samples above or below specified thresholds between ELISA and the multiplex assay. Percentage recovery of specific antibodies was determined by recalculating the concentration of each reference standard obtained by extrapolating its response (MFIs) through the fitted curve. The RE of each standard point was as follows: %RE = [(recalculated value – assigned value)/assigned value] x 100%.

Results:

The specificity of the assay was >92% for all tested antibodies and the coefficient of variation for intra- and inter-assay ranged between 5-22% for individual assays. The correlation coefficients of individual ELISAs to multiplex Luminex assay was 0.97, 0.93, 0.97, 0.99 and

0.72 for DT, TT, PT, FHA and HBsAg respectively; supplementary Figure 1. There was greater than 92% agreement in proportion of children with antibody threshold above sero-protective titers for DT, TT and HBsAg and complete concordance for the proportion of samples that were sero-positive for PT and FHA between the Luminex and uniplex assays.

Discussion:

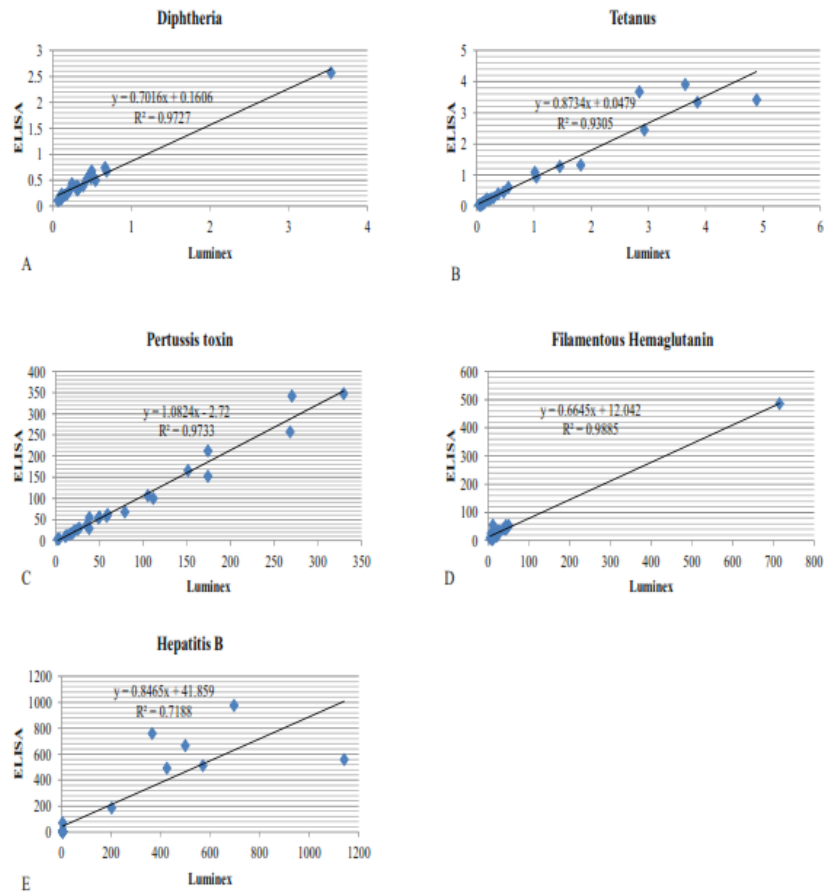
We describe for the first time the determination of anti-HBs with a multiplex platform. We also show that a single reference serum can be used to verify all the test antibodies. Commercially available antigens with high purity were utilized to ensure antigen availability for future testing. The major advantage of this multiplex assay was the need for small sample volumes (5µl) compared to ELISAs which ranged from 10µl to 100µl of sample. In addition, reduction of assaying time with the multiplex assay, that required 2.5 hours to test 38 samples for all five vaccine antigens simultaneously; instead of the 10.5 hours required for ELISAs. Studies have demonstrated the ability of ELISAs to accurately determine vaccine responses [5], but with advances in the vaccinology fields of new vaccines with increased number of combined antigens the use of ELISA has become increasingly unsuitable.[6] The ability to effectively multiplex a range of biological analytes has been shown for cytokines,[7] bacterial pathogen nucleic acid [8] as well as antigen and antibodies to specific pathogens [4, 6, 9]. The optimal bead conjugation concentration and procedure was found to be similar to those reported for elsewhere.[9] MFIs generated from all five conjugated microsphere bead regions showed linearity over a seven step 4-fold reference serum dilution range, the consequence of this increase in range was the resultant increase in accuracy, a reduction in the number of sample dilutions required and the ability to detect much lower antibody levels. MFI comparisons between uniplex and pentaplex assays allowed for determination of interference between the microsphere bead regions. Multiplexing did not affect specificity

and sensitivity of the assay when compared to monoplexing. The multiplex assay was found to be reproducible, with minimal variability inter- and intra assay, and correlated well with commercially available ELISAs.

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Supplementary Figure 1: Correlation between ELISA and Luminex assays.

Appendix 2:

Effect of in-utero HIV exposure and antiretroviral treatment strategies on measles susceptibility and immunogenicity of measles vaccine

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Introduction: The high burden of maternal HIV-infection in sub-Saharan Africa may affect measles control. We evaluated the effect of in-utero HIV-exposure and anti-retroviral treatment (ART) strategies on measles antibody kinetics prior and following measles vaccination.

Methods: Infants aged 6–12 weeks were enrolled. This included HIV-uninfected infants born to HIV-uninfected (HUU) and HIV-infected mothers (HEU). Additionally, we enrolled perinatal HIV-infected infants with CD4% equal or greater than 25% randomized to deferred-ART until clinically or immunologically indicated (Group-3) or immediate-ART initiation (Group-4). Group-4 was further randomized to interrupt ART at 1 year (Group-4a) or 2 years of age (Group-4b). Additionally, a convenience sample of HIV-infected infants with CD4⁺ less than 25% initiated on immediate-ART was enrolled (Group-5). Measles immunoglobulin-G antibodies were quantified by an indirect enzyme immunoassay with titers 330 mIU/ml or more considered 'sero-protective'. The referent group was HUU-children.

Results: The proportion with sero-protective titers at 7.3 weeks of age was higher in HUU (65.2%) compared with any HIV-infected group (range: 16.7–41.8%), but dropped to less than 17% in all groups at age 19.6 weeks. Twenty-eight weeks following the first measles vaccine, Group-4a was less likely to have sero-protective titers (79.3%) as compared to HUU (91.1%; $P < 0.0001$), Group-3 (95.7%; $P = 0.003$) or Group-4b (92.1%; $P = 0.018$). Although the proportion with sero-protective levels were similar between groups immediately postbooster dose, this was lower in HEU (79.6%; $P = 0.002$) and Group-4a (80.3%; $P = 0.010$) compared with HUU (94.3%) 41-weeks later.

Conclusion: Greater waning of immunity among HIV-infected children in whom ART was interrupted and in HEU following a booster-dose, indicate the possible need for further measles-booster doses after 2 years of age in these children.

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Introduction

Improved immunization of children against measles has reduced measles-associated mortality globally over the past decade [1,2]. Sporadic measles outbreaks persist in many settings, disproportionately involving children too young to be vaccinated [3]. Twenty-four percent of cases in a recent measles outbreak in South Africa were aged less than 9 months [4]. A greater proportion of measles-associated hospitalization occurs in children aged less than 9 months among HIV-infected (33%) compared with HIV-uninfected African children (23%) [5].

Factors contributing to the sporadic outbreaks of measles include vaccine coverage below the 95% threshold required to interrupt wild-type virus circulation in communities, pockets of susceptible individuals who remain unimmunized or have sub-optimal immune responses to vaccine and reduced transplacental measles antibody transfer to fetuses as maternal immunity is now mainly vaccine induced [2,3,6]. Furthermore, maternal HIV-infection has been associated with lower measles antibody titers [7], impaired transplacental antibody transfer to the fetus and more rapid decay of maternal-derived antibodies in HIV-infected children [7–10].

A recent meta-analysis identified a limited number of studies evaluating the immunogenicity of measles vaccine following primary vaccination in predominantly antiretroviral treatment (ART)-naïve HIV-infected children [11]. None of the studies were undertaken in the context of early initiation of ART in HIV-infected children [12]. The meta-analysis reported that 59% [95% confidence intervals (95% CIs), 46–71%] of HIV-infected children were sero-positive after receiving standard-titer measles vaccine at 6, 9 or 12 months of age without any differences by age of vaccination. Fewer HIV-infected children (40%) remained protected compared with HIV-exposed, uninfected children 12 months postvaccination [11]. A higher proportion of older HIV-infected children (78–89%) developed sero-protective titers when vaccinated post immune-reconstitution following ART initiation [13,14]. Despite sub-optimal immune responses and higher rates of vaccine failure in HIV-infected children, this was not considered a major hurdle in measles control and elimination because of the high mortality rate in the absence of ART [15]. Improved survival of HIV-infected children on ART may, however, paradoxically impair measles control because of sub-optimal immune responses to vaccine [16].

The objectives of our study were: evaluate the effect of in-utero HIV exposure and timing of ART initiation to measles susceptibility prior to vaccination; examine the association of HIV exposure and different ART strategies on the kinetics of measles antibody following the primary and booster doses of measles vaccine.

Methods

Study population

The study was conducted on archived serum samples from children in whom the effects of HIV exposure and timing of ART initiation on immune responses to pneumococcal conjugate vaccine were evaluated [17]. Infants between 6 and 12 weeks of age, were enrolled from April 2005 to June 2006 into five groups: HIV-uninfected children born to HIV-uninfected mothers with negative HIV-enzyme linked immunosorbent assay (ELISA) at enrolment (Group-1; HUU); HIV-exposed, uninfected children born to HIV-infected mothers in whom HIV-PCR was negative at enrolment (Group-2; HEU). In parallel, the study co-enrolled HIV-infected children already enrolled into the 'Children with HIV Early Antiretroviral (CHER)' study as described [18]. These included perinatal HIV-infected infants aged less than 12 weeks with CD4% equal to or greater than 25% who were randomized to deferred ART (Group-3) or immediate ART (Group-4) with interruption at approximately 1 year (Group-4a) or 2 years of age (Group-4b). ART initiation and re-initiation were based on WHO immunologic criteria (CD4% < 25%; < 20% after infancy) or CDC severe B or C clinical disease. Additionally, a convenience sample of HIV-infected infants with CD4⁺ less than 25% initiated on ART at enrolment (Group-5) were recruited. The first-line ART regimen in CHER included zidovudine, lamivudine and lopinavir-ritonavir. Following an interim analysis of the CHER study in which progression to AIDS or death was greater in Group-3 compared with Group-4, the Data, Safety and Monitoring Board recommended that all Group-3 children not yet on ART be immediately started on treatment [18]. In addition to other childhood vaccines administered as described [17], children received the Schwarz-strain measles vaccine (Rouvax; Aventis, France) at age 38–42 weeks and a booster dose at age 64–76 weeks.

Laboratory assays

Clotted blood samples were centrifuged and serum archived at –70°C until processing at the Respiratory and Meningeal Pathogens Research Unit, Johannesburg, South Africa. Samples were analyzed for measles specific IgG antibodies with a commercial indirect ELISA kit (Enzygnost, Dade Behring, Marburg, Germany) and results calibrated against the international reference preparation of measles antibody. Antibody titer levels were calculated using the α -method and presented in milli-International Units per millilitre (mIU/ml) per manufacturer recommendation. Assays with titers less than 150 mIU/ml were considered as negative [equivalent to < 0.1 optical density (OD)], 150–329 mIU/ml as equivocal (equivalent to 0.1–0.2 OD) and equal to or greater than 330 mIU/ml as sero-protective (equivalent to > 0.2 OD) per manufacturers criteria. For this study, sero-positivity was defined as antibody titers equal to or

greater than 150 mIU/ml. Samples for which the OD value was less than 0.1 were assigned a titer of half the detection limit of the assay (i.e. 75 mIU/ml) when calculating geometric mean titers (GMT).

Measles antibody titers were analyzed twice prevaccination at study enrolment (age 6–12 weeks) and approximately 20 weeks of age. Furthermore, measles antibody was measured prior to and 2 weeks after the booster dose, as well as at approximately 2 years of age. No blood was planned *a priori* immediately prior to the first dose of measles-vaccine or anytime soon thereafter.

Statistical analysis

Data was analyzed using GraphPad Prism 5a (GraphPad Software Inc, La Jolla, California, USA). GMTs and 95% CIs were calculated following $\log_{10}$ transformation of antibody titers. Comparison of GMTs between groups was done by unpaired *t*-test on $\log_{10}$ transformed data. Correlation between measles antibody titer and CD4⁺ percentage was measured using Spearman's correlation coefficients. Proportions of children were compared with chi-square test or Fischer exact test when needed. A *P*-value of 0.05 or less was considered statistically significant. The HUU children were the referent group. Comparisons between the ART strategies in HIV-infected children were also undertaken. Due to the limited number of participants in Group-5, statistical comparisons were not undertaken between this and other Groups.

Ethics

The study was approved by the Human Research Ethics Committee (HREC) of the University of the Witwatersrand (M080966). The study from which samples were obtained was approved by HREC, the Ethics Committee of Stellenbosch University, Medicine Control Council (South Africa) and the Division of AIDS of the NIH and was registered under Clinical Trials number (NCT00099658).

Results

Samples were available for 528 (91.3%) of 578 children enrolled into the parent study from the initial time-point. The number of samples available at each time-point in the various groups is detailed in Fig. 1. Of the 176 Group-4 children with samples available at the time of the booster dose, 87 had been assigned to discontinue ART at age 12 months and 89 were scheduled to interrupt ART at age 24 months. Forty-eight percent (*n* = 251) of children were male and 90.9% (*n* = 480) black-African descent. The mean age of children at each study time-point, at the time of primary measles vaccination and booster dose was similar between groups (Table 1).

