B and T cell phenotype and function in children with perinatally acquired HIV-1 infection

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Declaration

I, Alasdair R J Bamford, confirm the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Date: 8th October 2012

Abstract

Untreated HIV infection is well known to have profound effects on CD4 T cell phenotype and function, ultimately leading to CD4 T cell depletion, AIDS and death. HIV is also known to affect B cell function and phenotype, resulting in impaired and dysregulated humoral immunity. Studies in HIV infected adults have demonstrated that Highly Active Antiretroviral Therapy (HAART) only partially restores B cell phenotype and function. HIV infected adults and children on HAART remain at persistently high risk of pneumococcal disease and mount suboptimal responses to pneumococcal vaccines. The nature of persistent indirect effects of HIV infection on B cell immunity remain under investigated in the paediatric population.

Using flow cytometry we have shown that children and adolescents with perinatally acquired (paHIV) have persistent abnormalities in B and T cell phenotype despite fully suppressive HAART. Pneumococcal serotype specific IgG, acquired through natural exposure, was present at baseline, although at a lower concentration than in healthy controls for some serotypes. Vaccination with 13-valent pneumococcal conjugate vaccine (PCV13) was immunogenic, however serological responses to certain serotypes were impaired in comparison to healthy controls. *In vitro* whole blood cytokine responses to vaccine carrier protein were present at baseline and increased following immunisation, although the diversity and magnitude of antigen specific cytokine release was restricted in comparison to healthy controls. A greater proportion of life spent with undetectable viral load was associated with a more intact B cell phenotype and more robust serotype specific IgG vaccine responses for some vaccine serotypes. Nasopharyngeal pneumococcal carriage isolates were detected in a small proportion of children with paHIV, all of which were non-PCV13 serotypes.

These results, while providing an insight into the immunogenicity and mechanism of action of conjugate vaccines outside of the infant period, have wider implications for pneumococcal vaccination and HAART treatment practices for children and adolescents with paHIV.

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Abbreviations

AHC	Adult healthy control
AIDS	Acquired immune deficiency syndrome
AN(C)OVA	Analysis of (co)variance
APC	Antigen presenting cell
ART	Antiretroviral therapy
AZT	Azidothymidine (zidovudine)
BCR	B cell receptor
BLR1	Burkitt's lymphoma receptor 1 (CXCR5)
BPSU	British Paediatric Surveillance Unit
CBMC	Cord blood mononuclear cells
CCR	CC chemokine receptor
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CHC	Child healthy control
CHIPS	Collaborative HIV Paediatric Study
CI	Confidence interval
CP	HIV infected child patient
CR	Complement receptor
CRF	Case report form
CRM197	Cross reacting material 197
CRP	C-reactive protein
CSR	Class switch recombination
CV	Coefficient of variation
CVID	Common variable immunodeficiency
CXCR	CXC chemokine receptor
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific ICAM3-grabbing non-integrin
ESPID	European Society for Paediatric Infectious Diseases
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunosorbant SPOT
EMA	European Medicines Agency
FDA	Food and Drug Administration
FMO	Fluorescence minus one
FSC	Forward scatter
gp120	Glycoprotein 120
GC	Germinal center
GCP	Good Clinical Practice
GMC	Geometric mean concentration
HAART	Highly active antiretroviral therapy
HEU	HIV exposed uninfected
Hib	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPA	Health Protection Agency
HTLV-III	Human T-lymphotropic virus- III
ICOS	Inducible T-cell costimulator (CD278)
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
IQR	Interquartile range
LAV	Lymphadenopathy-associated virus
LLQ	Lower limit of quantification
LTA	Lipoteichoic acid
LPS	Lipopolysaccharide
MALT	Mucosa associated lymphoid tissue
MCRN	Medicines for Children Research Network
Med dif	Median difference

MenC	*Neisseria meningitidis* serogroup C
MFI	Mean fluorescence intensity
MHRA	Medicines and Healthcare Products Regulatory Agency
MHC	Major histocompatibility complex
MSD	Mesoscale discovery
Nef	Negative regulatory factor
NHS	National Health Service
NIHR	National Institute of Health Research
NK	Natural killer cells
NKT	Natural killer T cells
NLR	NOD-like receptor
NNRTI	Non nucleoside reverse transcriptase inhibitor
NOD	Nucleotide oligomerisation domain
NP	Nasopharyngeal
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor
NSHPC	National Study of HIV in Pregnancy and Childhood
NWLCRN	North West London Comprehensive Local Research Network
OPA	Opsonophagocytic assay
paHIV	Perinatally acquired HIV
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PCV7	7-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PENTA	Paediatric European Network for Treatment of AIDS
PHA	Phytohaemagglutinin
PI	Protease inhibitor
PMA	Phorbol myristate acetate
PMTCT	Prevention of Mother to Child Transmission
PPV	Pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptor
PTI	Planned treatment interruption
R&D	Research and development
REC	Research ethics committee
RMAN(C)OVA	Repeated measures AN(C)OVA
RPMI	Roswell Park Memorial Institute medium
RR	Relative risk
SAC	*Staphylococcus aureus* Cowan
SBA	Serum bactericidal activity
SI	Stimulation index
SSC	Side scatter
STGG	Skim-milk tryptone-glucose-glycerin
TCR	T cell receptor
TD	T cell dependent
T_{FH}	Follicular T helper cell
Th	T helper cell
Th1	T helper 1 cell
Th2	T helper 2 cell
Th17	T helper 17 cell
TI	T cell independent
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TREC	T cell receptor excision circle
Treg	Regulatory T cell
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organisation
VE	Vaccine efficacy
VL	Viral load

“In our endeavors to recall to memory something long forgotten, we often find ourselves upon the very verge of remembrance, without being able, in the end, to remember.”

- **Edgar Allen Poe**, from “Ligeia”. 1938

“Academicians are more likely to share each other's toothbrush than each other's nomenclature.”

- **M. Michael Cohen, Jr**., from “The Child With Multiple Birth Defects.” 1982.

“Pneumococcus is altogether an amazing cell. Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs biochemical feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death. Furthermore, living or dead, whole or in part, on entering the animal body Pneumococcus starts a train of impulses, stimulating all the reactions grouped under those inclusive phenomena known as immunity.”

- **Benjamin White,** from “The Biology of Pneumococcus” 1938.

"It has all been most interesting."

- the last words of **Lady Mary Wortley Montagu,**
(writer, poet, socialite, feminist and early supporter of vaccination) 1762.

Chapter 1: Background

1.1 Perinatally acquired Human Immunodeficiency Virus (paHIV) infection and Acquired Immune Deficiency Syndrome (AIDS)

1.1.1 *Global epidemiology*

Since the first cases of paediatric AIDS were described in the early 1980's [1, 2] childhood HIV infection and AIDS have escalated to become a global challenge. The latest Joint United Nations Programme on HIV/AIDS (UNAIDS) report estimates that in 2011, approximately 330 000 (280 000- 380 000) children were born with HIV bringing the total number of children under 15 estimated to be living with HIV to 3.4 million (3.1 million- 3.9 million). The estimated number of AIDS related deaths in this age group in 2011 was 230 000 (200 000- 270 000). The vast majority of the new infections in children occurred in sub-Saharan Africa. This is a reflection of the overall burden of HIV infection in women of childbearing age in this region and limited provision of interventions targeted at prevention of mother to child transmission (PMTCT) [3].

HIV transmission from mother to infant (vertical transmission) can occur antenatally, perinatally or postnatally [4]. The risk of vertical transmission can be reduced from as high as 40% with no intervention to around 1% by implementing various measures including appropriate antenatal antiretroviral therapy (ART), intrapartum ART, elective caesarean section in cases with detectable maternal viral load, early neonatal post exposure prophylaxis and exclusive formula feeding [5, 6]. Unfortunately multiple factors contribute to suboptimal implementation of these interventions in resource poor countries. In West and Central Africa region, for example, estimated ART coverage rates for PMTCT are still as low as 27% (23-30%) [3].

Highly active antiretroviral therapy (HAART) has been shown to lead to decreased morbidity and mortality in paediatric HIV in both resource rich [7-10] and resource poor settings [11-14] however access to HAART for children is limited in many of the countries with the highest requirement. Access for those children in need of ART (see Section 1.1.6 for discussion on treatment guidelines) was estimated to be only 28% (25-32%) globally in 2011, with some countries estimated to be achieving access rates as low as 8% [3]. Difficulties in diagnosis of HIV infection, rapid disease progression and problems in developing appropriate drug formulations for children all contribute to coverage figures that are frequently worse than those observed for adults in the same geographical region [3, 15].

Despite these issues, globally, the overall rate of new HIV infections in children is fortunately dropping. The death rate due to AIDS is decreasing and the overall number of children living with HIV is starting to plateau [3]. As access to ART increases in resource poor settings the numbers of children surviving to older childhood and adolescence will increase and problems currently affecting HIV infected children and adolescents in the resource rich setting will become increasingly relevant.

1.1.2 *UK epidemiology*

The British Paediatric Surveillance Unit (BPSU) has collected data on paediatric HIV and AIDS since 1986 [16]. In 1989 the National Study of HIV in Pregnancy and Childhood (NSHPC) was established for the UK and Ireland. This is a confidential national reporting scheme for pregnancies in HIV infected women (through obstetric services), babies born to HIV infected women (through BPSU) and other children with HIV infection and AIDS (also through BPSU). The NSHPC collect demographic data and limited follow up information on HIV exposed and infected children [17, 18]. In 2000 the Collaborative HIV Paediatric Study (CHIPS) was established to collect data on children treated in Paediatric European Network for Treatment of AIDS (PENTA) associated centres in the UK and Ireland [18, 19]. CHIPS currently collects long term clinical and immunological follow up data on all HIV-infected children reported to the NSHPC [17, 18].

The number of children under NSHPC/CHIPS follow-up steadily increased from the 1980's until around 5 years ago when numbers plateaued and have subsequently decreased (Fig. 1).

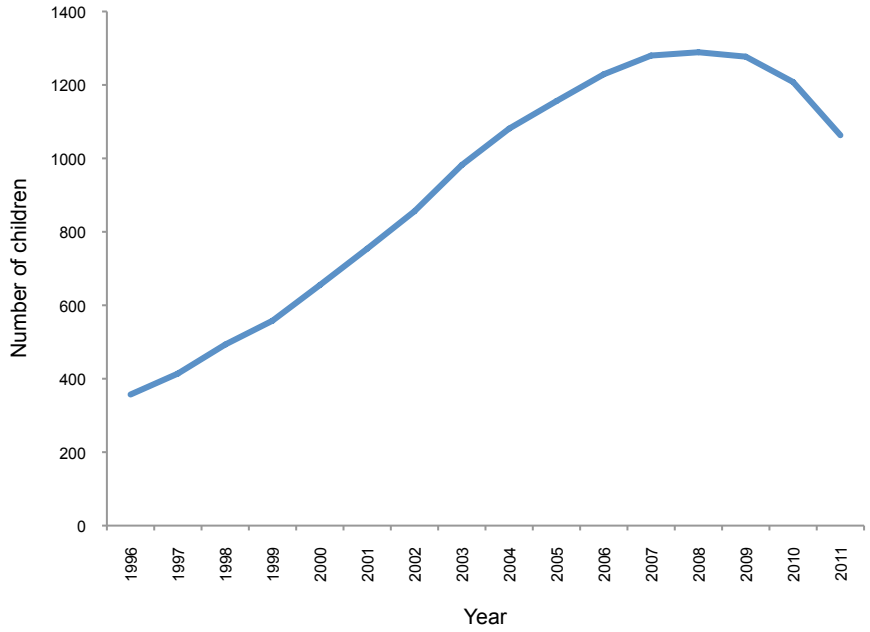


Figure 1. Number of HIV-1 infected children registered in NSHPC/CHIPS follow-up in the UK and Ireland by year (adapted from data provided by CHIPS: CHIPS report 2012)

This pattern can be mainly attributed to the introduction of HAART as standard therapy for children in 1997, introduction of voluntary antenatal HIV testing for pregnant UK women in 2000, effective implementation of PMTCT protocols leading to a reduction in the rate of vertical transmission for babies born in the UK to around 1% [5, 19] and a gradual increase in the number of adolescents transitioning to adult services [20]. Since the introduction of HAART for children, there has been a substantial decrease in the mortality associated with paediatric HIV infection [19, 21]. The median age of the cohort has steadily increased, the age distribution is shifting upwards (Fig. 2) and the number of children who have transitioned or are due to transition to adult services is also steadily increasing [20].

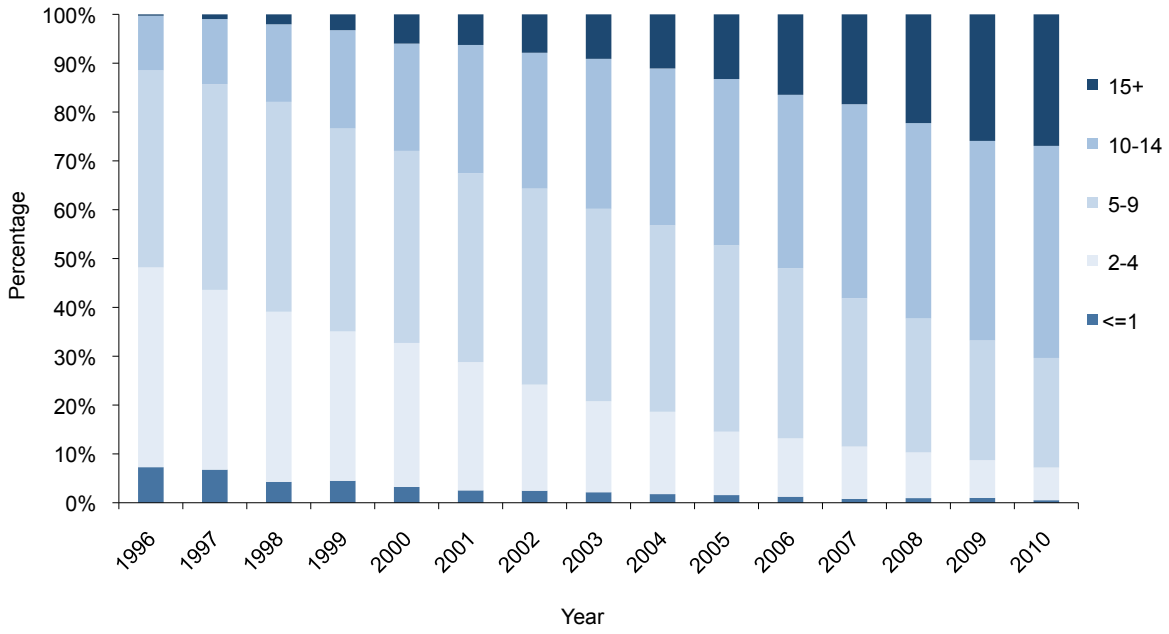


Figure 2. Age distribution of HIV-1 infected children in NSHPC/CHIPS follow-up in the UK and Ireland by year (adapted from data provided by CHIPS: CHIPS report 2011-2012)

The large majority of children have vertically acquired HIV-1 infection. Of the 1188 children currently under NSHPC/CHIPS follow up 51% are female, 51% were born abroad, 80% are of Black African ethnicity and 51% are followed up in a London clinic (Data provided by CHIPS: CHIPS report 2011-2012)

1.1.3 Cluster of differentiation (*CD)4 T cell immunology and paediatric HIV infection*

HIV-1 is a species of retrovirus in the genus lentivirus [22], first identified in 1983 [23] and proven to be the causative agent in AIDS in 1984 [24, 25]. Prior to 1986 HIV virus was known by other names including human T-lymphotropic virus- III (HTLV-III) or lymphadenopathy-associated virus (LAV) [26]. There are currently two known species of HIV: HIV-1 and HIV-2. The virological and immunological profile and pattern of disease associated with HIV-2 infection is somewhat different to that associated with HIV-1, HIV-2 having a lower rate of disease progression and a frequently milder disease course [27]. HIV-1 is the predominant species in the world today, whereas HIV-2 is found mainly in certain West African Countries (e.g. Guinea Bissau) or countries with close ties to these regions (e.g. Portugal) [28]. There are 140 cases of HIV-2 currently reported in the UK [29]. 39 pregnancies in 28 HIV-2 infected women have been reported to the NSHPC, with no known mother to child transmission [30] and there are no reported cases of paediatric HIV-2 in the UK (personal communication with CHIPS/NSHPC). The work in this project is therefore solely related to paediatric HIV-1 infection. HIV-1 will be referred to as HIV unless otherwise stated.

HIV mainly gains entry to target cells through glycoprotein 120 (gp120), a viral envelope glycoprotein, binding to CD4 molecules on the target cell surface [31]. CXC chemokine receptor (CXCR) 4 and CC chemokine receptor (CCR) 5 are important co-receptors for cell entry [32, 33]. *In vivo* HIV infection is therefore predominately restricted to cells expressing these molecules i.e. CD4+ T cells, monocyte/macrophages and dendritic cells [34, 35], although other mechanisms of cell entry exist e.g. Fc

receptor, dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) and complement receptors (CR) [36, 37]. The immunopathology of HIV infection is brought about primarily through the virus's effect on infected CD4+ T cells, although there are many indirect effects of infection on other aspects of host immunity.

CD4+ T cells are a subset of CD3+ lymphocytes, classically referred to as T helper cells (Th). They are termed "helper" cells as their main role is to provide antigen specific "help" to other arms of the immune response e.g. B Cells, macrophages. They express a T cell receptor (TCR) and CD4, which acts as a co-receptor. They recognise antigen, in the context of major histocompatibility complex (MHC) class II molecules expressed on the surface of professional antigen presenting cells (APCs) [38]. $CD4^+$ T cells can be divided into subsets based on expression of certain cell surface molecules, cytokine production and characteristic transcription factors. These include classical T helper 1 (Th1) and T helper 2 (Th2) subsets in addition to more recently defined T helper 17 (Th17), regulatory T cells (Tregs) and follicular T helper cells (T_{FH}). It has been proposed that CD4+ cells are induced to follow a specific developmental pathway depending on cytokine exposure and patterns of co-stimulation at the time of antigen presentation and during subsequent development. In this way the CD4+ T cell response is thought to adapt according to the nature of the invading pathogen/ immune response required (Fig. 3) [39].

Subset	Polarising cytokines	Transcription factor	Homing receptors	Effector cytokines	Target cell	Function
Th1	IL12, IFNγ	T-bet	CXCR3	IFNγ	Macrophages	Bacteria
Th2	IL4	GATA-3	CCR4/CRTh2	IL4, Il5, IL13	Eosinophils	Parasites
Th17	IL1β, IL6, TGFβ	ROR-γT	CCR6/CCR4	IL17, IL22	Neutrophils	Fungi
Treg	?	FOXP3	CCR7/CCR6	TGFβ	DC/ T cells	Regulation
Tfh	IL21	Bcl-6	CXCR5	IL21	B cells	Antibodies

Figure 3. A simplified overview of CD4+ T cell subsets. CD4+ T cells can be divided into subsets according to characteristic polarising cytokines, transcription factors, homing receptors and effector cytokines. It is proposed that each subset influences predominately one cell type and has a characteristic role to play in host defence (adapted from Sallusto et al [39]).

The mechanisms by which HIV infection alters CD4+ T cell number and function are still incompletely understood, however processes such as direct cytopathic effect of viral proteins, apoptosis thorough immune activation, destruction of lymphoid tissue, decreased thymic output, cell mediated anti-CD4 cytotoxicity, autophagy and anti-CD4 autoantibodies have all been implicated [36, 40, 41] It is generally agreed that immune activation is a key mechanism by which HIV has its effect both in the T cell compartment and more generally in the immune system as a whole [42].

The differential effect of HIV on CD4+ T cell subsets has been discussed for some time [36, 43-45] however changes in more recently defined CD4+ T cell subsets resulting from HIV infection are only starting to be investigated [36, 46-50]. CD4+ T cells originate in the bone marrow, develop in the thymus then circulate through peripheral blood and take up residence and circulate through secondary lymphoid structures such as the spleen, lymph nodes and mucosa associated lymphoid tissue (MALT) [51]. HIV infection impacts on

CD4+ T cell development and function in each of these sites, with different patterns of involvement observed at different stages of infection [52-61]. Horizontal transmission of HIV usually results in an insult to a fully developed immune system, whereas at the time when vertical transmission occurs (*in utero*, perinatally or postnatally) all aspects of immunity, including CD4+ T cells, are still developing [62]. The impact of HIV infection on the developing, immature immune system is different to that seen following horizontal transmission. This is reflected in the pattern of disease, virology and immunology observed in paediatric infection [63-65].

With respect to the immunological characteristics of paediatric infection, exposure to maternal infection, even without transmission, has an effect on immune phenotype at birth and during infancy. In comparison to unexposed healthy infants, various studies on cord blood mononuclear cells (CBMC) and/or peripheral blood mononuclear cells (PBMC) from HIV exposed uninfected infants (HEU) have been shown to have lower CD4+ T cell number and/or percentages [66-71]. PBMC from HEU have been shown to produce less interleukin (IL)2 (a Th1 cytokine) on stimulation with the mitogen phytoheamagglutinin (PHA) [72], however intracellular IL2 producing CD4+ T cells were more frequent in HEU PBMC on stimulation with the mitogen phorbol myristate acetate (PMA) and ionomycin. Higher production of IL10 (Th2 cytokine) has been demonstrated in HEU CBMC stimulated with PHA [73], whereas CBMC stimulated with anti-CD3 and anti-CD28 have been shown to produce less IL10 and more interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) (Th1 cytokines) but only in HEU of mothers with detectable viraemia [74]. CBMC from HEU produced less IL12 (monocyte derived Th1 cytokine) when stimulated with *Staphylococcus aureus* Cowan (SAC) when compared to unexposed controls. The pattern of maturation of the IFNγ response to whole blood stimulated with PHA seen over the first 6 weeks of life in unexposed infants was shown to be absent in a proportion of HEU, many of whom had relatively high IFNγ responses at birth [75]. These at times conflicting observations could be partially explained by the different mitogen/ T cell stimulating condition used, or different patient group demographics. Although somewhat contradictory, these studies clearly demonstrate differences in immune phenotype between HEU and unexposed controls.

Furthermore, expression of CD38 (a T cell activation marker) on CD4+ T cells of HEU is increased in the first year of life [72, 76]. CD4+ T cells isolated from HEU cord blood have also been shown to be more prone to apoptosis [77]. HEU have a lower proportion of naive and a higher proportion of memory CD4+ T cells at birth [67, 76], a pattern that may persist into later childhood [66]. Lower CD4 + T cell T cell receptor excision circle (TREC) levels, demonstrated at birth, suggesting a decrease in thymic output compared to healthy unexposed controls, may contribute to these findings. Although many of these abnormalities might be attributed to the effects of HIV infection in the mother, as more and more infants are exposed to ART as part of PMTCT programmes the potential impact of maternal ART and infant postexposure prophylaxis on these immune parameters must also be borne in mind [70, 71].

Investigating the implications of these subtle variations in immune phenotype will be of increasing importance as the numbers of infants being born uninfected through successful PMTCT programmes increases globally. Indeed, the increased morbidity observed in some studies of HEU [78-81] may be

partially attributable to altered immune function, and there may well be implications for routine vaccine responses [82].

As observed in adults, if left untreated, vertically transmitted HIV infection leads to a progressive decline in CD4+ T cell number and percentage [83], over and above that which is normally observed in early childhood [84, 85] or HEU [70, 85, 86]. Plasma viral load is usually relatively low at birth (unless transmission has occurred in utero) then subsequently rises rapidly in the first 2 months of life after which there is a steady decline in plasma viral RNA levels [87-89]. This is in contrast to the pattern observed in primary horizontal HIV infection in adults in whom there is usually an initial rapid rise in viral load followed by a similarly rapid fall in viral load to a much lower steady state [52, 90]. The cause of this drop in viraemia after primary infection in the absence of ART is not completely understood, however it is thought to be due mainly to acquired HIV specific CD8+ T cells responses [91-93], bringing viraemia to a lower set point and possibly related to an overall decrease in the number of cells capable of supporting active infection having an effect on viral replication potential [52, 90]. In addition, innate immunity, B cells, antibody and even CD4+ T cells themselves are all thought to have a role in controlling virus and determining this viral load set point [63, 65, 94-97].

As well as differences in viral load kinetics, differential effects of HIV on a child's developing immune system are reflected in characteristic patterns of CD4+ T cell depletion and disease progression. Following vertical infection, around 25% of children if untreated will rapidly progress to AIDS in the first year of life and 40% will have an AIDS diagnosis by the age of 4 years [98]. This is in contrast to observations in adults in whom a much lower proportion rapidly progress and a longer AIDS free period is the norm [99].

Many aspects of early life immunity [100, 101] may contribute to the different viral kinetics and immunology observed following vertical transmission. Certain aspects of infant immunology may be protective, whereas others may be detrimental [15, 102]. The thymus is relatively bulky and active in early life [103], which may well contribute to new variant-specific cytotoxic T cell responses more commonly observed in children than adults following viral escape mutation [104]. This relatively preserved thymic activity in infancy is also thought to have implications for immune reconstitution following initiation of HAART (see below). Despite the potential advantages of having an active thymus, the observed increased severity of untreated disease in infants suggests that, in the absence of HAART, the disadvantages of being an infant by far outweigh the benefits. Indeed, an active thymus may even be detrimental to the HIV infected infant, serving as a large source of cells vulnerable to infection [105] and the immaturity of the immune system as a whole during infancy [62] may well have far reaching implications for disease outcome.

Other viral and host factors may hinder effective control of infant infection. Transmission occurs more frequently in the context of high maternal viral load [5, 97]. Certain human leukocyte antigen (HLA) class I types are associated with better or worse viral control [106-108]. It follows that infants at greater risk of infection are also more likely to have inherited an HLA class I allele associated with poorer viral control [107, 108]. Moreover, infants are infected with virus that has existed in the context of the maternal and often paternal immune system. The transmitting virus may have evolved mutations specifically adapted to

existence in a host with a specific HLA class I genotype. Infants therefore inherit a virus that may have already adapted to escape control by the HLA class that they have also inherited from their parents [107, 109].

Differences between adult and paediatric HIV infection also become apparent when commencing ART. When starting treatment, dynamics of T cell recovery and immune reconstitution observed in peripheral blood are different from those observed in adults. In addition to the subsets mentioned above, CD4+ T cells can be further divided by expression of surface markers into naive, effector memory and central memory populations [110]. In children commencing ART there is initial replenishment of naive CD4 cell numbers and subsequent restoration of cells with memory phenotype [111]. This is in contrast to the pattern in adults in whom there is initial replenishment of the memory CD4+ T cell pool followed by gradual increases in naive cell numbers [112]. In healthy individuals, thymic function naturally decreases with age as demonstrated by studies quantifying TRECs, a marker used to assess thymic activity [103]. HIV leads to decreased thymic function in both children and adults [102, 103]. The different pattern of CD4+ T cell subset recovery observed in children is thought to be a reflection of relatively preserved thymic function and output potential in children [67, 95, 102, 113-116].

Changes in HIV specific CD4+ and CD8+ T cell populations occur after commencing effective HAART. In children HIV specific CD4+ T cell responses increase, whereas the magnitude of HIV specific CD8+ T cell responses decrease over time. The breadth of these CD8+ T cell responses is however maintained [95]. Once more, differences in the potential for immune restoration are observed between adults and children, children having a much higher proportion of HIV specific CD4+ T cells in peripheral blood than adults following initiation of therapy [117].

Despite these differences in the immunological response to HAART, the pattern of virological response is somewhat similar in adults and children. A characteristic biphasic virological response is observed with an initial rapid decrease in plasma viral load followed by a slower exponential decrease. The clearance rates observed for adults and children are roughly equivalent. It has been proposed that this biphasic response could reflect death rates of different susceptible cell populations i.e. initially productively infected cells, subsequently long lived/latently infected cells [118, 119].

1.1.4 *B cell immunology and paediatric HIV-1 infection*

Although the impact of untreated HIV infection on CD4+ T cells is the main driving force for HIV associated disease, the disruptive effect that infection has on other aspects of immunity should not be underestimated. There is little evidence to suggest that HIV can infect B cells *in vivo* however infection has wide ranging consequences for B cell phenotype and function. Following the dramatic success of HAART in restoring T cell populations, it is increasingly important to investigate the possible reversible and irreversible B cell deficits that are brought about by HIV [120].

B cells are lymphocytes with an essential role in the production of antibody and humoral immunity. They characteristically express a B cell receptor (BCR) and mostly develop in the bone marrow, from where they

emigrate in the form of transitional B cells [121, 122]. Transitional B cells mature in the periphery into naive mature B cells expressing surface bound immunoglobulin D and M (IgD/IgM) [122] and following antigenic challenge have the potential to differentiate into antibody secreting plasma cells (via a plasmablast stage) or memory B cells [121, 123-125].

Plasma cells secrete antibody and can either be short-lived or long-lived depending on the nature of antigenic challenge, T cell help, BCR affinity and the developmental state of the B cell from which they arise [126, 127]. They can develop following the germinal centre (GC) reaction (see below) or independently of this process [127-130].

Memory B cells can be defined as "long-lived quiescent B cells expressing somatically mutated IgV genes that are capable of eliciting rapid and robust responses compared with corresponding antigen-inexperienced naive B cells" [124]. They can be subdivided into various subsets according to surface marker expression. The origin and classification of these subsets is a matter of debate, with some confusion attributable to differences between mice and humans [131-133], however two main subsets are relatively well defined in humans; class switched and IgM memory B cells [124, 134].

B cells can respond to antigen by producing antibody through either T cell dependent (TD) or T cell independent (TI) mechanisms. TD antigens are classically proteins that can be processed and presented in the context of MHC class II molecules on the surface of APCs. TI antigens can be divided into TI type 1 and TI type 2 antigens. TI type 1 antigens, such as lipopolysaccharide (LPS), polyclonally stimulate B cells to produce antibody predominately via toll-like receptor interaction. TI type 2 antigens are usually larger repetitious molecules such as bacterial cell wall polysaccharides that stimulate antigen specific B cells through direct BCR cross-linking [128, 135].

Class switched memory B cells mainly develop following the GC reaction, whereby antigen specific B cells come into contact with helper T cells specific for the same antigen in GCs in secondary lymphoid tissue. The helper T cells responsible for this B cell help are termed follicular T helper cells (T_{FH}) [136-144]. The interaction between T_{FH} and B cell in the GC microenvironment results in class switch recombination (CSR), whereby the immunoglobulin isotype changes from IgM/IgD to an alternative one e.g. IgG, and affinity maturation, whereby, through the process of somatic hypermutation, the affinity of the antibody for the antigen is optimised. The GC reaction ultimately results in either long-lived plasma cells that migrate to settle in niches in the bone marrow [128], secreting antibody long term or class switched memory B cells that persist and circulate awaiting secondary antigenic challenge following which they can rapidly differentiate into antibody secreting plasma cells [123].

The origin of IgM memory B cells in humans is debated. It now seems clear that memory B cells which express cell surface IgM may not result from a single developmental pathway. It is currently thought that a proportion of them develop independently of the GC reaction, responding to TI antigens such as bacterial cell wall polysaccharides by rapidly differentiating into plasma cells secreting IgM [145]. On the other hand, it has been proposed that IgM memory B cells can also develop as a result of the germinal centre reaction

minus CSR, these being true IgM memory B cells. A final possibility it is that they may result from a separate antigen independent B cell lineage. These are thought to represent a more innate like B cell subset, equivalent to marginal zone and B1a cells in mice (see below). The debate is ongoing [146-148].

A further subset of B cells termed B1 cells, which can be further subdivided into B1a and B1b cells have been well characterised in the mouse, and are also thought to represent more innate-like B cells, responding to TI antigens [133]. Recent reports of an identifying marker for this subset in humans [149] met with some controversy [150-152]. As they are still not clearly defined as a population in humans, they will not be discussed further in the context of this work. See Fig. 4 for an overview of human B cell development.

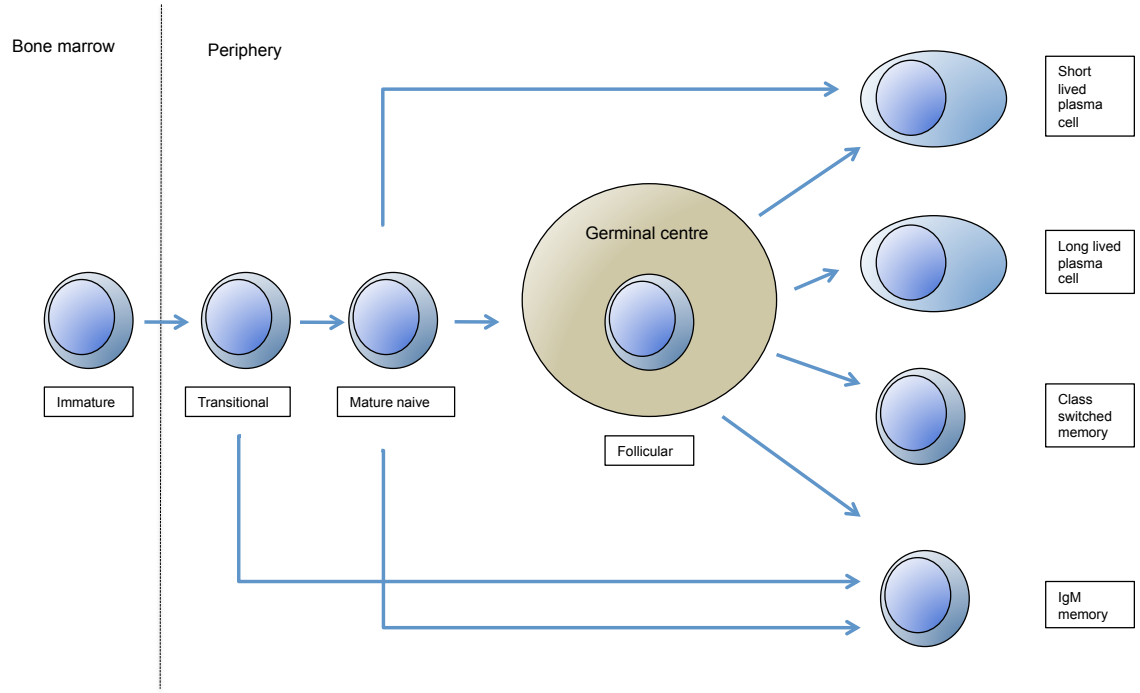


Figure 4. Simplified overview of peripheral B cell development. Immature B cells leave the bone marrow as transitional cells. These develop into naive mature B cells. Once activated these cells can either develop into short lived antibody secreting plasma cells or enter the germinal centre and develop into either class switched memory B cells or long lived antibody secreting plasma cells which migrate to persist in bone marrow niches. IgM memory B cells may develop through a number of proposed pathways including an antigen independent pathway direct from transitional cells, an extrafollicular antigen dependent pathway, or through the germinal centre reaction.

Just as with T cell immunity, B cell immunity is functionally immature at birth and develops into the adult phenotype over the course of childhood [62, 153]. Overall circulating B cell counts are highest in infancy then gradually decrease throughout childhood and adolescence. As overall B cell numbers decrease so do the absolute numbers of memory B cells. The proportion of B cells that are IgM and class-switched memory B cells gradually increases with age as the proportion of naive B cells correspondingly decreases [84, 154].

The proportion of B cells with transitional phenotype is highest in infancy then decreases with age [84]. Antibody responses to both protein/TD and especially polysaccharide/TI antigens are weaker and shorter lasting in infancy. The latter observation is thought to contribute to the increased vulnerability of children of this age to bacterial pathogens such as *Streptococcus pneumonia*, *Neisseria meningitidis* and *Haemophilus influenzae* all of which have a coating polysaccharide cell wall [153, 155].

Since the very earliest days of HIV research, abnormalities of the B cell compartment and humoral immunity were noted to be a prominent feature of HIV/AIDS in both adults [156] and children [157]. Untreated adult HIV infection is associated with a number of B cell and humoral abnormalities [120]. The observed abnormalities are listed in Box 1. Many of these abnormalities may be reversed by HAART, however certain aspects of memory B cell phenotype and function persist.

Hypergammaglobulinaemia
↑ polyclonal B cell activation
↑ cell turnover
↑ autoantibodies
↑ B cell malignancy

↑ activation markers
↑ plasmablasts
↑ immature/transitional B cell
↑ activated mature B cell
↑ exhausted/ tissue like B cell

↓ memory B cell numbers
↓ memory B cell function

Box 1. B cell abnormalities observed in HIV infection

In 2001 De Milito et al demonstrated that adult HIV infection is associated with decreased memory B cell number and percentage, irrespective of whether the patient is on HAART [158], a finding that was subsequently confirmed by Chong et al in 2004 [159]. De Milito's group went on to demonstrate that abnormalities in B cell number and phenotype occur early in primary HIV infection and are potentially reversible at this stage through the early use of HAART [160]. D'Orsogna et al demonstrated chronic HIV infection to be associated with decreased total B cell numbers but to have no effect on total memory B cell percentages. Class switched memory B cells were decreased in both treatment naive patients and those on HAART. They also demonstrated decreased IgM memory B cells in a subset of patients with low CD4 count <300 cells/μl, whereas patients on HAART had equivalent numbers to healthy controls, suggesting HAART restored this population [161]. In contrast to these findings Hart et al demonstrated that HIV infected adults have decreased memory B cell percentages, irrespective of treatment status. IgM memory B cell

percentages were lower than in healthy controls in both treatment naive patients and those on HAART, and this correlated with poor responses to the TI pneumococcal polysaccharide vaccine (PPV). Approximately 25% of patients had a significant reduction in class switched memory B cell percentage in both patient groups and this was associated with poor responses to TD tetanus vaccination [162]. More recently Grevenynghe et al have again confirmed decreased circulating memory B cells percentages in HAART treated aviraemic adults compared to healthy controls, but when examining memory subsets, only class-switched memory B cells were found to be significantly lower [163]. Johannesson et al did not include a healthy control group but did find adults on suppressive HAART to have a significantly higher class switched memory B cell count than HAART naive patients, but no difference in class switched proportion or IgM memory cell count or proportion [164]. Although these studies are in some aspects contradictory and do not investigate the effect of treatment longitudinally, observations together indicate that initiation of HAART in chronic adult HIV infection may not fully restore B cell abnormalities established early in infection and have implications for optimising treatment strategies and optimal vaccination strategies.

The Moir group have published extensively on B cell abnormalities in HIV infected adults [120, 165]. Rather than using IgD/IgM to classify memory subsets they have employed CD21 (Complement Receptor 2 (CR2)) to identify populations of circulating $CD21^{lo}$ B cells that are observed with increased frequency in adult infection. They also demonstrated this $CD21^{lo}$ population to be made up of transitional B cells ($CD10^{+}CD27^{-}CD21^{lo}$), activated mature B cells ($CD10^{-}CD27^{+}CD21^{lo}$), plasmablasts ($CD10^{-}CD27^{++}CD21^{lo}$) and a newly characterised subset with an exhausted phenotype termed “tissue like/exhausted” memory B cells ($CD10^{-}CD27^{-}CD21^{lo}$) [120]. Recent longitudinal data from the Moir group in adults commencing HAART in early and chronic HIV infection, have again suggested that early HAART may prevent irreversible changes in B cell phenotype [165]. A summary of the abnormalities of these B cell subsets in adult HIV infection is shown in Fig. 5.

The B cell compartment in HIV infected children has been less extensively investigated. Jacobsen et al found that HIV infected children on HAART with undetectable viral load had reduced IgD^{+} memory B cell percentage compared to those on treatment with detectable viral load, treatment naive patients and healthy controls [166]. Ghosh et al demonstrated decreased class switched and IgM memory B cell percentage and number in HIV infected children when compared to healthy controls. In contrast to the findings of Jacobsen et al, children with an HIV viral load (VL) >500 Eq/ml had lower class switched memory B cell counts than children with VL<500 Eq/ml (the patients presumably on treatment although this is not reported) [167].

Pensieroso et al found total memory B cell percentages in HIV infected children who started treatment early to be equivalent to those in age matched healthy controls, whereas patients who started HAART later had lower memory B cell percentages than healthy controls. The early treatment group was also found to have relatively preserved responses to common vaccine antigens as measured by serum antibody and antigen specific B cell enzyme linked immunosorbant SPOT (ELISPOT). IgM and class switched memory B cell populations were not significantly different between HIV infected children and healthy controls, irrespective of treatment status [168]. Iwajomo et al studied B cell immune phenotype in a cohort of East African HIV infected children who were not on HAART. They found decreased proportions of naive mature and resting

memory B cells and correspondingly increased activated mature B cells. In addition they found a decrease in IgM memory B cell numbers compared to healthy controls [169]. These results together, as with those in adults are somewhat contradictory and are not longitudinal, however they strongly suggest that abnormalities in B cell memory phenotype and function occur in paediatric infection, and that early HAART may prevent or reverse changes that at a later stage may be irreversible.

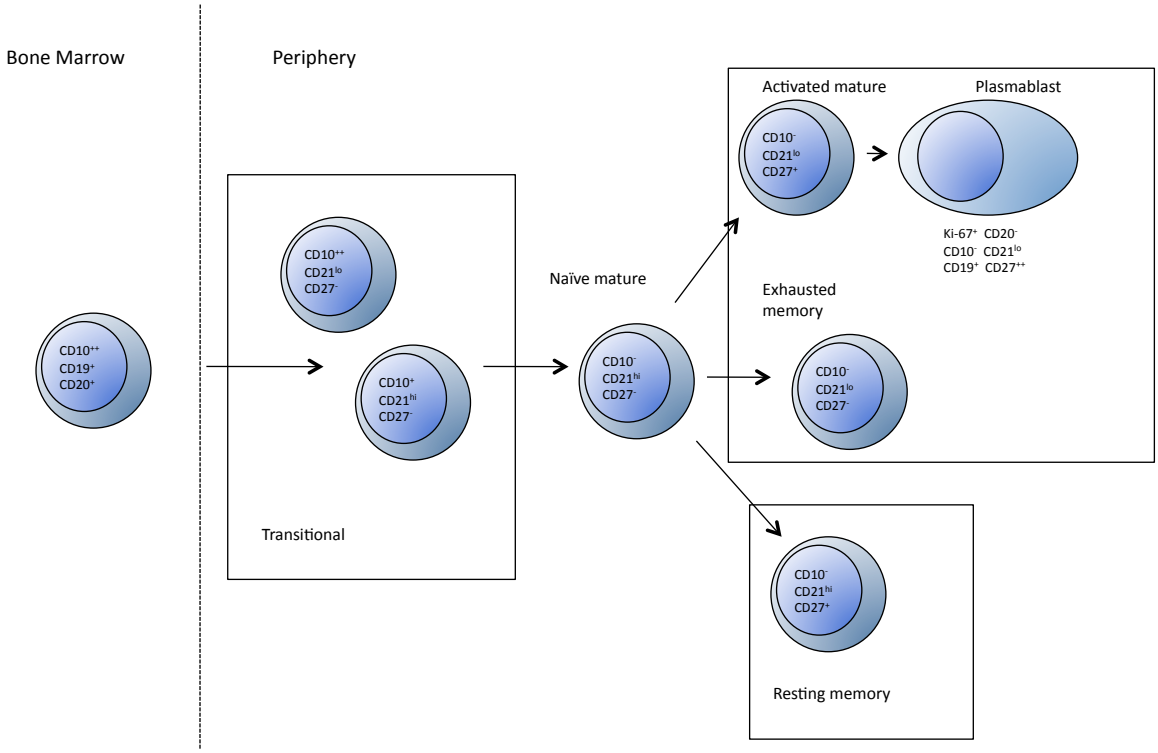


Figure 5. Summary of B cell phenotypic abnormalities observed during HIV infection. Cells in boxes are seen to change in proportion during chronic infection. Transitional cell numbers increase with CD4+ T cell lymphopenia. Exhausted and activated mature memory B cells and plasmablasts are increased in association with viraemia. Resting memory B cells are decreased during HIV infection. (hi = high, lo = low). (Adapted from review by Moir et al 2009 [120])

B cells play an essential part in the response to many routine vaccinations administered during childhood [170]. In addition, it is becoming increasingly apparent that B cells are not only important as a source of antibody but that they also have an immunomodulatory role through antigen presentation to T cells, costimulation and cytokine production [171, 172]. As HAART allows survival into adult life following vertical infection, it is important to ensure adequate protection against vaccine preventable diseases. The impact of

HIV on a child's developing B cell immunity must be borne in mind when planning treatment and vaccination schedules.

1.1.5 *Innate immune dysfunction in paediatric HIV-1 infection*

HIV is also known to disrupt innate immune cells and pathways [65, 173]. Decreased natural killer (NK) cell, natural killer T (NKT) cell and dendritic cell (DC) number and function have all been demonstrated in paediatric infection, especially in children with detectable viral load and low CD4+ T cell counts [173-175]. These deficits are only partially restored by HAART [173, 174]. Cytokine production by PBMC in response to Toll-like receptor (TLR) agonists is decreased in children on HAART [173]. Decreased neutrophil count and abnormalities of neutrophil function have been demonstrated in untreated HIV infected children [176-178], however it should be noted that antiretroviral treatment can itself contribute to decreased neutrophil counts both in HIV infected and HEU children [178]. Increased complement activation and consumption have been demonstrated in children with HIV [179], although the ability of HIV patient serum to opsonise pneumococci with complement *in vitro*, at least in adults, does not seem to be compromised [180]. Disruptions of many other aspects of innate immune function have been demonstrated in adults but are yet to be explored in children. Similarly the effect of HAART in restoring innate immune function in children is only beginning to be investigated [65].

1.1.6 *Staging and treatment of paediatric HIV-1 infection*

The first specific antiretroviral drug, zidovudine (AZT), was approved for use in humans in 1987. The efficacy of AZT as monotherapy was limited, often rapidly resulting in HIV viral drug resistance mutations and loss of virological suppression. Since then, a range of antiretroviral medications have been developed which are divided into classes of drug with differing mechanism of action. Since 1997 the use of combination therapy also known as HAART, employing drugs from 2 or more different classes has resulted in the potential for long term virological suppression (<50 viral RNA copies/ml peripheral blood), maintained CD4 T cell count, substantially decreased morbidity and increased survival in both adult and paediatric cohorts. Briefly, the classes of currently available antiretroviral medications include: inhibitors of viral reverse transcriptase (nucleotide/nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI)), inhibitors of viral protease (protease inhibitors (PI)), inhibitors of integration into the host genome (integrase inhibitors), inhibitors of gp41 mediated viral/host cell fusion (fusion inhibitors), and drugs which prevent viral co-receptor binding via CCR5 blockade (CCR5 receptor antagonists). Efficacy of HAART is assessed clinically and by monitoring peripheral blood HIV viral load (using PCR) and CD4 count (using flow cytometry) [500, 501].

Different groups recommend different staging and treatment strategies for paediatric HIV. World Health Organisation (WHO) guidelines are more tailored for use in developing countries. Centers for Disease Control and Prevention (CDC) guidelines are used mainly in North America and PENTA guidelines are followed in the UK and Europe. For the purposes of this report the PENTA guidelines will be referred to unless otherwise stated [181]. CDC and WHO staging are listed in Appendix 1 and 2. The PENTA treatment recommendations are summarised in Table 1. Guidance on appropriate drug treatment regimens to use is listed in Appendix 3.

HAART has resulted in a dramatic decrease in morbidity and mortality in vertically acquired HIV in the UK [10, 19, 21]. Recently it has become clear that all infants diagnosed with HIV should be commenced on HAART [7, 11, 181]. Potential drug toxicities of HAART are many and varied [181]. An important question that is yet to be answered is whether HAART can be safely interrupted following initiation in infancy in order to minimise long-term potential drug toxicity. Evidence for the possible detrimental effects of treatment interruption in HIV infected adults has been provided by the Strategies for Management of Antiretroviral Therapy (SMART) study group's large randomised controlled trial of CD4 count guided treatment interruption. An excess of opportunistic disease and death from any cause was observed in those randomised to treatment interruption [182]. Subsequent analysis of stored samples showed IL6 and D-dimer levels to be strongly related to all-cause mortality, treatment interruption being associated with an increase in these markers [502]. IL6 and high sensitivity CRP (hsCRP) were independently predictive of opportunistic disease [503]. Compared to healthy controls, adults with HIV had higher levels of hsCRP, IL6 and D-Dimer, even when on fully suppressive HAART [504], these markers being associated with an increased risk of cardiovascular disease [505]. A similar (although smaller) trial of treatment interruption in children with paHIV (PENTA 11) has found no excess morbidity or mortality in those randomised to treatment interruption, despite evidence of immune activation being demonstrated at the cellular level [183,184, 506]. A preliminary report on immunological and virological effects of PTI in HIV infected children showed increased viral load and CD4 cell activation and a decrease in CD4 cell number. The relative proportions of CD4 cell subsets changed initially but quickly returned to baseline. [183, 185]. This is an area of ongoing research in paediatric HIV (PENTA 11 and CHER trial) [7, 183, 184]. The effect of treatment interruption on B cell immunology in children has not as yet been reported.

A further important question is whether to raise the CD4 cut off for initiation of treatment in children diagnosed outside of the infant period, in line with current CDC paediatric guidelines [186] or possibly even to consider starting treatment in all children irrespective of immune/virological status, in line with current CDC adult guidelines [187]. Increasing evidence of detrimental effects of ongoing viral replication and the immunological benefits of earlier HAART initiation must be considered in the context of potential drug toxicity, logistic, financial and epidemiological factors in both resource rich and resource poor settings. Both these key questions are subject to ongoing debate.

Age	Means of assessment	Treatment guidance
0-11 months	Clinical	Treat all
	Immunological	Treat all
	Virological	Treat all
12-35 months	Clinical	Treat CDC stage B or C/ WHO stage 3 or 4
	Immunological (CD4%/count)	Treat <25% or <1000 cells/μl
	Virological	Consider >100 000 copies/ml
36-59 months	Clinical	Treat CDC stage B or C/ WHO stage 3 or 4
	Immunological (CD4%/count)	Treat <20% or <500 cells/μl
	Virological	Consider >100 000 copies/ml
5 years +	Clinical	Treat CDC stage B or C/ WHO stage 3 or 4
	Immunological (CD4%/count)	Treat <350 cells/μl
	Virological	Consider >100 000 copies/ml

Table 1. Table summarising PENTA treatment guidelines for HIV infected children.

1.1.7 *Vaccine responses in paediatric HIV-1 infection*

Vaccine responses in healthy infants are generally less robust than in older children and young adults [153]. Many studies have demonstrated impaired responses to a variety of vaccines in the context of paediatric HIV infection [188-191]. In HIV infected children on HAART, the basic quantification of T and B cell numbers and phenotype as well as vaccine responses show residual deficits in humoral and cellular immunity, despite recovery of CD4+ T cell numbers and suppression of viral load [168, 188-190, 192-205]. Many routine vaccines require T cell help for B cells for an effective serological response and the establishment of immune memory. There are many points along the pathway from innate immunity and antigen presentation to acquired cell mediated and humoral response that are potentially disrupted by HIV, and not completely restored by HAART. It is possible that HAART may fail to restore number and/or function of the T cell subset necessary for B cell help, namely follicular T helper (T_{FH}) cells. This in conjunction with persistent B cell defects could explain observed poor vaccine responses. Irreversible disruption of the T_{FH} compartment may well contribute to the persistent observed B cell abnormalities described above.

Forster et al were the first to describe a circulating subset of CD4+ T cells expressing cell surface Burkitt's lymphoma receptor 1 (BLR1) [206]. BLR1 is a chemokine receptor, later renamed CXCR5, and is now known to be one of the characteristic surface markers used to identify T_{FH} [136, 137, 139]. Strictly speaking T_{FH} by definition should be localised to the lymph node, however a proportion of circulating CD4+ T cells have T_{FH} phenotypic characteristics and *in vitro* functional properties [207-209]. It has been proposed that these cells are a circulating equivalent of true T_{FH} and are a reflection of GC function [141]. These circulating cells will be referred to as T_{FH}-like cells in this report.

Forster et al also showed in 1997 that HIV infected adults with lower peripheral blood CD4+ T cell counts had a higher proportion of CD45RO+ memory cells but a lower proportion of CD45RO+ expressing CXCR5 [210]. There have been few further studies of this circulating subset in the context of adult or paediatric HIV until a recent study by Rabian et al, which showed that in HIV infected adults vaccinated with a pneumococcal conjugate vaccine (PCV - see Section 1.3), higher baseline circulating T_{FH}-like cell numbers correlated with more sustained *in vitro* cellular responses to diphtheria toxin [48]. The carrier protein for the PCV is cross-reacting material 197 (CRM197), which is a modified diphtheria toxin [211, 212]. This result suggests that a subset of HIV infected children may respond sub-optimally to vaccination with a conjugate vaccine and that responses may correlate with circulating T_{FH}-like cell number. The group did not report any correlation between treatment history and T_{FH}-like cell numbers nor did they compare with healthy controls. Pallikuth et al subsequently found no difference in the frequency of T_{FH}-like cells in HIV infected adults compared to healthy controls. However, successful antibody response to influenza vaccine was associated with an increase in circulating T_{FH}-like cells 1 month post-immunisation in a subset of HIV infected adults and healthy controls [47]. To our knowledge there is no published data on circulating T_{FH}-like cells in HIV infected children.

1.2 *Streptococcus pneumoniae*

Streptococcus pneumoniae (also known as pneumococcus) is a gram-positive diplococcus with a polysaccharide capsule, which can be found naturally colonising the upper respiratory tract, especially in young children [213, 214]. Capsular polysaccharide helps to differentiate pneumococci into 46 serogroups and 93 currently recognised serotypes [215]. Not all serotypes are potentially invasive and only a relatively small proportion of serotypes commonly cause disease in humans [216].

1.2.1 *Global epidemiology*

Streptococcus pneumoniae is a leading cause of paediatric morbidity and mortality throughout the world, causing disease ranging from otitis media, to pneumonia, meningitis and septicaemia [216, 217]. O'Brien et al applied comprehensive, clear and rigorous methods to estimate the burden of pneumococcal infection in children under 5 at global, regional, sub-regional and country level using systematic, transparent methods including a comprehensive literature review. The report highlights the importance of this infection throughout the world as a leading cause of paediatric morbidity and mortality. It estimates that in 2000, approximately 14.5 million episodes of serious pneumococcal disease occurred globally in children aged 1-59 months (uncertainty range 11.1-18.0 million). Pneumococcal disease caused 826 000 deaths (582 000- 926 000) in this age group, of which 91 000 (63 000- 102 000) were HIV-positive and 735 000 (519 000- 825 000) were HIV-negative. Of the deaths in HIV-negative children over 61% occurred in 10 African and Asian countries. The 735 000 (519 000- 825 000) deaths in HIV-negative children accounts for 11% (8-12%) of the 6.6 million total deaths in this age group in that year. (Fig. 6) It should be noted that this analysis includes data on meningitis, pneumonia and non-meningitis, non-pneumonia invasive disease (defined as isolation of *S pneumoniae* from a normally sterile body fluid). Of these 3 outcomes pneumonia was by far the most common. The study did not include data on upper respiratory tract infection such as otitis media attributable to *S. pneumoniae*. The authors concluded that these are likely to be underestimates of overall diseases

burden due to limited reporting and culturing practices, alongside a substantial burden of non-severe/non-invasive pneumococcal infection [218]. Incidence of invasive pneumococcal disease (IPD) varies substantially by age, genetic background, socioeconomic status, immune status and geographical location [219].

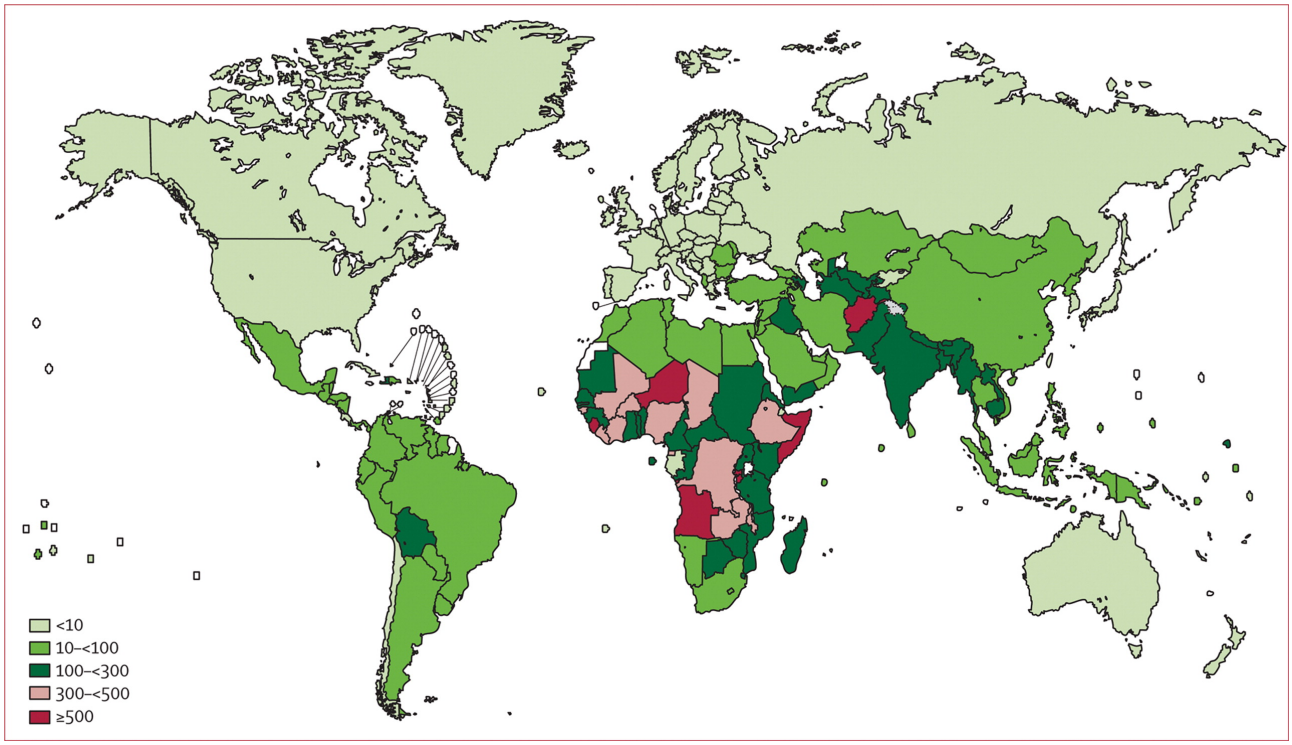


Figure 6. HIV negative pneumococcal deaths in children aged 1–59 months per 100 000 children younger than 5 years (Figure from O'Brien et al 2009 [218])

1.2.2 *UK epidemiology*

Streptococcus pneumoniae is one of the most frequent causes of invasive bacterial diseases in children in the UK. It has been reported to be the second most common bacterial cause of death in previously healthy children [220]. 7-valent PCV (PCV7 – see Section 1.3.1) was introduced into the national infant schedule in 2006. The epidemiology of pneumococcal disease in England and Wales from 1996-2006 has recently been summarised in a report by the Health Protection Agency (HPA) [221]. The burden of disease was considerable with peaks in the incidence of IPD in children under 2 years and adults over 75 years. Over the 10-year period overall incidence in all age groups ranged from 8.8 to 11.9 per 100 000/year. Incidence in under 2 year olds in 2005/2006 was 41.4/100 000. There was seasonal variation in reporting with peaks in the winter months. A diagnosis of meningitis was most common in children under 1 year (30% of IPD). For children under 2 years, 74% of isolates were PCV7 vaccine serotypes. The study noted that there were natural variations in serotype frequency over the 10-year period prior to introduction of PCV7, which should be taken in to consideration when monitoring the impact of vaccination introduction [221].

Following the introduction of universal immunisation with PCV7 in the UK there was a dramatic reduction in the rates of IPD in both children under two and older unvaccinated age groups (through herd immunity). In

contrast to this there was a significant increase in the incidence of IPD due to non-vaccine serotypes in all age groups, a phenomenon referred to as serotype replacement. The decrease in overall rates of IPD, although still significant, was therefore less substantial than if assessing vaccine serotypes alone [222]. 13-valent PCV (PCV13) replaced PCV7 in early 2010. There is less published data available regarding changes in IPD since then. Miller et al have reported early data for the UK, indicating that PCV13 has resulted in similar reductions in IPD due to the extra 6 serotypes in the vaccinated age group [223].

Since the introduction of PCV7, the HPA has reported weekly cumulative incidence of IPD in the UK according to age and PCV7 serotype vs. non-PCV7 serotype and subsequently PCV13 serotype vs. non-PCV13 serotype. From current graphs it appears that there has been a sustained decrease (almost elimination) of IPD due to PCV7, ongoing decreases in IPD due to extra PCV13 serotypes in vaccinated age groups (as reported by Miller et al) and a downwards trend in PCV13 serotypes in older age groups indicating successful herd protection. It is too early to say whether the phenomenon of replacement is occurring to the same extent as occurred with PCV7 [224]. Further effectiveness data is eagerly awaited.

1.2.3 *Pneumococcal immunity*

Pneumococcal virulence mechanisms are many and varied. The two most important virulence factors for invasive disease are thought to be the polysaccharide capsule and pneumolysin. Capsular polysaccharide prevents mucosal clearance, is antiphagocytic, sterically inhibits complement and immunoglobulin binding to host receptors, restricts autolysis and reduces antibiotic exposure. Pneumolysin is an exotoxin that is cytolytic, inhibits epithelial cell ciliary action, impairs respiratory burst, activates complement and stimulates cytokine and chemokine production. Other virulence factors include surface adhesins, anticomplement factors, metal binding transporters, bacterocidin and biofilm formation [219, 225, 226].

Most elements of the immune system, to a lesser or greater extent, may contribute to protection against *Streptococcus pneumoniae*. Antibacterial peptides such as β-defensins, lactoferrin and cathelicidin have all been shown to have anti-pneumococcal properties [227, 228]. The innate immune system relies on an array of receptors that recognise certain relatively conserved bacterial molecules. These receptors are called pattern recognition receptors (PRRs) and the structures they recognise are called pathogen-associated molecular patterns (PAMPs) [229]. PRRs can be extracellular, membrane-bound or cytoplasmic. Currently recognised PRRs include TLRs, C-type lectin receptors, scavenger receptors, complement receptors, NOD-like receptors (NLR), complement, pentraxins (e.g. C-reactive protein (CRP)) and collectins (e.g. mannose binding lectin (MBL)) [230-232]. A number of human PRRs recognise pneumococcal PAMPs. TLR 2,4 and 9 recognise pneumococcal lipoteichoic acid (LTA), pneumolysin and DNA respectively [219]. The increased susceptibility to IPD observed in individuals with deficiency in TLR signalling pathway might reflect the importance of TLRs in protection against pneumococcus in humans [233, 234]. Other PRRs specifically implicated in protection against pneumococcus include complement, soluble CD14, CRP, surfactant protein-D (SP-D (a collectin)), NOD2 (an NLR) and macrophage receptor with collagenous structure (MARCO - a macrophage scavenger receptor) [219, 235].

It is generally accepted that host defence against pneumococcus is primarily mediated by opsonisation and phagocytosis [236, 237]. Animal models of pneumococcal infection have demonstrated that alveolar macrophages are the initial cell responsible for phagocytosis of pneumococci infecting the lung. Neutrophils are then recruited and quickly take over as the main phagocytic cell, the role of macrophages then changing to clearance of apoptotic neutrophils [238, 239]. Human neutrophils readily kill pneumococci *in vitro* [240]. Indeed the ability of human neutrophils to kill pneumococci is the basis of the pneumococcal opsonophagocytic assay (OPA). This assay uses an immortalised human neutrophil cell line to assess the functional ability of human antibody to cause opsonophagocytosis and killing of pneumococci (see Section 1.3.3) [241]. Defects in the complement pathway predispose to IPD in humans, further evidence of the importance of opsonisation [236]. With respect to macrophages, it has been shown in adults that alveolar macrophages produce inflammatory cytokines including IL8, a neutrophil attractant chemokine in response to pneumococci [242]. It is clear that there is an essential close collaboration between phagocytic cells, complement pathways and antibody in the human response to pneumococcal infection that contributes to protection as well as pathogenesis of disease [219, 235-237]. Furthermore, studies in mice suggest that NKT cells and $\gamma\delta$ T cells may promote neutrophil-mediated protection against pneumococcal infection through interferon gamma and TNFα production respectively [243, 244].

Capsular polysaccharide is predominately a TI antigen, in that production of antibody by B cells against capsular polysaccharide does not require T cell help [245]. Recently, however, it has become apparent that CD4+ T cells do, nevertheless, play a role in pneumococcal immunity, particularly in relation to carriage [226]. It is generally agreed that nasopharyngeal colonisation is a necessary precursor to IPD [213, 246, 247]. Studies in mice have shown that CD4+ T cells infiltrate lung tissue infected with pneumococci and that pneumolysin stimulates CD4+ T cell chemotaxis [235, 248]. It appears that CD4+ T cells provide protection against colonisation in both an antibody dependent and independent mechanism. Antibody deficient mice were protected from subsequent pneumococcal colonisation following intranasal challenge with live pneumococci, whereas CD4+ T cell deficient mice were not [249]. In studies in humans, antibody responses to pneumococcal protein antigens have been shown to be T-cell dependent, primarily through IL10 and IFNγ production [250]. Malley et al have subsequently hypothesised that pneumococcal protein specific Th17 cells may be the CD4+ T cells responsible for protection against pneumococcal colonisation through IL17 mediated neutrophil recruitment [251].

CD8+ T cells recognise antigen in the context of MHC class I and therefore are primarily involved in response to pathogens accessing the cytosolic compartment such as viruses and intracellular bacteria [38]. There is limited data on their importance in protection against pneumococus [252] and they are not currently considered to play a major role in response to pneumococcal vaccines. For these reasons, while acknowledging that they play a key role in the immune response to HIV infection, they are not a main focus of this work.

Antibody in serum (IgM and IgG) and secreted at mucosal sites (IgA) are considered to be a major factor in defence against pneumococcal colonisation and infection. In support of this, patients with defective antibody production/ or hypogammaglobulinaemia (e.g. common variable immunodeficiency (CVID)) are vulnerable to

pneumococcal infection [253] and serum therapy, popular in the early 20th century, is effective against pneumococcal infection [254]. Anticapsular polysaccharide antibodies are thought to be of particular significance. Indeed, levels of serum anticapsular IgG are used as a read out of polysaccharide containing pneumococcal vaccine efficacy [255] (see Section 1.3.3).

A proportion of serum IgM that can bind capsule polysaccharides is present without prior antigen exposure, even at birth. These polyreactive antibodies have broad antibacterial activity and are termed natural antibody [256, 257]. The source of natural antibody has been shown to be B1a and marginal zone cells in mice. The cell subset responsible for natural antibody production in humans is not clear, however IgM memory B cells are thought to contribute [125]. It has been proposed that natural antibodies are an important first line defence against bacterial infection, bridging the gap between innate and acquired immunity [257, 258].

Anticapsular antibody is also produced following exposure to capsular polysaccharide antigen, either through natural exposure (colonisation [259] or infection [260]), or through vaccination (polysaccharide or conjugate [261]). Antibody is initially produced by short-lived plasma cells that develop from naive mature B cells following primary antigen exposure. Depending on the nature of the antigen, primary exposure can lead to GC formation and differentiation into memory B cells or long-lived plasma cells that subsequently home to the bone marrow and secrete antibody [123, 126, 129, 130]. (See Section 1.1.4) Memory B cells provide a more rapid and robust response following secondary antigen exposure [123]. The relative contribution of each of the B cell subsets in protection against pneumococcus is not completely clear and most likely changes with age and antigen exposure.

Children under 2 years of age have higher rates of pneumococcal colonisation [262] and are especially vulnerable to IPD [221]. Children have a relative insufficiency of IgG2 antibody, which is the main subclass of IgG induced by natural exposure and polysaccharide vaccination in adults [263, 264]. This supports the possibility that inadequate antibody responses to pneumococcal capsular polysaccharide may well be contributory to increased vulnerability in this age group. Relatively deficient antipneumococcal immune responses can be related to the general immaturity of the immune system during infancy [153]. More specifically, children under 2 develop poor responses to TI polysaccharide antigens when compared with TD protein antigens [265]. One proposed reason for this is functional immaturity of the spleen, in particular a region called the marginal zone, in which there is an accumulation of IgM memory B cells (see Section 1.1.4 for discussion on origin of this B cell subset) It has been proposed that early life immaturity of the splenic marginal zone (including decreased expression of the complement receptor CD21 on IgM memory B cells) corresponds to an equivalent immaturity of the IgM memory B cell compartment and therefore impairment of antipolysaccharide antibody responses [148, 266]. The observation that asplenic patients are more susceptible to IPD adds weight to this theory [267]. Circulating IgM memory B cells are however present at birth, be it in smaller numbers, and in some asplenic patients [148] so it seems unlikely that the relationship is as straightforward as this and that the increased risk of pneumococcal colonisation and IPD in early childhood is more likely to be multifactorial [153].

It has been demonstrated that the age specific incidence of IPD decreases prior to the natural acquisition of putative protective levels of anticapsular polysaccharide antibody. This suggests that other factors such as antipneumococcal protein antibodies or T-cell immunity are important in protection against IPD and take time to develop in early life, most likely as a result of antigen exposure during carriage in infancy [249]. Antibodies against pneumococcal proteins common to all serotypes have been shown to be protective against pneumococcal carriage. Novel vaccination strategies employing pneumococcal proteins are currently under investigation [251, 268].