The CD4⁺ cell percentage and counts at enrolment and subsequent visits in HIV-infected children are summarized in Table 1 and supplementary-Table S1 (<http://links.lww.com/QAD/A321>). At a mean age of 19.6 weeks, CD4⁺% were higher in Group-4 (41.6%) compared with Group-3 (29.9%; *P* = 0.0001) and Group-5 (31.5%; *P* = 0.003) children (Table 1). Similarly, CD4⁺% remained higher at the time of the first vaccine dose in Group-4 (39.1%) compared with Group-3 (31.0%; *P* = 0.0001) and Group-5 (30.6%; *P* = 0.011). Group-4a had lower CD4⁺% (27.8%) when measured 2 weeks postbooster dose compared with Group-3 (34.5%; *P* = 0.0001) and Group-4b (36.2%; *P* = 0.0001) (Table 1). The median age of ART initiation in Group-3 was 25.0 (18.4–40.0) weeks, and 65.3% (49/75) in this group had been initiated on ART by the time they received the first measles vaccine and 68 (98.6%) of the remaining 69 had been initiated on ART by the time of their booster dose of measles vaccine. Also, 67 (77.0%) of 87 Group-4a children had been re-initiated on ART by the time of their booster vaccine dose.

Prevaccination measles antibody kinetics

The proportion of HUU children who were sero-positive (78.3%) or had sero-protective titers (65.2%) was similar to HEU infants, but higher than in Group-3 (56.3 and 37.9%, respectively) and Group-4 (59.3 and 41.8%) (Fig. 2 and supplementary-Table S2, <http://links.lww.com/QAD/A321>). Also, GMTs were higher in HUU (963 mIU/ml) compared to Group-3 (657 mIU/ml, *P* = 0.04; Fig. 2) at enrollment. By 19.6 weeks of age, the proportion of children with sero-positive and sero-protective titers among HUU had declined to 28.9 and 16.7%, respectively; both higher than in Group-3 (5.7 and 0%) and Group-4 (16.1 and 7.2%) (Fig. 2 and supplementary-Table S2, <http://links.lww.com/QAD/A321>). There was no difference in the fold-decrease of antibody titers in HUU (0.49) compared with HEU (0.39, *P* = 0.21), Group-3 (0.31, *P* = 0.97) and Group-4 (0.42, *P* = 0.96) between enrollment and 19.6 weeks of age (supplementary-Table S2, <http://links.lww.com/QAD/A321>).

Antibody kinetics postprimary and booster doses of measles vaccine

The first measles vaccine was administered at 39.7 (SD $\pm$ 2.3) weeks of age. Twenty-eight weeks thereafter (study visit 3), the proportion of HUU children with sero-positive (93.8%) or sero-protective titers (91.1%) was similar to other groups, except for a lower proportion of Group-4a (79.3%; *P* < 0.0001) having sero-protective titers (Fig. 3 and supplementary-Table S3, <http://links.lww.com/QAD/A321>). This occurred despite 77.0% (67 of 87) Group-4a children already having been re-initiated on ART at this stage. Group-4a were also less likely to have sero-protective titers compared to Group-3 (95.7%; *P* = 0.003) and Group-4b (92.1%; *P* = 0.018;

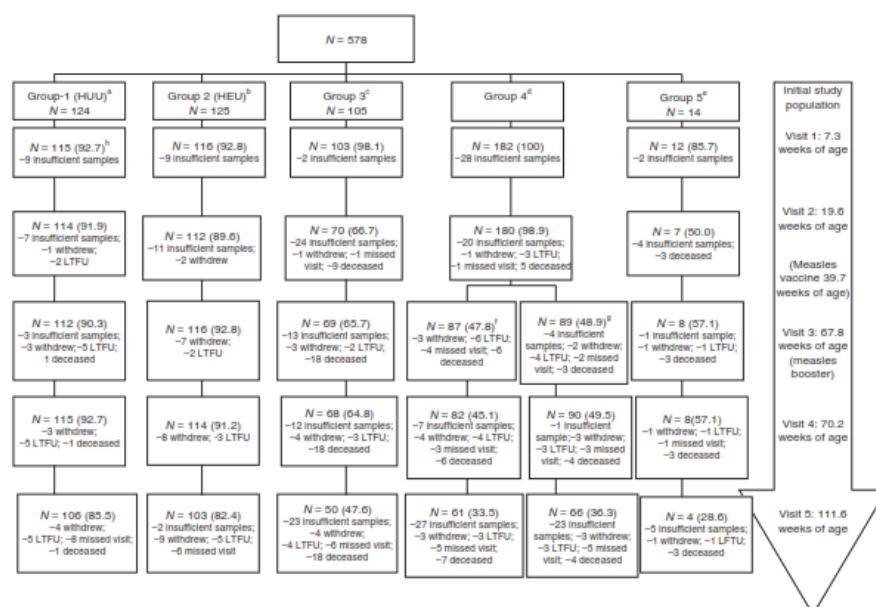


Fig. 1. Subject follow-up during the duration of the study. ^aHUU: HIV-uninfected children born to HIV-uninfected mothers (Group-1); ^bHEU: HIV-uninfected children born to HIV-infected mothers (Group-2); ^cGroup-3: HIV-infected children on deferred ART; ^dGroup-4: HIV-infected children on immediate ART; ^eGroup-5: HIV-infected children with CD4% less than 25% on immediate ART; ^fGroup-4a: HIV-infected children who interrupted ART at 12 months of age; ^gGroup-4b: HIV-infected children who continued ART till 24 months of age. Insufficient sample: sample unavailable for further testing; LTFU: loss to follow-up due to relocation or the child stopped the study without reason; Withdrawal: parent or guardian withdrew informed consent; Missed visit: children missed a scheduled visit; number of participants who left the study (LTFU, withdrawals, deceased) are represented as cumulative at each visit. ^hNumber in parenthesis are percentages of total enrolled; Group-4a and Group-4b combined: at visit 3 96.7%, at visit 4 94.5% and at visit 5 69.8%.

Fig. 3); and also had lower GMTs compared with each of the other Groups at this time-point (Fig. 3).

The booster dose was given at 67.8 (SD $\pm$ 4.4) weeks of age, after which the proportion of children with sero-protective titers was equal to or greater than 90.2% and did not differ between groups when evaluated 1.9 weeks (SD $\pm$ 0.1) later (Fig. 3 and supplementary-Table S3, <http://links.lww.com/QAD/A321>). The GMTs postbooster were higher in HUU (3124 mIU/ml) compared with HEU (2532 mIU/ml, $P=0.015$) and Group-4a (1435 mIU/ml, $P\leq 0.0001$) (Fig. 3). GMTs in Group-4a also remained lower compared with Group-3 (3254 mIU/ml, $P<0.0001$) and Group-4b (2740 mIU/ml, $P=0.002$) postbooster dose (Fig. 3). The proportion of Group-5 children with sero-protective titers was 100% postprimary and postbooster vaccine.

Persistence of antibody was evaluated 41.4 (S.D. $\pm$ 0.3) weeks following the booster dose, at approximately 111

weeks of age, by when HUU were more likely to have sero-protective titers (94.3%) compared with HEU (79.6%; $P=0.002$) and Group-4a (80.3%; $P=0.010$) (Fig. 3 and supplementary-Table S3, <http://links.lww.com/QAD/A321>). Although 75.4% (46/61) of Group-4a children had been re-initiated on ART by this time-point, they remained less likely to have sero-protective titers compared to Group-3 (96.0%; $P=0.013$) and similarly to Group-4b (86.4%; $P=0.360$). At the same time-point, GMTs were higher in HUU (2248 mIU/ml) compared to HEU (1773 mIU/ml, $P=0.004$) and Group-4a (1156 mIU/ml, $P<0.0001$) (Fig. 3). Group-4a also had lower GMTs compared with Group-3 (2577 mIU/ml) $P<0.0001$ and Group-4b (1924 mIU/ml, $P=0.009$).

Discussion

To our knowledge, this is the first study which systematically evaluated the kinetics of measles antibody

Table 1. Age and CD4⁺ lymphocyte percentage at different study time-points.

Characteristics	Group-1 (HUU) ^a	Group-2 (HEU) ^b	Group-3 ^c	Group-4 ^d	Group-5 ^e	Total	
Enrolment	N = 115	N = 116	N = 103	N = 182	N = 12	N = 528	
Mean age, weeks $\pm$ SD	6.9 $\pm$ 1.0	7.3 $\pm$ 1.0	7.3 $\pm$ 1.1	7.4 $\pm$ 1.3	8.4 $\pm$ 1.5	7.3 $\pm$ 1.2	
Mean CD4 ⁺ cell % $\pm$ SD	NA	NA	36.5 $\pm$ 1.1	35.4 $\pm$ 0.6	22.0 $\pm$ 1.9	NA	
Premeasles vaccination	N = 114	N = 112	N = 70	N = 180	N = 7	N = 483	
Mean age, weeks $\pm$ SD	19.3 $\pm$ 1.7	19.5 $\pm$ 1.1	19.7 $\pm$ 1.7	19.7 $\pm$ 1.6	20.7 $\pm$ 1.5	19.6 $\pm$ 1.6	
Mean CD4 ⁺ cell % $\pm$ SD	NA	NA	29.9 $\pm$ 1.0 ^f	41.6 $\pm$ 0.7 ^f	31.5 $\pm$ 2.1	NA	
1st measles vaccine	N = 115	N = 116	N = 75	N = 182	N = 10	497	
Mean age, weeks $\pm$ SD	38.9 $\pm$ 1.0	39.4 $\pm$ 1.6	40.8 $\pm$ 3.9	39.8 $\pm$ 2.0	41.5 $\pm$ 2.9	39.7 $\pm$ 2.3	
Mean CD4 ⁺ cell % $\pm$ SD	NA	NA	31.0 $\pm$ 0.9 ^f	39.1 $\pm$ 0.6 ^f	30.6 $\pm$ 2.7	NA	
Measles booster	N = 112	N = 116	N = 69	Group-4a ^g N = 87	Group-4b ^h N = 89	N = 8	N = 481
Mean age, weeks $\pm$ SD	67.2 $\pm$ 5.0	67.1 $\pm$ 2.2	69.3 $\pm$ 7.7	68.0 $\pm$ 1.9	67.8 $\pm$ 2.1	67.9 $\pm$ 1.5	67.8 $\pm$ 4.4
2 weeks postbooster	N = 115	N = 114	N = 68	N = 82	N = 90	N = 8	N = 477
Mean age, weeks $\pm$ SD	69.3 $\pm$ 5.4	69.4 $\pm$ 2.8	72.1 $\pm$ 6.8	69.7 $\pm$ 2.2	69.3 $\pm$ 2.2	69.4 $\pm$ 1.5	70.2 $\pm$ 5.4
Mean CD4 ⁺ cell % $\pm$ SD	NA	NA	34.5 $\pm$ 0.9 ⁱ	27.8 $\pm$ 0.9 ^j	36.2 $\pm$ 0.9 ^j	29.4 $\pm$ 3.4	NA
6 weeks postbooster	N = 115	N = 114	N = 68	N = 82	N = 90	N = 8	N = 477
Mean age, weeks $\pm$ SD	69.3 $\pm$ 5.4	69.4 $\pm$ 2.8	72.1 $\pm$ 6.8	69.7 $\pm$ 2.2	69.3 $\pm$ 2.2	69.4 $\pm$ 1.5	70.2 $\pm$ 5.4

NA, not available.

^aHUU, HIV-uninfected children born to HIV-uninfected mothers (Group-1).^bHEU, HIV-uninfected children born to HIV-infected mothers (Group-2).^cGroup-3, HIV-infected children on deferred ART.^dGroup-4, HIV-infected children on immediate ART.^eGroup-5, HIV-infected children with CD4% less than 25% on immediate ART.^f $P \leq 0.001$ comparing Group-3 vs. Group-4.^gGroup-4a, HIV-infected children who interrupted ART at 12 months of age.^hGroup-4b, HIV-infected children who continued ART till 24 months of age.ⁱ $P \leq 0.001$ comparing Group-3 vs. Group-4a and Group-4a vs. Group-4b. N = total number of children with available sample.

pre- and post-vaccination in HIV-infected children managed according to the current WHO recommendations of immediate initiation of ART upon diagnosis of HIV in infants; [12] and also the first study to examine the effect which interruption of ART in healthy 1-year old immunologically stable HIV-infected children had on measles antibody kinetics. Significant findings of our study included the high susceptibility to measles infection as early as 7 weeks of age in the majority of HIV-infected children and in more than 83% of all groups by 20 weeks of age. Also, interruption of ART in HIV-infected children (Group-4a) was associated with them being less likely to have sero-protective levels 28-weeks following the primary dose of measles-vaccine and less likely to maintain sero-protective titers 9.5 months after the booster dose. Furthermore, there was also waning of immunity in HEU children 9.5 months postbooster indicated by a lower proportion with sero-protective titers and lower GMTs compared with HUU. This despite, the proportion of HEU with sero-protective titers being similar compared with HUU children following the initial and immediately following the booster dose.

This high susceptibility to measles in children under 6 months is corroborated by other African studies in HIV-infected (85–91%) and HEU children [19,20], and more recently among healthy infants in developed countries [3]. In developed countries, the increased susceptibility to measles infection among young infants has been attributed to decrease in passive immunity

acquired from transplacental maternal measles antibody transfer, because of lower measles antibody levels in pregnant women who acquired immunity from vaccination rather than by natural infection [21]. Additional factors, which may contribute to the increased immunological susceptibility to measles infection in infants born to HIV-infected mothers compared with HUU infants, include lower measles antibody titers and impaired transplacental transfer of antibody in HIV-infected mothers [9]. Also, there may be greater decay of antibody in HIV-infected children [22]. The observation that GMTs and proportion of children with sero-protective titers were similar between HUU and HEU children suggests that factors other than solely maternal HIV positivity contributed to increased susceptibility to measles at an earlier age in HIV-infected children. Possible reasons include that mothers' of peripartum HIV-infected children had higher HIV viral load, which has been associated with increased perinatal HIV transmission and inversely associated with efficiency of trans-placental transfer of measles antibody [7,23,24].

The WHO has recommended that in settings of high HIV prevalence, infants should receive two doses of measles vaccine at 6 and 9 months of age [2]. This recommendation has, however, not been widely implemented; for example in South Africa the initial dose is still scheduled for 9 months of age. Reasons for this include the inherent low immunogenicity of measles vaccine in younger children, coupled with interference in the immune response by prevailing maternal-derived measles

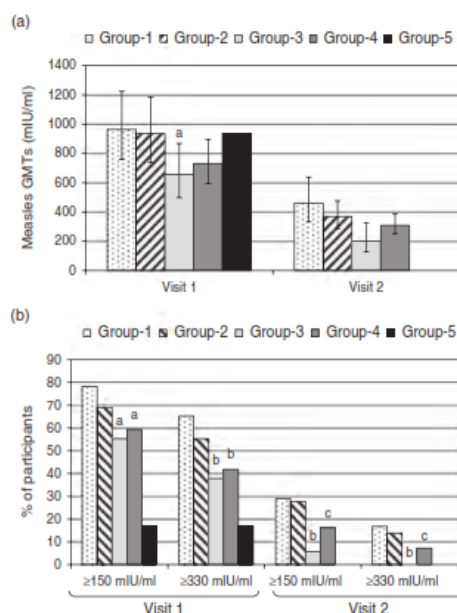


Fig. 2. Geometric mean titers and proportion of infants with sero-positive and sero-protective levels of measles antibody premeasles vaccination. Group-1(HUU): HIV-uninfected children born to HIV-uninfected mothers; Group-2 (HEU): HIV-uninfected children born to HIV-infected mothers; Group-3: HIV-infected children on deferred ART; Group-4: HIV-infected children on immediate ART; Group-5: HIV-infected children with CD4% less than 25% on immediate ART. (a) Measles geometric mean titers at visit 1 and visit 2. Vertical bars represent 95% CI. * P -value = 0.04 in relation to Group-1. (b) Proportion of infants sero-positive (≥ 150 mIU/ml) and sero-protected (≥ 330 mIU/ml) at visit 1 and visit 2. * P -value = 0.001 in relation to Group-1; ^b P -value < 0.001 in relation to Group 1; ^c P -value = 0.01 in relation to Group-1.

antibody [25–27]. Based on our study, the low prevalence of maternal-derived antibody in young infants (<30% at 20 weeks of age) supports immunization at 6 months of age. A recent report from Guinea-Bissau indicated that vaccination as early as 4.5 months of age using the Edmonston–Zagreb measles vaccine strain induced sero-protective antibody levels in 77% of infants, as well as being associated with 94% efficacy against measles infection [28].

A limitation of our study includes that immune response following the first dose of measles vaccine was only evaluated 8 months after vaccination, as earlier evaluation had not been planned *a priori* in the parent study. Consequently, although we were unable to undertake a direct comparison of immune responses in the overall

Group-4 compared with other Groups, Group-4b may be representative of the initial Group-4 cohort at least until 2 years of age. Persistence of antibody levels up until prior to the booster dose was similar in HUU compared with Group-4b, as well as the surviving children in Group-3. This indicated that the timing of ART did not affect immune responses to measles vaccine in those HIV-infected children with CD4⁺ more than 25% at enrolment. We have, however, previously reported higher early-childhood mortality rate in Group-3 compared with Group-4 children [18]. Additionally, 100% of Group-5 children also had persistence of sero-protective titers 8 months postprimary dose. The good immune responses in HIV-infected children on ART corroborate reports that initiation of ART in the first year of life allows the normal development of T-cell and B-cell immunity [29–31].

Although the proportion of Group-4a with sero-protective titers 2-weeks following the booster dose of vaccine was similar (>90%) to the other Groups, their GMTs were lower compared with other Groups immediately postbooster dose. Furthermore, greater waning of immunity was observed in Group-4a following the initial and after the booster-dose, despite them being carefully monitored for clinical and immunological disease progression following ART interruption at 12-months of age, resulting in ART being re-initiated in 77% of them at the time of the booster dose. CD4⁺ cell counts and percentage were however lower in Group-4a compared with Group-3 and Group-4b immediately following the booster dose. This indicated that interruption of ART in Group-4a resulted in waning of immunity, including possibly decline in anamnestic responses to measles. This may be explained by the deterioration of antigen specific pools of memory B cells in the absence of ART [31]. The proportion of Group-4a who had sero-protective titers at approximately 2-years of age was, however, higher compared with ART-naïve HIV-infected children who had been vaccinated at 6 and 9 months of age and assessed at 20 months of age (41%) as reported by Fowlkes *et al.* [32].

In exploring the effect of HIV exposure on persistence of measles antibody following vaccination, we observed that GMTs were higher in HEU compared with HUU children prior to the booster dose. Lower prevaccination antibody levels in HEU children have been previously reported to be associated with more robust immune responses to some vaccines during early childhood [33]. This was, however, unlikely to be the reason for the higher GMTs in HEU in our study as prevaccination measles antibody levels were similar to that of HUU children. The clinical relevance of the higher GMTs in HEU is unclear as the proportion of children with sero-protective titers was similar between the two groups. The higher GMTs in HEU children prior to the booster dose is also difficult to explain in the context of lower GMTs

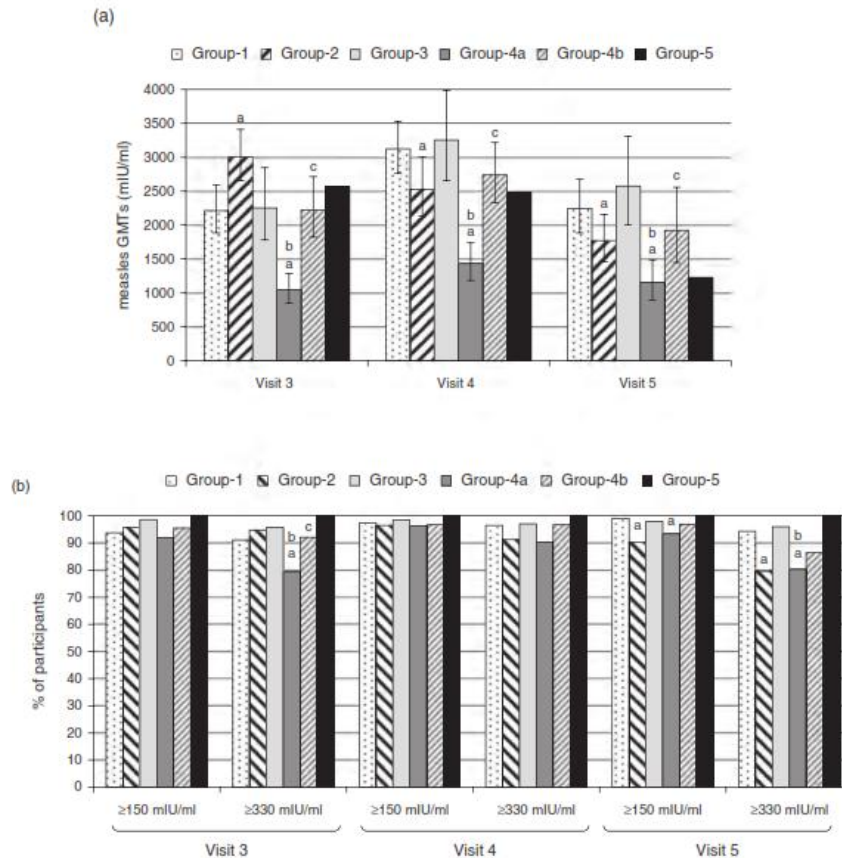


Fig. 3. Geometric mean titers and proportion of infants with sero-positive and sero-protective levels of measles antibody following primary and booster vaccine. Group-1(HUU): HIV-uninfected children born to HIV-uninfected mothers; Group-2 (HEU): HIV-uninfected children born to HIV-infected mothers; Group-3: HIV-infected children on deferred ART; Group-4: HIV-infected children on immediate ART; Group-5: HIV-infected children with CD4% less than 25% on immediate ART. Group-4a: HIV-infected children who interrupted ART at 12 months of age; Group-4b: HIV-infected children who continued ART till 24 months of age. (a) Measles geometric mean titers at visit 3 (prebooster), visit 4 (1.9 weeks postbooster) and visit 5 (41.4 weeks postbooster). Vertical bars represent 95% confidence interval. ^a*P*-value <0.02 in relation to Group-1; ^b*P*-value <0.001 in relation to Group-3; ^c*P*-value <0.01 in relation to Group-4a. (b) Proportion of infants sero-positive (≥ 150 mIU/ml) and sero-protected (≥ 330 mIU/ml) at visit 3, visit 4 and visit 5. ^a*P*-value <0.04 in relation to Group-1; ^b*P*-value <0.02 in relation to Group-3, ^c*P*-value = 0.02 in relation to Group-4a.

being observed 2-weeks thereafter in this Group compared with prebooster levels, as well as relative to HUU children postbooster. Lower GMTs persisted in HEU compared with HUU until 9.5 months postbooster, at which time HEU were also less likely to be sero-positive or have sero-protective titers. The underlying immune aberration which may cause waning of immunity in HEU, as well as the need for

further booster doses of vaccine in these children remain to be explored.

Limitations of our study included only having a convenience sample of HIV-infected children with CD4⁺ less than 25% at screening (i.e. Group-5). Nevertheless, the observations in this group were similar to those of HIV-infected children on ART (i.e. Group-4b),

suggesting that CD4⁺ percentage categorization at enrolment did not have an effect of immune responses to measles vaccine in children who had been initiated on ART upon confirmation of being HIV-infected. Furthermore, our study involved measuring measles antibody by ELISA as a surrogate of protection, which have been shown to miss low antibody level responses [34], rather than the plaque reduction neutralization assay which correlates more strongly with protection [35]. We may therefore have over-estimated the protection against measles infection, particularly among HIV-infected children in whom poorer IgG avidity may affect neutralizing activity which has, however, only been observed in the absence of ART [36].

In conclusion, our study indicates that persistence of maternal measles antibody does not any longer appear to be an impediment to measles vaccination by 6 months of age, possibly with the Edmonston–Zagreb vaccine strain, irrespective of the child's HIV infection status [11]. Reasonably good responses have been observed in ART-naïve HIV-infected children when immunized with an Edmonston–Zagreb vaccine strain as early as 6 months, followed by a booster dose at 12 months of age [37]. Sustainability of protection and need for further doses of measles vaccine in HIV-infected children in whom ART has been interrupted and in HEU remains to be explored, as does the issue of sustainability of protection in HIV-infected children who are maintained on ART.

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Conflicts of interest

There are no conflicts of interest.

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Table S1: CD4+ lymphocyte count at separate study time-points

Characteristics	Group-1 (HUU) ^a	Group-2 (HEU) ^b	Group-3 ^c	Group-4 ^d		Group-5 ^e	Total
Enrolment	N=115	N=116	N=103	N=182		N=12	N=528
Median CD4 ⁺ x10 ⁶ cells/mL (IQR) ^f	NA	NA	2303 (2090-2516)	2304 (2142-2468)		1617 (1208-2027)	NA
Pre measles vaccination	N=114	N=112	N=70	N=180		N=7	N=483
Median CD4 ⁺ x10 ⁶ cells/mL (IQR)	NA	NA	1805 (1586-2025) ^g	2346 (2202-2491) ^g		2065 (1412-2718)	NA
1st Measles vaccine	N=115	N=116	N=75	N=182		N=10	497
Median CD4 ⁺ x10 ⁶ cells/mL (IQR)	NA	NA	1683 (1521-1846) ^g	2252 (2113-2391) ^g		1802 (1457-2149)	NA
				Group-4a ^h	Group-4b ^h		
2 weeks post-booster	N=115	N=114	N=68	N=82	N=90	N=8	N=477
Median CD4 ⁺ x10 ⁶ cells/mL (IQR)	NA	NA	1823 (1657-1987) ^g	1364 (1212-1517) ^g	1770 (1597-1942) ^g	1434 (872-1995)	NA

^aHUU: HIV-uninfected children born to HIV-uninfected mothers (Group-1); ^bHEU: HIV-uninfected children born to HIV-infected mothers (Group-2); ^cGroup-3: HIV-infected children on deferred ART; ^dGroup-4: HIV-infected children on immediate ART; ^eGroup-5: HIV-infected children with CD4% <25% on immediate ART; ^fps0.001 comparing Group-3 vs. Group-4; ^gGroup-4a: HIV-infected children who interrupted ART at 12 months of age; ^hGroup-4b: HIV-infected children who continued ART till 24 months of age; ⁱps0.001 comparing Group-3 vs. Group-4a to and Group-4a vs. Group-4b. N= total number of children with available sample; SD= standard deviation; IQR= inter-quartile range (25-75th Centile); NA= not available

Table S2: Geometric mean titers and proportion of infants with sero-positive and sero-protective levels of measles antibody pre-measles vaccination

Visit	Measure	Group-1 (HUU) ^a	Group-2 (HEU) ^b	p-value HUU vs. HEU	Group-3 ^c	Group-4 ^d	Group-5 ^e	p-value HUU vs. G3	p-value HUU vs. G4	p-value G3vs. G4
Visit 1 (Mean age: 7.3 weeks)		N=115	N=116		N=103	N=182	N=12			
	GMT (95% CI) ^f	963 (759-1223)	935 (737-1186)	0.861	657 (497-869)	731 (596-896)	938 (43-20623)	0.04	0.08	0.54
	N (%) ≥150mIU/ml ^g	90 (78.3)	80 (69.0)	0.109	57 (55.3)	108 (59.3)	2 (16.7)	0.001	0.001	0.62
	N (%) ≥330mIU/ml ^h	75 (65.2)	64 (55.2)	0.119	39 (37.9)	76 (41.8)	2 (16.7)	0.0002	<0.0001	0.52
Visit 2 (Mean age: 19.6 weeks)		N=114	N=112		N=70	N=180	N=7			
	GMT (95% CI)	461 (332-639)	369 (287-476)	0.284	202 (126-324)	309 (249-385)	0 (0)	0.09	0.05	0.16
	N (%) ≥150mIU/ml	33 (28.9)	31 (27.7)	0.832	4 (5.7)	29 (16.1)	0 (0.0)	<0.0001	0.01	0.01
	N (%) ≥330mIU/ml	19 (16.7)	16 (13.8)	0.621	0 (0.0)	13 (7.2)	0 (0.0)	<0.0003	0.01	0.02
	GMT fold-change ⁱ	0.49	0.39	0.205	0.31	0.42	1.0	0.969	0.964	0.935

^aHUU: HIV-uninfected children born to HIV-uninfected mothers (Group-1); ^bHEU: HIV-uninfected children born to HIV-infected mothers (Group-2); ^cGroup-3: HIV-infected children on deferred ART; ^dGroup-4: HIV-infected children on immediate ART; ^eGroup-5: HIV-infected children with CD4% <25% on immediate ART; ^fGMT (95% CI) = geometric mean titer with 95% confidence interval in parenthesis; ^g≥150mIU/ml= proportion sero-positive for measles antibody; ^h≥330mIU/ml= proportion with sero-protective titers; ⁱFold-change in GMT from visit 1 to visit 2.