1.2.4 *Streptococcus pneumoniae infection in the context of paediatric HIV infection*

Children and adults with untreated HIV infection are at increased risk of pneumococcal infection. The incidence of pneumococcal bacteraemia or invasive disease is up to 40-fold higher in HIV infected children compared to uninfected children. Reports of mortality rates from IPD are variable but it seems that they are generally the same as uninfected children. The introduction of HAART has been associated with a decreased incidence of IPD in HIV infected children and adults, however rates are still higher than in the uninfected population [269-272]. There is only limited published data on the effect of HAART on pneumococcal colonisation in HIV infected children. Very high rates of carriage were found in HIV infected East African children and were not significantly different between HAART treated and untreated children [273]. Lower rates of carriage were reported in the South American setting, but again no significant effect of HAART on carriage status was shown [274].

The potential reasons for the increased risk of pneumococcal disease despite restoration of peripheral blood CD4 cell numbers in HIV infected children are many. As discussed above, almost all aspects of a child's immunity are affected by HIV infection, and many of these are involved in defence against pneumococcal infection. For example, HAART may not completely restore phagocytic cell function [275] and may actually contribute to phagocyte dysfunction and depletion [71]. Adult and child NK cell numbers and phenotype and observed changes in adult $\gamma\delta$ mucosal and circulating cell populations are not completely restored by HAART [173, 276, 277]. NKT and dendritic cell numbers are lower in children on HAART than healthy controls [173] and abnormalities in complement and MBL pathways in adults have been shown to persist on treatment [278]. The persistent B cell abnormalities on HAART have been discussed in Section 1.1.4.

HAART's ability to restore individual CD4+ T cell subsets in children has not been explored in detail. In particular, with respect to pneumococcal immunity, the potential for HAART to restore Th17 and T_{FH} cell number and function has not been fully addressed. When considering the reasons for impaired pneumococcal immunity (both naturally acquired and through immunisation) and the persistent B cell abnormalities observed in children on HAART, it is important to explore the contribution that a possible disruption in T_{FH} cell number and T_{FH}-B cell helper function might be making.

1.3 Pneumococcal conjugate vaccines

1.3.1 *History*

The first attempts at vaccination against pneumococcal disease with a whole cell vaccine took place in South Africa 100 years ago by a team based in the Inoculation Department of St Mary's Hospital London [279, 280]. This team was lead by Sir Almroth Wright, and included Leonard Colebrook, William Parry Morgan and Robert Dodgson, all of whom were alumni of St Mary's Hospital Medical School [281-283]. Following this a number of studies were undertaken using pneumococcal polysaccharide vaccine, however it was not until 1945 that well designed randomised trials definitively demonstrated efficacy of polysaccharide vaccine against invasive disease [279]. Initially a hexavalent vaccine was marketed. This was replaced in 1977 by a 14-valent vaccine, with demonstrated efficacy of 76%-92% in South African adult miners. In 1983 the 23-valent polysaccharide vaccine (PPV) was introduced in the USA and was introduced into the UK schedule for adults over 65 years old in 2003 [155, 284]. The true efficacy of the 23-valent polysaccharide vaccine in adults is not completely clear, and somewhat controversial however it probably has an efficacy of approximately 50-70% in preventing pneumococcal bacteraemia, but limited/no efficacy against non-bacteraemic pneumococcal pneumonia [285] [284, 286, 287]. PPV is a TI antigen and therefore does not generally lead to the establishment of immune memory and is ineffective in children under 2 years of age [288], an age group most at risk of invasive disease [221].

The inadequacies of polysaccharide vaccines has lead to the introduction and development of vaccines consisting of bacterial polysaccharide conjugated to a carrier protein, so called conjugate vaccines. The first conjugate vaccines were developed in the 1929, when Avery et al showed that conjugation of pneumococcal polysaccharide to a protein carrier enhanced immunogenicity [289]. *Haemophilus influenzae* type b (Hib) conjugate vaccines have been available since the 1980's and have been recommended in the UK routine vaccination schedule since 1992. They have proven to be highly effective in reducing the disease burden of this previously common pathogen. The next conjugate vaccine to be introduced into the UK schedule was the *Neisseria meningitidis* serogroup C (MenC) conjugate vaccine in 1999. This vaccine has led to a similarly profound reduction in the incidence of invasive serogroup C meningococcal disease. The most recent addition to the conjugate vaccine armoury is the pneumococcal conjugate vaccine, first recommended in the UK for use in certain at risk populations in 2002 and subsequently for universal use in 2006 [284]. Until April 2010, the pneumococcal conjugate vaccine in use in the UK was PCV7 (Wyeth). The 7 serotypes covered by PCV7 caused approx 70% of invasive pneumococcal disease in children under 5 in the UK prior to its universal recommendation in 2006 [290]. Due to limitations in the demonstrated effectiveness of the vaccine as a result of serotype replacement [222, 291], a 13- valent conjugate vaccine (PCV13 – Wyeth) has now been licensed and is recommended in the UK national schedule [284].

1.3.2 *Mechanism of action*

The pneumococcal conjugate vaccine consists of pneumococcal polysaccharide conjugated to a protein carrier called CRM197, a modified diphtheria toxin [155, 288]. The mechanism of action and reasons for the immunological advantages of the pneumococcal conjugate vaccine over polysaccharide vaccines are incompletely understood, but it is thought that the presence of a protein attached to the polysaccharide

converts the TI antigen into a TD one. Most polysaccharide antigens cannot be processed and presented by MHC class II on the APC surface [292]. It has been proposed that B cells with a BCR specific for the polysaccharide internalise the vaccine and process the CRM197 for presentation in the context of MHC class II. T cells possessing a TCR specific for CRM197 epitopes then provide T cell help for the polysaccharide specific B cell through cytokine production and costimulation, allowing GC formation and establishment of B cell memory [155, 288]. This hypothesis is supported by studies in humans that demonstrate CRM197 specific CD4+ T cell responses in conjunction with polysaccharide specific B cell responses and antibody production following immunisation with conjugate vaccines [293, 294]. Recent evidence, however from murine studies suggests that this model might not be the whole story, and that parts of the polysaccharide bound to CRM197 may be presented as glycopeptides in the context of MHC class II [295, 296]. The importance of this in the mechanism of action of conjugate vaccines in humans is yet to be established. It is interesting to note that the long held belief that TI antigens cannot lead to the establishment of immune memory or long lived plasma cells has also recently been called into question in murine models of pneumococcal immunity with some evidence also in humans [297, 298].

1.3.3 *Immunological correlates for efficacy of PCV*

Efficacy is a measure of how well a vaccine works. Strictly speaking true vaccine efficacy (VE) is calculated from the results of randomized, placebo-controlled clinical trials and is defined as the percentage reduction in the incidence of a disease in the vaccinated (I_{vac}) compared to the incidence of the disease in the unvaccinated (I_{unv}):

$$VE = \frac{I_{unv} - I_{vac}}{I_{unv}} x\,100$$

or alternatively represented as:

$$VE = \left(1 - \frac{I_{vac}}{I_{unv}}\right) x\,100 = \left(1 - RR\right) x\,100$$

where RR = Relative risk of disease.

Vaccine effectiveness, often confused with vaccine efficacy is also a measure of how well a vaccine works. However, it is a measure of how well a vaccine works when used in the target population. It takes into account both direct and indirect effects of vaccination and is calculated using results of observational epidemiological studies e.g. case-control studies [299].

Four phases of clinical study are required for vaccine development and licensing. Phase I studies involve small numbers of adult subjects and give preliminary data on safety and immunogenicity. Phase II studies involve larger numbers and are used to investigate optimal dosing and formulation and to give further information on immunogenicity and safety. Phase III studies include larger numbers and usually include key

efficacy trials. The results of these efficacy trials will determine licensing decisions by regulatory bodies such as the European Medicines Agency (EMA) or the Food and Drug Administration (FDA). Post licensing, phase IV studies are large-scale epidemiological studies designed to assess effectiveness and safety [300-302].

In order for a vaccine to be licensed for clinical use clinical efficacy should ideally have been demonstrated [255]. This is not, however, essential and vaccines may be licensed on the basis of immunogenicity data alone [300]. Immunogenicity in this instance is used as a surrogate for vaccine efficacy. This is usually only possible if a proven correlation between an immunological endpoint and clinical efficacy has previously been demonstrated or following seroepidemiological studies. Immunological endpoints that have been used for this purpose for licensure of vaccines against Hib, MenC and *Streptococcus pneumoniae* and include both quantitative and functional serological responses [255, 303-305].

One way to assess immunity following conjugate vaccination is to measure serotype specific IgG using enzyme linked immunosorbant assay (ELISA) [255]. It is not necessarily the case that all measured serotype specific antibody is functional. The antibody may not bind antigen or the strength of binding may not be optimal. Avidity assays measure the strength of antigen/antibody binding. Patient serum is added to antigen-coated plates. The measured amount of antibody bound after incubating with serial dilutions of ammonium thiocyanate is then used to calculate an avidity index [255, 306].

In addition to the strength of antibody binding, the ability of antibody to elicit killing of bacteria is of fundamental importance. The way in which this can be assessed is by an OPA in the case of pneumococcal immunity or the serum bactericidal activity (SBA) in the case of Haemophilus and Meningococcus. OPA involves incubating serial dilutions of patient plasma, together with complement and the pneumococcal serotype of interest. The maximum dilution of serum that leads to 50% phagocytosis or killing is then used to calculate the opsonophagocytic index [241, 255]. Immunological memory can be demonstrated by enhanced quantitative antibody responses or antibody avidity following booster dose of the vaccine when compared to age matched non-primed subjects [307].

Following a WHO consultation in 2003 a series of recommendations were made which are used to set the immunological criteria for demonstrating non-inferiority of new conjugate vaccines against existing vaccines for protection against invasive pneumococcal disease. The protective antibody concentration was calculated from pooled efficacy and immunogenicity trials based on the assumption that if a vaccine has for example 80% efficacy, then 80% of vaccinated individuals should have serum antibody concentrations above the level of protection. A cumulative distribution curve of serum antibody concentrations one month after a three-dose course of vaccination, pooled from three PCV7 efficacy studies therefore allowed estimation of the protective threshold of 0.35 μg/ml [308].

This cut off is accepted bearing in mind some assumptions:

- antibody concentration at the 1 month time point after vaccination predicts protection
- that protection against disease is a step-wise function (when in reality it is continuous)
- that protective levels are similar across serotype

It should also be noted that this cut off does not in reality relate to individual protection or risk of disease, it is purely used as a cut off for showing non-inferiority when comparing pneumococcal conjugate vaccines efficacy against *invasive* pneumococcal disease [255]. This serological cut off is the primary end point for current efficacy trials. Secondary end points that are also required include demonstration of opsonophagocytic activity and also immunological memory following boosting [255, 307].

The WHO recommendations have been very useful in expediting the licensure of novel higher valency pneumococcal conjugate vaccines, however there is ongoing debate about whether the 0.35mcg/ml cut off is an adequate indicator of efficacy, especially in HIV infected children. It is generally accepted that an OPA titre would be a more useful and accurate measure of immunity, however due to problems with standardisation of this assay, primary endpoints using OPA are yet to be established [309].

1.3.4 *Immunogenicity, efficacy and effectiveness of PCV*

The immunogenicity, efficacy and effectiveness of PCV7 and other PCVs with differing carrier proteins and valencies against IPD, acute otitis media and pneumonia has been demonstrated in a number of different settings in varying schedules [310-315]. Efficacy for vaccine serotype IPD ranges from 77%-97%, 35-89% for all serotype IPD, 21-37% for all cause pneumonia and 6-7% for all cause AOM [316]. A Cochrane review has summarised the findings from PCV efficacy trials in HIV negative children less than 2 years of age to date. The pooled vaccine efficacy estimate for vaccine serotype IPD was 80% (95% confidence interval (CI) 58%-90%, $p<0.0001$); all serotypes-IPD, 58% (95% CI 29%-75%, $p=0.001$); WHO X-ray defined pneumonia was 27% (95% CI 15%-36%, $p<0.0001$); clinical pneumonia was 6% (95% CI 2%-9%, $p=0.0006$); and all-cause mortality, 11% (95% CI -1% - 21%, $p=0.08$) [317].

The EMA extensively reviewed the information available on PCV13, as part of the licensing procedures in Europe. Fourteen clinical trials in Europe, India and the USA, including one study from the UK, supplied data on safety and immunogenicity. The report summary concluded that the vaccine is immunogenic, as assessed by IgG ELISA and OPA for all 13 serotypes, and leads to the establishment of immune memory, as demonstrated by an anamnestic response following a booster dose. PCV13 is slightly less immunogenic than PCV7 overall, and OPA values were relatively low for 3 of the 6 new additional serotypes, however the clinical relevance of these findings and potential impact on efficacy are unknown. The safety profile is comparable to that of PCV7, with no new risks identified. Marketing authorisation was granted and the PCV13 has now superseded PCV7 in the UK schedule for all indications [284, 318].

Several groups have assessed *in vitro* responses of PBMC when stimulated with the vaccine antigen CRM197 by measuring cytokine responses either by ELISPOT assay or by measuring cytokine

concentrations in culture supernatants. Kamboj et al measured CRM197 specific IL2, 4, 5, 10 and IFNγ production by PBMC using ELISPOT pre and post immunisation with Hib/CRM197 conjugate vaccine, and found CRM197 induced a mixed Th1/Th2 response, numbers of IL2 and IL5 secreting cells correlating with levels of anti-Hib polysaccharide IgA (Kamboj 2001). The same group also measured CRM197 specific production of the same cytokines (plus IL6) by PBMC, using ELISPOT and ELISA following vaccination with PCV. A mixed Th1/Th2 responses was again observed but no correlation with polysaccharide specific antibody responses (Kamboj 2003). Vernacchio et al measured IL4, 5, 10, 13 and IFNγ in PBMC culture supernatants following immunisation with PCV and various doses of adjuvant, and found mixed Th1/Th2 responses with differing dose dependent effects of adjuvant concentration on cytokine responses (Vernacchio 2002). A neonatal/infant study by Van den Biggelaar et al measured the concentrations of IL5, 6, 10, 13, IFNγ and TNFα in PBMC culture supernatants when stimulated with CRM197, after 2 different schedules of PCV administration (neonatal vs. infant). Again, a mixed Th1/Th2 response was demonstrated, however the responses were skewed towards Th2 in the neonatal cohort [319].

In addition to classically Th1/Th2 related cytokines, IL21 is particularly of interest in the context of our study. IL21 is one of the characteristic cytokines produced by T_{FH} cells in the germinal centre and is a key cytokine responsible for allowing class-switch recombination and somatic hypermutation [320]. IL21 has been found to be at lower concentrations in serum samples from adult HIV infected patients compared to HIV uninfected controls. These low levels correlate with low CD4 count and are partially restored with HAART. HIV infection of CD4 cells *in vitro* leads to a decrease in IL21 production capabilities [321, 322]. Pallikuth et al demonstrated successful antibody responses to an influenza vaccine to be associated with an increase in serum IL21 1 month post immunisation in both healthy controls and a subset of HIV infected patients [323].

1.3.5 *Immunogenicity, efficacy and effectiveness of PCV in the context of HIV*

The immunogenicity and efficacy of PCV, in the context of both adult and paediatric HIV, has been extensively reviewed by Bliss et al in 2007 and subsequently updated by Nunes et al in 2012. Safety data from all available studies in HIV infected children suggest that PCV is well tolerated with an acceptable rate of adverse reactions. One five-year follow up study in Africa found lower CD4 percentage in children receiving PCV compared to placebo although the significance of this has not been established. No studies in children have reported effects on viral load although 2 studies in adults reported no changes in viral load with administration of PCV. In terms of immunogenicity, it should be noted that an equivalent immunological correlate of vaccine efficacy to that used by the WHO (described in Section 1.3.3) has not been established for HIV infected children [270, 271].

Eleven immunogenicity studies in children were reviewed from the USA, Europe and South Africa [270, 271]. For reasons that are not clear, one study from South America was not included in the most recent review despite fulfilling the search criteria [324] and a further study from Thailand has been published subsequent to review publication [325]. There is a great deal of variation in study setting, age group, vaccine valency, schedule used, immunological end-points, proportion of children on HAART, inclusion of uninfected control group and use of avidity/OPA. In summary, however, evidence to date indicates that PCV is immunogenic in children with HIV, but less so than in uninfected children. For children not on HAART, quantitative (IgG

ELISA) and qualitative (OPA) primary antibody responses are less robust and shorter lasting than in healthy children, and anamnestic responses are also reduced. For children on HAART it appears that quantitative responses are improved and may be equivalent to those in uninfected children, but the quality of antibody response as assessed by OPA is still not as strong and poorly correlates with IgG ELISA [270, 324, 326]. The reasons behind the deficient antibody responses to PCV in children both on and off HAART have not been investigated in detail in paediatric HIV infected children.

Only the South African studies including children mainly not on HAART have formally assessed clinical efficacy [312, 327]. Vaccination with a nine-valent PCV provided protection against vaccine-serotype invasive disease (65% [95% CI 24–86%; *p*=0·006]), however this was lower than in uninfected children (83% [39–97%; *p*=0·003]). A significant reduction in pneumonia was only demonstrated in uninfected children (2–35%; *p*=0·03). 5-year follow-up demonstrated a substantial reduction in efficacy against vaccine-type IPD in HIV infected children when compared with uninfected controls (38·8% [–7·8% to 65·2%]) vs. (77·8% [34·4–92·5%]) [270, 312, 327].

Steenhoff et al showed no further reduction in incidence of IPD in a cohort of HIV infected children following the introduction of PCV7 into the national schedule in the USA, although conclusions are limited due to relatively small sample size and rates of IPD [328]. In contrast, the potential indirect effect of universal PCV7 vaccination in protecting families infected/affected by HIV was demonstrated in a study by Flannery et al, which demonstrated a reduction in overall IPD, but especially vaccine serotype, in HIV infected adults since the introduction of PCV7 into the US national schedule [329]. A recent randomised double-blind placebo controlled trial in mainly HIV infected adolescents (>15 years) and adults, the majority of whom were not on HAART, investigated the efficacy of PCV7 in preventing IPD in patients that had previously had documented IPD. The study gave an estimated vaccine efficacy in this context of 74% (95% CI, 30-90%) [330].

To our knowledge, there are at present no published reports of PCV13 immunogenicity, efficacy or effectiveness in HIV infected individuals.

1.3.6 *Using PCV as a tool to probe T/B cell interaction and function in paediatric HIV infection*

As described above, pneumococcal conjugate vaccine is thought to act as a TD antigen, enlisting T cell help in the establishment of B cell antibody responses. This project aimed to investigate the reasons for immune dysfunction of HIV infected children both on and off HAART by using administration of PCV to probe T and B cell responses to vaccine antigens. Antibody responses to vaccine capsular polysaccharides as well as carrier protein specific T cell responses were measured, alongside detailed peripheral blood B and T cell immunophenotyping in order to better understand the reasons for B cell immune dysfunction in this vulnerable group and to further delineate possible mechanisms of action of pneumococcal conjugate vaccine outside of the infant period.

Chapter 2: Hypothesis and aims

2.1 Hypothesis

Irreversible loss of circulating T_{FH}-like cells is associated with impaired B cell memory responses in HIV infected children.

2.2 Aims

1. To characterise peripheral blood B and T cell phenotype in HIV infected children compared to healthy child and young adult controls.
2. To assess associations between clinical parameters such as treatment history and detectable viral load and B and T cell phenotype.
3. To assess stability of B and T cell subsets over time.
4. To assess serum serotype specific pneumococcal IgG concentrations by ELISA pre, 1 month post and 6 months post immunisation with PCV13 in children with paHIV compared to healthy controls.
5. To assess *in vitro* cytokine responses following whole blood culture with the vaccine carrier protein CRM197 and to test for correlations between balance of cytokine production and vaccine immunogenicity.
6. To assess serum IL21 levels pre and post immunisation, and to assess correlation with serotype specific vaccine immunogenicity.
7. To assess NP carriage pre and post immunisation, effects of immunisation on carriage and effects of carriage on immunogenicity.

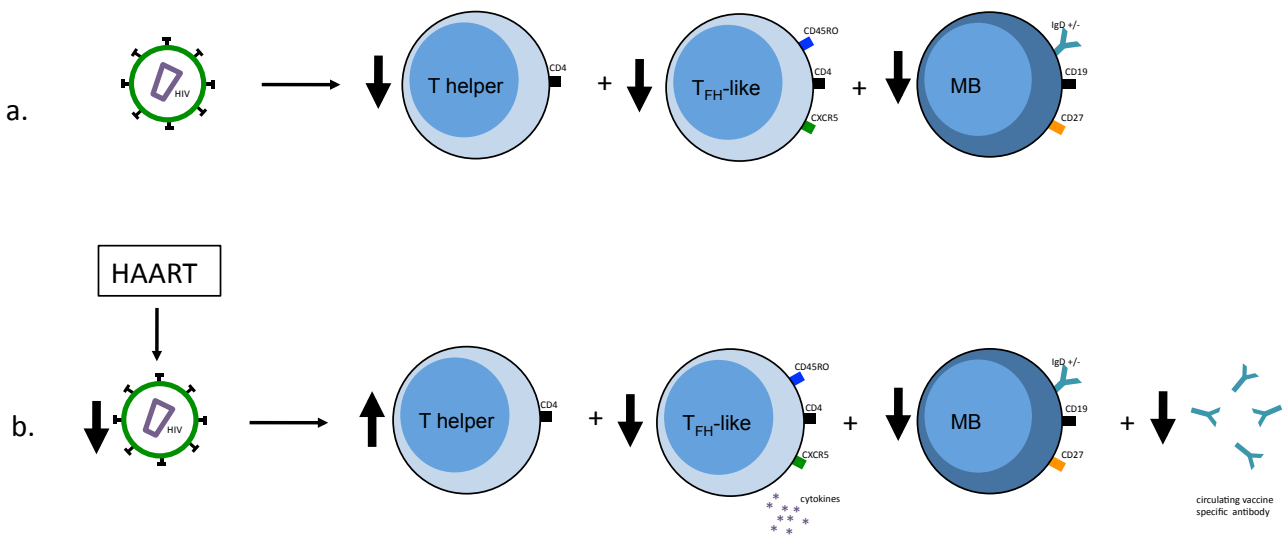


Figure 7. Diagram summarising the main study hypothesis. a) Ongoing HIV viraemia results in reduced circulating $CD4^+$ T cell numbers, including circulating T_{FH}-like (identified as $CD4^+$, $CD45RO^+$, $CXCR5^+$). This is associated with decreased memory B cell numbers (identified as $CD19^+CD27^+$). b) suppression of HIV viraemia results in increased circulating $CD4^+$ T cells, however we hypothesise that the circulating T_{FH}-like and memory B cell populations are incompletely restored and that this is associated with impaired antibody response to vaccine antigens. (HAART= Highly Active Antiretroviral Therapy, MB = memory B cell)

Chapter 3: Materials and methods

3.1 Recruitment and consent

3.1.1 *Study setting*

The study took place in the setting of the HIV Family Clinic at St. Mary's Hospital. This clinic has been established for over 15 years and is one of the largest in the UK. It has followed over 300 children with HIV infection and currently actively cares for over 120 children and their families. All children are seen at 3-monthly intervals or more often, as clinically indicated. Regular blood samples are taken for the monitoring of HIV disease, such as CD4 count and viral load measurements.

3.1.2 *Ethical considerations*

Ethical approval to perform the study was obtained from the Riverside Research Ethics Committee, Charing Cross Hospital, London (REC reference number: 09/H0706/23). Local Site Specific Approval was obtained from the Research and Development Office, St Mary's Hospital, London (R&D reference number: 08/GC/004). Documents (including substantial amendment for PCV13) are included in Appendix 4 and 5.

3.1.3 *Funding*

Research was fully funded by a National Institute of Health Research (NIHR) Research Training Fellowship competitively awarded to Dr Alasdair Bamford. Additional funding for consumables was also competitively awarded to Dr Bamford by the European Society for Paediatric Infectious Diseases (ESPID). Vaccine and vaccine carrier protein were generously provided by Wyeth and Pfizer.

3.1.4 *Study design*

Intervention cohort:

HIV infected child patients (CP) consenting to be vaccinated had blood samples taken at baseline for T and B cell immunophenotyping, serological studies, and cytokine release assay. They then received one dose of PCV13 (Pfizer, Batch number: E27415). The next blood sample was taken 1 month post immunisation for serology and cytokine release assay and at 6-9 months for T and B cell immunophenotyping, serological studies, and cytokine release assay. Blood sampling was timed to coincide with routine lymphocyte quantification and VL assessment via the National Health Service (NHS) laboratory. Those consenting to have nasopharyngeal (NP) carriage swabs taken had swabs taken at baseline and 6-9 months later.

HIV infected controls:

CP consenting to have just blood monitoring had blood taken at two time-points, baseline and 6-9 months later but did not receive PCV13. As for the intervention cohort, blood sampling was timed to coincide with routine lymphocyte quantification and VL assessment via the NHS laboratory. Those consenting to have NP carriage swabs taken had swabs taken at baseline and 6-9 months post immunisation.

Child healthy controls (CHC):

Healthy children matched for age and sex and consenting to be in the study had blood sampling at one time point only for T and B cell immunophenotyping, serological studies and cytokine release assay. They did not receive PCV13. Lymphocyte quantification was performed via NHS laboratory services (see Section 2.2.3).

Adult healthy controls (AHC):

Adults consenting to be in the study had blood and NP swabs taken and received PCV13 as for the intervention cohort. Lymphocyte quantification was performed via NHS laboratory services. See Fig. 8 for flow chart of study design.

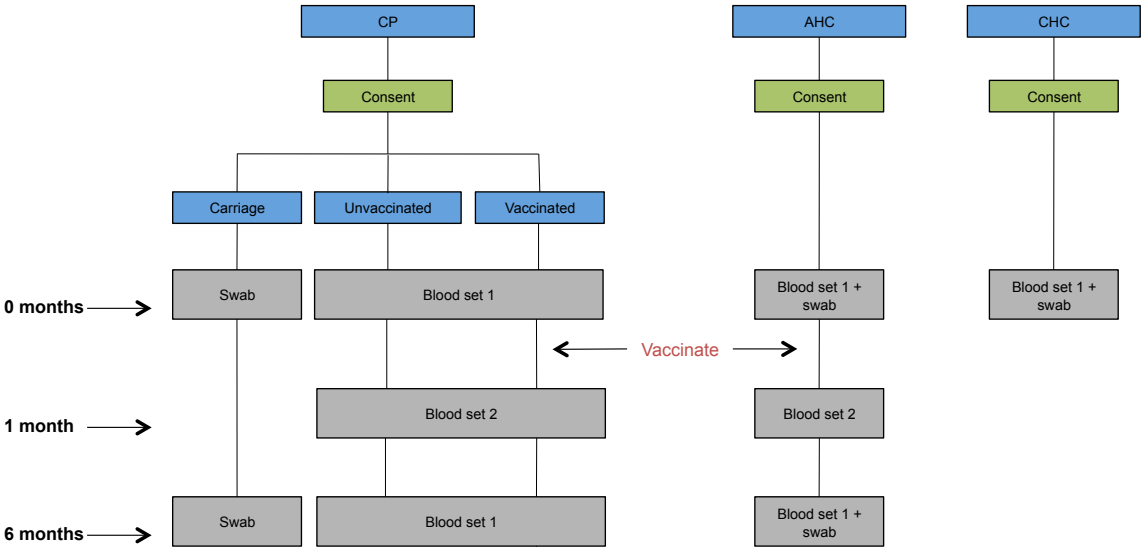


Figure 8. Flow chart for all study participants. Blood set 1 = Serology, flow cytometry, cytokine release assay. Blood set 2 = Serology, cytokine release assay. (CP= child patient, AHC = adult healthy control, CHC=child healthy control)

3.1.5 *Study populations*

The HIV Family Clinic cohort includes 130 children. CHC were recruited from otherwise healthy children attending for pre-operative assessment or GP phlebotomy services at St Mary's Hospital. AHC were recruited from staff and students of Imperial College Healthcare NHS Trust and Imperial College London. None of the study subjects had received PCV13 prior to study entry.

3.1.6 *Recruitment*

Recruitment took place between 07/08/09 and 10/05/11. Parents and children attending St Mary's family clinic were approached for inclusion in the study. Otherwise healthy children and their parents attending for pre-operative assessment or GP phlebotomy sessions requiring routine blood tests were approached for recruitment as child healthy controls. Adult healthy controls were recruited through poster advertisement within Imperial College Healthcare NHS Trust and Imperial College London.

3.1.7 *Consent*

In line with ethical requirements, parents of all children included in the study provided informed consent for study entry. Those children deemed competent provided informed consent. The opportunity to provide informed assent was given to children deemed not competent to give consent. If there was disagreement between child and parent about inclusion in the study they were no longer eligible for study entry. If a child was competent to provide informed consent but unaware of their HIV diagnosis, they were ineligible for inclusion in the study. Informed consent was provided by adult healthy controls. All consent forms and information sheets were designed accordingly and in an age-appropriate from.

3.1.8 *Inclusion/exclusion criteria*

HIV INFECTED PCV13 RECIPIENTS:

Inclusion criteria

- Any patient attending St Mary's Hospital paediatric HIV clinic

Exclusion criteria

- Patients younger than 2 months or older than 18 years
- Patients with other additional forms of immune suppression e.g. malignancy or medication
- Patients with previous reaction to PCV13 or any of its components
- Patients who are pregnant or planning to become pregnant
- Patients competent to give consent but unaware of HIV diagnosis
- Disagreement between child and parent about inclusion in the study

HIV INFECTED (UNVACCINATED) CONTROLS:

Inclusion criteria

- All patients attending St Mary's Hospital paediatric HIV clinic

Exclusion criteria

- Patients younger than 2 months or older than 18 years
- Patients with other additional forms of immune suppression e.g. malignancy or drugs
- Patients who are pregnant or planning to become pregnant
- Patients competent to give consent but unaware of HIV diagnosis
- Disagreement between child and parent about inclusion in the study

CHILD HEALTHY CONTROLS:

Inclusion criteria

- Healthy children in the care of St Mary's having blood taken for another clinical reason

Exclusion criteria

- Age younger than 2 months or older than 18 years
- Known immunosuppression or immunodeficiency
- Febrile illness
- Pregnancy

ADULT HEALTHY CONTROLS:

Inclusion criteria

- Age between 18 and 30 years

Exclusion criteria

- Previous reaction to PCV13 or any of its components
- Known immunodeficiency or immunosuppression
- Febrile illness
- Pregnancy or planned pregnancy

3.1.9 *Demographic and clinical information*

For CP, background demographic and clinical information, including vaccination and treatment history were obtained from parent interview, clinical notes, GP records, hospital pathology records and Parent-Held Child Health Record or "Red Book". For CHC, background demographic and clinical information, including vaccination history were obtained from parent interview, hospital notes, GP records and Red Book. For AHC, background demographic and clinical information, including vaccination history were obtained from interview and/or GP records. All information was recorded in Case Report Forms (CRFs). Any changes in clinical status or adverse events were recorded in the CRF at all subsequent study participant contacts.

3.1.10 *Vaccine and vaccination*

At the time of study planning and during initial stages of the research PCV7 was recommended as part of the national schedule. PCV7 for the study was kindly supplied by Wyeth Pharmaceuticals. As of April 2010, PCV13 was recommended for all indications in the UK schedule. As a result, recruitment of subjects to be vaccinated was on hold while a major amendment to the ethical approval was applied for and approved, and a supply of PCV13 was kindly supplied by Pfizer. Prior to the change in national guidelines a total of 8 healthy adult controls had been recruited and vaccinated with PCV7.

1 dose (0.5ml) of Prevenar (PCV7) contains:

Pneumococcal polysaccharide serotype 4	2 µg
Pneumococcal polysaccharide serotype 6B	4 µg
Pneumococcal polysaccharide serotype 9V	2 µg
Pneumococcal polysaccharide serotype 14	2 µg
Pneumococcal oligosaccharide serotype 18C	2 µg
Pneumococcal polysaccharide serotype 19F	2 µg
Pneumococcal polysaccharide serotype 23F	2 µg

All polysaccharides are conjugated to the CRM197 carrier protein and adsorbed on aluminium phosphate (0.5 mg) [331].

1 dose (0.5ml) of Prevenar 13 (PCV13) contains:

Pneumococcal polysaccharide serotype 1	2.2 µg

Pneumococcal polysaccharide serotype 3	2.2 µg
Pneumococcal polysaccharide serotype 4	2.2 µg
Pneumococcal polysaccharide serotype 5	2.2 µg
Pneumococcal polysaccharide serotype 6A	2.2 µg
Pneumococcal polysaccharide serotype 6B	4.4 µg
Pneumococcal polysaccharide serotype 7F	2.2 µg
Pneumococcal polysaccharide serotype 9V	2.2 µg
Pneumococcal polysaccharide serotype 14	2.2 µg
Pneumococcal polysaccharide serotype 18C	2.2 µg
Pneumococcal polysaccharide serotype 19A	2.2 µg
Pneumococcal polysaccharide serotype 19F	2.2 µg
Pneumococcal polysaccharide serotype 23F	2.2 µg

All polysaccharides are conjugated to CRM197 carrier protein and adsorbed on aluminium phosphate (0.125 mg aluminium) [332] (Additional serotypes to PCV7 in italics). Both vaccines were administered as an intramuscular injection into the anterolateral aspect of the thigh for infants and into the deltoid muscle for older children and adults.

3.1.11 *Safety monitoring*

All study subjects receiving vaccine were provided with detailed contact information and were asked to contact the research team or clinical team should they suffer AE following vaccination. All AEs were recorded in the CRF. A history of adverse events was sought at each subsequent contact. Any serious adverse events were reported according to Good Clinical Practice (GCP) guidance to Imperial College London, vaccine manufacturer, funding body and research ethics committee, as well as via standard national Medicines and Healthcare Products Regulatory Agency (MHRA) “Yellow card” reporting system.

2.2 Plasma viral load and lymphocyte quantification

3.2.1 *Blood sampling*

All blood samples for CP and CHC were taken at the same time as other routine blood tests by the attending clinician or trained phlebotomist. Blood samples from AHC were taken by trained medical or laboratory staff. A minimum of 5mls and a maximum of 10mls extra blood were taken for all investigations. At least 1ml of blood was collected in a preservative free lithium heparin containing Vacutainer (BD) for the cytokine release assay. At least 2ml of blood was collected in EDTA containing Vacutainer (BD) for flow cytometry. At least 2ml of blood was collected in a SST II Advance Vacutainer (BD) for serological analysis.

3.2.2 *HIV viral load*

Plasma HIV RNA viral load (VL) were measured as part of routine care using a branched chain DNA assay (Siemens Systems) via St Mary’s Hospital NHS Virology Laboratory. The assay was carried out according to manufacturers instructions giving a detection range of 50-500000 copies/ml.