Table S3: Measles-antibody titers following primary and booster-vaccine stratified by HIV-exposure and HIV-infection status

Measure	Group-1 (HUU) ^a	Group-2 (HEU) ^b	p-value HUU vs. HEU	Group-3 ^c	p-value HUU vs. Group-3	Group-4a ^d	p-value HUU vs. Group-4a	Group-4b ^e	p-value HUU vs. Group-4b	Group-5 ^f	p-value Group-3 vs. Group-4a	p-value Group-3 vs. Group-4b	p-value Group-4a vs. Group-4b
Visit 3	N=112	N=116		N=69		N=87		N=89		N=8			
GMT (95% CI) ^g	2212 (1888-2591)	3009 (2653-3412)	0.008	2253 (1782-2847)	0.893	1044 (849-1283)	<0.0001	2224 (1822-2717)	0.963	2566 (1188-5542)	<0.0001	0.936	<0.0001
N (%) ≥150mIU/ml ^h	105 (93.8)	111 (95.7)	0.512	68 (98.6)	0.127	80 (91.9)	0.623	85 (95.5)	0.587	8 (100.0)	0.06	0.278	0.330
N (%) ≥330mIU/ml ⁱ	102 (91.1)	110 (94.8)	0.267	66 (95.7)	0.246	69 (79.3)	<0.0001	82 (92.1)	0.116	8 (100.0)	0.003	0.368	0.018
Visit 4	N=115	N=114		N=68		N=82		N=90		N=8			
GMT (95% CI)	3124 (2763-3533)	2532 (2134-3005)	0.015	3254 (2656-3988)	0.717	1435 (1185-1740)	<0.0001	2740 (2334-3217)	0.190	2480 (1175-5233)	<0.0001	0.181	0.002
N (%) ≥150mIU/ml	112 (97.4)	110 (96.5)	0.692	67 (98.5)	0.611	79 (96.3)	0.673	87 (96.7)	0.760	8 (100.0)	0.408	0.46	0.908
N (%) ≥330mIU/ml	111 (96.5)	104 (91.2)	0.095	66 (97.1)	0.844	74 (90.2)	0.069	87 (96.7)	0.955	8 (100.0)	0.096	0.89	0.091
Fold-change ^j Visit 3 to Visit 4	1.4	0.95	0.0003	1.44	0.697	1.37	0.969	1.23	0.067	0.96	0.761	0.250	0.144
Visit 5	N=106	N=103		N=50		N=61		N=66		N=4			
GMT (95% CI)	2248 (1888-2676)	1773 (1459-2157)	0.004	2577 (2005-3311)	0.379	1156 (898-1488)	<0.0001	1924 (1448-2557)	0.331	1230 (253-5990)	<0.0001	0.143	0.009
N (%) ≥150mIU/ml	105 (99.1)	93 (90.3)	0.005	49 (98.0)	0.584	57 (93.4)	0.040	64 (96.9)	0.309	4 (100)	0.249	0.729	0.349
N (%) ≥330mIU/ml	100 (94.3)	82 (79.6)	0.002	48 (96.0)	0.079	49 (80.3)	0.010	57 (86.4)	0.071	4 (100)	0.013	0.013	0.360
Fold-change Visit 4 to Visit 5	0.72	0.70	0.809	0.79	0.482	0.80	0.637	0.70	0.288	0.50	0.854	0.837	0.669

^aHUU: HIV-uninfected children born to HIV-uninfected mothers (Group-1); ^bHEU: HIV-uninfected children born to HIV-infected mothers (Group-2);

^cGroup-3: HIV-infected children on deferred ART; ^dGroup-4a: HIV-infected children who interrupted ART at 12 months of age; ^eGroup-4b: HIV-

infected children who continued ART till 24 months of age; ^fGroup-5: HIV-infected children with CD4% <25% on immediate ART; ^gGMT (95% CI)=

geometric mean titer with 95% confidence interval in parenthesis; ^h≥150mIU/ml= proportion sero-positive for measles antibody; ⁱ≥330mIU/ml=

proportion with sero-protective titers; ^jFold-change in GMT.

APPENDIX 3: OTHER TRIALS REFERRED TO IN THE STUDY

HREC reference number: 040703

Abbreviated title: CIPRA 2

Trial title: A randomized trial to evaluate strategies for providing antiretroviral therapy to infants shortly after primary infection in a resource poor setting.

Date of initial approval: Ethics: 24 August 2004 MCC: 21 September 2004

HREC reference number: 040704

Abbreviated title: CIPRA 4

Trial title: Evaluation of quantitative and qualitative antibody responses to *Streptococcus pneumoniae* and *Haemophilus influenza* type B conjugate vaccines amongst HIV-1-exposed-infected children that are receiving vs. those not receiving antiretroviral therapy, as well as among HIV-1-exposed-uninfected children and HIV-1-unexposed-uninfected children.

Date of initial approval: Ethics: 30 August 2004 MCC: 8 November 2004

HREC reference number: 031013

Abbreviated title: Durability study

Trial title: Durability of antibody response and measurement of anamnesis responses to a nanovalent Pneumococcal Conjugate Vaccine among HIV-infected and uninfected children

Date of initial approval: 08 December 2003

APPENDIX 4: METHODOLOGY SECTION

5.1. STUDY VACCINE FORMULATION.

The vaccines' antigen composition is summarized in the Table 5.1 below. Commercial batches of CombAct-Hib were manufactured by Sanofi Pasteur, France the vaccine was prepared at the time of injection by reconstituting freeze-dried PRP-T with DTwP suspension (0.5ml). Heberbiovac HB was obtained commercially and was manufactured by HerberBiotec S.A., Cuba and came in 0.5ml vials. Commercial Live-attenuated measles vaccine (Rouvax®) was manufactured by Aventis, France and was reconstituted with 0.5ml injection water prior to use.

Table 5.1: Antigen composition of study vaccines

Antigen	CombAct-Hib	Heberbiovac HB	Rouvax
Purified DT	≥30IU	-	-
Purified TT	≥60IU	-	-
wPertussis	≥4 IU ^a	-	-
Purified recombinant HBsAg	-	10µg	-
PRP-T	10µg	-	-
Schwarz strain of live-attenuated measles virus	-	-	1000 CCID ₅₀ ^b

Footnote: CombAct-Hib, Heberbiovac HB and Rouvax administered as 0.5 mL doses. ^a A *Bordetella pertussis* inactivated suspension, potency measured by the Kendrick test (mice intracerebral challenge assay). ^b CCID₅₀, 50% cell culture infective doses (viral infectious units). DT indicates diphtheria toxoid; TT, tetanus toxoid; wPertussis, whole cell pertussis; HBsAg, HeberBiotec S.A. recombinant hepatitis B surface antigen; PRP-T, *Haemophilus influenzae* type b polysaccharide conjugated to tetanus protein

5.2 EQUIPMENT, CONSUMABLES, CHEMICALS AND REAGENTS UTILIZED IN THE STUDY.

Table 5.2: List of equipment and consumables

Bio-Plex components	Manufacturer	Maintenance/servicing
Array reader	Bio-Rad Laboratories Hercules, CA	Daily
Plate reader	Bio-Rad Laboratories Hercules, CA	Daily
HTF system	Bio-Rad Laboratories Hercules, CA	Annually
PC with Bio-Plex manager software version 4.1.1	Bio-Rad Laboratories, Hercules, CA	NA
Waste bottle	Bio-Rad Laboratories Hercules, CA	Daily
Sheath fluid bottle	Bio-Rad Laboratories Hercules, CA	Daily
Bio-Plex vacuum pump and plate holder	Bio-Rad Laboratories Hercules, CA	Weekly and monthly
MCV Plate IV	Bio-Rad Laboratories Hercules, CA	NA

Other equipment	Manufacturer	
Ultra low freezer (-70°C)	SS engineering, Fontainebleau	NA
Refrigerator (+2 to +8°C)	Staycold, Parys South Africa	Annually
Freezer (-20°C)	Defy, Benrose South Africa	Annually
Orbital mixer	Thermo mixer Mxi4t, Fine PCR, Korea	Monthly
Ice flaker	Scotsman, Italy	
Micro-centrifuge	Boeco, Boeckel and Co, Germany	Monthly
Vortex	Fine vortex, Fine PCR, Velp Scientifica s.r.l., Italy	Annually
Timer	Bio-Rad Laboratories Hercules, CA	NA
Single channel pipettes: 5-50µl, 20-200µl, 200-1000µl, 1-10 ml	Nichipet, Japan	6 months
Multi-channel pipettes: 5-50µl, 50-300µl	Finnpipette, Thermoscientific,	6 months
Automatic pipettes: BIOHIT electronic 1200 single and multi-channel	Sartorius BIOHIT, Helsinki, Finland	6 months
pH meter and electrode	Jenway, England	monthly
Balance	Ohaus Corporation, USA	monthly
Microscope	Leica Microsystems, Heerbrugg	monthly
hemocytometer slide	Merck, Darmstadt Germany	NA
+37°C incubator	Scientific, South Africa	Every 2 years
+37°C incubator under 5% CO ₂	Sanyo Electric Biomedical, Japan	Every 2 years
Waterbath	Scientific South Africa	Annually
Autoclave	Gemmy Industrial Corp, Taiwan	Annually
Microplate reader	Labsystems Multiskan RC, USA	Annually
Microplate washer	Molecular Devices, CA USA	Annually
Liquid Nitrogen chamber	Vacuum Cryogenic Technology, South Africa	NA
Consumables	Manufacturer	Storage
Tips: 10, 100, 1000µl	AEC Amersham	RT
Tips: 5ml, 10ml and 25ml	LP Italiano Spa, Italy	RT
5ml tubes with lids	AEC Amersham	RT
1.5ml Microcentrifuge tubes	Whitehead Scientific	RT
Erlemeyer flasks	Schott Duran, Germany	RT
Graduated cylinders: 50ml, 100ml, 1000ml and 2000ml	Schott Duran, Germany	RT
50µl centrifuge tubes	Corning	RT
1000 and 2000ml bottles	Schott Duran, Germany	RT
Polypropylene cryovials (2ml)	Thermoscientific Nalgene, USA	RT
Stericup filter unit (500ml, 0.22µm)	Millipore, Massachusetts	RT
Culture flasks (160ml)	Nunc, Denmark	RT
Aluminium foil	Various	RT

Gloves	Medic-Dent™ Supermax, Malaysia	RT
Multi-well filter plates	Pall Life Sciences, USA	RT
96 well round bottom plate	Sterilin® Limited, UK	RT
96 well flat bottom plates with lids	Nunc, Denmark	RT

Table 5.3: List of antigens, chemicals and reagents for Luminex assay

Antigen and Beads	Manufacturer	Code/Batch	Stock concentration	Storage
Carboxylated microspheres (beads)	BioRad Laboratories, Hercule CA	Several	12x10^6 beads/ml	+2 to +8°C
Diphtheria toxoid (DT)	Staten Serum Institute, Denmark	D52-2	1911 Lf/ml	+2 to +8°C as powder -70°C reconstituted
Tetanus toxoid (TT)	Staten Serum Institute, Denmark	T177-1	774 Lf/ml	+2 to +8°C as powder -70°C reconstituted
Pertussis toxin (PT)	List biological Laboratories, Inc., USA	181209A1	50µg	+2 to +8°C as powder -70°C reconstituted
Filamentous Hemagglutinin (FHA)	List biological Laboratories, Inc., USA	17036AI	50µg	+2 to +8°C as powder -70°C reconstituted
Hepatitis B surface antigen (HBsAg)	Donated by Glaxo-Smith Kline biological SA, Belgium	08-011	14.99mg	+2 to +8°C
Conjugate				
R-phycoerthyrin (R-PE)-conjugated goat anti-human IgG (γ-chain specific)	Jackson ImmunoResearch Laboratories Inc. Westgrove, PA USA	109-115-098	0.5mg/ml	+2 to +8°C
Phosphate buffered saline 1x (PBS), pH 7.2±0.2	RMPRU	1xPBS		RT
Activation buffer	Manufacturer	Code/Batch	Storage	
1x PBS, pH6.1±0.2	RMPRU	1xPBS-6.1	RT	
N-hydroxy-sulphosuccinimide	Pierce, Rockland IL	sulfo-NHS	+2-+8°C	
1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide hydrochloride	Pierce, Rockland IL	EDC	+2-+8°C	
Washing Buffer				
1x PBS, pH 7.2±0.2	RMPRU	1xPBS	RT	

Tween 20 (T)	Merck	8.22184.0500	
Sodium Azide (N)	Sigma-Aldrich	S 2002-100G	
Microbead coating buffer			
1x PBS, pH 7.2±0.2	RMPRU	1xPBS	RT
Fetal Bovine Serum (F) (1% v/v)	Adcock Ingram	10270	+2 to +8°C
Sodium Azide (N) (0.05% w/v)	Sigma-Aldrich	S 2002-100G	RT
Assay buffer (1xPBS-FN)			
1x PBS, pH 7.2±0.2	RMPRU	1xPBS	RT
Fetal Bovine Serum (F) (10% v/v)	Adcock Ingram	10270	+2 to +8°C
Sodium Azide (N) (0.05% w/v)	Sigma-Aldrich	S 2002-100G	RT
Standards and controls			
In-house reference serum	RMPRU	Luminex ref	-70°C
Positive control (commercial pooled IgG- Hebagam)	Natal Bioproducts, South Africa	PC/2004/01P50	+2-+8°C
Negative control (1xPBS-FN)	RMPRU	1xPBS-FN	+2-+8°C

Table 5.4: List of chemicals and reagents for the Hib ELISA

Antigen	Manufacturer	Code/Batch	Storage
HbOHA (Hib polysaccharide conjugated to serum protein)	Wyeth vaccines, distributed by NIBSC	lot 17 #BAHBOHA)	-20°C
Coating buffer– PBS+Sodium azide (PBS-N)	RMPRU	PBS-N	RT
Dilution buffers			
1xPBS	RMPRU	1xPBS	RT
Fetal bovine serum (F 10% v/v)	Adcock-Ingram(Delta)	14-501-A1	-20°C
Washing reagents			
1xPBS	RMPRU	PBS-T	RT
Tween 20 (0.05% v/v)	Merck		
Distilled Water <0.2µS/cm	RMPRU		RT
Conjugate			
Goat Anti-human IgG Alkali Phosphatase	Sigma	A-3188	+4-8°C

conjugate			
Substrate			
p-nitrophenol phosphate disodium salt Hexahydrate 1.4 mg /ml in Carbonate buffer or p-nitrophenol phosphate disodium salt 1mg /ml in Carbonate buffer Carbonate buffer, pH 9.8	Fluka, Sigma Calbiochem, Merck RMPRU	#71768 #4876	+4-8°C +4-8°C +4-8°C
Stopping solution			
2-M NaOH	RMPRU		+22°C
Reference and controls			
Reference serum (Human anti- <i>H. influenzae</i> <i>type b</i>) High control (Commercial pooled IgG - Polygam) Low control (In house pool)	FDA, Bethesda Natal Bioproducts RMPRU	Lot 1983	-20°C -20°C -20°C
External Quality Control Sera			
QC plasma 1 (Human anti-Hib polysaccharide, 0.43µg/ml)	FDA, Bethesda	lot 1986A	-20°C
QC plasma 2 (Human anti-Hib polysaccharide, 0.22µg/ml)	FDA, Bethesda	lot 1986B	-20°C
QC plasma 3 (Human anti-Hib polysaccharide, 0.11µg/ml)	FDA, Bethesda	lot 1986C	-20°C
QC plasma 4 (Human anti-Hib polysaccharide, 0.65µg/ml)	FDA, Bethesda	lot 1986D	-20°C
QC plasma 5 (Human anti-Hib polysaccharide, 4.00µg/ml)	FDA, Bethesda	lot 1987E	-20°C

Table 5.5: List of reagents and consumable for Hib bactericidal assay

Reagents	Manufacturer	Storage
H. influenza Type B, strain B3292	Donated by Sandra Romero-Steiner, CDC Atlanta	-70°C
Hank's Balanced Salt Solution w/o Ca ²⁺ and Mg ²⁺ (HBSS ⁻)	Life Technologies	+2-+8°C

10% Gelatine stock solution	RMPRU	+2-+8°C
100 x Ca ²⁺ /Mg ²⁺ stock solution	RMPRU	+2-+8°C
Dilution buffer		
44.5ml HANKS + 0.5ml Ca ⁺⁺ and Mg ⁺ + 5ml 1% gelatin	RMPRU	+2-+8°C
Baby Rabbit Complement (BRC)	Peel-Freez Biologicals	-70°C
Media		
Brain Heart Infusion (BHI) Broth	Difco	+2-+8°C
Fildes Enrichment	BBL™	+2-+8°C
Chocolate Agar	NHLS media	+2-+8°C

5.3. DETAILED STANDARDS OF OPERATION (SOP) FOR MEASUREMENT OF QUANTITATIVE AND FUNCTIONAL ANTIBODY RESPONSES.

5.3.1. Measurement of DT antibodies by quantitative ELISA (Anti-diphtheria toxoid IgG enzyme immunoassay (MK014))

Principle of the assay:

Microwells are pre-coated with diphtheria toxoid antigen. The calibrators, control and diluted samples are added to the wells and antibodies recognizing the diphtheria toxoid antigen bind during the first incubation. After washing the wells to remove unbound proteins, purified peroxidase labelled rabbit anti-human IgG conjugate is added. The conjugate binds to the captured human antibody and the excess unbound conjugate is removed by a further wash step. The bound conjugate is visualized with 3', 3', 5, 5' tetramethylbenzidine (TMB) substrate which gives a blue reaction product, the intensity of which is proportional to the concentration of antibody in the sample. Phosphoric acid is added to each well to stop the reaction. This produces a yellow end point colour, which is read at 450nm.

Materials

Components of anti-diphtheria toxoid IgG enzyme immunoassay (MK014) kit

Materials supplied	Composition
QC certificate	Indicates expected performance of the batch and is lot specific
Diphtheria toxoid coated wells	12 break apart 8 well strips coated with diphtheria toxoid derived from <i>Corynebacterium diphtheria</i> .

Sample diluent	50ml buffer for sample dilution. Ready to use
Wash buffer	50ml of 20-fold concentrated buffer for washing the wells. Add 50ml wash buffer to 950ml of distilled water (1 in 220 dilution) and store at room temperature.
Diphtheria toxoid IgG calibrators	7 bottles each containing 1.2ml diluted human serum, with the following concentrations of anti-diphtheria toxoid antibody: 3, 1, 0.33, 0.111, 0.037, 0.012, 0.004IU/ml. Ready to use
Diphtheria toxoid IgG high control	1 bottle containing 1.2ml of diluted human serum containing anti-diphtheria toxoid. The expected value is given on the QC card. Ready to use
Diphtheria toxoid IgG high control	1 bottle containing 1.2ml of diluted human serum containing anti-diphtheria toxoid. The expected value is given on the QC card. Ready to use
Diphtheria toxoid IgG conjugate	1 bottle containing 12ml of purified peroxidase labelled antibody to human IgG. Ready to use
TMB substrate	1 bottle containing 14ml TMB substrate. Ready to use
Stop solution	1 bottle containing 14ml of 3M phosphoric acid. Ready to use

Working phase

One hundred microliters (μ l) of calibrator (NIBSC diphtheria antitoxin reference preparation 00/496, range: 0.004-3 IU/ml), controls (high control range; 0.6-1.0 IU/ml and low control range: 0.1-0.2 IU/ml) and diluted sample (1:100 pre-dilution) was dispensed into appropriate antigen pre-coated wells (MK014) and incubated at room temperature for 30 minutes. After incubation the microwell plates were washed three times with 350 μ l of wash buffer (Working wash buffer was prepared by 1:20 dilution) per well, using an automated plate washer (EM-96PW-TECAN-1; Australia). After the final wash, the plates were inverted and tapped dry, before proceeding to the next step. One hundred microliters of purified peroxidase labelled rabbit anti-human IgG (γ chain specific) conjugate was dispensed into each well and incubated for 30 minutes at room temperature. The plates were washed as mentioned previously. After the wash step, 100 μ l of TMB substrate (para-nitrophenylphosphate, solvent free buffer and <0.1% sodium azide as preservative) was dispensed into each well, and incubated at room temperature in the dark for 30 minutes. To stop the reaction 100 μ l of stop solution (3M phosphoric acid) was added to each microwell, this resulted in a colour change from blue to yellow. The reaction was read at 450

nanometers (nm) on a plate reader (Labsystems Multiskan RC; USA) within 30 minutes of stopping of the reaction.

Interpretation of results

In order to accept the assay as valid, the following criteria have to be met:

- a. Reader settings: In order to accurately measure low samples, the manufacturer advised not to subtract blank optical density (OD) readings from the results, in addition ODs should be read at 450nm without the use of a reference filter.
- b. Quality control (QC): In order to accept the assay as valid; all the following criteria must be met:
 1. Calibrators and controls should have been included in each run.
 2. The value obtained for each control should be in the range specified on the supplied QC Certificate, which were 0.1-0.2IU/ml for the low control and 0.6-1.0IU/ml for the high control.
 3. The curve shape should have been similar to the calibration curve, shown on the QC Certificate (Figure 5.1).

If the above criteria are not met, the assay is invalid and the test should be repeated.

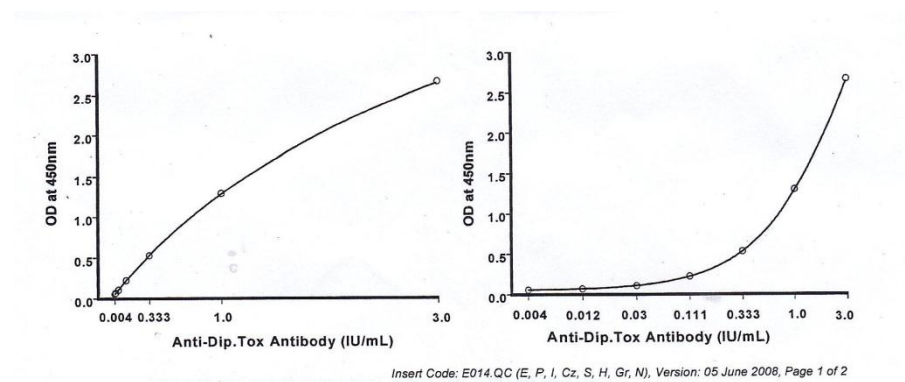


Figure 5.1: Two examples of calibration curves from the QC Certificate supplied by the manufacturer.

- c. Calculate mean optical densities: For each calibrator, control and sample the mean OD was calculated of the duplicate readings. The percentage coefficient of variation (%C.V.) for each duplicate OD should have been less than 15%.
- d. Plot calibration curve: The calibration curve was plotted automatically by use of GraphPad Prism 5a (GraphPad Software Inc, La Jolla, California) as follows: by plotting the anti-DT IgG antibody concentration on the log scale against the OD on the linear scale for each calibrator by choosing the curve fit that best fits the data.
- e. Treatment of anomalous point: If any one point does not lie on the curve, it can be removed. If the absence of this point means that the curve has a shape dissimilar to that of the sample calibration curve, or more than one point appears to be anomalous, then the assay should be repeated.
- f. Calculation of the control values: The anti-DT concentration of the controls should be read directly from the calibration curve and the values should be within the given ranges of the QC Certificate.
- g. Calculation of antibody levels in diluted samples: The anti-DT concentration of the diluted samples was read directly from the calibration curve. The calibrator values were adjusted by a factor of 100 to account for the 1:100 dilution of the sample, therefore no further correction was required.

5.3.2. Measurement of TT antibodies by quantitative ELISA (VaccZyme™ anti-tetanus toxoid IgG EIA (MK010)).

Principle of the assay

Microwells are pre-coated with tetanus toxoid antigen. The calibrators, control and diluted samples are added to the wells and antibodies recognizing the tetanus toxoid antigen bind during the first incubation. After washing the wells to remove unbound proteins, purified peroxidase labelled rabbit anti-human IgG conjugate is added. The conjugate binds to the captured human antibody and the excess unbound conjugate is removed by a further wash step. The bound conjugate is visualized with TMB substrate which gives a blue reaction product, the intensity of

which is proportional to the concentration of antibody in the sample. Phosphoric acid is added to each well to stop the reaction. This produces a yellow end point colour, which is read at 450nm.

Materials

Components of VaccZyme™ Anti-tetanus toxoid IgG EIA (MK010) kit.

Materials supplied	Composition
QC certificate	indicates expected performance of the batch and is lot specific
Diphtheria toxoid coated wells	12 break apart 8 well strips coated with tetanus toxoid derived from <i>Clostridium tetani</i> .
Sample diluent	50ml buffer for sample dilution. Ready to use
Wash buffer	50ml of 20-fold concentrated buffer for washing the wells. Add 50ml wash buffer to 950ml of distilled water (1 in 220 dilution) and store at room temperature.
Tetanus toxoid IgG calibrators	7 bottles each containing 1.2ml diluted human serum, with the following concentrations of anti-tetanus toxoid antibody: 7, 2.33, 0.78, 0.26, 0.09, 0.03, 0.01IU/ml. Ready to use
Tetanus toxoid IgG high control	1 bottle containing 1.2ml of diluted human serum containing anti-diphtheria toxoid. The expected value is given on the QC card. Ready to use
Tetanus toxoid IgG high control	1 bottle containing 1.2ml of diluted human serum containing anti-diphtheria toxoid. The expected value is given on the QC card. Ready to use
Tetanus toxoid IgG conjugate	1 bottle containing 12ml of purified peroxidase labelled antibody to human IgG. Ready to use
TMB substrate	1 bottle containing 14ml TMB substrate. Ready to use
Stop solution	1 bottle containing 14ml of 3M phosphoric acid. Ready to use

Working phase

One hundred microliters of calibrator (NIBSC tetanus antitoxin reference preparation 76/589; measurable range: 0.01-7 IU/ml), controls (High control (range: 1.05-1.75 IU/ml) and low control (range: 0.15-0.35 IU/ml) and diluted sample (pre-diluted 1:100) were dispensed into appropriate wells and incubated at room temperature for 30 minutes. After incubation the plates were washed three times with 350µl of wash buffer per well, using an automated plate washer (EM-96PW-TECAN-1; Australia) to remove all unbound antibodies. After the final wash, plates were inverted and tapped dry, before proceeding to the next step. One hundred microliters purified peroxidase labelled rabbit anti-human IgG (γ chain specific) conjugate was dispensed into each well and incubated for 30 minutes at room temperature. The plates were washed as previously

described. After the wash step, 100µl of TMB substrate was dispensed into each well, and incubated at room temperature in the dark for 30 minutes. To stop the reaction 100µl of stop solution (3M phosphoric acid) was added to each well, this resulted in a colour change from blue to yellow. The reaction was read at 450nm with a plate reader (Labsystems Multiskan RC; USA) within 30 minutes of stopping the reaction.