3.2.3 *Lymphocyte quantification*

Quantification of lymphocyte subsets were undertaken in the Department of Immunology, St Mary's Hospital and the Department of Immunology, Chelsea and Westminster Hospital using 4 colour flow cytometry. Briefly, whole blood (EDTA) was stained with monoclonal antibodies (CD45-FITC, CD4-RD1, CD8-ECD, CD3-PC5, CD56-RD1, CD19-ECD, CD16-PE (Beckman Coulter)) and evaluated using an FC500 flow cytometer (Beckman Coulter).

3.3 Immunophenotyping of B and T cell subsets by flow cytometry

3.3.1 *Lymphocyte populations of interest*

We identified T and B cell subsets from whole blood using 6 colour flow cytometry and the following surface markers [120, 140, 145]:

Memory T cells:	CD3+ CD4+ CD45RO+
Follicular T helper-like cells (T_{FH}-like):	CD3+ CD4+ CD45RO+ CXCR5+
$ICOS^+$ T_{FH}-like:	CD3+ CD4+ CD45RO+ CXCR5+ ICOS+
Transitional B cells:	CD19+ CD10+ CD27-
Plasmablasts:	CD19+ CD10- IgD- CD27++
Naive B cells:	CD19+ CD10- IgD+ CD27-
IgD^+ memory B cells:	CD19+ CD10- IgD+ CD27+
IgD^- memory B cells:	CD19+ CD10- IgD- CD27+
Double negative B cells:	CD19+ CD10- IgD-CD27-
Naive mature B cells	CD19+ CD10- CD21+CD27-
Resting memory B cells	CD19+ CD10- CD21+ CD27+
Activated mature B cells	CD19+ CD10- CD21- CD27+
Exhausted/Tissue like memory B cells:	CD19+ CD10- CD27- CD21-

3.3.2 *Lymphocyte immunophenotyping method*

For each subject, 150μl of whole blood was added to 8 flow cytometry tubes (BD) (4 x B cell tubes and 4 x T cell tubes). Samples were washed twice in 2ml of phosphate buffered saline (PBS) (Oxoid) (5 mins x 400g). Cells in B cell tubes were then resuspended in 100μl of near-IR-fluorescent reactive dye (Invitrogen) diluted 1:1600 in PBS. Cells in T cell tubes were resuspended in 100μl of red-fluorescent reactive dye (Invitrogen) diluted 1:1600 in PBS. All tubes were then incubated in the dark at room temperature for 30 minutes. All tubes were subsequently washed once in 1ml PBS (5 mins x 400g). Monoclonal antibodies were added to each tube as per Table 2. Tubes were incubated in the dark at room temperature for 30 minutes.

Lysis of erythrocytes and fixation of staining was performed using an automated TQ Prep System (Beckman Coulter). Cells were washed twice in 2ml PBS (5 mins x 400g) and resuspended in 300μl PBS. Acquisition was performed on an LSR II (BD Biosciences). A minimum of 10000 CD19 events and 30000 CD4 events were acquired for the B and T cell tubes respectively. Data was analysed using FlowJo software v.9.4 (Tree Star).

Tube	Antibody	Volume
T	CD3 APC-Cy7^	5μl
	CD4 PE-Cy7*	10μl
	CXCR5 Axf647^	5μl
	ICOS PE^	20μl
	CD45RO FITC^	10μl
	CD14 ECD*	5μl
	CD19 ECD*	5μl
TmCXCR5	CD3 APC-Cy7^	5μl
	CD4 PE-Cy7*	10μl
	ICOS PE^	20μl
	CD45RO FITC^	10μl
	CD14 ECD*	5μl
	CD19 ECD*	5μl
TmCD45RO	CD3 APC-Cy7^	5μl
	CD4 PE-Cy7*	10μl
	CXCR5 Axf647^	5μl
	ICOS PE^	20μl
	CD14 ECD*	5μl
	CD19 ECD*	5μl
TmICOS	CD3 APC-Cy7^	5μl
	CD4 PE-Cy7^	10μl
	CXCR5 Axf647^	5μl
	CD45RO FITC^	10μl
	CD14 ECD*	5μl
	CD19 ECD*	5μl
B	IgD FITC*	2μl
	CD21 PE^	5μl
	CD27 APC^	5μl
	CD19 PE-Cy7*	5μl
	CD10 PerCP°	10μl
	CD3 APC-Cy7^	5μl
BmCD27	IgD FITC*	2μl
	CD21 PE^	5μl
	CD19 PE-Cy7*	5μl
	CD10 PerCP°	10μl
	CD3 APC-Cy7	5μl
BmCD21	IgD FITC*	2μl
	CD27 APC^	5μl
	CD19 PE-Cy7*	5μl
	CD10 PerCP°	10μl
	CD3 APC-Cy7^	5μl
BmCD10	IgD FITC*	2μl
	CD21 PE^	5μl
	CD27 APC^	5μl
	CD19 PE-Cy7*	5μl
	CD3 APC-Cy7^	5μl

Table 2. List of monoclonal antibodies used for lymphocyte immunophenotyping *Beckman coulter, ^BD biosciences, °ExBio (APC=Allophycocyanin, APC-Cy7=Allophycocyanin-cyanine 7, Axf647=Alexafluor-647, PE=Phycoerythrin, FITC=Fluorescein isothiocyanate, ECD=Electron Coupled Dye, PE-Cy5=Phycoerythrin-Cyanine 7, PerCP=Peridinin chlorophyll protein)

3.3.3 *Optimisation of flow cytometry method*

Some of the populations of interest (especially $ICOC^+$ T_{FH}-like cells) are relatively rare in whole blood. For this reason it is important to ensure that there are adequate controls in the flow cytometry panels to ensure that the cells detected are a true population [333, 334]. There follows a discussion on the controls that have been incorporated into the flow cytometry methods in order to ensure data collected is of a high quality.

Titration of monoclonal antibodies:

Monoclonal antibody titration experiments were performed for each monoclonal antibody used. For each titration experiment, the monoclonal antibody of interest was added to 150μl of whole blood at a range of volumes, the top volume being that recommended by the manufacturer, subsequent volumes determined by serial halving of this volume. Optimum volume of monoclonal antibody to be used was determined as the volume leading to the largest value for the ratio of mean fluorescence intensity (MFI) of the positive population to MFI of the negative population [334].

Compensation controls: compensation beads vs. stained cells:

In order to accurately compensate for fluorescence spillover it is relatively common practice to use single stained cells as compensation controls. This is only really accurate, however, if using the same monoclonal antibody/fluorochrome combination in the single stain compensation tube as in the complete panel. If the expression of the marker on the cell type of interest is not clearly positive or negative, this becomes inaccurate. In this instance, it is possible to use the same fluorochrome conjugated to an antibody specific for a surface antigen with a clear positive/negative population (e.g. CD8). However this adds an element of inaccuracy as the fluorochrome may behave differently when bound to another antibody or if the manufacturing process is not the same. This is especially true for tandem dyes. Compensation beads provide a solution to this problem as they are designed to bind any fluorochrome/antibody combination with a distinct positive and negative population. This allows use of the same monoclonal antibodies as in the panel, irrespective of the pattern of expression on cells [333, 334]. Compensation beads (BD) were therefore used as compensation controls for flow cytometry in this project.

Gating controls: Fluorescence minus one (FMO) vs. isotype:

Gating controls are necessary when there is no clear division of positive from negative expression of a cell surface marker. For isotype control gating, an antibody that is not specific for any human marker, labelled with the same fluorochrome as that used in the flow panel, is employed to detect any nonspecific binding and therefore a true boundary between positive and negative expression. Historically this has been the main method used for gating. However more recently the accuracy of this technique has been called into question. The monoclonal antibody/fluorochrome combination used in the isotype control may be similar to the antibody used in the panel, but it is not exactly the same and therefore patterns of non-specific binding will not be identical. In addition, unless the experiment consists of a separate tube for each antibody of interest, in which only the relevant antibody is replaced by the corresponding isotype control, the effect of fluorescence spillover from other antibodies in the panel will not be accounted for [333, 335].

FMO controls are considered to be a solution to this problem and there use is generally encouraged. An FMO control consists of a tube which lacks the antibody of interest. This is used to gate a true negative population and accounts for fluorescence spillover from other fluorochromes in the panel. It could be argued that this does not detect non-specific binding, however it is thought that if an antibody has been titrated accurately there should be negligible background staining. Moreover, spillover induced background is a more prominent feature of multicolour flow cytometry [333, 335]. FMO controls are therefore used as gating controls in this project.

Internal negative control:
If there is a population within the cell type of interest that is negative for the marker of interest, this can serve as an internal negative control. By using this approach the internal negative cells have been exposed to all the same conditions as the cell of interest including the monoclonal antibody of interest. Any non-specific binding of the antibody will be present on internal negative and the fluorescence spillover effects will also be equivalent. An internal negative control is therefore included in the T cell flow cytometry panel.

Internal positive control:
When a marker is rarely expressed (e.g. CD10 on B cells), it is important to assess that the antibody/fluorochrome specific for that marker is still functioning. CD10 is also expressed on granulocytes [336]. For this reason CD10 staining of granulocytes (identified through forward scatter (FSC) vs. side scatter (SSC)) was confirmed for B cell tubes.

Live/dead discrimination and dump channel:
Non-specific binding of monoclonal antibodies to dead cells can lead to significant measurement errors. A viability dye, such as an amine reactive dye, can be utilised to identify and gate out dead cells. These dyes penetrate the dead cell's damaged membrane and bind to amine groups in the cytoplasm. They also bind to amine groups on the cell surface but at a much lower intensity. Their use has been validated for cell surface immunophenotyping [337, 338] and they are therefore included in our staining methods. Irrelevant cell populations can be excluded from analysis using a dump channel, which contains antibodies specific for unwanted cells labelled with fluorochromes which all read in the same fluorescence channel [337-339]. For this reason the T cell panel in this project includes anti-CD19 and anti-CD14 labelled with a fluorochrome that reads in the same fluorescence channel as the amine reactive dye. The B cell panel includes anti-CD3 labelled with a fluorochrome that reads in the same fluorescence channel as the amine reactive dye.

Doublet discrimination:
Cells can adhere to each other to form clumps or doublets, therefore producing inaccurate results. Doublets can be reliably gated out by plotting FSC area vs. FSC height, as the readout for these values from a single cell should be proportional [340]. A doublet discrimination stage is therefore included in the gating strategy in this project.

3.3.4 *Gating strategy*

Figures 9 and 10 provide an overview of the gating strategies used throughout this project.

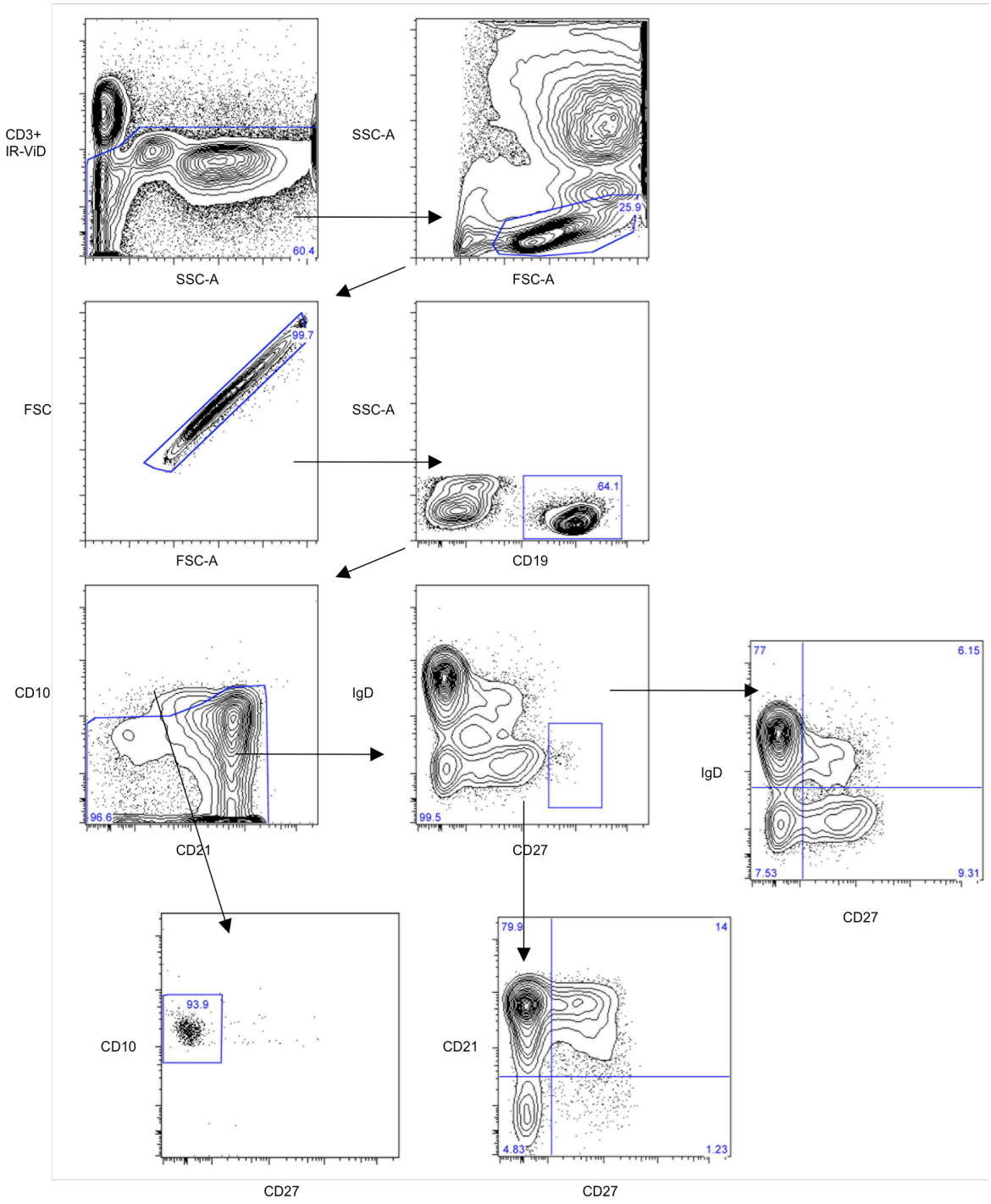


Figure 9. Gating strategy for identification of B cell subsets. Following identification of live CD3- cells. CD19+ B lymphocytes were identified by sequentially gating FSC-A vs SSC-A, FSC-A vs FSC-H and CD19 vs SSC-A.CD10+CD27- (transitional B cells) and IgD-CD27++ (plasmablasts) were then quantified.

The remaining cells were subdivided in 2 different ways: CD27 vs IgD and CD27 vs CD21 resulting in the identification of 8 further subsets as described in Section 2.3.1. Controls were utilised as described in Section 2.3.3.

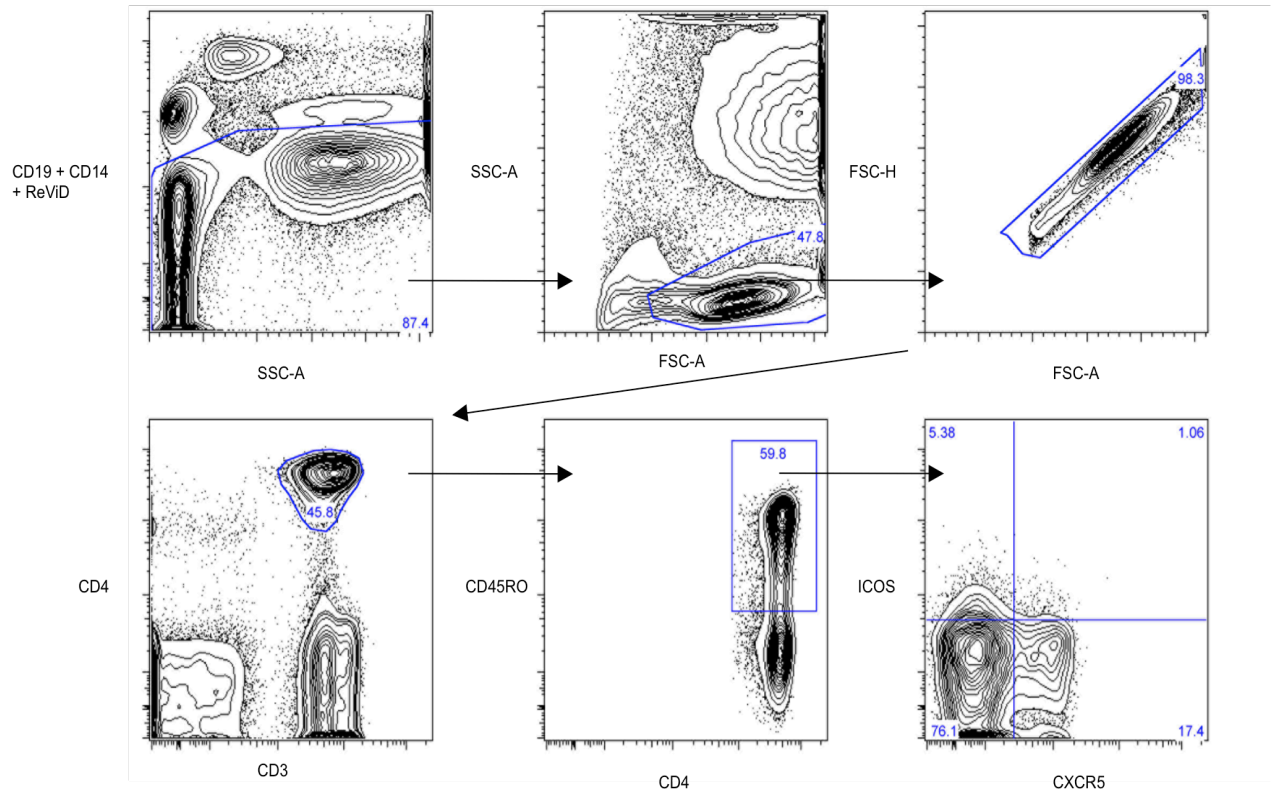


Figure 10. T cell gating strategy. Following identification of live CD19- CD14- cells. CD4+ T lymphoctyes were identified by sequentially gating FSC-A vs SSC-A, FSC-A vs FSC-H and CD3 vs CD4.

Following identification of CD45RO+ CD4+ T cells, T_{FH} like cells were identified and characterised by expression of CXCR5 and ICOS. Controls were utilised as described in Section 2.3.3.

3.4 Whole blood cytokine release assay

In order to investigate T cell responses to the PCV13 carrier protein, CRM197, a novel whole blood cytokine release assay was developed. Whole blood was chosen over PBMC as the use of whole blood is more likely to represent the natural in vivo environment in which antigen is encountered [341] and is better suited to the often small amounts of blood obtainable from young children and infants.

3.4.1 *Time course experiments*

An initial time course experiment was performed in order to identify the optimum concentration of CRM197 and time points for harvesting culture supernatants. Whole blood (Lithium heparin) from a healthy adult control was diluted 1:10 in RPMI (Sigma Aldrich). 180μl of dilute whole blood solution was added to individual wells in a 96 well tissue culture plate (Nunc) containing either 20μl RPMI (Sigma Aldrich) (negative control), 20μl of a 10μg/ml solution anti-CD3 monoclonal antibody (Mabtech) in RPMI (positive control - final concentration 1μg/ml), or 20μl of CRM197 in RPMI at a concentration of 20, 100, 200, and 300μg/ml (final concentrations 2,10,20 and 30μg/ml respectively). Samples were then incubated in a humidified incubator, at 37°C in a 5% CO_2 atmosphere. Culture supernatants were harvested daily from day 1 to 6 then stored at -80°C for subsequent cytokine analysis, as described below.

3.4.2 *Cytokine measurement in whole blood culture supernatants using Mesoscale Discovery (MSD) platform*

The cytokine content of culture supernatants were measured with MSD ultrasensitive cytokine detection plates (MSD). A Th1/Th2 10-plex kit was used for the initial time course experiment / assay development. The cytokines measured by this kit include IL1β, IL2, IL4, IL5, IL8, IL10, IL12p70, IL13, IFNγ and TNFα. A custom made 5 plex kits measuring IL1β, IL5, IL10, IL13 and IFNγ was used for assessing supernatants from the main study. The assay works on the basic principle of an ELISA with plates precoated with spots of a range of cytokine specific capture antibodies. A detection antibody bound to a marker with electrochemiluminescent properties is then used to detect bound cytokine. An electric current is passed through the plate and light emitted is detected with a camera specific for the type of assay.

The kits were processed as per manufacturers instructions. Briefly, culture supernatants were defrosted to room temperature. Samples were prepared for the calibration curve by taking the provided 10000pg/ml cytokine standard solution and performing serial 1:4 dilution. The 96 well MSD plate was then blocked by adding 25μl of blocking solution to each well and incubating for 30 minutes with vigorous shaking at room temperature. 25μl of each calibrator solution or culture supernatant was added to separate wells with one duplicate for each sample. Samples were then incubated for 2 hours with vigorous shaking at room temperature. The plate was then washed 3 times with PBS + 0.05% Tween-20 (Oxoid) 25μl of detection antibody solution was then added to each well, followed by 2 hour incubation with vigorous shaking. After 3 further washes, 150μl of read buffer was then added to each well and the plate was read immediately on the SECTOR® imager (MSD). The lower limit of quantification (LLQ) for the assay was set at the lowest point on the standard curve with a coefficient of variation (CV) reliably less than 25% and a back calculated cytokine

concentration within 20% of the actual calibrator concentration. Readings below this were reported as half this value.

3.4.3 *Serum IL21 measurement using MSD platform*

Prototype MSD plates for IL21 detection were used to measure IL21 in serum samples. The plates were manufactured by MSD using commercially available IL21 ELISA reagents (eBioscience). Serum samples were defrosted to room temperature. Samples were prepared for the calibration curve by taking the 10000pg/ml cytokine solution (eBioscience) and performing 1:4 serial dilutions in ELISA assay diluent (EAD) (eBioscience). The 96 well MSD plate was blocked by adding 200μl EAD to each well and incubating for 1 hour at room temperature. The plate was then washed 3 times with PBS + 0.05% Tween-20. 100μl of calibrators or serum sample were added to separate wells with one duplicate per sample. Samples were incubated over night at 4°C then washed 3 times with PBS + 0.05% Tween-20. 25μl of biotin labelled IL21 detection Ab (eBioscience) (2μl/ml) was added to each well then incubated for 1 hour with vigorous shaking at room temperature. Plates were then washed 3 times and 25μl streptavidin/SULFO-TAG™ (MSD) added to each well. Following a further 3 washes, 150μl of read buffer was then added to each well and the plate was read immediately on the SECTOR® imager (MSD). The LLQ for the assay was set as described in Section 3.4.2. Readings below this were reported as half this value.

3.5 Assessment of pneumococcal serology

3.5.1 *Sample preparation*

Blood collected in SST II Advance Vacutainer (BD) was centrifuged for 10 minutes at 1400g. Serum was aspirated and separated into 500μl aliquots for subsequent storage at -80°C.

3.5.2 *Anticapsular IgG ELISA*

Pneumococcal capsular polysaccharides specific IgG for all strains contained in the PCV13 was measured by ELISA after absorption with cell wall and 22F polysaccharide as previously described, and described in detail by the Goldblatt laboratory (see Appendix 6). Briefly, sera are mixed before analysis with an absorbent containing cell wall polysaccharide (CPS) (Statens Serum Institute) and 22F capsular polysaccharide (American Type Culture Collection (ATCC)) to neutralize antibody binding to CPS and other common contaminants present in the pneumococcal polysaccharide coating antigens. ELISA microtitre plates (Greiner) are coated with pneumococcal polysaccharide by adsorbing individual pneumococcal polysaccharide serotype antigens (ATCC) to ELISA plates. Dilutions of absorbed sera are then added to the ELISA plates. The serotype specific antibody bound to the ELISA plate is detected with goat anti-human IgG antibody (all 4 IgG subclasses) conjugated with alkaline phosphatase (Biosource), followed by addition of the substrate, p-nitrophenyl phosphate (Sigma). The optical density of each well is measured at 405 nm and 620 nm using a Multiskan EX microtitre plate reader (Thermo Life Sciences). By comparing the optical density of the sample wells to that of the standard, human anti-pneumococcal reference serum 007sp (FDA), the level of antibody in the human serum can be calculated.

3.6 Pneumococcal nasopharyngeal carriage detection

Nasopharyngeal swabs were collected and processed according to the WHO working group standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae* [342].

3.6.1 *NP swab technique*

Samples were obtained with a deep nasopharyngeal swab using a paediatric size rayon tipped swab (Medical Wire and Equipment). The study subject's head was tipped back slightly and the swab passed directly backwards, parallel to the floor of the nasopharynx. The swab was left for five seconds and rotated through 180 degrees before removing. Once collected the swab tip was cut off using sterile scissors into a 2ml microtube (Sarstedt) containing skim-milk tryptone-glucose-glycerin (STGG) transport medium, prepared according to instruction provided by the CDC (see Appendix 7). The sample was then transported to the lab, vortexed then separated into 2 x 1ml aliquots (1 containing the original swab tip) and stored at -80°C.

3.6.2 *Isolation of Streptococcus pneumoniae from NP swabs*

Once 10 or more samples were collected and stored, samples were further processed in batches. The aliquots not containing the original swab tip were transported to Imperial College Healthcare NHS Trust Department of Microbiology, Charing Cross Hospital. Samples were defrosted and vortexed and 50 μl of vortexed specimen inoculated onto Columbia blood agar plus aztreonam selective plates (Oxoid) (primary plate) and incubated overnight at 37°C in 3-10% CO_2. From the primary plate, 2 presumptive pneumococcal colonies were streaked on to a Columbia blood agar plate (Oxoid). An optichin disc (Oxoid) was placed on the plate and again incubated overnight as above. From the optichin plate, confirmed pneumococcal colonies were streaked on to a Columbia blood agar plate to make a lawn, then incubated overnight as above. Pure growth was harvested with a plastic inoculation loop (Medical Wire & Equipment) and dispensed into STGG storage medium, prepared according to protocol provided by the CDC (see Appendix 7) and stored at -80°C for subsequent serotype identification (see below).

3.6.3 *Serotype identification*

Pneumococcal isolates were transported to the HPA Respiratory and Systemic Infection Laboratory (RSIL) where samples were serotyped using their standard latex agglutination method. Samples were defrosted to room temperature, subcultured on Columbia blood agar plates (HPA media services) with an optochin disc (MAST) and cultured over night at 35°C in 4-5% CO_2. If there was pure growth Todd-Hewitt broth (HPA media services) was inoculated with a heavy inoculum of fresh pneumococcal culture and incubated overnight at 35°C in 4-5% CO_2. The cultures were then centrifuged at 1500rpm for 30 minutes. Supernatants were removed leaving a few drops in each tube and the cell pellets resuspended in the remaining small amount of fluid. The suspension was used to perform slide agglutination using pooled and individual pneumococcal polysaccharide antisera (Statens Serum Institute) in a stepwise identification process. 10μl of bacterial suspension was placed on a glass slide using a plastic pipette. Antisera were added using a 1μl plastic loop and mixed briefly. Agglutination was classified as a positive result. If there was no agglutination with any antiserum, bile solubility was assessed. If bile soluble the sample was reported as non-typeable (NT).

3.7 Statistical analysis

The analysis of the data has been guided by statistical advice received from the Department of Statistics at Imperial College (Dr Elena Kulinskaya) and Department of Statistics at Institute of Child Health (Dr Deborah Ridout). Analysis of and extrapolation from existing data in the literature [198, 343] confirmed that our study would be adequately powered to detect differences in serum antibody pre and post vaccination and between groups according to the proposed stratifications. All analysis was performed by Alasdair Bamford using Stata IC version 12.1 for Mac (StataCorp) and Prism 5 for Mac OS X (GraphPad), with the guidance of Dr Deborah Ridout of the Institute of Child Health statistical advisory service. Specific analytical methods are described for each chapter. Prior to data analysis an analysis plan was formulated. In summary, data was initially inspected and assessed for completeness. Summary statistics were produced and the distribution of continuous data was assessed using both visual and statistical methods for assessing normality. Data was analysed using parametric tests when possible, using log transformation of data when appropriate and necessary. Linear regression and one way and repeated measures analysis of variance and covariance were used as appropriate following assessment for violation of test assumptions. When necessary, non-parametric tests were employed. A significance level of $p<0.05$ was considered significant while acknowledging that a lower cut off might be considered more appropriate in the context of multiple comparisons (as discussed in Section 6.5).

Chapter 4: B and T cell phenotype

4.1. Introduction

4.1.1 *B cell phenotype in perinatally acquired HIV (paHIV)*

There are few published papers describing the phenotype of B cells in the context of paediatric HIV, with partially contradictory results [166-169, 344]. Nevertheless it is now clear that paHIV is associated with B cell phenotypic abnormalities both on and off HAART. How these phenotypic abnormalities impact upon antibody responses to vaccine antigens, and how treatment history impacts upon potential for B cell immune preservation/reconstitution remains to be explored [168, 169].

More is known about B cell phenotypic abnormalities and their association with impaired vaccine responses in adults. The published literature confirms that some but not all of these abnormalities may be reversed by HAART and that earlier HAART may increase potential for maintenance of normal B cell phenotype and function [120, 160, 165, 188]. It is well known that there are differences in the immunological and virological features of paHIV and adult infection and caution must therefore be taken when making extrapolations from adult data to the paediatric population [111, 344]. PaHIV results in an insult to a relatively naive and still developing immune system, which can in turn be directly related to differences in the dynamics of viral replication, immunosuppression, clinical progression and response to HAART.

4.1.2 *Circulating T_{FH}-like cells in paHIV*

Despite contributing up to around 10% of circulating $CD4^+$, to our knowledge there is very little data concerning circulating T_{FH}-like cells in children in health or disease [209, 345] and no published data on T_{FH} in paHIV. This is perhaps unsurprising considering the ongoing debate over the last 15 years concerning their relationship to true lymphoid tissue T_{FH} [136, 137]. It is now increasingly clear that they are directly related to their GC counterparts [207, 208, 346] and data is emerging regarding the effect of HIV on this subset in adults and the resulting implications for T dependent vaccine responses [46-48, 347, 348].

4.1.3 *Relevance to pneumococcal immunity*

Children with paHIV are particularly susceptible to pneumococcal infection and remain at increased risk of invasive disease even when on suppressive HAART. Pneumococcal conjugate vaccines have been shown in both adults and children with HIV, to provide further protection against invasive disease, albeit less so than in healthy children in the context of paediatric infection [270, 271]. Antibody response to pneumococcal conjugate vaccines is key to their efficacy and it is generally accepted that conjugate vaccines result in the provision of T cell help to pneumococcal polysaccharide specific B cells [155]. It is thought that through this mechanism they are more effective at promoting class-switch recombination, affinity maturation and the establishment of immune memory in comparison to polysaccharide vaccines, at least in young children. However serum IgG responses to PCV in children with paHIV are less robust than in healthy children, most likely as a result of a combination of effects on T and B cell phenotype and function [270, 271].

By using multicolour flow cytometry we have characterised B and T cell subsets in peripheral blood of children with paHIV, healthy children and healthy young adults. We hypothesise that these subsets in particular, are likely to be of importance in the response to PCV and in the following chapter we will investigate how any observed alteration in cell phenotype impacts upon serological response to vaccination.

4.2 Chapter aims

i) To compare lymphocyte, B and T cell subset phenotype of children with paHIV with that of healthy children and healthy young adults

iii) To compare lymphocyte, B and T cell subsets phenotypically between healthy young adults and children with paHIV pre and post immunisation with PCV13

iv) To investigate how clinical history impacts upon T and B cell phenotype in paHIV

v) To investigate correlation between T cell and B cell phenotype in children with paHIV

4.3 Methods

A general overview of methods used to immunophenotype T and B cell subsets is provided in Section 2.3. The following is a further in-depth discussion of the choice of markers and the gating strategies used for investigation of B and T cell phenotypes applied throughout the work of this thesis. The following considerations were of importance when designing the flow cytometry panel used in this study:

1. Published and generally accepted methods for B cell immunophenotyping
2. Published observations of B cell abnormalities in HIV infected adults and children
3. Current practices regarding quality control in flow cytometry
4. Limitations of the available flow cytometer
5. Availability of fluorochrome/antibody combinations and fluorescence spillover
6. The use of small volumes of whole blood
7. Cost

4.3.1 *B cell gating strategy*

Immunophenotyping of human B cells has lagged behind that observed in the T cell field. However, in recent years circulating human B cell subsets have been characterised in increasing detail and with increasing complexity [349-351]. As with other lymphocyte subsets, there still remains a certain lack of consensus regarding the ontogenic relationship between observed cell types, nomenclature and the relationship between observed phenotype and function.

IgD and CD27 have classically been used to identify the following subsets of circulating $CD19^+$ B cells [145]:

- IgD^+CD27^- (naive)
- IgD^+CD27^+ (IgD^+ memory)
- IgD^-CD27^+ (class switched memory)
- IgD^-CD27^- (double negative)

IgD^+ memory B cells have been shown to predominantly consist of cells co-expressing IgD and IgM, leading some to use them as a surrogate for IgM^+ memory B cells. This classification system is complicated by the fact that circulating populations of $IgD^+IgM^-CD27^+$ and $IgD^-IgM^+CD27^+$ have been characterised, although they are relatively minor populations [349, 350, 352]. For some time CD27 was considered to be a definitive memory marker, however $CD27^-IgD^-$ memory cells have now also been identified and characterised in healthy human subjects [146, 353].

This classification system was included for reasons of consistency and comparability with published data in healthy and diseased adults and children and the nomenclature described above will also be used, accepting its limitations.

Circulating transitional B cells, and their subsets, may be identified using various combinations of markers and are characteristically $CD38^{+}CD24^{+}CD10^{+}$ [354]. For logistic reasons it was desirable to use only one marker to identify this subset and therefore CD10 was chosen. This marker has previously been used to investigate transitional B cell numbers in HIV infected children and adults [355] [169, 344, 356] [357]. CD10 also identifies a minor subset of "germinal centre founder" B cells, a proportion of which are $CD27^{+}$ [165, 355, 358], for this reason transitional cells were identified as $CD19^{+}CD10^{+}CD27^{-}$.

Circulating plasmablasts may be identified in a number of ways including $CD27^{++}CD38^{++}$IgD [349]. For the purposes of this study they were identified as $CD19^{+}CD10^{-}$ IgD^{-} $CD27^{++}$ [350, 352].

Moir et al have extensively investigated B cell phenotype in adults with HIV [120]. Part of their work has involved the characterisation of a $CD21^{lo}$ population demonstrated to be abnormally increased in this patient group. Through a series of carefully designed experiments they have shown that this $CD21^{lo}$ can be further subdivided into various subsets. $CD27^{+/++}CD21^{lo}$ cells have been characterised as activated mature B cells and plasmablasts [120]. $CD27^{-}CD21^{lo}$ cells include a subset of transitional cells [122, 355] but also a newly defined subset of cells with an exhausted phenotype, termed "exhausted" or "tissue-like" memory B cells. Phenotype and function of the latter subset has been extensively characterised using other specific surface markers such as Fc-receptor-like-4 (FCRL4) [359]. We have found it difficult to define a consistent gating strategy, using FMO controls, for a "lo" subset. For this reason this subset, which from now on will be referred to as tissue like/ exhausted memory B cells, were identified as $CD27^{-}CD21^{-}$. Activated mature B cells were accordingly defined as $C27^{+}CD21^{-}$.