Interpretation of results

In order to accept the assay as valid, the following criteria had to be met:

- a. Reader settings: In order to accurately measure low samples, the manufacturer advised not to subtract blank OD readings from the results, in addition ODs should be read at 450nm without the use of a reference filter.
- b. QC: In order to accept the assay as valid; all the following criteria must have been met:
 1. Calibrators and controls must have been included in each run.
 2. The value obtained for each control should have been in the range specified on the supplied QC Certificate, which were 0.15-0.35 IU/ml for the low control and 1.05-1.75 IU/ml for the high control.
 3. The curve shape should have been similar to the calibration curve, shown on the QC Certificate (Figure 5.2).

If the above criteria were not met, the assay was invalid and the test should have been repeated.

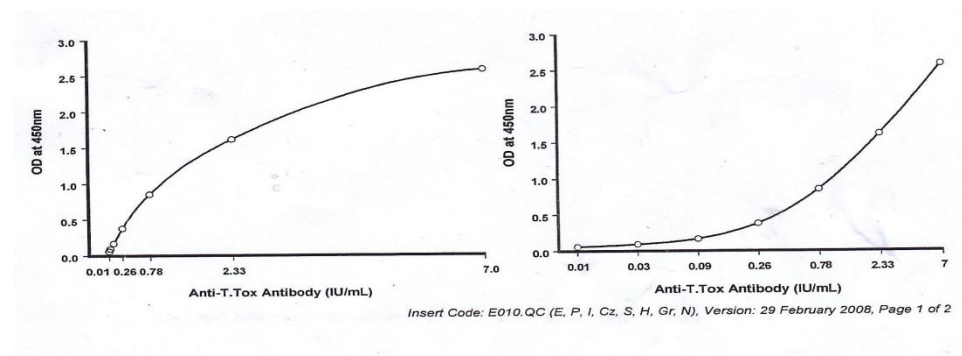


Figure 5.2: Two examples of the anti-TT calibration curves from the QC Certificate supplied by the manufacturer.

c. Calculate mean optical densities: For each calibrator, control and sample the mean OD was calculated of the duplicate readings. The %C.V. for each duplicate OD should have been less than 15%.

d. Plot calibration curve: The calibration curve was plotted automatically by use of GraphPad Prism 5a (GraphPad Software Inc, La Jolla, California) as follows: by plotting the anti-TT IgG antibody concentration on the log scale against the OD on the linear scale for each calibrator by choosing the curve fit that best fits the data.

e. Treatment of anomalous points: If any one point does not lie on the curve, it can be removed. If the absence of this point means that the curve has a shape dissimilar to that of the sample calibration curve, or more than one point appears to be anomalous, then the assay should be repeated.

f. Calculation of the control values: The anti-TT concentration of the controls should be read directly from the calibration curve and the values should be within the given ranges of the QC Certificate.

g. Calculation of antibody concentration in diluted samples: The anti-TT concentration of the diluted samples was read directly from the calibration curve. The calibrator values were adjusted by a factor of 100 to account for the 1:100 dilution of the sample, therefore no further correction was required.

5.3.3. Measurement of PT and FHA antibodies by quantitative ELISA (SERION ELISA classic *Bordetella pertussis* IgG (ESR120G)).

Principle of the assay

Microwells are pre-coated with pertussis toxin and filamentous hemagglutinin antigens, which constituted the solid phase. The standard serum, control and diluted samples are added to the wells and antibodies recognizing these antigens bind during the first incubation. After washing the wells to remove unbound proteins, purified alkaline phosphatase is added. The conjugate

binds to the captured human antibody and the excess unbound conjugate is removed by a further wash step. The bound conjugate is visualized with para-nitrophenylphosphate (pNPP) substrate which gives a yellow reaction product, the intensity of which is proportional to the concentration of antibody in the sample. Sodium hydroxide is added to each well to stop the reaction. This produces a yellow end point colour, which is read at 405nm with a reference wavelength between 620nm and 690nm.

Materials

Components of SERION ELISA classic *Bordetella pertussis* IgG (ESR120G) kit.

Material	Composition
Quality control certificate with standard curve and evaluation table	quantification of antibodies in IU/ml or U/ml
PT and FHA coated microtiter strips	Break apart microtiter test strips with 8 antigen coated single wells (altogether 12 strips), the coating material is inactivated.
Standard serum	Human serum in phosphate buffer with protein; negative for anti-HIV, HBsAg, and anti-HCV antibodies; preservative <0.1% sodium azide; colouring aminaranth O. Ready to use
Negative control serum	Human serum in phosphate buffer with protein negative for anti-HIV, HBsAg, and anti-HCV antibodies; preservative <0.1% sodium azide; colouring Lissamin green V. Ready to use
Anti-human IgG conjugate	Anti-human IgG from goat (polyclonal), conjugated to alkaline phosphatase, stabilized with protein stabilization solution; preservatives: 0.01% methylisothiazole, 0.01% Bromnitrodioxide. Ready to use
Washing solution concentrate	Sodium chloride solution with Tween 20, 30nM Tris, preservative: <0.1% sodium azide.
Dilution buffer	Phosphate buffer with protein and Tween 20, preservative: <0.1% sodium azide and 0.01g/L Bromophenol blue sodium salt.
Stopping solution	1.2N sodium hydroxide
Substrate	para-nitrophenylphosphate, solvent free buffer, preservative: <0.1% sodiumazide

Working phase

Prior to running the test, patient samples were diluted as follows: 10µl patient sample in 1000µl dilution buffer (phosphate buffer with protein and Tween 20, <0.1% sodium azide and 0.01g/l bromophenol blue sodium salt as preservative) and mixed thoroughly to prepare a homogenous solution. One hundred microliters of duplicate pre-diluted sample, negative control (human

serum in phosphate buffer with protein and <0.1% sodium azide as preservative and Lissamin green V as colouring) and Standard serum (human serum in phosphate buffer with protein, <0.1% sodium azide as preservative and amaranth O as colouring) were dispensed into appropriate wells pre-coated with PT and FHA, and incubated for 60 minutes under humid conditions at 37°C. Wells were washed three times with 300µl of wash buffer (chloride solution with Tween-20, 30mM Tris and <0.1% sodium azide as preservative) per well. After the wash step, 100µl of Anti-human IgG-conjugate (anti-human-IgG from goat (polyclonal), conjugated to alkaline phosphatase, stabilized with protein stabilization solution, 0.01% methylisothiazolone and 0.01% bromnitrodioxane as preservative) was added to all wells except for the substrate blank well, and incubated at 37°C for 30 minutes under humid conditions. Plates were washed three times as previously described. Followed by the addition of 100µl of substrate (para-nitrophenylphosphate, solvent free buffer and <0.1% sodium azide as preservative) into all the wells including the substrate blank well, and incubated at 37°C for 30 minutes under humid conditions. The reaction was stopped by the addition of 100µl of stopping solution (1.2N sodium hydroxide) and the plates were gently shaken. The plates were read at 405nm with a plate reader (Labsystems Multiskan RC; USA) against the substrate blank.

Interpretation of results

Substrate blank must be OD <0.25. The mean OD value of the standard serum must be within the validity range (OD 0.48-1.63), which was given on the lot specific QC certificate (Lot number: SKY.CX) of the kit (after subtraction of the substrate blank). The percentage variation of OD was to be less than 20%. Antibody concentration in the samples was calculated using the SERION *evaluate*-Software, which operates by inputting the 4-parameters and the reference value of the standard serum, antibody activity were calculated online.

5.3.4. Measurement of HBsAg antibodies by quantitative ELISA (IMMUNOLISA TM HBSAB, ORGANICS).

Principle of the assay

Microtiter plates are coated with a preparation of highly purified hepatitis B surface antigen (HBsAg) that in the first incubation with sample specifically captures anti-HBs antibodies to the

solid phase. After washing captured antibodies are detected by an HBsAg labelled with peroxidase that specifically binds the second available binding site of these antibodies. The enzyme specifically bound to wells, by acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the amount of anti-HBs in the sample and can be detected by an ELISA reader. The amount of antibodies may be quantified by means of a standard curve calibrated against the WHO reference preparation. Samples are pre treated in the wells with a specimen diluent able to block interference present in vaccinated individuals.

Materials

Components of ImmunoLISATM HBsAb, Organics kit.

Materials	Composition
Microtiter plate	8x12 microwell strips coated with purified heat-inactivated HBsAg of both subtypes (ad and ay) from human origin.
Calibration curve	Standard curves, derived from anti-HBs positive plasma titrated on WHO standard for anti-HBs 91 st reference preparation 1977, lot 17-2-77), ranging: Cal1=0mIU/ml; Cal2=10mIU/ml; Cal3=50mIU/ml; Cal4=100mIU/ml; Cal5=250mIU/ml. Contains human serum proteins, 5% BSA, 10nM phosphate buffer (pH7.4±0.1), 0.09% sodium azide and 0.1% Kathon GC as preservative.
Wash buffer concentrate	20x concentrated solution. Once diluted, the wash solution contains 10nM phosphate buffer (pH7.0±0.2), 0.05% Tween 20 and 0.1% Kathon GC as preservative.
Enzyme conjugate	Contains inactivated purified HBsAg of both subtypes ad and ay, labelled with horse radish peroxidase (HRP), 5% BSA, 10nM Tris buffer (pH6.8±0.1), 0.3mg/ml entamicine sulphate and 0.1% Kathon GC as preservatives.
Chromogen/substrate	Contains a 50nM citrate-phosphate buffered solution pH3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine (TMB) and 0.02% hydrogen peroxide.

Stop solution	Contains 0.03M sulphuric acid solution.
Specimen diluent	10mM Tris buffered solution pH 7.4±0.1, 0.09% sodium azide as preservative.
Control serum	Contains fetal bovine serum proteins, human anti-HBsAg antibodies at about 50±10% WHO mIU/ml. 0.3mg/ml gentamicin sulphate and 0.1% Kathon GC as preservatives

Working phase

Test strips pre-coated with highly purified HBsAg from subtypes *ad* and *ay* of human origin and reagents were brought to room temperature. Fifty microliters of specimen diluent (10mM Tris buffered solution pH 7.4 ±0.1, 0.09% sodium azide) was dispensed into all wells except the blank wells. One hundred microliters of all calibrators (five standards that contains human serum proteins, 5% bovine serum albumin, 10mM PBS pH 7.4 ±0.1, 0.09% sodium azide and 0.1% Kathon GC as preservatives. With a measurable range of 0-250mIU/ml), controls (Positive control: Inactivated human Anti-HBs positive serum; Negative control: serum non-reactive for hepatitis B virus (HBV) markers in buffer; both controls had protein stabilizers and 0.003% Gentamycin and 0.01% Thimerosal as preservatives) and samples were dispensed into appropriate wells and incubated for 60 minutes at 37°C. After incubation the plates were washed four times with 350µl per well of wash buffer (10mM PBS pH 7.0 ±0.2, 0.05% Tween-20 and 0.1% Kathon GC as preservative). One hundred microliters of enzyme conjugate (inactivated purified HBsAg of *ad* and *ay*, labelled with horse radish peroxidase, 5% BSA, 10mM Tris buffer pH 6.8 ±0.1, 0.3 mg/ml gentamicin sulphate and 0.1% Kathon GC) was added to each well except for the blank wells and incubated for 60 minutes at 37°C. The plates were washed as described previously. One hundred microliters of chromogen/substrate mixture (50mM citrate-phosphate buffered solution pH 3.5-3.8, 4% dimethylsulphoxide, 0.03%tetra-methyl-benzidine (TMB) and 0.02% hydrogen peroxidase (H₂O₂)) was dispensed into each well including the blank wells and incubated at room temperature for 20 minutes in the dark. After the final incubation 100µl of stop solution (0.3M sulphuric acid) was added to each well and the reaction was read at 450nm with a plate reader (Labsystems Multiskan RC; USA).

Interpretation of results

In order to accept the test as valid the following criteria in the list below should be met.

Quality checks for validity of test.

Parameters	Requirements
Blank wells	<0.100 OD at 450nm
Calibrator 0 WHO mIU/ml	<0.200 OD at 450nm after blanking
Calibrator 10 WHO mIU/ml	OD 450nm higher than the OD 450nm of the calibrator 0 mIU/ml + 0.100
Calibrator 250 WHO mIU/ml	>1.500 OD at 450nm
Control Serum	OD 450nm = OD 450nm Cal 50mIU/ml $\pm$ 10%
Coefficient of variation	<30% for the calibrator 0mIU/ml

Samples with a concentration lower than 10mIU/ml were considered negative for anti-HBs.

Samples with a concentration higher than 10mIU/ml were considered as positive for anti-HBs.

5.3.5. LUMINEX® bead-based multiplex assay

Principle of the assay

Luminex colour-codes microbeads called microspheres into 100 distinct sets. Each bead set can be coated with an antigen, antibody or reagent specific to a particular bioassay, allowing the capture and detection of multiple analytes from a single sample. The principle of the assay is that colour-coded microspheres pre-coated with analyte specific antigen or capture antibody for the analyte of interest are added to the micro-filter plates. Sample to be tested is added to the microspheres, Biotinylated detection antibodies specific to the analyte of interest are added and form an antigen antibody sandwich. Followed by, the addition of phycoerythryn (PE)-conjugated streptavidin. The beads are read on a dual-laser flow-based detection instrument, such as the Luminex 200™ or Bio-plex 100™ analyzer. One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of the bound analyte.

Equipment and consumables

Listed in section B.2, under the subtitle: List of equipment and consumables.

Reagents

Listed in section 5.2, under the subtitle: List of antigens, chemical and reagents for Luminex assay

Reagent and antigen preparation

- a. DT and TT antigens were prepared by converting limit of flocculation (Lf) per ml to micrograms per ml, where 0.332Lf/ml is equivalent to 1µg/ml. Followed by diluting the antigens in 1xPBS (pH7.2) to a final coating concentration of 25µg/ml.
- b. PT and FHA were prepared by dissolving the lyophilized powder in 1 ml sterile distilled water and shaken till all the powder was dissolved, the stock concentration was 50µg/ml for each antigen. From this the antigens were diluted in 1xPBS to a final concentration of 5µg/ml.
- c. HBsAg was used straight from the stock solution without any further dilution, with a working concentration of 14.99 µg/ml of antigen in 1x PBS.
- d. Activation buffer was prepared with 1xPBS (pH 6.1) containing 5mg EDC and 5mg Sulfo-NHS per ml.
- e. Washing buffer was prepared with 1x PBS (pH 7.2), 0.05% Tween 20 (v/v), and 0.05% sodium azide (w/v).
- f. Blocking buffer was prepared with 1x PBS (pH 7.2), 1% FBS (v/v) and 0.05% sodium azide (w/v).
- g. Assay buffer was prepared with 1x PBS (pH 7.2), 10% FBS (v/v) and 0.05% sodium azide (w/v).

Activation carboxylated microsphere beads and conjugation of Antigens to activated beads

Coupling of DT, TT, PT, FHA and HBsAg to activated microspheres was achieved by using a two step carbodiimide reaction.

Activation of beads

Five hundred microliters of vigorously vortexed carboxylated microspheres (12.5×10^6 beads/ml) were measured into sterile 1.5ml microcentrifuge tubes; the microsphere solution was washed twice with wash buffer. The supernatant was removed after each washing step and discarded. Five hundred microliters of the activation buffer was added to the 1.5ml microcentrifuge tube containing the microspheres. The microsphere and activation buffer mixture was incubated at room temperature for 20 minutes under constant rotation in the orbital mixer at 25 revolutions per minute (rpm) to allow for activation of microspheres to occur. Activated microspheres were washed three times with 500µl of wash buffer followed by centrifugation at 12 000xg for 5 minutes per wash.

Conjugation of beads with antigen after bead activation

Microsphere regions were carefully chosen as to avoid regions being directly adjacent to each other: Microsphere regions for this assay were regions 63, 36, 66, 14 and 20 for DT, TT, PT, FHA and HBsAg respectively.

After the final wash step post-activation of beads, the supernatant was removed and discarded. Activated beads were re-suspended in 500µl 1x PBS (pH 7.2) with an antigen to bead ratio of 25µg/ 6.25×10^6 for DT and TT, 5µg/ 6.25×10^6 for PT and FHA and 14µg/ 6.25×10^6 for HBsAg. This mixture was subsequently incubated for 18 hours at room temperature in the dark under constant rotation in the orbital mixer at 25 rpm. The beads were washed three times with 500µl wash buffer and supernatant discarded. The beads were then stored in 500µl blocking buffer at 4°C in the dark until used.

Bead counting

This process was performed prior to storage of beads at 4°C as a means of quality control. Five microliters of the bead suspension was diluted in 45µl of blocking buffer and mixed gently by rocking microcentrifuge tube up and down for 10 seconds. Ten microliters of the bead solution was placed on a hemocytometer slide, and the number of beads was counted in each quadrant

under 40 x magnifications on a microscope. The bead count was determined by the following formula: grid 1 + grid 2+ grid 3 + grid 4 x 10 000/0.4 = number of beads/ml. The bead count should be between 1-1.27 x 10⁷ beads/ml, the number of beads was captured after each conjugation reaction.

Measurement and quantification of DT, TT, PT, FHA and HBsAg specific IgG antibody concentration by Luminex assay

The purpose of the method was to determine antigen specific IgG antibody concentration in serum samples.

Reference serum, sample and control preparation

In-house reference sera, serum samples and controls were brought to room temperature before use. The reference serum was diluted with assay buffer in duplicate with a starting dilution of 1/50 in a seven step four fold dilution series. Serum samples were diluted with assay buffer in duplicate to 1/100. The positive control was diluted in assay buffer in duplicate to 1/2000 and the negative control was run as is.

Working phase

Ninety six well multiscreen HTS filter plate was wet with 100µl of assay buffer, and allowed to stand for 30 seconds, the excess liquid was filtered by placing the plate on the plate holder with vacuum pump at 5Hg (the pressure should not exceed 5Hg as there was a risk of puncturing the filter wells). Fifty microliters of activated beads at 5000 beads/region were added to each well. Fifty microliters of reference sera, sample and controls were added to each well in the following plate layout (Figure 5.4).

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	R4 3200	S 1	S 5	S 9	S 13	S 17	S 21	S 25	S 29	S 33	S 37
B	B	R4 3200	S 1	S 5	S 9	S 13	S 17	S 21	S 25	S 29	S 33	S 37

C	R7 204800	R3 800	S 2	S 6	S 10	S 14	S 18	S 22	S 26	S 30	S 34	S 38
D	R7 204800	R3 800	S 2	S 6	S 10	S 14	S 18	S 22	S 26	S 30	S 34	S 38
E	R6 51200	R2 200	S 3	S 7	S 11	S 15	S 19	S 23	S 27	S 31	S 35	PC 2000
F	R6 51200	R2 200	S 3	S 7	S 11	S 15	S 19	S 23	S 27	S 31	S 35	PC 2000
G	R5 12800	R1 50	S 4	S 8	S 12	S 16	S 20	S 24	S 28	S 32	S 36	NC
H	R5 12800	R1 50	S 4	S 8	S 12	S 16	S 20	S 24	S 28	S 32	S 36	NC

Figure 5.4: Multiplex Plate layout. B= blank; R= reference sera; S= sample, PC= Hebagam IM positive control; NC= negative control

The plate was covered in foil and incubated for an hour in the orbital mixer at 600rpm. The plate was washed three times with 100µl of washing buffer and vacuum filtered at 5Hg. R-PE conjugated anti-human IgG was diluted to 1/200 and 50µl was added to each well and incubated as previously described for 30 minutes. Plate was washed three times as previously described, the beads were re-suspended in 130µl wash buffer and transferred to 96 well round bottom plates for analysis on the Bio-Plex 200 in combination with Bio-Plex manager software version 4.1.1 (Bio-Rad Laboratories, Hercules, CA).

Interpretation of results

For each analyte, the median fluorescent intensity (MFI) was converted to IU/ml or mIU/ml by interpolation from a 5-parameter logistic standard curve (log-log) for every bead region/reference. The microsphere immunoassay (MIA) results were determined from the mean of the two sample dilutions if their MFI signals were on the linear part of the reference curve.

5.3.6. Measurement of *Haemophilus influenzae* type b PRP-t antibodies with in-house ELISA

Principle of the method

The Hib ELISA is a solid phase indirect sandwich ELISA. The Hib antigen was pre-coated onto microtiter plates, which act as the solid phase. Hib specific antibodies in the sample were bound to the antigens on the surface of the well. The antibody-antigen complex was detected by adding

conjugate. Substrate was added to the antigen-antibody conjugate complex and measured colometrically at 405nm.

Chemicals and Reagents

Listed in section 5.2, under the subtitle: *List of chemicals and reagents for the Hob ELISA*

Reagent preparation:

- a. The reference serum, anti-*H.influenzae* type b capsular polysaccharide serum, lot 1983 (NIBSC) was dissolved in sterile, pyrogen free water (1ml/ampoule). The mixture was incubated overnight at 4°C to ensure complete dissolving of the reagent. The reference serum was stored in 1ml aliquots at -20°C.
- b. Quality control plasma (Human anti-Hib polysaccharide), was reconstituted in 1ml sterile distilled water and stored at -20°C.
- c. The low positive control was an in-house pooled control serum which was stored at -20°C in 1ml aliquots.
- d. High positive control – Polygam (Natal Bioproducts Institute, Durban South Africa) was a preparation of purified IgG from pooled human serum. Packaged in vials of 1g of lyophilized powder and stored at -20°C. Polygam was reconstituted in 50ml of sterile saline/vial and agitated for 15 minutes to dissolve the powder and stored at -20°C.
- e. Hib polysaccharide conjugated to serum protein (HboHA) was reconstituted in 1ml of sterile water to a final concentration of 1 mg/ml. The mixture was incubated for 30 minutes at room temperature or until the HboHA was completely dissolved. The antigen was stored at -70°C in aliquots.
- f. PBS-F was made by adding 10% thawed fetal bovine serum to sterile 1x PBS. The solution was stored for a maximum of 5 days at +2-8°C and when it became cloudy it was discarded.
- g. An appropriate amount of pNPP substrate powder: p-nitrophenol phosphate disodium salt 1mg/ml or p-nitrophenol phosphate disodium salt Hexahydrate 1.4mg/ml was weighed out. The substrate powder was added to 0.05M carbonate buffer (pH 9.8) and swirled to dissolve, substrate was prepared 10-15 minutes prior to use.

Antigen coating of wells in the microtiter plates

Ninety six well microtiter plates were coated with 1µg/ml HboHA antigen diluted in PBS-N. One hundred microliters of the diluted antigen was pipetted into each well, the plates were covered with clear plate cover tape and incubated at 37°C over night (Coated plates were stored at +2-+8°C for maximum of five days). The plates were washed with a Skanwasher 400, using a programme that dispenses 300µl/well of PBS-T and washed four times

Blocking-step

One hundred microliters of PBS-F was aliquoted into each well; the plates were then covered and incubated for one hour at 37°C. The plates were emptied by inverting the plates and the liquid poured out and the plates were patted dry with a hand towel.

Working phase

Reference sera, controls and samples were tested as true duplicates; two identical dilutions were made up, in separate tubes which were distributed into two wells in a row allocated to a patient/control/reference as a form of internal quality control to minimize dilution and pipetting errors. The starting dilution for samples was 1:100 in 1x PBS-F. The starting dilution for the high positive control was 1:300 and the low positive control starting dilution was 1:100. The plate layout is shown in figure 5.5.

ELISA sample layout												
	1	2	3	4	5	6	7	8	9	10	11	12
A	High control three fold				Low Control three fold				Sample 1 three fold			
B	1:100	1:300	1:900	1:2700	1:100	1:300	1:900	1:2700	1:100	1:300	1:900	1:2700
C	Reference serum two fold dilution							Blank	Sample 2 three fold			
D	1:100	1:200	1:400	1:600	1:800	1:1600	1:3200	Blank	1:100	1:300	1:900	1:2700
E	Sample 3 three fold				Sample 4 three fold				Sample 5 three fold			
F	1:100	1:300	1:900	1:2700	1:100	1:300	1:900	1:2700	1:100	1:300	1:900	1:2700
G	Sample 6 three fold				Sample 7 three fold				Sample 8 three fold			
H	1:100	1:300	1:900	1:2700	1:100	1:300	1:900	1:2700	1:100	1:300	1:900	1:2700

Figure 5.5: Plate layout for sample dilution for Hib ELISA

Samples and controls: 150µl/well of the starting dilution was added to the first sample rows and 100µl/well of PBS-F to the other wells. Doubling dilutions using 50µl (creating a 1:3 dilution) leaving final volume of 100µl/well. The last 50µl removed from the last row was discarded with the tips.

Reference serum: the starting dilution was 1:600, in the first two wells, 200µl of reference serum was dispensed, and in the remaining wells 100µl of PBS-F was dispensed. One hundred microliters from the first well was transferred to the next well to have the 2 fold dilution series. The 100µl from the last wells was discarded to have the final volume of 100µl/well. For reagent control (blank), PBS-F was distributed into the blank wells (100µl/well). Plates were then covered and incubated for two hours at 37°C.

Plates were washed four times with 300µl PBS-T on the Skanwasher 400, plates were patted dry. One hundred microliters of HboHA conjugate at 1:1000 dilution was distributed into each well, covered and incubated for two hours at 37°C. Plates were then washed three times with 300µl/well of PBS-T and twice with distilled water on the Skanwasher 400. One hundred microliters of pNPP substrate was distributed into each well, the plates were incubated at room temperature in the dark for one hour. The reaction was stopped by the addition of 50µl/well of 2M NaOH. The plates were read with a microtiter plate reader (Multiscan II plus) at a wavelength of 405nm.

Interpretation of results

Results were calculated with the CDC programme ELISA for windows version 2.00 (U.S. Department of Health and Human Services, CDC, Atlanta). The reference curve should give an OD reading of >0.2 and 2.5, the plate was discarded if the reference readings did not fall within the set limits. The sample was considered positive when the 1/100 dilution gave an OD reading >0.2. The control values should have been within the calculated alarm limits as set out by RMPRU in order to except serum results for the plate.

5.3.7. Measurement of serum bactericidal activity specific for *Haemophilus influenzae* type b.

Principle of Assay

This protocol was based on the original method by Schlesinger et al (1992) and later modified by Romero-Steiner et al (2001). It reproducibly measures the functionality of serum antibodies against *Haemophilus influenzae* type b. In this assay, serum samples were serially diluted in a microtitre plate, and a known concentration of live bacteria was added to each well. An active source of complement was added to induce killing of opsonized bacteria. After a fixed time point, the bacteria from each well were plated out to determine the viable count. The serum bactericidal activity was determined by the concentration of serum which was able to produce 50% killing of bacteria in relation to complement control. This assay measured functional binding, as opposed to ELISA which measures total binding.

Reagents

Listed in section 5.2, under the subtitle: *List of reagents and consumable for Hib bactericidal assay*

Reagent preparation

All reagents and media were brought to room temperature.

- a. Dilution buffer: 44.5ml HANKS with 0.5ml Ca^{++} and Mg^{++} 5ml 1% gelatin.
- b. Complement source: Baby rabbit serum was used as the complement source. Batches of complement were pre-screened for a batch that had a killing effect in the presence of anti-PRP antibodies, but had no killing effect on Hib in the absence of opsonising antibodies. Frozen stocks of complement were aliquoted in 1.2ml aliquots and stored at -70°C .

Haemophilus influenzae type b frozen stock preparation.

An overnight culture of *Haemophilus influenzae* Type b, strain GB3292 was prepared. One hundred millilitres of BHI broth was prepared and autoclaved at 121°C for 15 minutes, it was allowed to cool and 2% Fildes Enrichment media was added. From the overnight culture two to three colonies were isolated and inoculated into the Fildes enrichment BHI broth and incubated at 37°C . The culture was checked regularly by measuring the optical density at 600nm, until the growth phase of the strain was 0.45 (mid-log growth phase). Once that phase had been reached 15% glycerol was added to the broth and mixed well. Zero point five millilitres of the mixture was aliquoted into cryotubes and subsequently flash frozen in an ethanol bath and dry ice, the frozen aliquots were stored at -70°C . Before use 50 μl of the frozen culture was plated onto chocolate agar plates and incubated overnight at 37°C in 5% CO_2 , this was done to check the purity of the culture. After 24 hours at -70°C bacterial viability was calculated from the stored aliquots in order to determine loss of viability overtime.