4.3.2 *T cell gating strategy*

As discussed in Chapters 1 and 2 the main hypothesis of this study relates to the subset of circulating T_{FH}-like cells. This subset is a relatively recently characterised helper T cell subset [137]. As with the circulating B cell subsets described above definitive phenotypic and functional characterisation of lymphoid tissue T_{FH} and their circulating counterparts are still subject to much debate in the literature. However it is generally now agreed that circulating T_{FH}-like cells are a subset directly related to T_{FH} in lymphoid tissue and that they are identified by expression of CXCR5 [207, 208, 346]. Various other markers have been employed including CD57, ICOS, PD-1, CD40L and OX40, but CXCR5 is the surface marker most consistently used to identify this circulating subset in humans [207, 208, 346]. For the purposes of this study circulating T_{FH}-like cells are defined as $CD4^{+}CD45RO^{+}CXCR5^{+}$. Inducible T-cell costimulator (ICOS) was initially included as a subset specific marker (as it is expressed at high levels by lymphoid T_{FH}), however since devising the panel its utility as a specific marker in *circulating* T_{FH} has been disproved [136, 137, 207, 208]. In order to focus on the project main hypothesis and to limit complexity of data analysis the T helper cell subsets of interest have been defined as $CD4^{+}CD45RO^{+}$ ($CD45RO^{+}$ T helper cells), $CD4^{+}CD45RO^{+}CXCR5^{+}$ (circulating T_{FH}-like cells) and $CD4^{+}CD45RO^{+}CXCR5^{+}ICOS^{+}$ ($ICOS^{+}$ circulating T_{FH}-like cells).

The overall gating strategy is shown in Figs. 9 and 10 in Chapter 2.

4.3.3 *Statistical analysis*

For baseline comparisons, Chi^2 and Fisher's exact test were used to compare proportions. Mann-Whitney U test was used to compare baseline age and for contrasting baseline cell subset data between child and adult groups. Hodges-Lehman median differences with 95% confidence intervals were also calculated. For direct comparison of cell subsets from the two groups of children, data was log transformed and compared using linear regression analysis to allow for adjustment for age and to assess age*group interactions. Wilcoxon-Sign rank was used to assess for changes in cell subsets at baseline compared to 6 months post immunisation. Hodges-Lehman median difference and 95% confidence interval were also calculated. Linear regression was used to assess for effects of clinical history on subsets, after log transformation, within the CP group, and also to assess for correlation between T and B cell subsets.

4.4 Results

3.4.1 *Recruitment*

An overview of study numbers and recruitment is shown in Fig. 11.

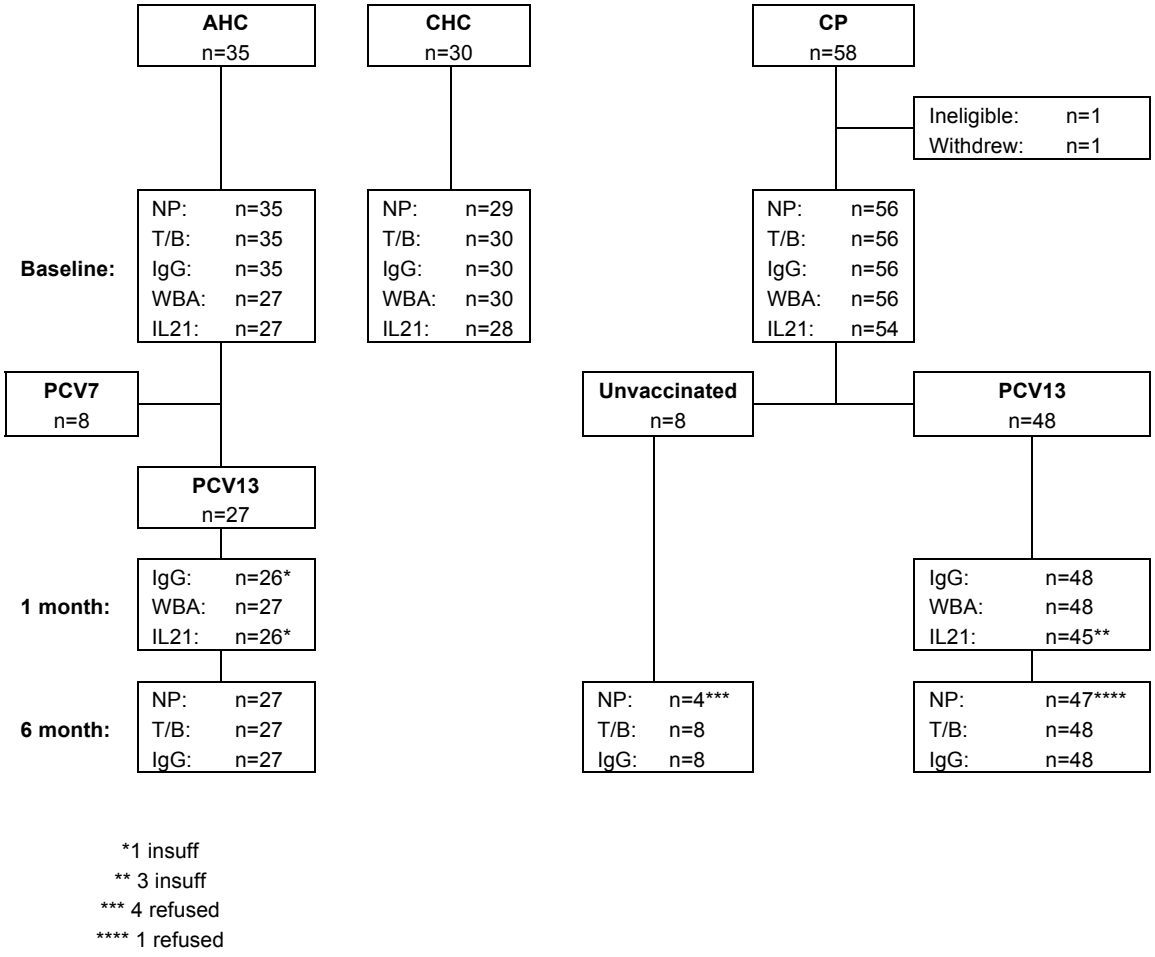


Figure 11. Overview of recruitment to study (AHC = Adult Healthy Control, CHC = Child Healthy Control, CP = Child Patient, NP = nasopharyngeal swab, T/B = immunophenotyping, IgG = pneumococcal serology, WBA = whole blood assay, IL21 = serum IL21 determination)

4.4.2 *Baseline characteristics*

The baseline characteristic of those included in the assessment of B and T cell phenotype are summarised in Table 3.

		CHC (n=30)		AHC (n=27)		CP (n=56)	
Sex	Female	14	(46.7)	16	(59.3)	25	(44.6)
	Male	16	(53.3)	11	(40.7)	31	(55.4)
Ethnicity	White	4	(13.3)	19	(70.4)	2	(3.6)
	Mixed	0	(0)	2	(7.4)	3	(5.4)
	Asian	0	(0)	4	(14.8)	3	(5.4)
	Black Caribbean	4	(13.3)	0	(0)	2	(3.6)
	Black African	13	(43.3)**	0	(0)	45	(80.4)*
	Other	9	(30)	2	(7.4)	1	(1.8)
Born in UK		29	(96.7)**	20	(74.1)***	28	(50)
Age/months		107.5	(19-195)	322†	(245-359)	151.5ϕ	(12-209)
Nadir CD4%		-	-	-	-	13	(0-48)
Proportion of life undetectable		-	-	-	-	0.47	(0-0.98)
VL <50 copies/ml		-	-	-	-	44	(78.6)
Treated 1st year		-	-	-	-	10	(17.9)
Treated 1st 2 years		-	-	-	-	15	(26.8)
Lymphocyte subsets	lymphcells/μl	2139	(1666-3414)	1833ϕζ	(1377-2125)	2390.5 ‡	(1764-3300.5)
	CD3 cells/μl	1575.5	(1166-2486)	1464ζ	(1094-1706)	1889.5 ‡	(1345.5-2516.5)
	CD3 %	71.6	(66.6-78.3)	74.7	(70.1-79.1)	74.35	(68.9-79)
	CD4 cells/μl	833.5	(647-1391)	803	(694-1061)	855 ‡	(525.5-1133.5)
	CD4 %	43.1	(36.7-46.5)	43.8ζ	(39.8-50.6)	34.55 ‡	(27.2-40.8)
	CD8 cells/μl	529.5	(356-907)	500ζ	(383-557)	895.5 §°	(626-1070.5)
	CD8 %	23.8	(21.6-27.2)	26.3ζ	(22.2-28.9)	32.7 ‡	(28.6-42.8)
	CD19 cells/μl	446.5	(272-679)	255ϕζ	(153-326)	403 §	(267.5-573.5)
	CD19 %	18.2	(14.5-24.2)	11.4ϕζ	(8.9-16.8)	17.05 §	(13-20.9)
	CD56 cells/μl	134.5	(82-205)	173ζ	(96-263)	119 ‡	(72-186)
	CD56 %	5.4	(2.9-8.2)	9.4ϕζ	(6.5-12.7)	4.4	(2.8-6.9)

Table 3. Baseline characteristics for all study participants assessed by flow cytometry. Sex, ethnicity, UK origin, HIV viral load status, treated in the 1st year of life and treated in the 1st 2 years of life are presented as number and percentage. Age, nadir CD4% and proportion of life undetectable are presented as median and range. Lymphocyte subsets are presented as median and interquartile range. Parameters for which significant differences between groups were found are highlighted in blue. (CHC= child healthy control, AHC= adult healthy control, CP = child patient, VL = HIV viral load, PCV7 = 1-valent pneumococcal conjugate vaccine, PPV= pneumococcal polysaccharide vaccine)

* Proportion significantly different: CP vs. CHC (p<0.005)

** Proportion significantly different: CHC vs. AHC (p<0.005)

*** Proportion significantly different: AHC vs. CP (p<0.05)

† Median significantly different from CP and CHC (p<0.001)

ϕ Median significantly different from CHC (p<0.05)

ζ Median significantly different from CP (p<0.05)

‡ Significant age*group interaction when comparing CHC vs. CP (p<0.05)

§ Significant independent age effect when comparing CHC vs. CP (p<0.05)

° Significant independent group effect when comparing CHC vs. CP (p<0.05)

4.4.3 *Demographic information*

Chi^2 or Fisher's exact test were used as appropriate to compare proportions in each group.

The proportions that were male did not differ by group: 53.3%, 40.7% and 55.4% for CHC, AHC and CP respectively (p=0.444). The proportions that were of black ethnicity differed between groups being higher in CP (84%) compared to CHC (56.6%) (*p*=0.006) which in turn was higher when compared to AHC (0%) (*p*< 0.001). The proportions born in the UK also differed between groups being higher in CHC (96.7%) compared to AHC (74.1%) (*p*=0.021) which in turn was higher when compared to CP (50%) (*p*=0.037). As the age distributions were non-parametric and not amenable to uniform transformation Mann-Whitney U test was employed to compare ages between groups. As expected from the study design the age of AHC (median= 322 months) was higher when compared to CHC (median = 107.5 months) (*p*<0.001) and CP (median = 151.5 months) (*p*<0.001). CHC median age was lower than CP (*p*=0.009).

4.4.4 *Lymphocyte subsets*

Children with paHIV have significantly different lymphocyte subsets when compared to young adult healthy controls

The data for each of lymphocyte subsets were skewed. Log transformation normalised the data, however non-homogeneity of variance precluded use of parametric t-test for comparisons of CP and CHC with AHC. Mann-Whitney U test was used to detect differences in median lymphocyte subset absolute numbers and percentages. Hodges-Lehman median differences with 95% CI were calculated. The results of this analysis are shown in Table 4.

	CHC vs. AHC			CP vs. AHC		
	Med diff	95%CI	*p*	Med diff	95%CI	*p*
Lymph abs	470.50	-1.00 - 1111.00	0.0449	557.00	237.00 - 929.00	0.0029
CD3 abs	231.00	-64.00 - 690.00	0.1048	438.00	203.00 - 739.00	0.0021
CD3%	-2.85	-6.60 - 1.20	0.1873	-0.50	-3.80 - 2.80	0.7706
CD4 abs	64.00	-115.00 - 291.00	0.4196	7.00	-164.00 - 183.00	0.8918
CD4%	-3.50	-6.92 - 0.50	0.0747	-11.20	-15.10 - -7.00	<0.0001
CD8 abs	63.00	-58.00 - 243.00	0.2915	394.00	283.00 - 511.00	<0.0001
CD8%	-1.20	-3.80 - 1.20	0.2987	7.90	4.90 - 11.90	0.0001
CD19 abs	198.50	86.00 - 330.00	0.0003	158.00	88.00 - 236.00	0.0001
CD19%	6.10	2.70 - 9.38	0.0002	4.48	2.20 - 7.00	0.0009
CD56 abs	-43.00	-93.00 - 5.00	0.0709	-53.00	-95.00 - -3.00	0.0232
CD56%	-3.90	-5.90 - -1.70	0.0011	-4.50	-6.50 - -2.80	<0.0001

Table 4. Results of comparison of lymphocyte subsets of each of the child cohorts with the adult cohort. Percentages are of absolute lymphocyte count. Regarding absolute cell counts: CHC had a significantly higher lymphocyte and B cell counts than AHC, whereas CP had higher absolute lymphocyte, T cell, CD8 cell and B cell count. Regarding percentages: CHC had higher B cell percentage and lower NK cell percentages, whereas CP had higher B cell and CD8 percentages and lower CD4 and NK cell percentages (Lymph= lymphocytes, abs = absolute count in cells/μl, Med diff = median difference, CI = confidence interval,

As can be seen from Table 4, a greater number of differences were observed when comparing CP to AHC than when comparing CHC to AHC. Regarding absolute cell counts: CHC had a significantly higher lymphocyte and B cell counts than AHC, whereas CP had higher absolute lymphocyte, T cell, CD8 cell and B

cell count. Regarding percentages: CHC had higher B cell percentage and lower NK cell percentages, whereas CP had higher B cell and CD8 percentages and lower CD4 and NK cell percentages. Results are also shown in Figure 12. Further direct comparison of CHC with CP group is described below.

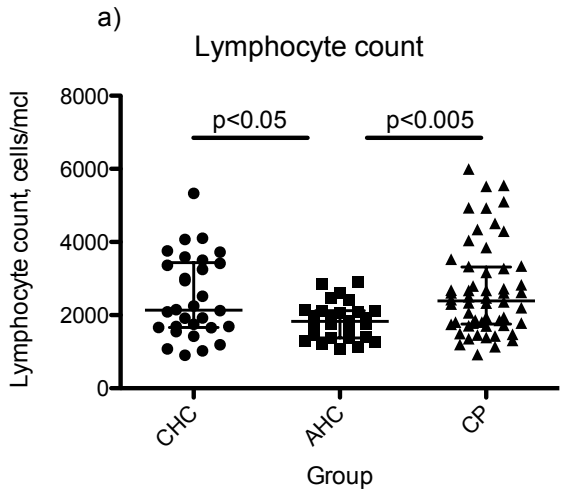
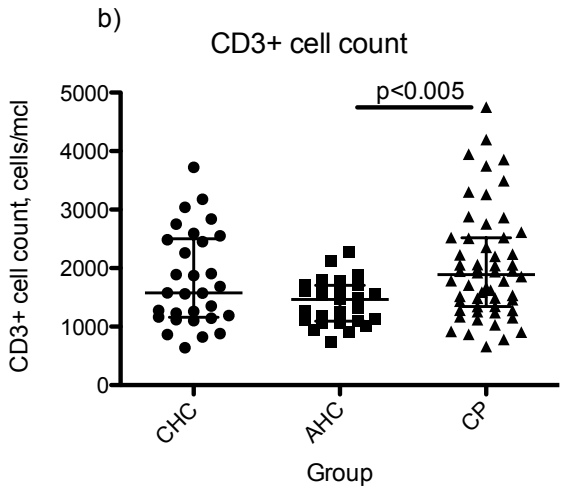
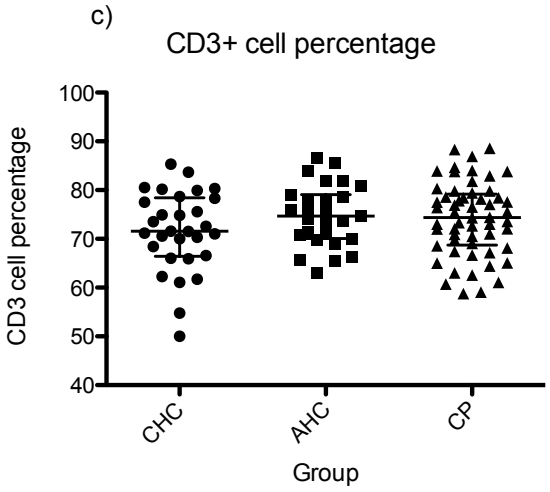
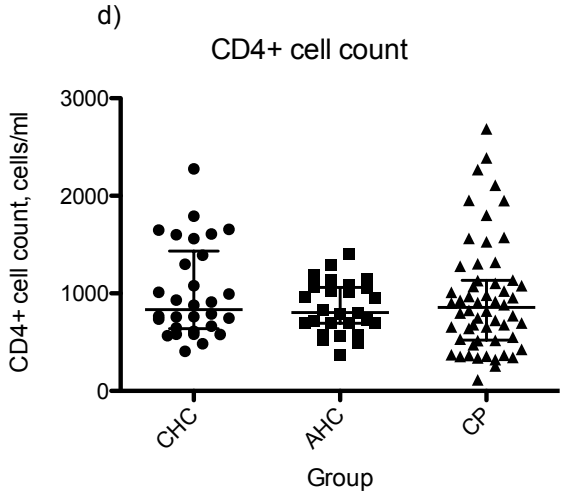
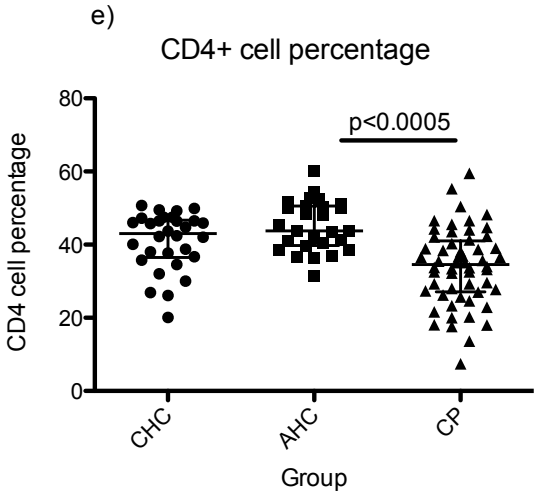


Figure 12 a-k) Scatter plots illustrating absolute counts and percentages for lymphocyte subsets in each group. a) overall lymphocyte counts, b) CD3+ cell counts, c) CD3+ cell % d) CD4+ cell count e) CD4+ cell % f) CD8+ cell count g) CD8+ cell % h) CD19+ cell count i) CD19 cell % j) CD56+ cell counts k) CD56 cell %. Median and interquartile ranges are shown. Significance level is shown where significant differences occurred at the p<0.05 level. Regarding absolute cell counts: CHC had a significantly higher lymphocyte and B cell counts than AHC, whereas CP had higher absolute lymphocyte, T cell, CD8 cell and B cell count. Regarding percentages: CHC had higher B cell percentage and lower NK cell percentages, whereas CP had higher B cell and CD8 percentages and lower CD4 and NK cell percentages (CHC= child healthy control, AHC = Adult healthy control, CP= Child patient)









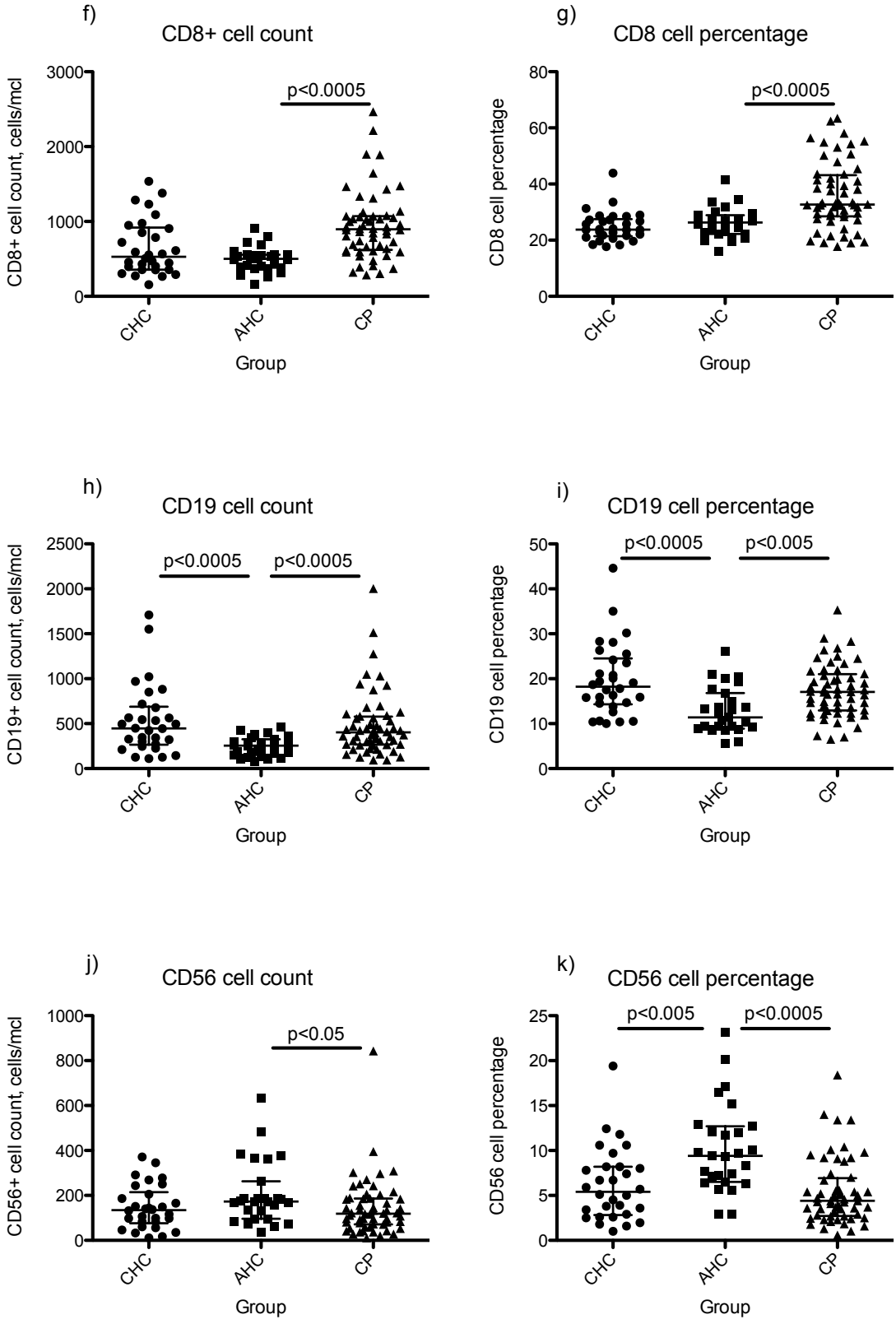


Figure 12 continued.

Children with paHIV have significantly different lymphocyte subsets when compared with healthy children despite effective HAART

To compare baseline lymphocyte subsets between CP and CHC groups we performed linear regression analysis to allow adjustment for age and to investigate any age*group interactions. All subset data was log transformed prior to analysis and ages were converted to years for ease of interpretation. The results of these comparisons are shown in Table 5.

	Age*Group			Age			Group		
	antilog coef	antilog 95% CI	*p* value	antilog coef	antilog 95% CI	*p* value	antilog coef	antilog 95% CI	*p* value
Lymp abs	0.962	0.927 - 0.999	0.043						
CD3 abs	0.954	0.919 - 0.991	0.017						
CD3%				1.004	0.999 - 1.009	0.151	1.026	0.975 - 1.080	0.321
CD4 abs	0.916	0.874 - 0.959	<0.001						
CD4%	0.950	0.921 - 0.981	0.002						
CD8 abs				0.961	0.939 - 0.985	0.002	1.690	1.349 - 2.117	<0.001
CD8%	1.035	1.006 - 1.064	0.017						
CD19 abs				0.926	0.899 - 0.953	<0.001	1.142	0.869 - 1.501	0.337
CD19%				0.973	0.956 - 0.989	0.002	0.963	0.818 - 1.133	0.642
CD56 abs	0.910	0.838 - 0.987	0.024						
CD56%				1.028	0.994 - 1.064	0.102	0.813	0.591 - 1.118	0.199

Table 5. Results of linear regression analysis comparing lymphocyte subsets for CHC with CP. Antilog regression coefficients are reported with 95% CI. When a significant age*group interaction was demonstrated, only the interaction coefficient is reported. When interaction was non-significant age and group effects are reported individually. Percentages are of absolute lymphocyte count. Significant age*group interactions were present for lymphocyte, T cell, CD4 cell and NK cells. The only subset for which there was a significant independent group effect after adjusting for age was CD8+ absolute count. There was no difference between groups after adjusting for age in $CD19^{+}$ cell absolute count. Age*group interactions were present for CD8% and CD4%.There was no difference in CD19 % or NK % after adjusting for age. (Lymph = lymphocyte, abs = absolute count in cells/μl, coef = regression coefficient, CHC = child healthy control, AHC = adult healthy control, CP = child patient)

Log transformation of a dependent variable affects the interpretation of regression coefficients. For this reason the antilog of the regression coefficient is reported. The independent variable, “group”, is a categorical variable and therefore the antilog of the regression coefficient is the ratio of the geometric mean of the dependent variable in the two compared groups. For example, from Table 5 it can be seen that there is a significant effect of group on CD8+ absolute count. The antilog of the regression coefficient is 1.69. This indicates that if age is kept constant the geometric mean of the absolute CD8+ absolute count is predicted to be 1.69 times higher in the CP group than the geometric mean of the CHC group. This effect is shown in Fig. 13A.

The independent variable, age, is a continuous variable. The antilog of the regression coefficient is the ratio of the predicted dependent variable following a unit increase in the independent variable. Using the absolute CD8+ cell count as an example again, it can be seen that there is a significant effect of age. The antilog of the regression coefficient is 0.961. Therefore for a one year increase in age there will be an approximately 4% decrease in the absolute CD8+ cell count. This again can be seen from Fig. 13A.

The interpretation of the antilog of the regression coefficient for the interaction term is slightly more complex. It is effectively a ratio of change per unit increase in age for one group vs. change per unit increase in age for the other group. For example, from Table 5 it can be seen that there is a significant age*group interaction for the $CD3^+$ cell count. The antilog of the regression coefficient, 0.954, is a measure of how different the change in $CD3^+$ cell count with age is for CP when compared to the change in $CD3^+$ cell count with age is for CHC. This difference in regression slopes can be seen from Fig. 13B.

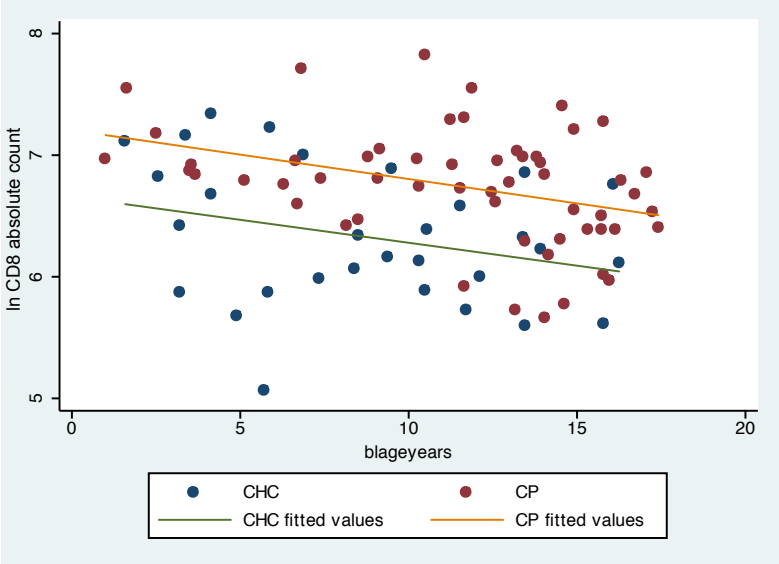


Figure 13A. Illustrative example of regression plots comparing log transformed CD8 cell counts between healthy controls and children with paHIV. Demonstrates separate age and group effects (CHC = child healthy controls, CP = child patients, blageyears = baseline age in years.

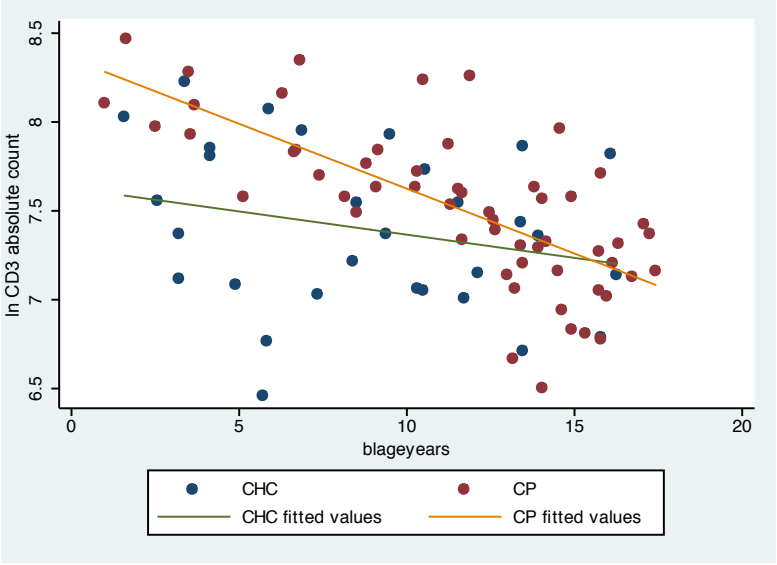


Figure 13B. Illustrative example of regression plots comparing log transformed CD3 cell counts between healthy controls and children with paHIV. Demonstrates age*group interaction (CHC = child healthy controls, CP = child patients, blageyears = baseline age in years)

Absolute cell counts:

Significant age*group interactions were present for lymphocyte, T cell, CD4 cell and NK cells. Lymphocyte counts were higher in younger children in the CP group than the CHC group, but more equivalent in the older children. A similar pattern was observed for $CD3^+$ absolute cell counts. For absolute $CD4^+$ cell count, younger CP had higher counts than CHC and older CP had lower counts than CHC. For absolute $CD56^+$ cell count, older children in the CP group tended to have lower cell counts than CHC of a similar age. The only subset for which there was a significant independent group effect after adjusting for age was CD8+ absolute count, with higher CD8+ counts in CP group than in CHC group. There was no difference between groups after adjusting for age in $CD19^+$ cell absolute count.

Subset percentages:

Age*group interactions were present for CD8% and CD4%. For CD8%, older CP had higher cell percentages than CHC of a similar age. And conversely, older CP had lower CD4% than CHC of a similar age. There was no difference in CD19% or NK cell % after adjusting for age.

All these effects remained significant at $p<0.05$ level after adjusting for detectable viraemia.

Overall these results indicate significant differences in lymphocyte subsets between children with paHIV and healthy children, the pattern of difference often varying with age. These observed differences are present despite the majority of the HIV infected children included in the study being on effective HAART with undetectable HIV viral load. Results are also shown in Figure 14.

a) Lymphocyte count

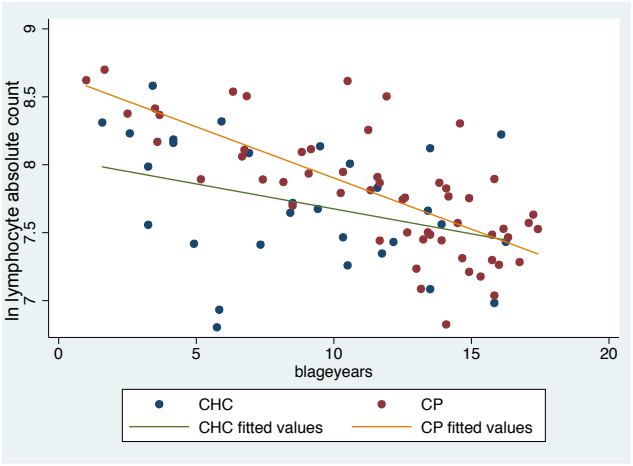
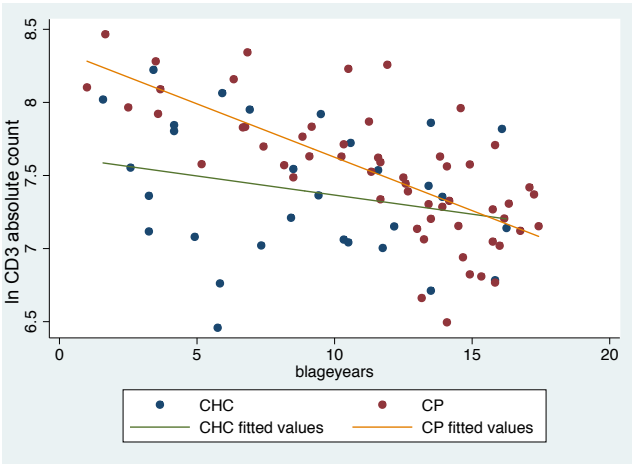
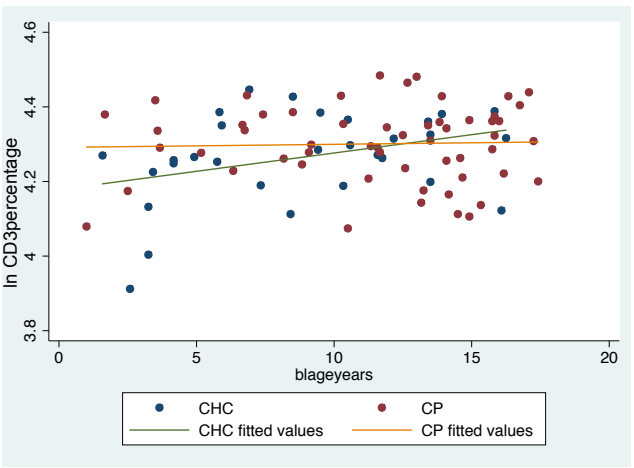


Figure 14. a-k) Regression plots comparing healthy children with children with paHIV. Significant age*group interactions were present for lymphocyte, T cell (CD3), CD4 cell, and NK cell (CD56) counts (p<0.05) and for CD4 cell percentage (p<0.005). Significant independent age effects were present for CD8 cell and B cell (CD19) counts and B cell percentage (p<0.005). Significant independent group effect was present for CD8 cell count (p<0.005). (CHC= child healthy control, CP = child patient, blageyears = baseline age in years).

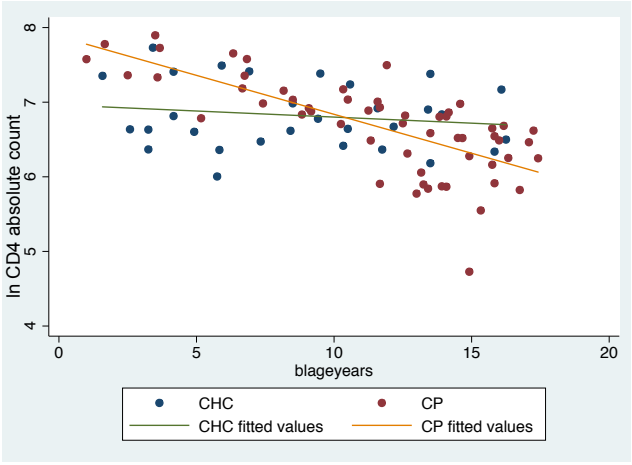
b) CD3+ cell count



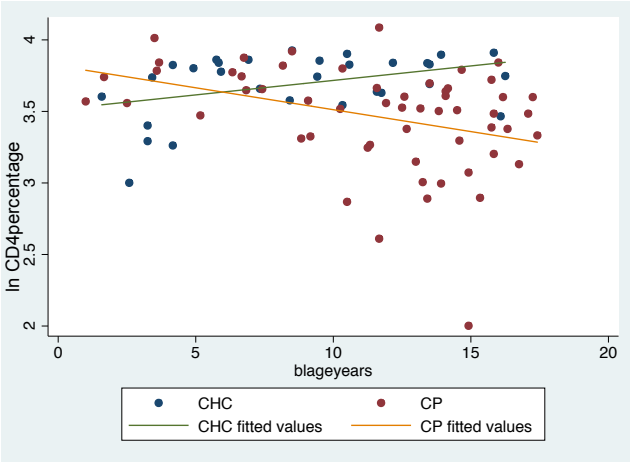
c) CD3+ cell percentage



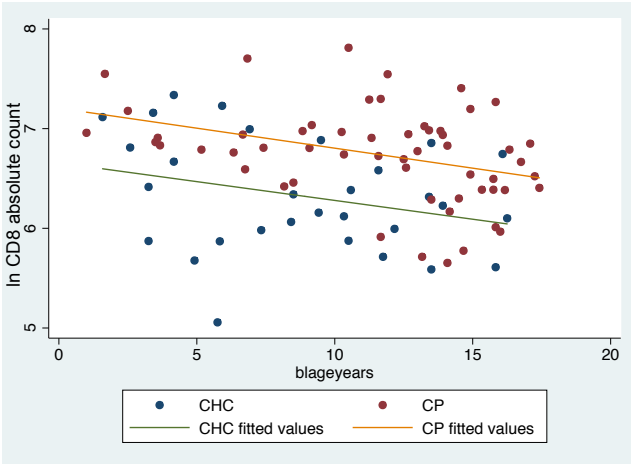
d) CD4+ cell count



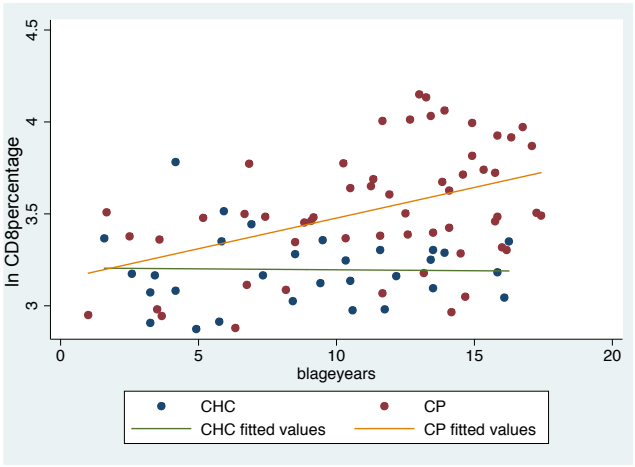
e) CD4+ cell percentage



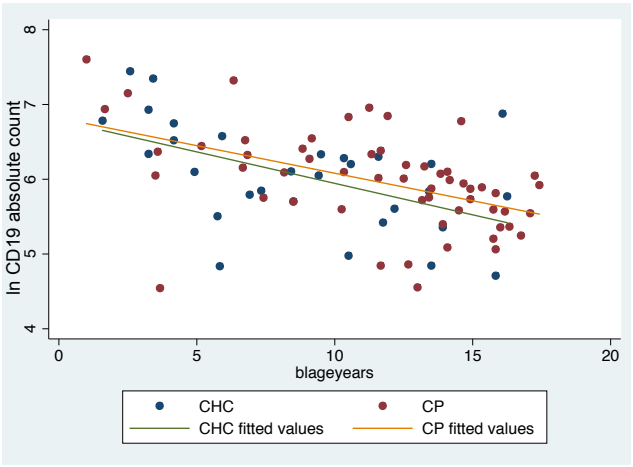
f) CD8+ cell count



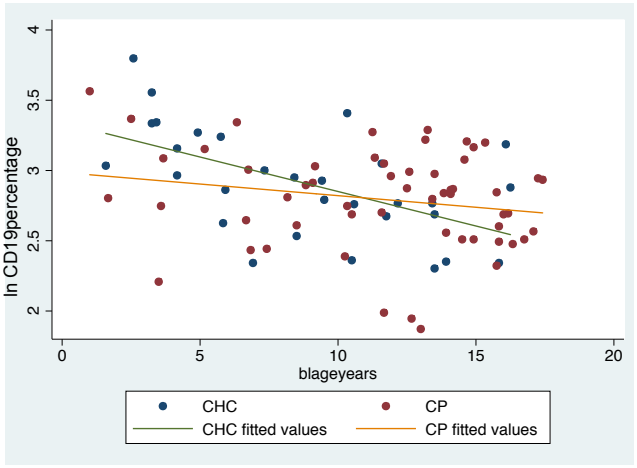
g) CD8+ cell percentage



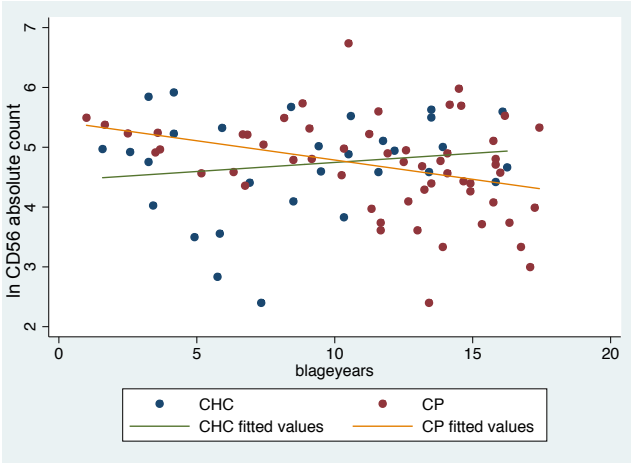
h) CD19+ cell count



i) CD19+ cell percentage



j) CD56+ cell count



k) CD56+ cell percentage

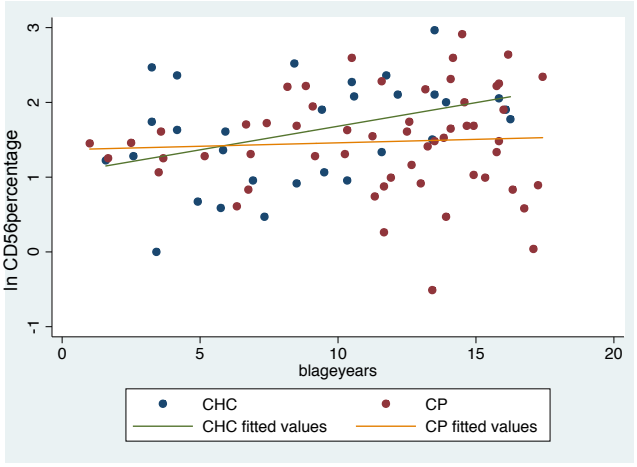


Figure 14. continued.

4.4.5 *B and T cell subsets*

Children with paHIV have significantly different B and T cell subsets when compared to healthy young adults

A summary of B and T cell subsets from each group is presented in Table 6. In order to limit the number of comparisons being made, only percentages of B and T cell subsets were analysed. As for lymphocyte subsets, B and T cell subset percentages for AHC were first compared with CP and CHC. Mann-Whitney U test was used to detect differences in median B and T cell subset percentages. Hodges-Lehman median differences with 95% CI were calculated. The results of this analysis are shown in Table 7.

For B cell subsets the differences between CP and AHC mirrored those observed between CHC and AHC. Median transitional cell percentages were higher in both child groups than AHC. When using CD21 vs. CD27 to subdivide B cells, median naive mature and tissue like/ exhausted cell percentages were higher in both child groups (CHC vs. AHC: p=0.0034 and p<0.0001 respectively. CP vs AHC: p<0.0001 and p<0.0001 respectively) and median resting memory percentages were lower (p≤0.0001). When using IgD and CD27 to subdivide B cells median IgD^+ memory and median class switched memory percentages were lower in both child groups compared to adults (CHC vs AHC: p=0.002. CP vs AHC: p<0.0001) and median naive and double negative B cell percentages were higher (CHC vs AHC: p=0.0034 and p=0.0484 respectively. CP vs. AHC: p<0.0001 and p=0.001 respectively). Median $CD45RO^+$ percentages were lower in both child groups compared to adults (p<0.0001) and median $CXCR5^+ICOS^+$ percentages were higher than in adults (CHC vs AHC: p=0.0002. CP vs. AHC: p=0.0017). Median $CXCR5^+$ percentages were higher in the CHC group compared to AHC (p=0.0002), however there was no difference when comparing CP with AHC (p=0.2474).

	CHC				AHC				CP			
	Med	95%CI	GM	95%CI	Med	95%CI	GM	95%CI	Med	95%CI	GM	95%CI
transitional %	1.26°	0.61 - 2.38	1.20	0.86 - 1.66	0.49	0.30 - 0.89	0.51	0.36 - 0.74	1.47°	0.85 - 1.95	1.34	1.09 - 1.65
plasmablast %	0.98	0.49 - 1.48	0.88	0.66 - 1.16	0.60	0.40 - 1.00	0.67	0.50 - 0.89	0.79	0.40 - 1.41	0.84	0.63 - 1.10
naive mature %	72.65°	66.20 - 79.40	71.83	68.40 - 75.42	63.50	50.90 - 75.40	60.61	55.43 - 66.28	76.55°	71.00 - 81.00	73.96	69.91 - 78.25
resting memory %	20.50°	14.00 - 27.60	18.77	15.95 - 22.09	33.50	22.50 - 46.00	31.15	25.76 - 37.65	15.50°	10.00 - 18.05	12.62*	10.78 - 14.78
activated mature %	2.01	1.47 - 2.50	2.06	1.62 - 2.64	1.78	1.19 - 2.33	1.78	1.49 - 2.14	1.66	1.12 - 3.44	1.89	1.46 - 2.46
exhausted/tissue like %	4.04°	2.32 - 5.84	3.76	2.99 - 4.72	1.41	1.20 - 1.77	1.56	1.31 - 1.86	4.40°	3.07 - 7.96	5.06	4.13 - 6.20
naive %	72.40°	63.70 - 79.80	70.38	66.55 - 74.44	60.80	47.60 - 71.00	57.94	52.41 - 64.05	76.70°	69.00 - 82.70	75.44*	72.78 - 78.20
IgD^+ memory %	11.30°	6.52 - 13.50	9.82	8.24 - 11.71	15.70	9.61 - 22.20	15.22	12.29 - 18.85	7.43°	4.79 - 10.20	6.60*	5.66 - 7.68
Switched memory %	11.50°	7.69 - 16.30	11.31	9.46 - 13.52	18.00	12.20 - 22.70	17.19	14.17 - 20.86	10.50°	6.59 - 12.75	8.71*	7.34 - 10.34
Double negative %	4.47°	3.14 - 6.32	4.69	3.86 - 5.70	3.57	2.36 - 4.67	3.49	2.88 - 4.24	5.52°	3.59 - 7.96	5.26	4.51 - 6.12
$CD45RO^+$%	31.30°	25.30 - 40.40	32.05	28.38 - 36.20	50.20	40.10 - 56.50	48.15	44.06 - 52.62	36.25°	27.55 - 43.00	34.10	31.01 - 37.51
$CXCR5^+$%	27.19°	20.71 - 32.90	26.26	23.69 - 29.10	19.49	15.11 - 22.78	17.22	13.23 - 22.41	23.77	12.65 - 29.41	17.36*	13.93 - 21.64
$CXCR5^+ICOS^+$%	0.80°	0.36 - 1.17	0.75	0.53 - 1.07	0.22	0.14 - 0.53	0.26	0.18 - 0.38	0.55°	0.27 - 1.53	0.59	0.43 - 0.82

Table 6. Summary of results for B and T cell subsets. Transitional ($CD19^+CD27^-CD10^+$) and plasmablast ($CD19^+CD10^-CD27^+IgD^-$) cells reported as a proportion of $CD19^+$ cells. Remaining B cell subsets reported as a proportion of $CD19^+$ after exclusion of $CD10^+$ and plasmablasts (naive mature ($CD19^+CD10^-CD27^-CD21^+$), resting memory ($CD19^+CD10^-CD27^+CD21^+$), activated mature ($CD19^+CD10^-CD27^+CD21^-$), exhausted/tissue like ($CD19^+CD10^-CD27^-CD21^-$), naive ($CD19^+CD10^-CD27^-IgD^+$), IgD^+ memory ($CD19^+CD10^-CD27^+IgD^+$), switched memory ($CD19^+CD10^-CD27^+IgD^-$), double negative ($CD19^+CD10^-CD27^-IgD^-$)). $CD45RO^+$ reported as a proportion of $CD4^+$. $CXCR5^+$ and $CXCR5^+ICOS^+$ reported as a proportion of $CD4^+CD45RO^+$. When comparing CP and CHC group with AHC group, significant differences were present for most subsets, consistent with published reference range data and expected changes with age. The magnitude of these differences was greater for the CP group than the CHC group (see Table 7). Of note $CXCR5^+$ cell percentages were significantly higher in the CHC group than AHC group, which was not the case for CP compared to AHC. When comparing CHC with CP group using geometric mean, significant differences in resting memory, naive, IgD^+ memory, switched memory and $CXCR5^+$ (T_{FH}-like) cells were observed after adjusting for age (p<0.05). (CHC= child healthy control, CP = child patient, AHC = adult healthy control, Med = median, GM= geometric mean)

° Significant difference in median when compared to AHC (p<0.05)

* Significant difference in geometric mean after adjusting for age (p<0.05)

	CHC v AHC			CP v AHC		
B cell subsets	Med diff	95%CI	*p*	Med diff	95%CI	*p*
transitional %	0.74	0.29 - 1.10	0.0005	0.86	0.52 - 1.12	<0.0001
plasmablast %	0.19	-0.03 - -0.03	0.2698	0.10	-0.10 - 0.39	0.4659
naive mature %	10.45	3.40 - 17.60	0.0034	14.75	7.20 - 21.40	<0.0001
resting memory %	-14.40	-21.10 - -7.30	0.0001	-20.30	-27.10 - -13.70	<0.0001
activated mature %	0.13	-0.34 - 0.67	0.5650	-0.04	-0.51 - 0.53	0.9033
tissue like/ exhausted %	2.26	1.17 - 3.53	<0.0001	2.80	1.97 - 4.25	<0.0001
naive %	11.80	4.40 - 19.40	0.0035	16.75	9.30 - 23.90	<0.0001
IgD^+ memory %	-6.49	-10.40 - -1.73	0.0021	-9.56	-5.20 - -13.60	<0.0001
switched memory %	-6.10	-9.90 - -2.30	0.0023	-8.46	-11.70 - -4.70	<0.0001
double negative %	0.96	0.01 - 2.08	0.0484	1.85	0.84 - 2.99	0.0010
T cell subsets						
$CD45RO^+$ %	-15.80	-21.70 - -10.10	<0.0001	-13.35	-18.5 - 8.00	<0.0001
$CXCR5^+$ %	8.02	4.34 - 11.69	0.0002	3.17	-1.64 - 7.15	0.2474
$CXCR5^+ICOS^+$ %	0.45	0.18 - 0.70	0.0002	0.29	0.12 - 0.51	0.0017

Table 7. Results of comparison of B and T cell subsets of each of the child cohorts with the adult cohort. Transitional ($CD19^+CD27^-CD10^+$) and plasmablast ($CD19^+CD10^-CD27^+IgD^-$) cells reported as a proportion of $CD19^+$ cells. Remaining B cell subsets reported as a proportion of $CD19^+$ after exclusion of $CD10^+$ and plasmablasts (naive mature ($CD19^+CD10^-CD27^-CD21^+$), resting memory ($CD19^+CD10^-CD27^+CD21^+$), activated mature ($CD19^+CD10^-CD27^+CD21^-$), exhausted/tissue like ($CD19^+CD10^-CD27^-CD21^-$), naive ($CD19^+CD10^-CD27^-IgD^+$), IgD^+ memory ($CD19^+CD10^-CD27^+IgD^+$), switched memory ($CD19^+CD10^-CD27^+IgD^-$), double negative ($CD19^+CD10^-CD27^-IgD^-$)). $CD45RO^+$ reported as a proportion of $CD4^+$. $CXCR5^+$ and $CXCR5^+ICOS^+$ reported as a proportion of $CD4^+CD45RO^+$. When comparing CP and CHC group with AHC group, significant differences were present for most subsets, consistent with published reference range data and expected changes with age. The magnitude of these differences was greater for the CP group than the CHC group. Of note $CXCR5^+$ cell percentages were significantly higher in the CHC group than AHC group, which was not the case for CP compared to AHC. Median values for each subset are reported in Table 6. (Lymph= lymphocytes, abs = absolute count in cells/μl, Med diff = median difference, CHC = Child healthy control, AHC = Adult healthy control, CP = Child patient)

Overall both healthy children and children with paHIV had a similar pattern of differences in median T and B cell subsets when compared to healthy young adults. However these differences tended to be more extreme for the CP group and in the case of $CXCR5^+$ T cells there is divergence in that healthy children had a higher percentage when compared to adults whereas the HIV cohort showed no difference. Further direct comparison of CHC with CP group is discussed below.

Children with paHIV have significantly different B and T cell subsets when compared with healthy children despite effective HAART

As when comparing baseline lymphocyte subsets between CP and CHC groups linear regression analysis was used to compare B and T cell subsets adjusting for age and to investigate any age*group interactions. All subset data was log transformed prior to analysis and ages were converted to years for ease of interpretation. The results of these comparisons are shown in Table 8.

No significant age*group interaction effects were observed. The antilog regression coefficients reported in Table 8 can be interpreted as described in Section 4.4.4. After adjusting for age there were significant

differences in B cell subsets. Using CD27 vs. CD21 to subdivide B cells, resting memory percentages were lower in patients than healthy controls. When using CD27 vs. IgD to subdivide B cells, both IgD^+ and class switched memory percentages were lower and naive percentages were higher in patients than healthy controls. Only the difference between class switched memory percentages lost significance at the $p<0.05$ level after adjusting for detectable viraemia (VL>50 RNA copies/ml).

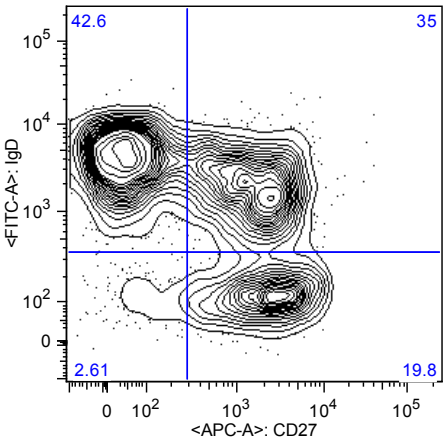
	Age			**Group**		
	antilog coef	antilog 95% CI	*p*	antilog coef	antilog 95% CI	*p*
transitional %	0.942	0.907 - 0.979	0.003	1.312	0.914 - 1.885	0.139
plasmablast %	1.066	1.019 - 1.115	0.006	0.805	0.526 - 1.233	0.315
naive mature %	0.994	0.985 - 1.003	0.178	1.047	0.960 - 1.143	0.296
resting memory %	1.002	0.975 - 1.029	0.886	0.669	0.518 - 0.864	0.002
activated mature %	1.014	0.971 - 1.059	0.517	0.884	0.585 - 1.333	0.551
tissue like/ exhausted %	0.998	0.964 - 1.034	0.929	1.352	0.967 - 1.892	0.078
naive %	0.997	0.990 - 1.004	0.431	1.080	1.011 - 1.153	0.023
IgD^+ memory %	0.994	0.968 - 1.021	0.670	0.682	0.529 - 0.879	0.004
switched memory %	1.024	0.994 - 1.054	0.112	0.724	0.551 - 0.952	0.021
double negative %	1.012	0.984 - 1.040	0.404	1.086	0.838 - 1.409	0.527
$CD45RO^+$ %	1.041	1.025 - 1.056	<0.001	0.957	0.831 - 1.101	0.535
$CXCR5^+$ %	0.970	0.938 - 1.003	0.072	0.717	0.523 - 0.985	0.040
$CXCR5^+ICOS^+$ %	0.927	0.878 - 0.977	0.006	0.961	0.579 - 1.593	0.875

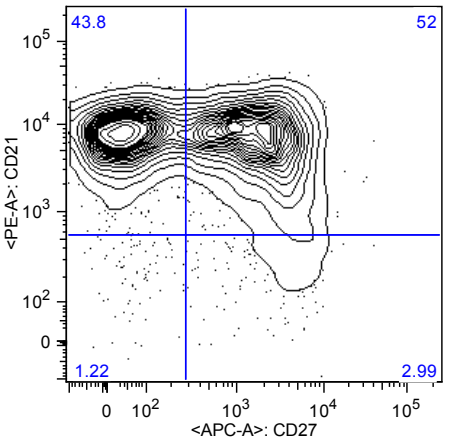
Table 8. Results of linear regression analysis comparing B and T cell subsets for CHC with CP. Antilog regression coefficients are reported with 95% CI. Transitional ($CD19^+CD27^-CD10^+$) and plasmablast ($CD19^+CD10^-CD27^+IgD^-$) cells reported as a proportion of $CD19^+$ cells. Remaining B cell subsets reported as a proportion of $CD19^+$ after exclusion of $CD10^+$ and plasmablasts (naive mature ($CD19^+CD10^-CD27^-CD21^+$), resting memory ($CD19^+CD10^-CD27^+CD21^+$), activated mature ($CD19^+CD10^-CD27^+CD21^-$), exhausted/tissue like ($CD19^+CD10^-CD27^-CD21^-$), naive ($CD19^+CD10^-CD27^-IgD^+$), IgD^+ memory ($CD19^+CD10^-CD27^+IgD^+$), switched memory ($CD19^+CD10^-CD27^+IgD^-$), double negative ($CD19^+CD10^-CD27^-IgD^-$)). $CD45RO^+$ reported as a proportion of $CD4^+$. $CXCR5^+$ and $CXCR5^+ICOS^+$ reported as a proportion of $CD4^+CD45RO^+$.When comparing CHC with CP group using geometric mean, significant differences in resting memory, naive, IgD^+ memory, switched memory and $CXCR5^+$ (T_{FH}-like) cells were observed after adjusting for age ($p<0.05$). Geometric means for each subset are reported in Table 6. (Lymph = lymphocyte, abs = absolute count in cells/μl, coef = regression coefficient, CHC = child healthy control, AHC = adult healthy control, CP = child patient)

After adjusting for age, geometric mean $CXCR5^+$ percentages were lower in CP than in CHC. This remained significant at the $p<0.05$ level after correcting for detectable viraemia.

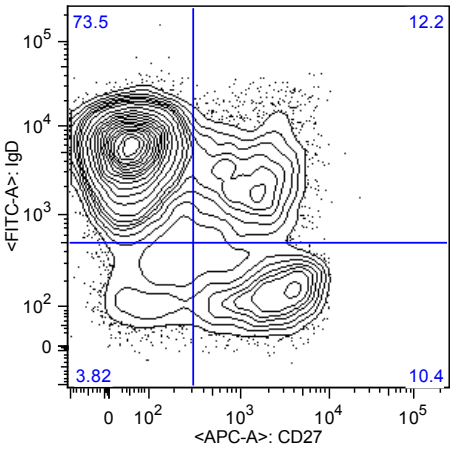
In summary, these results show that there are abnormalities of B cell subset distribution in children with paHIV when compared to healthy children. Memory B cell percentages are lower and naive B cell percentages are correspondingly higher. In addition to the relative deficiency in memory B cell subsets, there is a significantly lower proportion of T_{FH}-like cells in children with paHIV compared to healthy children. These differences are present despite the majority of the infected children being on effective HAART and remain significant after adjusting for detectable viraemia. Representative contour plots for gating CD27 vs. CD21 and CD27 vs IgD for $CD19^+CD10^-$B cells are shown in Figure 15.

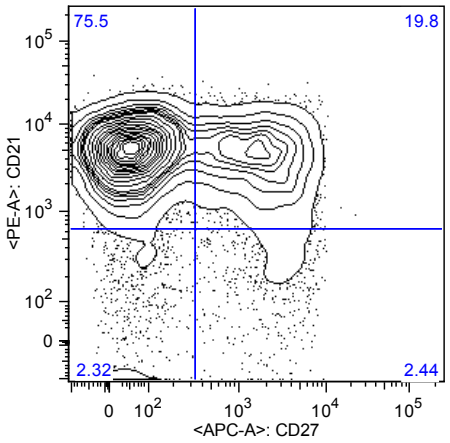
a) AHC



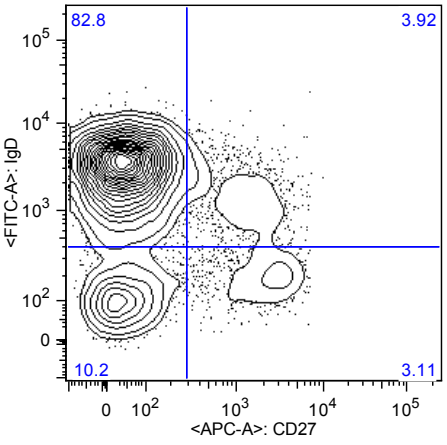


b) CHC





c) CP



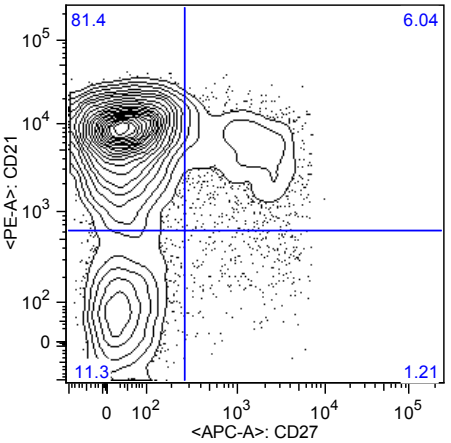


Figure 15 a-c. Typical contour plots from a) a healthy adult (AHC), b) a healthy child (CHC) and c) a child with paHIV (CP). Key differences between the groups when gating CD27 vs. CD21 and CD27 vs. IgD on $CD19^{+}CD10^{+}$ lymphocytes are illustrated. Healthy children have higher proportions of naive B cell subsets ($CD27^{-}$) and a correspondingly lower proportion of memory subsets ($CD27^{+}$) compared to healthy adults. Children with paHIV have even lower proportion of memory subsets compared to healthy controls.

4.4.6 *Stability of subsets over time*

There was no loss to follow and data was complete for all study subjects.

Baseline lymphocyte, B cell and T cell subsets were compared with the same parameters 6 months following vaccination for the child patient and adult healthy control groups. Data did not fit assumptions for parametric paired comparisons therefore non-parametric Wilcoxon Signed Rank test was used. Hodges-Lehman median paired differences with 95% CI were also calculated. Results are reported in Tables 9 and 10.

Secondary to study design and logistic considerations the number of days from baseline to follow up flow cytometric assessment was longer for CP (median= 259 days) than AHC (median= 210 days) (median difference = 52.5 days (95%CI: 34-72 days) $p<0.05$.

AHC	Baseline (median)	6 months (median)	med diff	95% CI	*p*
Lymph abs	1833.0	1787.0	-85.5	(-328.0-158.5)	0.456
CD3 abs	1464.0	1347.0	-39.0	(-206.5-146.0)	0.701
CD3 %	74.7	74.5	0.9	(-0.7-2.4)	0.244
CD4 abs	803.0	795.0	-9.8	(-105.0-108.0)	0.885
CD4 %	43.8	47.4	0.0	(-2.2-2.2)	0.970
CD8 abs	500.0	489.0	-1.8	(-56.5-68.0)	0.943
CD8 %	26.3	25.1	0.8	(-0.3-1.5)	0.150
CD19 abs	255.0	208.0	-9.5	(-38.0-18.0)	0.435
CD19 %	11.4	11.9	-0.6	(-1.4-0.2)	0.170
CD56 abs	173.0	168.0	-8.2	(-58.0-43.0)	0.614
CD56 %	9.4	9.0	-0.4	(-2.5-1.4)	0.631

CP					
Lymph abs	2349.5	2189.0	242.8	(84.0- 414.5)	0.005
CD3 abs	1854.5	1654.5	202.8	(72.5-319.5)	0.004
CD3 %	75.5	76.2	-0.9	(-2.4-0.5)	0.230
CD4 abs	855.0	797.0	66.8	(9.5- 133.0)	0.024
CD4 %	34.6	36.1	-1.6	(-2.7--0.5)	0.007
CD8 abs	913.5	791.0	106.5	(37.0- 180.0)	0.009
CD8 %	33.0	34.8	0.4	(-1.3-1.5)	0.542
CD19 abs	403.0	357.5	47.0	(10.0- 85.0)	0.020
CD19 %	17.3	16.6	0.6	(-0.4-1.6)	0.200
CD56 abs	119.0	123.5	1.5	(-12 - 13.5)	0.802
CD56 %	4.4	5.1	-0.5	(-2.5-1.4)	0.123

Table 9. Lymphocyte subsets at baseline compared with those at 6 months post immunisation for CP and AHC. Wilcoxon Signed Rank test was used to compare paired data and Hodges-Lehman median paired differences with 95% CI were calculated. Significant decreases are highlighted in red, significant increases, in green. There was no significant change in lymphocyte subsets over 6 months in the AHC group. Observed changes in lymphocyte subsets over 6 months in the CP group were as might be expected with increasing age and ongoing antiretroviral therapy. (abs= absolute cell count in cell/μl, med dif = median difference, CP= Child patient, AHC= Adult healthy control)

Young adult lymphocyte subset absolute numbers remained stable whereas those in children with paHIV changed over 6 months following vaccination

AHC lymphocyte subset absolute numbers and percentages did not significantly change over 6 months.

Only vaccinated children in the CP group were included for longitudinal subset analysis.

CP absolute lymphocyte count decreased over 6 months (med diff = 242.8 cells/μl (95%CI: 84-441.5 cells/μl, *p*=0.005)). The absolute CD4 count decreased (med diff = 66.8 cells/μl (95%CI: 9.5-133cells/μl), *p*=0.024)) as did the CD8 count (med diff = 106.5 cells/μl (95%CI: 37-180 cells/μl), *p*=0.009)) and CD19 count (med diff = 47.0 cells/μl (95%CI: 10-85 cells/μl), *p*=0.02).

CP lymphocyte percentages did not significantly change over 6 months apart from CD4%, which increased (med diff = -1.6% (95%CI: -2.87- -0.5) *p*=0.007).

AHC	Baseline (median)	6 months (median)	med diff	95% CI	*p*
B cell subsets					
transitional %	0.5	0.4	0.1	(0.0-0.3)	0.108
plasmablast %	0.6	0.8	0.0	(-0.3-0.3)	0.971
naive mature %	62.0	59.6	2.3	(0.4-4.1)	0.023
resting memory %	35.3	37.6	-1.8	(-3.4-0.1)	0.061
activated mature %	2.0	2.2	0.0	(-0.6-0.2)	0.471
tissue like/ exhausted %	1.4	1.5	0.1	(-0.4-0.2)	0.728
naive %	60.4	57.8	1.7	(0.3-3.3)	0.017
IgD^+ memory %	15.9	16.5	-1.1	(-2.4-0.2)	0.093
switched memory %	19.5	19.1	-1.0	(-2.1-0.1)	0.072
double negative %	3.6	3.4	-0.1	(-0.2-0.4)	0.631
T cell subsets					
$CD45RO^+$ %	50.2	48.9	-1.3	(-3-0.3)	0.075
$CXCR5^+$ %	19.5	19.7	1.4	(-2-4.2)	0.269
$CXCR5^+ICOS^+$ %	0.2	0.2	0.1	(0.0-0.27)	0.164

CP					
B cell subsets					
transitional %	1.5	0.9	0.4	(0.2-0.7)	0.001
plasmablast %	0.9	0.8	-0.2	(0.0-0.49)	0.073
naive mature %	76.4	75.8	1.4	(-0.15-2.94)	0.086
resting memory %	15.2	16.7	-1.3	(-2.22--0.35)	0.008
activated mature %	2.0	2.1	0.0	(-0.4-0.3)	0.927
tissue like/ exhausted %	4.4	5.1	0.1	(-1.3-1.5)	0.963
naive %	76.4	74.0	1.8	(0.4-3.3)	0.021
IgD^+ memory %	8.0	7.6	-0.5	(-1.1-0.3)	0.211
switched memory %	10.5	11.9	-0.9	(-1.6-0.0)	0.049
double negative %	5.3	5.6	-0.4	(-0.1-0.0)	0.062
T cell subsets					
$CD45RO^+$ %	36.0	35.7	-0.3	(-1.5-1.1)	0.659
$CXCR5^+$ %	23.6	24.1	-2.4	(-5.4-0.1)	0.050
$CXCR5^+ICOS^+$ %	0.6	0.5	0.3	(0.1-0.5)	0.012

Table 10. B and T cell subsets at baseline compared with those at 6 months post immunisation for CP and AHC. Wilcoxon Signed Rank test was used to compare paired data and Hodges-Lehman median paired differences with 95% CI were calculated. Transitional ($CD19^+CD27^-CD10^+$) and plasmablast ($CD19^+CD10^-CD27^+IgD^-$) cells reported as a proportion of $CD19^+$ cells. Remaining B cell subsets reported as a proportion of $CD19^+$ after exclusion of $CD10^+$ and plasmablasts (naive mature ($CD19^+CD10^-CD27^-CD21^+$), resting memory ($CD19^+CD10^-CD27^+CD21^+$), activated mature ($CD19^+CD10^-CD27^+CD21^-$), exhausted/tissue like ($CD19^+CD10^-CD27^-CD21^-$), naive ($CD19^+CD10^-CD27^-IgD^+$), IgD^+ memory ($CD19^+CD10^-CD27^+IgD^+$), switched memory ($CD19^+CD10^-CD27^+IgD^-$), double negative ($CD19^+CD10^-CD27^-IgD^-$)). $CD45RO^+$ reported as a proportion of $CD4^+$. $CXCR5^+$ and $CXCR5^+ICOS^+$ reported as a proportion of $CD4^+CD45RO^+$.Significant decreases are highlighted in red, significant increases, in green. Only minor changes in proportion of B and T cell subsets occurred over 6 months and these were as might be expected with aging: lower proportions of naive subsets and higher proportions of memory subsets. (med diff = median difference, CP = Child patient, AHC = Adult healthy control)

Minor changes in B and T cell subset percentages occurred over 6 months following vaccination in both young healthy adults and children with paHIV.

B cell subset percentages remained stable on the whole over 6 months any significant changes being relatively slight.

For AHC, if classifying using CD27 vs. CD21, naive mature percentage decreased slightly (med diff= 2.3% (95%CI:0.4-4.1%) *p*=0.023). If using CD27 vs. IgD, naive percentage decreased slightly (med diff =1.7% (95%CI: 0.3-3.3%) *p*=0.017).

For CP, transitional cell percentage decreased (med diff=0.4% (95%CI: 0.2-0.7%) *p*= 0.001). If classifying using CD27 vs. CD21, resting memory percentage increased (med diff= -1.3% (95%CI: -2.2- -0.35%) *p*=0.008). If using C27 vs. IgD, naive percentage decreased (med diff = 1.8% (95%CI: 1.5-3.3%) p=0.021) and switched memory percentage increased (med diff = -.9% (95%CI: -1.64- -0.01%) *p*=0.049) although the latter was borderline significant.

T cell subsets percentages also remained stable apart from CP $CXCR5^{+}ICOS^{+}$ percentages, which decreased (med diff= 0.3% (95%CI: 0.06-0.53%) *p*=0.011).

There were no significant changes in any subset for the unvaccinated CP group over 6 months, although sample size was small and therefore conclusions are limited (data not included).

As can be seen from the confidence intervals for median differences the variation in subsets over time was relatively minor with more rare populations showing a greater degree of variation relative to their absolute percentages.

4.4.7 *Impact of treatment history on lymphocyte, T and B cell subsets in children with paHIV*

Multiple linear regression was employed to investigate the relationship between lymphocyte, B cell and T cell subsets and clinical history for the CP group, while adjusting for age. Clinical variables assessed included viral load >50 RNA copies/ml (yes/no), treatment started in the 1st year of life (yes/no), treatment started in the 1st two years of life (yes/no), nadir CD4 %, and proportion of life with viral load <50 RNA copies/ml expressed as a percentage. Subset absolute numbers and percentages were log transformed and therefore the antilog of regression coefficients and confidence intervals are reported and should be interpreted as described in Section 4.4.4. The results of this regression analysis for effects of detectable viral load and proportion of life undetectable are shown in Tables 11 and 12.

Alterations in lymphocyte subsets are associated with detectable viraemia, while higher CD4 percentages are associated with a higher proportion of life spent with an undetectable viral load.

From Table 11 it can be seen that after adjusting for age, having a detectable viral load was associated with significantly lower CD4+ and CD56+ cell counts (*p*<0.0001 and *p*=0.021 respectively) and percentages

(p<0.0001 and p=0.005 respectively) and higher CD8+ counts (p=0.002) and percentages (p<0.0001). A larger proportion of life with undetectable viral load was associated with higher CD4+ counts (p=0.001) and percentages (p<0.0001) and lower CD8+ counts (p=0.004) and percentages (p<0.0001) having adjusted for age. However after adjusting for detectable viraemia only a higher CD4 percentage was significantly associated with a longer proportion of life spent with undetectable viral load.

Treatment in the first year of life was also found to be associated with higher CD4 percentage after adjusting for age and detectable viraemia (antilog regression coefficient = 1.3233 (95%CI: 1.0848-1.6144) p= 0.0070). There was no association of nadir CD4% or treatment in the first 2 years of life with any lymphocyte subset after adjusting for age and detectable viraemia.

	VL detectable (yes/no)			Percentage of life VL undetectable		
	antilog coef	antilog 95%CI	*p*	antilog coef	antilog 95%CI	*p*
lymphocyte abs						
cd3 abs						
cd3%						
cd4 abs	1.696	1.312 - 2.191	<0.0001	1.007	1.003 - 1.012	0.0010
cd4%	1.765	1.489 - 2.092	<0.0001	1.008	1.005 - 1.011	<0.0001
cd8 abs	0.644	0.491 - 0.845	0.0020	0.994	0.989 - 0.998	0.0040
cd8%	0.668	0.565 - 0.789	<0.0001	0.995	0.992 - 0.997	<0.0001
cd19 abs						
cd19%						
cd56 abs	1.757	1.090 - 2.830	0.0210			
cd56%	1.854	1.210 - 2.840	0.0050			

Table 11. Results of separate linear regression analyses for viral load detectable and proportion of life undetectable after adjusting for age. Antilog of regression coefficients and 95% CI and corresponding *p* values are reported. Only results with a p value of <0.05 are shown for simplicity. After adjusting for age, having a detectable viral load was associated with significantly lower CD4+ and CD56+ cell counts and percentages and higher CD8+ counts and percentages.A larger proportion of life with undetectable viral load was associated with higher CD4+ count and percentage and lower CD8+ count and percentages, having adjusted for age. However after adjusting for detectable viraemia only a higher CD4percentage was significantly associated with a longer proportion of life spent with undetectable viral load. (abs = absolute cell count in cells/μl, VL = viral load, coef= regression coefficient)

Detectable viraemia is associated with higher proportions of activated and tissue like/ exhausted memory B cells whereas a higher proportion of life spent with undetectable viraemia is associated with higher IgD$^+$ and class switched memory B cells

From Table 12 it can be seen that after adjusting for age, and when subdividing B cells using CD21 vs. CD27, undetectable viral load was associated with lower proportions of activated mature and tissue like/ exhausted memory B cells (p= 0.003 and p<0.0001 respectively) and correspondingly higher proportions of resting memory and naive mature B cells (p=0.001 and p=0.025 respectively). When using IgD vs. CD27,

undetectable viral load was associated with higher class switched memory B cell proportions (p=0.048). Typical contour plots for CD27$^+$ vs. CD21 for a viraemic child compared to a child with VL < 50 copies/ml are shown in Figure 16 a) and b).

a) VL<50 copies/ml b) VL detectable

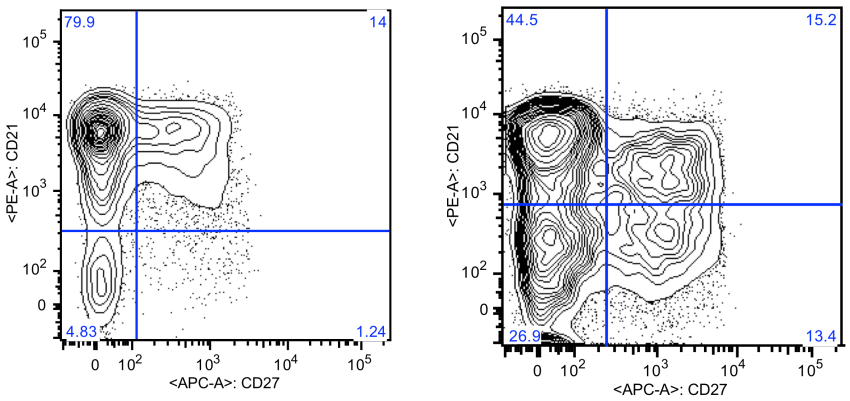


Figure 16 a) and b). Typical contour plots demonstrating the marked differences observed when plotting CD19$^+$CD10$^-$ cells as CD27 vs. CD21. Note how detectable viral load is associated with an over representation of CD21$^-$ populations ie. activated mature (CD27$^+$CD21$^-$) and tissue like/exhausted (CD27$^-$CD21$^-$) cells.

A larger proportion of life spent with undetectable viral load was associated with a higher proportion of resting memory, IgD$^+$ memory and class switched memory B cells (p<0.0001, p=0.014 and p=0.001 respectively). These associations remained significant at the p<0.05 level after adjusting for detectable viraemia. Lower tissue like/ exhausted memory B cells were also associated with a larger proportion of life spent undetectable (p=0.002), however this was non-significant at the p<0.05 level after adjusting for detectable viraemia.

No association was found between any B cell subset and treatment commenced in the 1st year of life, treatment commenced in the 1st two years of life or nadir CD4%.

Treatment in infancy and a larger proportion of life with undetectable viral load are associated with lower CD45RO$^+$ T helper cell proportions

As can be seen in Table 12, after adjusting for age, a larger proportion of life spent undetectable was associated with lower proportions of CD45RO$^+$ T helper cells (p=0.026). In addition treatment commenced in the first year of life was also associated with lower CD45RO$^+$ cell proportions (antilog regression coefficient = 0.7494 (95%CI: 0.5936-0.9460) p= 0.0160). These associations remained significant at the p<0.05 level after correcting for detectable viraemia. No other association was found between T cell subsets of interest and clinical parameters assessed.

In summary, the differences in T and B cell subsets observed in children with paHIV are more pronounced in children not on fully suppressive HAART. However as described above, abnormalities in B cell memory populations are observed even when children are successfully treated. More severe abnormalities are also associated with a longer proportion of life spent with a detectable viral load.

	VL detectable (yes/no)			Percentage of life VL undetectable		
	antilog coef	antilog 95%CI	*p*	antilog coef	antilog 95%CI	*p*
B cell subsets						
transitional %	0.584	0.360 - 0.948	0.0300			
plasmablast %						
naive mature %	1.167	1.020 - 1.335	0.0250			
resting memory %	1.863	1.302 - 2.666	0.0010	1.013	1.008 1.018	<0.0001
activated mature %	0.399	0.220 - 0.722	0.0030			
tissue like/ exhausted %	0.406	0.260 - 0.634	<0.0001	0.988	0.981 0.996	0.0020
naive %						
IgD^+ memory %				1.007	1.002 1.013	0.0140
switched memory %	1.517	1.003 - 2.294	0.0480	1.011	1.005 1.017	0.0010
double negative %						
T cell subsets						
$CD45RO^+$ %				0.997	0.994 0.999	0.0260
$CXCR5^+$ %						
$CXCR5^+ ICOS^+$ %						

Table 12. Results of separate linear regression analyses for viral load detectable and proportion of life undetectable after adjusting for age. Antilog of regression coefficients and 95% CI are reported. Transitional ($CD19^+CD27^-CD10^+$) and plasmablast ($CD19^+CD10^-CD27^+IgD^-$) cells reported as a proportion of $CD19^+$ cells. Remaining B cell subsets reported as a proportion of $CD19^+$ after exclusion of $CD10^+$ and plasmablasts (naive mature ($CD19^+CD10^-CD27^-CD21^+$), resting memory ($CD19^+CD10^-CD27^+CD21^+$), activated mature ($CD19^+CD10^-CD27^+CD21^-$), exhausted/tissue like ($CD19^+CD10^-CD27^-CD21^-$), naive ($CD19^+CD10^-CD27^-IgD^+$), IgD^+ memory ($CD19^+CD10^-CD27^+IgD^+$), switched memory ($CD19^+CD10^-CD27^+IgD^-$), double negative ($CD19^+CD10^-CD27^-IgD^-$)). $CD45RO^+$ reported as a proportion of $CD4^+$. $CXCR5^+$ and $CXCR5^+ICOS^+$ reported as a proportion of $CD4^+CD45RO^+$.Only results with a p value of <0.05 are shown for simplicity. Having a detectable viral load was associated with higher transitional, activated mature and tissue like/exhausted percentages and lower naïve mature, resting memory and switched memory percentages. A higher percentage of life spent with undetectable viral load was associated with higher resting memory, IgD^+ memory and switched memory percentages and lower $CD45RO^+$ T cell percentage.(abs = absolute cell count in cells/μl, VL = viral load, coef= regression coefficient)

4.4.8 *Correlation between T cell subset and B cell subset phenotype*

Linear regression of log transformed subset data was used to assess correlation between T cell subsets and B cell subsets in the CP group after adjusting for age. Log transformation of both the dependent and independent variable affects interpretation of the regression coefficient. If the regression coefficient = β, then a 10% increase in the independent variable would predict the dependent variable to increase by a factor of 1.10^β**.** For example, if the coefficient is 0.9 then a 10% increase in the independent variable predicts the dependent variable to increase by a factor of $1.10^{0.9}$ = 1.09 i.e. an approximately 9% increase. If the coefficient = 0.5 then a 10% increase in the independent variable predicts the dependent variable to increase

by a factor of $1.10^{0.5}$ = 1.05 i.e. an approximately 5% increase. Negative coefficients indicate a negative correlation between dependent and independent variable.

In children with paHIV, memory B cell proportions correlate with CD4 percentage

Out of the T cell subsets of interest ($CD4^+$, $CD4^+CD45RO^+$, $CD4^+CD45RO^+CXCR5^+$, $CD4^+CD45RO^+CXCR5^+ICOS^+$) only CD4 percentage was found to significantly correlate with B cell subsets after adjusting for age. CD4 percentage significantly correlated with resting memory B cell percentage (regression coefficient = 0.957, (95%CI: 0.564-1.350) $p<0.001$) tissue like/ exhausted memory B cell percentage (regression coefficient =-0.903, (95%CI: -1.459- -0.348) $p=0.002$) IgD^+ memory B cell percentage (regression coefficient =0.478, (95%CI: 0.042-0.915) $p=0.032$) and class switched memory B cell percentage (regression coefficient =0.791, (95%CI: 0.329-1.254) $p=0.001$). After correcting for detectable viraemia, the correlation with tissue like/ exhausted memory B cells was non-significant at the $p<0.05$ level. Overall this indicates that any given age a higher CD4 percentage is associated with a higher proportion of resting memory B cells (if using CD27 vs. CD21 to classify) and higher proportions of class switched and IgD^+ memory B cells (if using CD27 vs. IgD to classify).

4.5 Discussion

This work has expanded upon the few existing published reports on perturbations in B cell phenotype in children with paHIV. We have confirmed that abnormalities of B cell phenotype persist despite effective HAART and that this is impacted upon by the proportion of life spent with an undetectable viral load. We have also shown for the first time that circulating $CD45RO^+CXCR5^+$ follicular like T helper cells are decreased in children with HIV when compared to healthy controls.

Age related changes in lymphocyte subsets are well documented [84, 85, 154, 345, 356, 360, 361]. Changes in lymphocyte subset absolute number and proportion are most dramatic in the first 2 years of life, thereafter changes are more gradual through childhood, adolescence and in to early adulthood. Differences between the young adult and child groups were therefore to be expected.

The median ages of the CHC, CP and AHC group were 9, 13 and 27 years respectively. If the children in the CP group were not HIV infected, it would be reasonable to predict (in view of their slightly older age) that their lymphocyte subsets would be more similar group to those of the AHC group. However we have demonstrated that in the context of paHIV, lymphocyte subsets significantly differ from those of healthy young adults, much more so than expected due to differences in age. This is despite the majority of children (78.6%) being on fully suppressive HAART.

The healthy children in the study had a higher median absolute lymphocyte count, B cell count and B cell percentage and a lower median NK cell percentage than the young healthy adults. This is consistent with published data [84, 85, 154, 345, 356, 360, 361]. All of these differences were present in the HIV infected

children, however in addition they had a lower median CD4 percentage and absolute NK cell count and higher median CD8 absolute cell count and percentage. As well as being of relevance for the analysis of vaccine specific cytokine responses in Chapter 6, this is also indicative of persistent lymphocyte abnormalities in the HIV infected children on HAART.

Further evidence to this effect was provided by direct comparison of healthy children with HIV infected children. CD4 absolute cell counts are expected to decrease with age however the difference between younger and older children were much more extreme for HIV infected children, older HIV infected children having lower CD4 counts than uninfected children of a similar age. CD4 percentages are expected to be higher in older healthy children however the opposite was true of the HIV infected children. CD8 absolute cell counts are expected to be lower in older children. This was true for both healthy and HIV infected children, however the latter had consistently higher CD8 absolute cell counts than uninfected children throughout childhood. CD8 percentages are expected to remain relatively constant throughout childhood, if anything increasing slightly. This was found for healthy control children, however the CD8 percentages were much higher in older HIV infected children. NK cell numbers are expected to be slightly lower in older children. This pattern was observed in HIV infected children but to a greater extent than in healthy children. There were no significant differences between healthy and HIV infected children with respect to absolute B cell counts and percentages and NK percentages, which followed the expected age related patterns of change. [84, 85, 154, 345, 356, 360, 361].

Overall these observed differences in lymphocyte subsets are consistent with published data on immune reconstitution in HAART treated children. It has been demonstrated that despite suppressive HAART, some children continue to have lower CD4 percentages. It is also well recognised that CD8 cell counts and percentages remain elevated when the HIV viral load is undetectable [95, 362-366]. NK cells are less well studied in HIV infected children, with conflicting reports. It has been shown that there is no difference in NK cells between HIV infected and uninfected children, however, it has also been reported that NK cells numbers and proportions are lower than in healthy controls [173, 175]. Our data indicates lower absolute counts in older children but no differences in NK cell percentages, which may to some extent explain conflicting findings to date.

These differences in lymphocyte subset might be explained by viraemic children being included in the analysis. However these were relatively few in number and the age, group and interaction effects remained significant after adjusting for detectable viraemia.

There are many possible reasons for incomplete immune reconstitution on HAART. It is interesting to speculate that these persistent abnormalities may, to some extent, be related to the historical deferral of treatment until lower CD4 thresholds than is currently practice. Recent evidence suggests that this may be the case for ultimate CD4 count recovery potential in children with paHIV [367].

There is less published data on normal B cell subset variation with age, but proposed reference ranges are available for a proportion of the subsets assessed in this study. As with lymphocyte subsets there are

changes in absolute count and percentage throughout childhood and therefore it is expected that the 2 child groups will differ from the young adult group.

At time of writing there are published reference ranges for transitional cells and plasmablasts, and for subsets as defined by CD27 vs. IgD, albeit with slightly varying gating strategies and methods for reporting proportions (usually percentage of total B cells) [84, 154, 360, 368, 369]. However as yet there is to our knowledge, no published data on natural changes in subsets defined by CD27 vs. CD21 with age.

When comparing healthy young adults with healthy children, we observed differences in B cell subsets that are consistent with published reference ranges. Adults had higher proportions of IgD^+ and class switched memory and correspondingly lower proportions of naive and double negative B cells. Adults also had a lower proportion of transitional B cells. If subdividing B cells by CD27 and CD21 expression, children had higher proportions of naive and tissue like/ exhausted memory B cells than young adults and a lower proportion of resting memory B cells. There was no significant difference in the proportion of activated mature B cells. This pattern of differences in B cell subsets was also true for the HIV infected children when compared as a whole with the healthy young adults. These findings are consistent with both of the child groups having less $CD27^+$ memory B cells than young healthy adults.

Despite these apparent similarities when comparing the child groups with the young adult group individually, further direct comparison between the HIV infected and uninfected children revealed additional differences in B cell subset distribution. After adjusting for age, children with HIV had lower IgD^+, class switched and resting memory B cell proportions and higher naive B cell proportions, although the difference in class switched memory B cells was non-significant after correcting for viraemia.

Four other groups have investigated some or all of these populations in HIV infected children. Jacobsen et al [166] used IgD and CD27 to characterise B cell subsets. They reported lower IgD^+ memory B cell proportions in children with suppressed viral load on HAART, which is in agreement with our data. In contrast to our data they report no difference in these subsets when comparing viraemic children with healthy children, leading them to suggest that detectable virus may in some way sustain numbers of this subset. Our data are more in keeping with observations by our group in HIV infected adults, which show reduction in IgD^+ memory B cells proportions irrespective of treatment status [162]. Ghosh et al also reported decreased IgD^+ memory B cells in HIV infected children compared to healthy controls, however they do not report differences between viraemic and aviraemic children [167]. Iwajomo et al studied a large cohort of untreated HIV infected children in Africa and found there to be decreased IgD^+ memory B cells compared to healthy children, however they report analysis of only absolute cell counts rather than number which limits direct comparison [169].

Jacobsen et al found no significant difference in class switched memory B cell percentages between HIV infected and healthy children [166]. We have shown a lower proportion of switched memory percentages in children with HIV however this was non significant after adjusting for viraemia, detectable virus being associated with lower class switched memory B cells. This is more in keeping with Ghosh et al, who report

lower proportions of class switched memory B cells in children with HIV compared to healthy controls and lower class switched memory B cells to be associated with higher HIV viral load [167]. It is also consistent with Cagigi et al who have shown decreased proportions of class switched memory B cells in older HIV viraemic children but not those with undetectable virus [344]. Iwajomo et al found no difference in class switched memory B cells, however again it should be noted that they report absolute cell counts and not proportions [169].

In an adult study, our own group found low class switched memory B cells in a proportion of HIV infected patients, but there was no significant difference between viraemic and non-viraemic patients [162].

Only one paediatric study has reported on B cell subpopulations characterised using CD21 and CD27. Iwajomo et al report on a cohort of HIV infected children that were not as yet on HAART. They found decreased percentages of naive mature and resting memory and higher percentage of activated mature. These changes were evident even in children with relatively high CD4 percentages. They did not report on tissue like/ exhausted memory B cells [169].

We found resting memory percentages to be lower in the HIV infected children when comparing them with the healthy control children. However when evaluating the effects of detectable viral load on these subsets within the patient group we found viraemia to be associated with significantly higher activated mature B cells and tissue like/ exhausted B cell proportions and lower resting memory and naive mature B cell proportions which is consistent with the findings of Iwajomo et al [169].

When comparing the HIV infected group of children as a whole with healthy control children we found no difference in the proportion of transitional B cells. Within the HIV infected group there was a significant effect of viraemia, children with a detectable viral load having a higher proportion of transitional B cells. Cagigi et al demonstrated that viraemia was associated with a lack of physiological age related decline in transitional cells [344], consistent with our findings, whereas Iwajomo et al found no significant difference between viraemic HIV infected and control children [169]. Malaspina et al have shown transitional cell percentages to be increases in adults and to correlate with CD4 lymphopenia and raise serum IL7, leading them to hypothesise a causal relationship between low CD4 count and increased transitional cells. This is supported by the observations by the same group that these correlations are also observed in patients with idiopathic CD4 lymphocytopenia [355, 370]. Cagigi et al found that in children with paHIV, the age related physiological decrease in transitional cell proportions is lost and that this is related to detectable viraemia and did not correlate with CD4 count. Serum IL7 concentrations were decreased compared to healthy controls [344]. This suggests a possibly distinct mechanism for increased transitional cell proportions in children with paHIV.

Published reference ranges for $CD45RO^+$ cell percentages for the paediatric age range are available [84, 85]. These clearly show a natural increase in the proportion of $CD4^+$ that are $CD45RO^+$ throughout childhood. In accordance with this, our data show that both child groups had lower $CD45RO^+$ cell percentages than the young adult group. When comparing the HIV infected children with the healthy control children as a whole there were also found to be no significant differences. Studies in the pre HAART era

have shown that the proportions of $CD4^+$ cells positive for $CD45RO^+$ are higher for young children with HIV but lower for older children when compared with healthy controls [371, 372]. Later studies have shown that HAART treated children with a low CD4 count have a higher proportion of $CD45RO^+$ cells whereas children on HAART with a normal CD4 count have similar proportions to healthy controls [114, 373]. The fact that the majority of children have relatively preserved CD4 percentages (11 children less than 25%, 10 of whom have detectable viral load) could explain why there are no statistically significant differences in this marker. CD4 percent did, however show a strong negative correlation with CD45RO percent in the CP group (data not reported).

Only one paper to our knowledge has published reference ranges for $CD45RO^+CXCR5^+$ cells for children. Schatorjé et al demonstrated that the proportion of $CD4^+CD45RO^+$ cells that are positive for CXCR5 is stable at around 20% from around 2 years of age through to adult life if anything decreasing slightly with age [345]. Our data comparing the child groups with the young healthy adults shows them to have higher median proportions of this subset. Direct comparison of HIV infected children with healthy children demonstrated HIV infected children to have lower proportions of this subset after adjusting for age.

Of the clinical parameters examined for effects on lymphocyte and B and T cell subsets, detectable viraemia and the proportion of life with undetectable viral load appear to have the greatest impact. Detectable viraemia is associated with lower CD4 and NK cell percentages and higher CD8 percentages. It is also associated with higher proportions of transitional, activated mature and tissue like/ exhausted memory B cells and lower proportions of naive mature, resting memory and class switched memory B cells.

It is of particular interest that within the HIV infected group, having detectable viraemia does not have a significant effect on naive and IgD^+ memory percentages nor on $CD45RO^+CXCR5^+$ percentages. As mentioned above, these populations were lower in HIV infected children than healthy control children. This then reinforces the point that changes in these subsets are not fully corrected by suppressive HAART

A larger proportion of life spent with undetectable viral load is associated with a higher CD4 percentage, higher proportions of resting memory, IgD^+ memory and switched memory B cells and lower proportion of $CD45RO^+$ T cells. Treatment commenced in the first year of life was associated with higher CD4 percent and lower $CD45RO^+$ percent. As discussed above, publications to date have reported conflicting results with respect to the effects of viraemia on B cell subsets [166, 167, 169, 344]. Seen together with our results it now seems very clear that detectable viraemia has a substantial impact on B cell phenotype, specifically in relation to CD21 expression, activation and exhaustion, in much the same way has been shown in adults with HIV infection [120].

It has been suggested that for children with paHIV, the timing of HAART commencement and consequently the proportion of life spent with undetectable viral load impacts on potential for immune reconstitution of both the T cells compartment [367] and the B cell compartment [168, 203]. Similar findings in adults [165, 374], along side an increasing body of clinical evidence of benefit, have contributed to reconsideration of current American adult treatment guidelines and a decision to offer HAART to all patients irrespective of CD4 count/

stage of disease [375]. Our findings are consistent with a view that allowing a child to remain viraemic is associated with significant B cell phenotypic changes and that the longer a child is on suppressive HAART the better in terms of preventing possibly irreversible changes to B cell phenotype. This in turn is likely to have significant implications for the HIV infected child's potential to respond to vaccines dependent on antibody responses for protective efficacy.

As discussed in Section 1.1.4 the development of class switched memory B cells is dependent on T-B interaction in the germinal centres of secondary lymphoid organs. Follicular T helper cells, through direct cell contact and cytokine production contribute to the development of class switched, affinity matured B cells which exit the germinal centre reaction to become either memory B cells or long lived plasma cells. It therefore seems likely that HIV induced perturbations of T cell phenotype and function will have direct effects on B cell development and phenotype. Consistent with this view we have shown a higher CD4 percentage to be associated with higher resting memory B cells and class switched memory B cells (depending on how subsets are characterised). We have also shown that higher $CD4^+$ T cells are associated with a higher percentage of IgD^+ memory B cells, a subset not known to be dependent on T cells for their development. This is suggestive of the likely possibility that other factors contribute to B cell abnormalities in the context of HIV infection.

Lymphocyte, B and T cell subsets changed in frequency over time to a certain extent, more so for the HIV infected children than the healthy adults. It is not possible to determine the significance and cause of these changes, partially as a result of the fact numbers in the paediatric unvaccinated cohort were small and it was not a randomised, placebo controlled study. All of the observed changes are consistent with those expected with ongoing aging of the immune system and as described above, these changes are less rapid in early adulthood than in childhood. Other contributory factors in the child patient group are ongoing immune reconstitution as a result of HAART and effects of vaccination. This study was not designed to assess this, however these results are important to note when considering the results of antigen specific cytokine release assay in Chapter 6.

We have shown that these two flow cytometric panels allow assessment of some of the key T and B cell subsets thought to be involved in effective humoral immune responses and that these subsets remain relatively stable over a 6 months period in children infected with HIV. This suggests their use should therefore be encouraged in studies of antibody responses in children with not just HIV but other forms of congenital and acquired immune deficiency. In conjunction with flow cytometric techniques for detection of antigen specific T and B cell (e.g. tetramer staining and fluorescent labelled antigen respectively) it should be possible to elucidate the respective roles of the cell types involved in the response to T dependent antigens in humans.

HIV infection in humans, although not directly infecting B cells, is known to be associated with multiple B cell abnormalities including increased cell turnover [376], activation [159, 377], apoptosis [377, 378], exhaustion [359], cell death [376], terminal differentiation [158], immature phenotype [355], hypergammaglobulinaemia [1, 379], B cell malignancy [380] and autoimmunity [381, 382].

The reasons for B cell dysfunction and phenotypic abnormalities in HIV infection are still not completely delineated [40, 120, 383-385], however it is clear that it a multifactorial process.

Animal models can be employed to some extent to clarify the pathogenic mechanisms of immune dysfunction relating to humoral immunity observed in HIV infection. Recent studies of SIV infection in non-human primates have served to delineate in some detail the changes that occur in lymphoid architecture, T_{FH} and their associated B cells in lymph nodes and peripheral blood in the acute and chronic stages of infection [59] [386] [387-390]. A common finding of these studies and investigation of acute/early HIV infection in adult humans [160, 165], is that dramatic changes occur in B cell phenotype, function, activation status and distribution at the very earliest stages of retroviral infection, and that early ART, to some extent can reverse this process. On the other hand, starting HAART in chronic HIV infection leads to a less complete nomalisation of the B cell compartment [158, 159, 162, 165].

Since the earliest days of the HIV epidemic, HIV has been known to have direct effects on B cells [391]. HIV may have direct effects on B cells via complement bound to virus particles binding surface CD21, although the levels of cell associated virus are relatively low and therefore this is not likely to be a main contributing factor [392]. HIV associated proteins e.g. Negative Regulatory Factor (Nef) [393-395] and gp120 [396] have been shown to affect B cell function [383]. Disruption of secondary lymphoid tissue architecture and lymph node fibrosis has been shown in both SIV models [390] and human HIV infection [397, 398]. Altered germinal centre homing chemokine receptor expression on B cells has also been demonstrated [399]. In both children and adults, it is now generally accepted that a key contributory factor in progressive disruption of B and T cell immunity associated with HIV infection is generalised immune activation [40, 384, 400]. The precise mechanism of this immune activation is still not completely clear however there is a growing body of evidence supporting a role of translocation of microbial products such as LPS from the gut, homeostatic response to lymphopenia, direct viral effects and indirect viral effects through alteration of the cytokine milieu and cell-cell signalling [40, 383, 384, 401-403].

In the absence of HIV infection, there is increasing evidence of reciprocal relationship between B cell development and T cell development especially relating to T_{FH} in the context of the germinal centre [137, 404, 405]. It is therefore interesting to speculate that T cell independent abnormalities of B cell phenotype brought about by HIV infection, may in turn affect T_{FH} development. B cells already compromised by HIV infection prior to T cell contact would not only respond less effectively to help from (also impaired) $CD4^+$ T cells but also prevent effective further development of T_{FH}. Add to this the fact that the follicular dendritic cell network is also directly affected by HIV [406], then the 3 major players in the GC reaction are compromised before they have even encountered each other and one might predict that further development of both T and B cells within this microenvironment might be impaired. This concept can be expanded to hypothesise an important contribution of disrupted B cell effector/regulatory activity to T cell abnormalities in HIV pathogenesis [407].

Little is known about the role of T_{FH} in HIV infection in general and how they might be implicated specifically in relation to disruption/dysregulation of humoral immunity. In 1997, in one of the first papers to describe circulating $CD4^+CD45RO^+CXCR5^+$ cells, Forster et al found the proportion of this subset to be lower in adults with HIV than in healthy controls [210]. In the intervening 15 years there has been an ever-expanding literature characterising these cells in detail in the germinal centres of mice, accompanied by frequent debate on the relationship between these "true" follicular T helper cells and the circulating equivalent in humans. It has recently been more accepted that although phenotypically quite different to their lymph node resident counterparts, circulating T_{FH}-like cells are indeed directly related and therefore of relevance to both normal and dysregulated humoral immunity [207, 208, 346]. Despite the well-documented defects observed in humoral immunity in HIV infection, there has been very little further work until now investigating the effect of HIV on this subset in humans. Mounting evidence for their involvement in dysregulated humoral immunity in autoimmune diseases such as SLE and dermatomyositis [208, 408], further supports their likely importance in the context of paHIV.

Rabian et al, in a study of pneumococcal vaccines in HIV infected adults, found an association between higher T_{FH} cell counts and more robust *in vitro* cellular response to vaccine carrier protein, however they reported only circulating T_{FH}-like cell numbers and did not compare with healthy controls [48]. Pallikuth et al report no difference from healthy controls in the frequency of circulating T_{FH} (defined as $CD4^+CXCR5^+$) in chronically HIV infected adults with good CD4 counts and suppressed virus. They demonstrated that expansion of antigen specific IL21 producing T_{FH} with the capacity to provide B cell help one month post immunisation was a characteristic of those patients who responded successfully to 2009 H1N1/09 flu vaccine. In addition post vaccination T_{FH} frequency correlated positively with memory B cell frequency and H1N1 antibody titres [47].

Yue et al found there to be a higher proportion of IL21 producing CD4 cells in peripheral blood of HIV infected adults. These cells were of effector memory phenotype, enriched for CXCR5 and HIV specificity. They did not however report overall frequency of $CXCR5^+$ T helper cells [348]. Lindqvist et al report data on lymph node histology from chronically HIV infected adults compared to healthy controls. Interestingly, they found HIV infected lymph nodes to have high frequency of IL21 producing T_{FH} in lymph nodes irrespective of HAART status. These cells were found to be responsive to HIV antigens and their frequency correlated with higher plasma cell and germinal centre cell B cell and lower late memory B cell frequency (as classified by IgD and CD38) [46]. At the 2012 Conference on Retroviruses and Opportunistic Infections (CROI), Connick et al presented data on ex vivo infection of human lymphoid tissue with HIV and found $CXCR5^+$ T helper cells to be more permissive to HIV infection than $CXCR5^-$ T helper cells [409]. The Lindqvist study highlights the importance of considering the limitations of studies of peripheral blood immune phenotype and reminds us that changes in cell subsets in the periphery may represent redistribution rather than a change in overall cell numbers.

One clear limitation of our study is the choice of surface markers used to identify B cell and T cell subsets and the possibility that they may not reliably identify distinct subsets. The reasoning behind the choices of markers used and their limitations is discussed in Section 4.3, however it should be again noted that

additional transitional (CD24, CD38) and plasmablast (CD24, CD38, CD138) markers as well as markers of class switch, immune activation and exhaustion would have enhanced our B cell findings, allowing further detailed characterisation of the observed phenotypic abnormalities. The debate as to the origin and function of circulating $CD4^+CXCR5^+$ T cells is ongoing. Shane Crotty, a key player in the T_{FH} world, recently presented data at the Keystone Conference on Immunological Mechanisms of Vaccination (Ottawa, Canada, 2012), further characterising this subset with the addition of the markers PD-1 and CXCR3 to more specifically identify a true circulating counterpart to T_{FH} cells. We hope that our data might provide a piece of the jigsaw in the quest to understand this $CXCR5^+$ population and how it may or may not be involved in the response to T cell dependent vaccine antigens in both healthy and HIV infected individuals.

4.6 Conclusion

Taken together, findings from this work, other studies of humans HIV infection and non-human primate SIV models, are indicative of a complex interplay between HIV, T_{FH} and B cells in lymphoid tissue and peripheral circulation, to the detriment of humoral immunity, which is only partially corrected by HAART. Current evidence suggests there may be an accumulation of HIV specific and/or infected T_{FH} in lymph nodes, which correlates with altered lymph node B cell phenotype. This is reflected in altered T and B cell phenotype in the peripheral blood, which in turn can be correlated with impaired response to vaccination. To our knowledge, this is the first study of circulating T_{FH} in children with paHIV either in relation to B cell phenotype or vaccine responses. Our results not only emphasise that T and B cell abnormalities persist in children with HIV despite suppressive HAART, but also suggest that earlier and sustained HAART may well serve to maximise potential for normal immune development.

It is likely that the impact of HIV and HAART on the child's relatively naive and developing immune system will have implications for humoral immunity that may differ from the limited observations in adults to date. The majority of vaccinations are administered in childhood, and are expected to give life long protection against vaccine preventable disease [189]. This should be borne in mind when considering treatment options and timing of vaccinations in this especially vulnerable group.

The next chapter will examine how observed phenotypic differences in this cohort of children with paHIV relate to serological response to the 13-valent pneumococcal conjugate vaccine

Chapter 5: Pneumococcal serology

5.1 Introduction

5.1.1 *Pneumococcal vaccination and the HIV infected child*

HIV infected children are at increased risk of invasive pneumococcal disease. HAART reduces this risk to some extent yet it is still greater than that of the HIV uninfected population [270-272]. Pneumococcal polysaccharide vaccine has been available for many years and its use is currently recommended in the UK for children over the age of 2 years that are particularly at risk of pneumococcal disease, including those with HIV [284], despite a lack of evidence of efficacy in children with paHIV and very limited, and somewhat controversial evidence of efficacy in adults with HIV [270, 271]. Pneumococcal conjugate vaccines have proven efficacy when administered to HIV infected infants [312, 327] and adults [330]. They are therefore likely to be efficacious in older children and adolescents with paHIV.

5.1.2 *Immunogenicity in older children with paHIV: a special case?*

Studies of pneumococcal conjugate vaccines in HIV infected individuals of all ages have mainly shown that immunogenicity is impaired in comparison to healthy controls [271]. When considering the effects of HIV infection on responses to pneumococcal vaccines it is important to acknowledge that a different pattern of impairment may be present in older children with paHIV compared to HIV infected infants or adults.

The infant's immune system is relatively naive to pneumococcus and also functionally immature. The response to pneumococcal conjugate vaccine will be in the main part, a primary one, on the background of an immature immune system trying to cope with the frequently high viral loads and rapid progression associated with infant HIV infection. On the other hand, adults with horizontally acquired HIV become infected when they have already encountered and established a certain degree of immunity to many of the pathogenic pneumococcal serotypes. The response to pneumococcal vaccination in this context will be determined in part by the specific impact that HIV has on the fully developed and pneumococcal antigen experienced immune system. The older child with paHIV has survived infancy but their immune system has developed (and is still developing) under the influence of HIV, with or without the benefit of HAART. They will have developed a certain degree of pneumococcal immunity through natural exposure but this will have been modified by alterations of both the innate and acquired immune system caused by HIV. Any abnormality of immune phenotype found to be characteristic of this patient group, is likely to contribute to similarly characteristic responses to pneumococcal conjugate vaccine.

There are few studies of licensed pneumococcal conjugate vaccine in older HIV infected children and no published data to date on PCV13 in any HIV infected age group [271]. Through studying serological responses to PCV13 and relating them to abnormalities of immune phenotype in older children with paHIV, we hope to not only provide immunogenicity data, but also to use the vaccine as a probe in order to clarify the nature of the persistent defects of humoral immunity observed in children on HAART [168, 190].

5.2 Chapter Aims

i) To assess baseline serotype specific pneumococcal serology in children with paHIV compared to healthy children and healthy young adults using ELISA and to compare pre-existing functional immunity for selected serotypes using OPA.

ii) To assess immunogenicity of PCV13, 1 month post immunisation in children with paHIV compared to healthy young adults using ELISA.

iii) To assess persistence of serotype specific pneumococcal antibody, 6 months post immunisation in children with paHIV compared to healthy young adults using ELISA.

iv) To assess pneumococcal nasopharyngeal carriage in children with paHIV compared to healthy children and healthy young adults.

v) To investigate association between both clinical and immunological parameters and the serological response to PCV13 in children with paHIV.

5.3 Statistical analysis

For baseline comparisons, Chi^2 and Fisher's exact test were used to compare proportions. Mann-Whitney U test was used to compare baseline age and for contrasting baseline cell subset data between child and adult groups. Hodges-Lehman median differences with 95% confidence intervals were also calculated. For direct comparison of cell subsets from the two child groups, data was log transformed and compared using linear regression analysis to allow for adjustment for age and to assess age*group interactions. Mann-Whitney U test was used to compare time between vaccination and blood sampling between groups. Chi^2 and Fisher's exact tests were used to compare baseline proportions above a cut off antibody concentration between groups. One way analysis of variance (ANOVA) with Bonferroni correction was used to compare log transformed antibody concentrations at baseline between the 3 groups.

Chi^2 was used to compare proportions above a cut off antibody level between groups at each time point post immunisation and McNemar's test was used to detect changes in proportion from baseline within groups. Repeated measures ANOVA and analysis of covariance (ANCOVA), the latter including baseline concentration as a covariate, were used to compare log transformed antibody concentrations between and within group. Post hoc analyses for simple effects were then performed as appropriate. Linear regression was used to determine associations between clinical/immunological parameters and baseline/1 month antibody concentrations.

5.4 Results

There was no loss to follow up and only 1 sample out of 271 was insufficient in volume for analysis. This sample was from an adult healthy control one month post immunisation. This subject was therefore excluded from all serological comparisons.

5.4.1 Baseline characteristics

Baseline characteristics are shown in Table 13.

In view of exclusion of one adult healthy control and the intention to compare only the vaccinated groups directly, comparison of baseline characteristics was repeated as in Chapter 4. When comparing only the vaccinated children with child and adult healthy controls the pattern of differences were as in Chapter 4 apart from for absolute lymphocyte count comparison of CHC with CP. This showed separate age and group effects in place of an age*group interaction, total lymphocyte being higher in the patient group and decreasing with age in both groups.

Prior to this study, 10/56 (18%) and 2/56 (3.6%) of the children with paHIV had received 1 or 2 doses of PPV respectively. Median time since last dose of PPV was 46 months (range: 10-99 months). 1/56 (1.8%), 2/56 (3.6%) and 5/56 (8.9%) of children with paHIV had received 1,2 or 3 doses of PCV7 respectively. Median time since last dose of PCV7 was 16 months (range: 9-28 months). 2/30 (6.7%), 1/30 (3.3%), 6/30 (20%) of healthy control children had received 1, 2 or 3 doses of PCV7 respectively. Median time since last dose of PCV7 was 26 months (range: 12-99 months). No healthy young adult had received PCV or PPV and no study participant had ever received PCV13.

Using Mann-Whitney U test to compare timing of blood sampling in relation to vaccination, there was no significant difference in the timing of vaccination to 1 month follow up for CP (med= 32 days) compared to AHC (med = 32 days) (med diff = 1 day (95%CI: -2- 5 days) p=0.440) or of vaccination to 6 month follow up for CP (med = 199.5 days) compared to AHC (med=209) (med diff = 9 days (95%CI: -8-25 days) p=0.316).

5.4.2 *Baseline serology*

Reverse cumulative distribution (RCD) curves are a useful way of visually summarising complex serological data sets for different groups at different time points. They plot antibody concentration on the x axis and the proportion of subjects above this cut off on the y axis. RCD curves including baseline serum pneumococcal polysaccharide specific IgG concentrations for AHC, CHC and CP are shown in Figure 17.

Proportion in each group above a cut of 0.35μg/ml and 1μg/ml are reported in Tables 14 and 15. The rationale for using these cut-offs is discussed in Sections 1.3.3 and 5.5.

		CHC (n=30)		AHC (n=26)		CPv (n=48)		CPu (n=8)	
Sex	Female	14	(46.7)	15	(57.7)	21	(43.8)	4	(50)
	Male	16	(53.3)	11	(42.3)	27	(56.3)	4	(50)
Ethnicity	White	4	(13.3)	19	(73.1)	2	(4.2)	0	(0)
	Mixed	0	(0)	2	(7.7)	3	(6.3)	0	(0)
	Asian	0	(0)	3	(11.5)	2	(4.17)	1	(12.5)
	Black Caribbean	4	(13.3)	0	(0)	2	(4.17)	0	(0)
	Black African	13	(43.3)	0	(0)	38	(79.2)	7	(87.5)
	Other	9	(30)	2	(7.7)	1	(2.1)	0	(0)
Age/months		107.5	(19-195)	330	(245-359)	154	(12-209)	131	(81-189)
Lymphocyte subsets	lymph cells/μl	2139	(1666-3414)	1807.5	(1377-2115)	2349.5	(1764-3308.5)	2548	(1709.5-3000.5)
	CD3 cells/μl	1575.5	(1166-2486)	1389	(1094-1706)	1854.5	(1390.5-2518)	1897.5	(1215-2362.5)
	CD3 %	71.6	(66.6-78.3)	75.3	(70.1-79.1)	75.45	(68.9-79.6)	71.8	(65.8-74.9)
	CD4 cells/μl	833.5	(647-1391)	794	(694-1061)	855	(525.5-1118)	878.5	(565-1207)
	CD4 %	43.1	(36.7-46.5)	44.55	(39.8-50.6)	34.55	(27.2-40.8)	35.8	(27.9-42.2)
	CD8 cells/μl	529.5	(356-907)	497	(383-557)	913.5	(659-1074.5)	695.5	(579-952)
	CD8 %	23.8	(21.6-27.2)	26.25	(22.2-28.9)	32.95	(28.9-44.5)	31.7	(24.6-39.1)
	CD19 cells/μl	446.5	(272-679)	257	(153-326)	403	(266-570.5)	378.5	(267.5-622)
	CD19 %	18.2	(14.5-24.2)	12	(8.9-16.8)	17.3	(12.96-21.4)	16.8	(13.5-18.7)
	CD56 cells/μl	134.5	(82-205)	172.5	(96-188)	119	(72-184.5)	125.5	(68.5-318.5)
	CD56 %	5.4	(2.9-8.2)	9.35	(6.5-12.1)	4.35	(2.8-5.6)	7.35	(3.05-11.8)
Prev PCV7		9	(30.0)	0	(0)	7	(14.6)	1	(12.5)
Prev PPV		0	(0)	0	(0)	12	(25.0)	0	(0)
Nadir CD4%		-	-	-	-	13	(0-48)	15	(0-33)
Proportion of life undetectable		-	-	-	-	0.49	(0-0.98)	0.44	(0.03-0.94)
VL <50 copies/ml		-	-	-	-	34	(72.3)	7	(77.8)
Treated 1st year		-	-	-	-	7	(14.9)	3	(33.3)
Treated 1st 2 years		-	-	-	-	12	(25.3)	3	(33.3)

Table 13. Baseline characteristics for all study participants assessed for pneumococcal serology. Sex, ethnicity, HIV viral load status, treated in the first year of life, treated in the 1st 2 years of life, previous PCV7 and previous PPV are presented as number and percentage. Age, nadir CD4% and proportion of life undetectable are presented as median and range. Lymphocyte subsets are presented as median and interquartile range. (CHC= Child healthy control, AHC= Adult healthy control, CPv = Child patient (vaccinated) , CPu = Child patient (unvaccinated), VL = HIV viral load, PCV7 = 7-valent pneumococcal conjugate vaccine, PPV= pneumococcal polysaccharide vaccine)

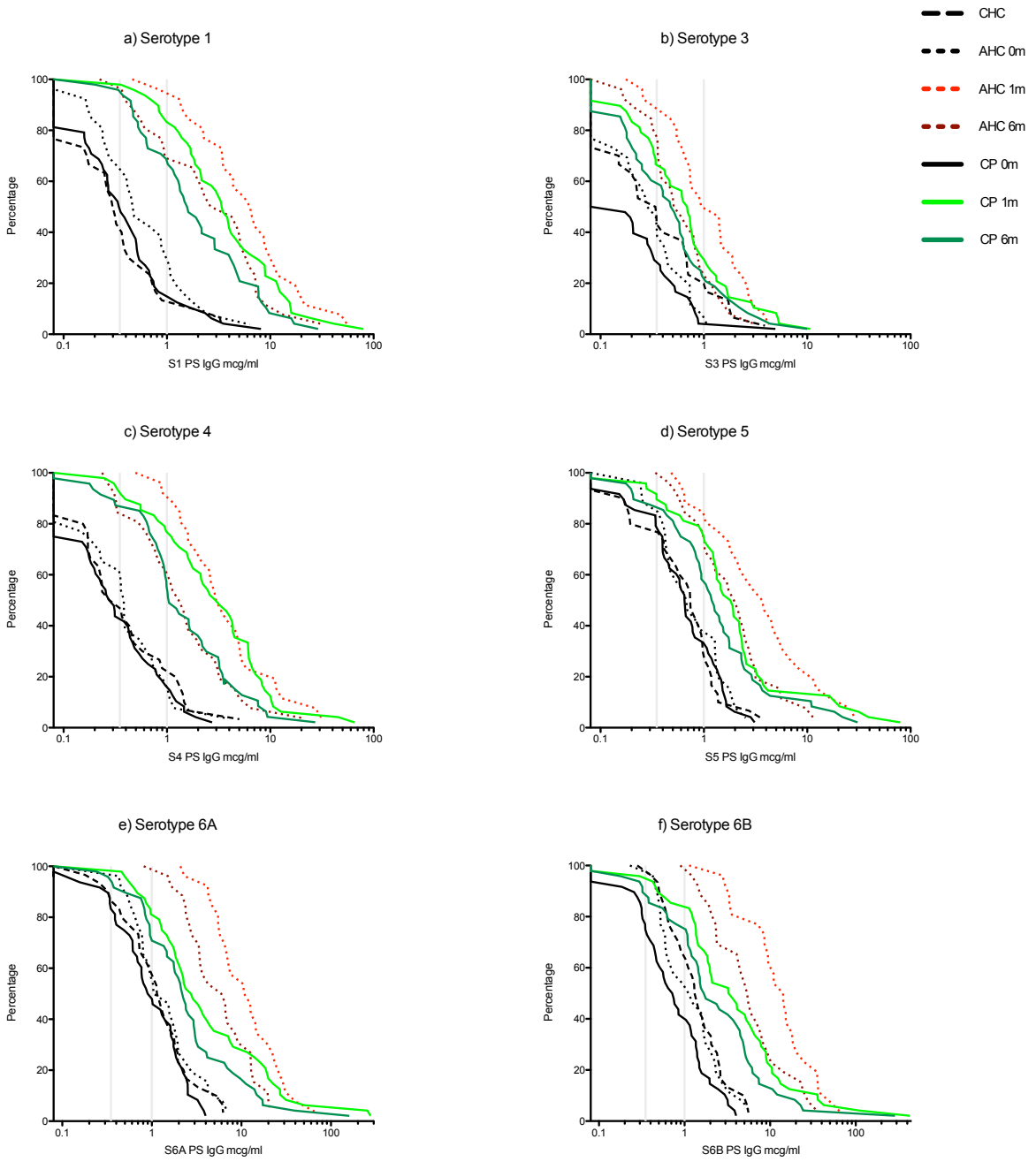


Figure 17 a-m). Reverse cumulative distribution curves showing the proportion of individuals above increasing concentrations of serotype specific antibody for healthy children, young healthy adults and children with paHIV. Baseline antibody concentration data is displayed in black. Post immunisation data for young healthy adults are shown in red and for HIV infected children, in green. Vertical grey lines indicate cut off of 0.35 μg/ml and 1 μg/ml. GMCs were higher at baseline for AHC than CP for 6/13 serotypes: 7F, 9V, 14, 18C, 19F and 23F ($p< 0.05$). Without adjusting for baseline antibody concentration, serotype specific IgGGMCs were higher in the AHC group than the CP group 1 month post immunisation for 8/13 serotypes: 6A, 6B, 7F, 9V, 14, 18C, 19A and 23F ($p<0.05$). Higher antibody levels persisted to 6 months for 5/13 serotypes: 6A, 6B, 7F, 18C, 23F. After adjusting for baseline antibody concentrations GMCs were significantly higher in AHC than CP for serotypes 6A, 6B and 23F at both the 1 month ($p<0.01$) and 6 month ($p<0.05$) time points. (S= serotype, mcg=μg, CHC=child healthy control, AHC= adult healthy control, CP = Child patient, 0m = baseline, 1m/6m = 1 month/ 6 months post immuniaation.) Continued on next page.

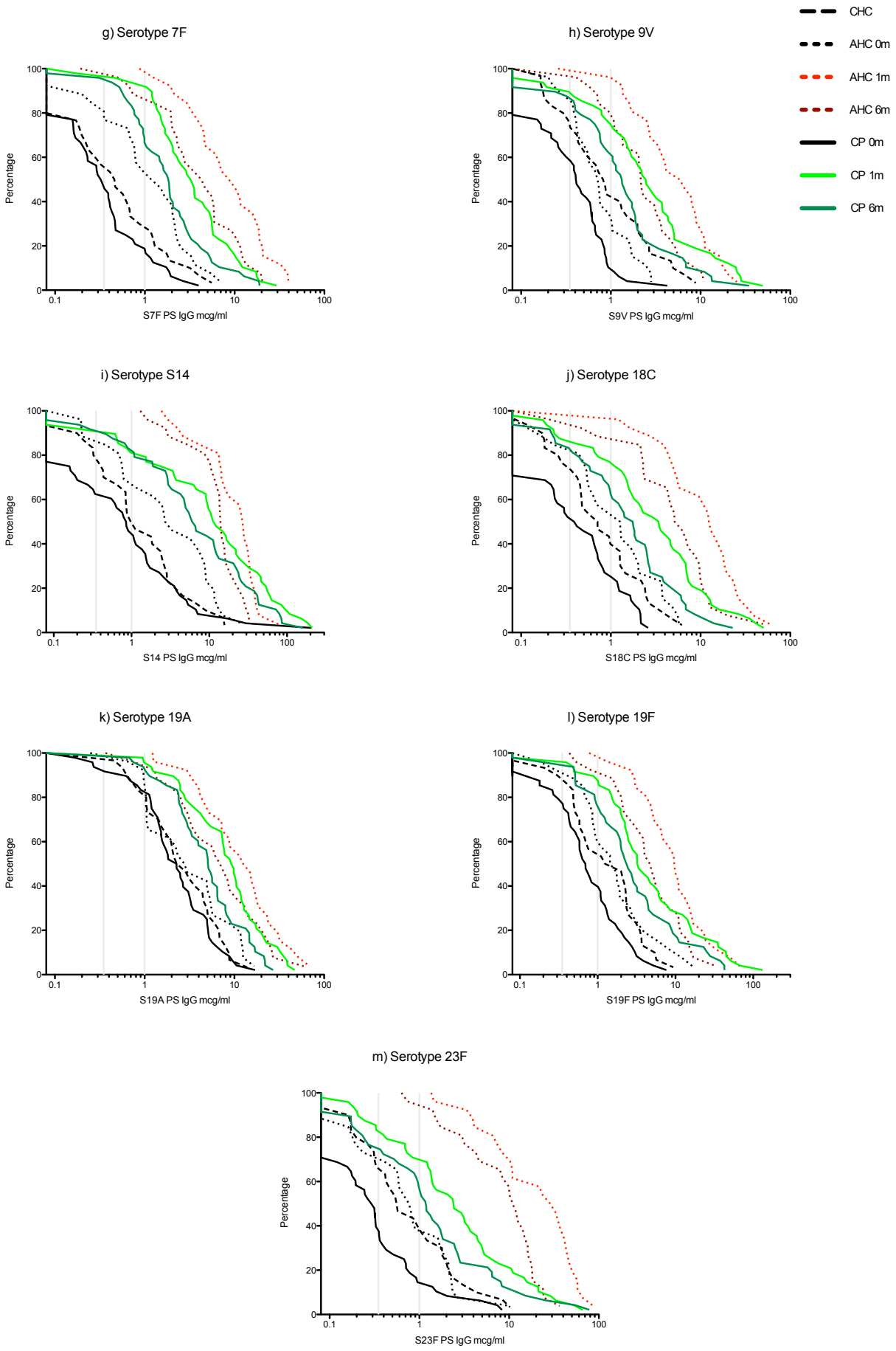


Fig. 17 Continued

Children with perinatally acquired HIV have similar serotype specific IgG concentrations to healthy controls at baseline for some but not all PCV13 serotypes

Proportions above each cut off were compared between groups using Chi^2 or Fisher's exact test as appropriate.

When comparing AHC to CP, a higher proportion of AHC were above 0.35μg/ml serotype specific IgG at baseline for 6/13 serotypes: **6B** (0.96 vs. 0.73 , *p*= 0.015), **7F** (0.77 vs. 0.46, *p*= 0.01), **9V** (0.85 vs. 0.58, *p*=0.021), **14** (0.85 vs. 0.6, *p*= 0.032), **18C** (0.81 vs. 0.50, *p*= 0.01) and **23F** (0.69 vs. 0.38, *p* = 0.009). When comparing CHC to CP, a higher proportion of CHC were above 0.35μg/ml serotype specific IgG at baseline for 3/13 serotypes: **6B** (0.97 vs. 0.73, *p*=0.008), **18C** (0.73 vs. 0.50, *p*= 0.042) and **23F** (0.63 vs. 0.38, *p*= 0.026).

There was no significant difference between the proportions above 0.35μg/ml for AHC compared to CHC.

	Proportion>0.35μg/ml												
	S1	S3	S4	S5	S6A	S6B	S7F	S9V	S14	S18C	S19A	S19F	S23F
CHC	0.40	0.40	0.47	0.77	0.83	0.97	0.53	0.73	0.77	0.73	0.97	0.87	0.63
AHC	0.62	0.38	0.58	0.85	0.96	0.96	0.77	0.85	0.85	0.81	0.96	0.89	0.69
CP	0.48	0.27	0.42	0.77	0.79	0.73	0.46	0.58	0.60	0.50	0.92	0.77	0.38
AHCvCP *p*	0.263	0.313	0.187	0.442	0.050	0.015	0.010	0.021	0.032	0.010	0.460	0.233	0.009
CHCvCP *p*	0.494	0.234	0.665	0.966	0.650	0.008	0.519	0.179	0.139	0.042	0.380	0.296	0.026
CHCvAHC *p*	0.108	0.906	0.410	0.455	0.122	1.000	0.066	0.305	0.455	0.511	0.918	0.839	0.642

Table 14. Proportion with serum serotype specific IgG concentration > 0.35 μg/ml at baseline. (S = serotype, CHC= child healthy control, AHC= adult healthy control, CP= child patient)

When comparing AHC to CP, a higher proportion of AHC were above 1μg/ml serotype specific IgG at baseline for 4/13 serotypes: **7F** (0.5 vs. 0.17, *p* =0.002), **9V** (0.31 vs. 0.08, *p*= 0.012), **18C** (0.5 vs. 0.25, *p*=0.030) and **23F** (0.35 vs0.13, *p*= 0.024). When comparing CHC to CP, a higher proportion of CHC were above 1μg/ml serotype specific IgG at baseline for 4/13 serotypes: **3** (0.16 vs. 0.02, *p*=0.029), **6B** (0.63 vs. 0.4, *p*= 0.041), **9V** (0.4 vs. 0.08, *p*=0.001) and **23F** (0.37 vs. 0.13, *p* =0.012).

There was no significant difference between the proportions above 1μg/ml for AHC compared to CHC.

	Proportion > 1μg/ml												
	S1	S3	S4	S5	S6A	S6B	S7F	S9V	S14	S18C	S19A	S19F	S23F
CHC	0.10	0.16	0.20	0.27	0.57	0.63	0.27	0.40	0.47	0.37	0.80	0.53	0.37
AHC	0.27	0.03	0.15	0.35	0.54	0.50	0.50	0.31	0.65	0.50	0.85	0.58	0.35
CP	0.15	0.02	0.15	0.31	0.48	0.40	0.17	0.08	0.44	0.25	0.81	0.40	0.13
AHCvCP *p*	0.196	1.000	0.926	0.768	0.626	0.388	0.002	0.012	0.075	0.030	0.716	0.136	0.024
CHCvCP *p*	0.556	0.029	0.532	0.666	0.452	0.041	0.287	0.001	0.801	0.272	0.892	0.235	0.012
CHCvAHC *p*	0.099	0.200	0.653	0.519	0.832	0.315	0.072	0.472	0.160	0.315	0.653	0.743	0.873

Table 15. Proportion with serum serotype specific IgG concentration > 1 μg/ml at baseline. (S = serotype, CHC= child healthy control, AHC= adult healthy control, CP= child patient)

Log transformed baseline serotype specific IgG concentrations were compared using ANOVA with Bonferroni correction for multiple comparison. Geometric mean concentrations (GMC) of IgG at baseline are reported in Table 16.

GMCs were higher for AHC than CP for 6/13 serotypes: **7F** (0.91 vs. 0.31 μg/ml, *p*= 0.001), **9V** (0.67 vs. 0.34 μg/ml, *p*= 0.024, **14** (2.3 vs. 0.73 μg/ml, *p*= 0.018), **18C** (0.9 vs. 0.35 μg/ml, *p*= 0.007), **19F** (1.43 vs. 0.66 μg/ml, *p*= 0.028) and **23F** (0.62 vs. 0.29 μg/ml, *p*= 0.049).

GMCs were higher for CHC than CP for 3/13 serotypes: **6B** (1.29 vs. 0.68 μg/ml, *p*= 0.009), **9V** (0.81 vs. 0.34 μg/ml, *p*= 0.001) and **23F** (0.65 vs. 0.29 μg/ml, *p*= 0.024).

GMC was higher for AHC than CHC for 1/13 serotypes: **7F** (0.91 vs. 0.41 μg/ml, *p*= 0.043)

	GMC μg/ml												
	S1	S3	S4	S5	S6A	S6B	S7F	S9V	S14	S18C	S19A	S19F	S23F
CHC	0.29	0.30	0.33	0.56	1.03	1.29	0.41	0.81	1.06	0.67	2.22	1.10	0.65
AHC	0.51	0.25	0.33	0.66	1.21	1.08	0.91	0.67	2.30	0.90	2.72	1.43	0.62
CP	0.35	0.18	0.29	0.58	0.82	0.68	0.31	0.34	0.73	0.35	1.97	0.66	0.29
Bonferroni *p*													
AHC v CP						0.108	0.001	0.024	0.018	0.007		0.028	0.049
CHC v CP						0.009	0.941	0.001	1.000	0.082		0.204	0.024
CHC v AHC						1.000	0.043	1.000	0.268	1.000		1.000	1.000

Table 16. Geometric mean concentration of pneumococcal polysaccharide serotype specific IgG for each group at baseline. One-way ANOVA on log transformed data was used to compare groups. Bonferroni corrected *p* values for post hoc comparisons are reported when result of one-way ANOVA was found to be significant. (GMC = geometric mean concentration, AHC = adult healthy control, CHC = child

5.4.3 *Response to vaccination*

The proportions above each cut off at 1 month and 6 month post immunisation are shown in Tables 17 and 18 and represented graphically in Figures 18 and 19.

McNemar's test was used to compare paired proportions above a cut off of 0.35 μg/ml and 1 μg/ml within groups at baseline vs. 1 month and baseline vs. 6 months post immunisation

PCV13 leads to a significant increase from baseline in the proportion above 1 μg/ml for all vaccine serotypes which persists to 6 months in children with paHIV

When comparing the proportion above a cut off of 0.35μg/ml at baseline with 1 month results there was a statistically significant increase for 11/13 serotypes for CP and 5/13 serotypes for AHC. In all cases where the increase in proportion was non-significant for the AHC group, the baseline proportion was already above 0.8 and increased to 1 or just below. This was true for serotype 19A for the CP group. For serotype 5 in the CP group the non-significant increase was from 0.77 at baseline to 0.88 at 1 month (*p*= 0.125).

When comparing baseline to 6 month proportions above 0.35 μg/ml a similar pattern was observed, however the difference from baseline became non significant for serotype 7F in the AHC group (0.77 vs. 0.96, *p*= 0.063) and 6A in the CP group (0.79 vs. 0.92, *p*= 0.109). The post immunisation proportions were above 0.8 for the majority of serotypes at both post immunisation time points for both groups.

There was a statistically significant increase in the proportion above 1μg/ml for all serotypes in both groups at the 1 month time point. The same was true for the 6 month time point apart from for serotype 3 in the AHC group (0.03 vs. 0.19, *p*=0.125) and for serotype 19A for both groups however in the latter case baseline proportions were above 0.8 and 6 month proportions remained above 0.9.

The proportion above a 1 μg/ml cut off are lower in children with paHIV compared to young healthy adults for almost half vaccine serotypes 1 month and 6 months following PCV13

Chi^2 was used to compare proportions above 0.35 μg/ml and 1 μg/ml cut offs between groups at 1 month and 6 months following immunisation.

A high proportion AHC and CP achieved serotype specific concentration greater than 0.35 μg/ml at both 1 month and 6 months post immunisation. The only serotype for which the proportion was higher in AHC than CP was 23F. This was true at 1 month and 6 months post immunisation: 1.00 vs. 0.81 (*p* = 0.023), 1.00 vs. 0.75 (*p* =0.006) respectively.

The proportions above a cut off of 1 μg/ml were significantly higher for AHC than CP at 1 month post immunisation for 6/13 serotypes: **6A** (1.0 vs. 0.81, *p*= 0.023), **6B** (1.0 vs. 0.83, *p*= 0.044), **9V** (0.92 vs. 0.73, *p*=0.048), **14** (1.0 vs. 0.79, *p*= 0.012), **18C** (0.96 vs. 0.75, *p*= 0.026), **23F** (1.0 vs. 0.69, *p*=0.001).

	S1			S3			S4			S5			S6A			S6B			S7F		
	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m
AHC	0.62	1.00	0.96	0.38	0.85	0.77	0.58	1.00	0.81	0.85	1.00	0.96	0.96	1.00	1.00	0.96	1.00	1.00	0.77	1.00	0.96
CP	0.48	0.98	0.94	0.27	0.65	0.58	0.42	0.92	0.83	0.77	0.88	0.85	0.79	0.98	0.92	0.73	0.94	0.88	0.46	0.96	0.94
CPvsAHC																					
p		1.000	1.000		0.068	0.110		0.291	0.782		0.085	0.247		1.000	0.291		0.548	0.085		0.538	1.000
bl vs post																					
AHC *p*		0.002	0.004		0.001	0.002		0.001	0.031		0.125	0.250		1.000	1.000		1.000	1.000		0.031	0.063
CP *p*		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		0.125	0.219		0.008	0.109		0.006	0.039		<0.001	<0.001

	S9V			S14			S18C			S19A			S19F			S23F		
	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m
AHC	0.85	0.96	0.96	0.85	1.00	1.00	0.81	0.96	0.92	0.96	1.00	1.00	0.89	1.00	1.00	0.69	1.00	1.00
CP	0.58	0.88	0.85	0.60	0.90	0.90	0.50	0.85	0.81	0.92	0.98	0.98	0.77	0.96	0.94	0.38	0.81	0.75
CPvsAHC																		
p		0.410	0.247		0.155	0.155		0.247	0.309		1.000	1.000		0.538	0.548		0.023	0.006
bl vs post																		
AHC *p*		0.250	0.250		0.125	0.125		0.125	0.250		0.317	0.317		0.250	0.250		0.008	0.008
CP *p*		<0.001	<0.001		<0.001	0.001		<0.001	<0.001		0.250	0.250		0.004	0.008		<0.001	<0.001

Table 17. Comparison between AHC and CP group for proportion above a cutoff of 0.35 μg/ml and within group comparison for baseline compared to 1 month and 6 months post immunisation. (S= serotype, bl= base line, 1m = 1 month post immunsation, 6m= 6 months post immunisation)

	S1			S3			S4			S5			S6A			S6B			S7F		
	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m
AHC	0.27	0.92	0.65	0.03	0.46	0.19	0.15	0.88	0.58	0.35	0.81	0.73	0.54	1.00	0.96	0.50	1.00	0.96	0.50	0.96	0.85
CP	0.15	0.81	0.67	0.02	0.29	0.21	0.15	0.75	0.52	0.31	0.73	0.56	0.48	0.81	0.71	0.40	0.83	0.75	0.17	0.92	0.65
CPvsAHC																					
p		0.309	0.911		0.144	0.870		0.169	0.644		0.452	0.154		0.023	0.010		0.044	0.026		0.651	0.068
bl vs post																					
AHC *p*		<0.001	0.002		0.001	0.125		<0.001	0.001		<0.001	0.002		<0.001	0.001		<0.001	<0.001		<0.001	0.004
CP *p*		<0.001	<0.001		<0.001	0.004		<0.001	<0.001		<0.001	<0.001		<0.001	0.003		<0.001	<0.001		<0.001	<0.001

	S9V			S14			S18C			S19A			S19F			S23F		
	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m
AHC	0.31	0.92	0.77	0.65	1.00	1.00	0.50	0.96	0.85	0.85	1.00	0.92	0.58	0.96	0.88	0.35	1.00	0.92
CP	0.08	0.73	0.60	0.44	0.79	0.81	0.25	0.75	0.60	0.81	0.94	0.92	0.40	0.88	0.75	0.13	0.69	0.56
CPvsAHC																		
p		0.048	0.152		0.012	0.023		0.026	0.032		0.548	1.000		0.410	0.169		0.001	0.001
bl vs post																		
AHC *p*		<0.001	<0.001		0.004	0.004		<0.001	0.004		0.046	0.157		0.002	0.008		<0.001	<0.001
CP *p*		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		0.031	0.125		<0.001	<0.001		<0.001	<0.001

Table 18 Comparison between AHC and CP group for proportion above a cut off of 1 μg/ml and within group comparison for baseline compared to 1 month and 6 months post immunisation. (S= serotype, bl= base line, 1m = 1 month post immunisation, 6m= 6 months post immunisation)