Viable counts

Chocolate agar plates were dried for 30 minutes in an air circulating 37°C incubator, once dry the

plate was divided into six sectors and labelled with the appropriate dilution 10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} . A sterile U-bottom 96 well plate was prepared by pipetting 180 μ l of sterile PBS into well B-F. Two hundred microliters of undiluted sample was aseptically pipette into well A as in Figure 5.6. Twenty microliters of the mixture in well A was pipetted into well B and mixed four times resulting in a ten-fold dilution. The transfer of 20 μ l into the remaining wells was repeated as previously stated. Twelve samples could be run on one plate. The bacterial dilutions were plated out onto labelled agar plates by dropping 20 μ l per drop from each dilution according to Figure 5.7. The plate was incubated overnight at 37°C; the colonies in each drop were counted the following day.

Calculation of CFU/ml

$(\text{Colony drop \#1} + \text{Colony drop \#2} + \text{colony drop \#3}) \times 50 \times \text{dilution factor}$

3

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Figure 5.6: Plate layout of 96 well U-bottom plate used for diluting bacterial strain for viable count.

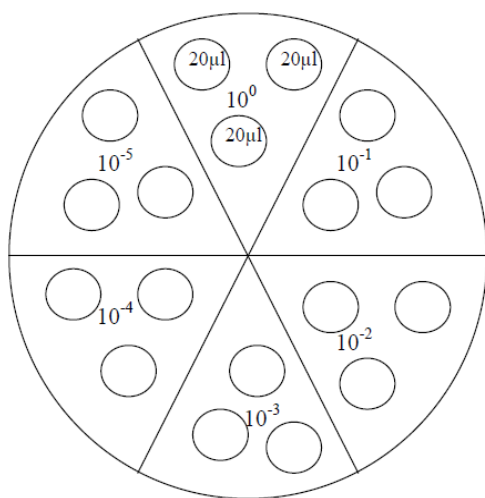


Figure 5.7: Agar plate layout

Quality control sera

QC serum was serum with a known reproducible titer of PRP antibodies and was included in each assay; one QC sample was included in each plate.

Sample preparation

Serum samples were heat inactivated at 56°C in a water bath for 30 minutes. The starting serum dilution was adjusted on the basis of ELISA antibody results as shown in the table below. When it was necessary samples were pre-diluted in dilution buffer.

Serum IgG concentration in µg/ml and the reciprocal dilution.

IgG concentration µg/ml (EIA)	Sample dilution
0-4	1/1
4-7	1/2
7-10	1/4
10-15	1/8
15-30	1/16
30-50	1/32
> 50	1/64

Serum samples were tested at a starting dilution of 1:8 (in plate final dilution) and diluted in a two-fold dilution scheme as shown in Figure B8. Twenty microliters of serum was aliquoted into the first wells (A1, A2.....A10), in duplicate with the last two wells being reserved for QC sample (A11 and A12). The remaining wells were filled with 10µl of dilution buffer (B1-12 to H1-12), were two-fold serial dilutions of sample were made using a multichannel pipette by transferring 10µl of sample and mixing well. Rows H9-12 was reserved for the complement control sample. All wells had a final volume of 10µl, row A was a 1:8 dilution once the remainder of the reagents were added. Row B=1:16, row C=1:32, row D=1:64, row E=1:128, row F=1:256, row G=1:512 and row H=1:1024. G11 and G12 are 1:512 dilutions for the QC sera, whereas H9 through H12 were the Complement control wells. The final volume per well at the end of the entire assay was 80µl.

Serum		Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		QC sera	
Dilution		1	2	3	4	5	6	7	8	9	10	11	12
1:8	A	All wells (except the Complement (C') control wells) contain: 10 µl Serum dilution 20 µl Bacteria dilution 10 µl Complement 40 µl Dilution buffer											
1:16	B												
1:32	C												
1:64	D												
1:128	E												
1:256	F												
1:512	G	C' control wells contain: 10 µl Dilution buffer 20 µl Bacteria dilution 10 µl Complement 40 µl Dilution buffer											
1:1024	H										C'	C'	C'

Figure 5.8: microliter plate format for Hib Killing assay

Pre-opsonization: Dilution of frozen bacterial stocks

The frozen bacterial stock was thawed at room temperature. It was then mixed well by vortexing. Dilutions were made (1×10^{-1} to 10^{-4} bacteria/ml) in dilution buffer. The required concentration for working dilution was 2000 bacteria/20 μ l in each well i.e. 10^5 bacteria/ml. The concentration of the bacteria stock was determined beforehand by viable counts. Plate T_0 controls were prepared as follows in microcentrifuge tubes:

T_0 plate: 20 μ l of the working dilution was added to 60 μ l of dilution buffer.

T_0-3 : 20 μ l of the 10^{-2} bacteria dilution was added to 60 μ l of dilution buffer.

T_0-4 : 20 μ l of the 10^{-3} bacteria dilution was added to 60 μ l of dilution buffer.

The dilutions were mixed by vortexing followed by transferring 10 μ l of the dilution onto three chocolate agar plates each labelled accordingly. This was repeated three to four times on the same plates. The plates were tilted at a 45° angle to allow the liquid to run slowly down the plates. The liquid was allowed to completely absorb into the agar plates before being inverted. The agar plates were incubated overnight at 37°C in 5% CO₂. Approximately 60 cfu/aliquot were expected on the T_0 plate after 18-48 hours of incubation. The average colony count was recorded for each T_0 control

Distribution to the wells

A multi-channel pipette was set to 20 μ l in order to add the diluted bacterial suspension to each of the 96 wells in the microtiter plate. A sterile reagent reservoir was used to hold the diluted bacterial suspension. The lid was replaced on the plate and the plate was incubated at 37°C in 5% CO₂ for 15 minutes. This step allows the pre-opsonization of bacteria with diluted sera.

Complement

Vials of complement were thawed immediately before use and should be at 4°C before they were added to the plates. Following pre-opsonization, 10 μ l of complement was added into each well.

Bacterial killing

Immediately after the complement was added, 40 μ l of dilution buffer was added into each well and tapped five times to allow for mixing of all reagents. The plate was covered and the plate incubated for one hour at 37°C in 5% CO₂ without shaking.

Viable counts

The surface of chocolate agar plates was allowed to dry by placing the plates in a 37°C forced air incubator and plates were labelled. The mixture in the 96 well plates after the one hour incubation was carefully mixed. Using a multichannel pipette 5 μ l of solution in each well was released onto pre-warmed chocolate agar plates. This was repeated for all the remaining columns, resulting in 12 chocolate agar plates per 96 well plates. The spots were allowed to air dry for 5 minutes; the plates were inverted and incubated at 37°C in 5% CO₂. A colony counter was used to count the colonies on the plates. Complement control counts and T₀ plate counts were expected to be close to each other at $\leq 20\%$.

Bactericidal titers

The bactericidal titer was defined as the reciprocal of the serum dilution with at least 50% killing, when compared to the average growth in the complement control wells (H9 through H12). Occasionally, sera with high titers needed to be re-tested at higher initial dilutions than 1:8 to determine the opsonic titer. Serum samples with opsonic titers <8 were reported as a titer of 4 for purposes of data analysis.

Quality Control

- a. Time zero (T₀) control: ~60 cfu/aliquot are expected.
- b. Complement control: The average growth of the four complement control wells should be close to the T₀ control a variation of $\leq 20\%$ is acceptable. Occasional growth in the complement control wells (doubling) is preferable to killing. This control is prepared within the plate and at the same time that the samples are run. If the assay consists of several microliter plates, use the average of the complement control wells in each plate to calculate the opsonic titers.

c. Quality control serum: A serum with known titer should be included in each assay to control plate to plate and day to day variation. Include one QC serum per plate (wells A11 and A12). Any high volume serum (with a known reproducible titer) can be used as a QC serum. QC serum should yield results within ± 1 dilution of previous average values (corresponds to a coefficient of variation of approximately 30%).

5.3.8. Quantitative measurement of measles virus specific antibodies with ENZYGNOST® anti-Masern-virus/ IgG

Principle of assay

Immunoglobulin G (IgG) antibodies in the test sample which are specific for measles virus were bound to the antigen coated onto the wells of the test plates. Anti-human IgG/POD conjugate was added and bound to these specific antibodies. The enzyme component of the conjugate catalyzed the working chromogen solution thus producing a blue colour. This reaction was terminated by the addition of stopping solution POD during which the colour changed to yellow. The intensity of the yellow colour was proportional to the activity of measles virus IgG in the sample. The activity was quantified in international units by calculating using the α -method.

Materials

Components of Enzygnost® Anti-Masern-Virus/ IgG kit.

Materials supplied	Composition
Enzygnost® Anti-Measles Virus/IgG test plate	Microtitration plate coated with inactivated measles virus antigen. The wells in the left row of each test strip were coated with antigen derived from permanent simian kidney cells infected with measles virus. While the wells on the right row were coated with non-infected cells (control antigen).
Anti-human IgG/POD conjugate	F(ab)' fragment of a rabbit antibody to human IgG, conjugated with peroxidase and contained in 0.05M Tris/HCL buffer solution, pH 7.2 with 0.5% polygeline. Dye max 60mg/L patent blue and max 440mg/L tartazine, preservative phenol 1g/L.
Conjugate buffer Microbial	37 mg EDTA in a litre of 0.01M phosphate-buffered solution, pH 7.2 with approximately 2% tetriconic and ~100mg/L gentamicin, ~6mg/L 5-chloro-2-methyl-isothiazole-3-one and ~2mg/L 2-methyl-4-isothiazole-3-one.
Anti-Measles Virus reference P/N	Human serum containing IgG antibodies to measles

	virus antigens, contained in Tris buffer solution (20mmol/L), pH 7.4 with ~60% humanalbumin® (5%). Preservatives, ~5mg/L amphotericin B and ~100mg/L gentamicin.
Sample buffer POD	0.3M Tris/HCL buffer solution, pH8.0-8.2, ~0.1% Tween 20, ~1% polygeline and ~ 5% Boviserin®. Preservatives ~5mg/L amphotericin b and ~ 100mg/L gentamicin.

Reagent Preparation

All reagents were brought to room temperature before the test procedure was initiated.

- a. Anti-human IgG/POD conjugate: was diluted 1:50 with conjugate buffer microbial, transfer 250µl of conjugate into a vial of 12.5ml of conjugate buffer microbial and shaken gently.
- b. Coloured sample buffer POD: 2.5ml of colour solution blue was added to 50ml of sample buffer POD and shaken gently.
- c. Washing solution POD: for each test plate 20ml of washing solution was added to 400ml of distilled water.
- d. Working chromogen solution: for each test plate 1ml of chromogen was added to 10ml of buffer/substrate TMB and stored protected from light.
- e. Specimen predilution: serum samples and anti-measles virus reference P/N were diluted in a ratio of 1:20 using coloured sample buffer POD. Therefore 20µl of sample into a dilution tube and added into 400µl of sample buffer POD and mixed well.

Working phase

Each well was filled with 200µl of non-coloured sample buffer POD. Twenty microliters of pre-diluted anti-measles virus reference P/N into the first pair of coated wells (A1= measles virus antigen; A2: Measles virus control antigen). Afterwards prediluted samples were added to the remaining wells. The plate was incubated at 37°C ±1°C for 60 minutes under humid conditions. Plates were washed 4 times with diluted wash buffer solution POD, they were not tapped dry. One hundred microliters of working anti-human IgG/POD was added to each well and sealed with foil and incubated at 37°C for 60 minutes under humid conditions. The wash step was repeated, after washing 100µl of working chromogen solution was added to each well and the

plate was sealed with foil and incubated for 30 minutes at room temperature in the dark. One hundred microliters of stopping solution POD was added to each well, the reaction was read at 450nm and referenced at 620nm.

Interpretation of results

a. Internal quality control: for all evaluations the values used were the sample absorbance values in the antigen well minus those in the control antigen well. These difference values were stated as ΔA .

b. Validation criteria: For evaluation, each ΔA value obtained per test with anti-measles virus reference P/N must be within the lot-dependent lower and upper margins stated on the barcode table of values. In addition, the individual ΔA values must not differ by more than 20% from the mean value calculated from these values. If these conditions were not met the test was considered as invalid and had to be repeated.

c. Measurement correction: For achieving optimal reproducibility of the results, the measurements require correction, both for the quantitative evaluation using the α -method and for the qualitative evaluation of the test. First, calculate the mean ΔA value for the Reference P/N. Then, divide the nominal value of the reference P/N by the calculated mean ΔA value, this gave the correction factor. This value was used to multiply the ΔA values.

Correction factor = nominal value / Mean ΔA of Reference P/N

d. Qualitative evaluation:

The following criteria were applied for qualitative evaluation:

Anti-Measles Virus/IgG negative	$\Delta A < 0.100$ (cut-off)
Anti-Measles Virus/IgG positive	$\Delta A > 0.200$
Anti-Measles Virus/IgG equivocal	$0.100 \leq \Delta A \leq 0.200$

e. Quantitative evaluation using the α -method: Samples with IgG antibody activities above the cut-off value were evaluated quantitatively with the aid of the α -method.

$$\text{Log}_{10} \text{ mIU/ml} = \alpha \times \Delta A^{\beta}$$

APPENDIX 5

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

R14/49 Simani

CLEARANCE CERTIFICATE

PROTOCOL NUMBER M080966

PROJECT

Effect of HIV Infection and Treatment
with Antiretroviral Therapy on
Immunological responses to Childhood
Vaccines

INVESTIGATORS

Miss OE Simani

DEPARTMENT

RMPRU

DATE CONSIDERED

08.09.26

DECISION OF THE COMMITTEE*

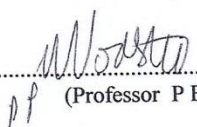
Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE

08.09.29

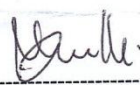
CHAIRPERSON


pp (Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor :

Dr SA Madhi



DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES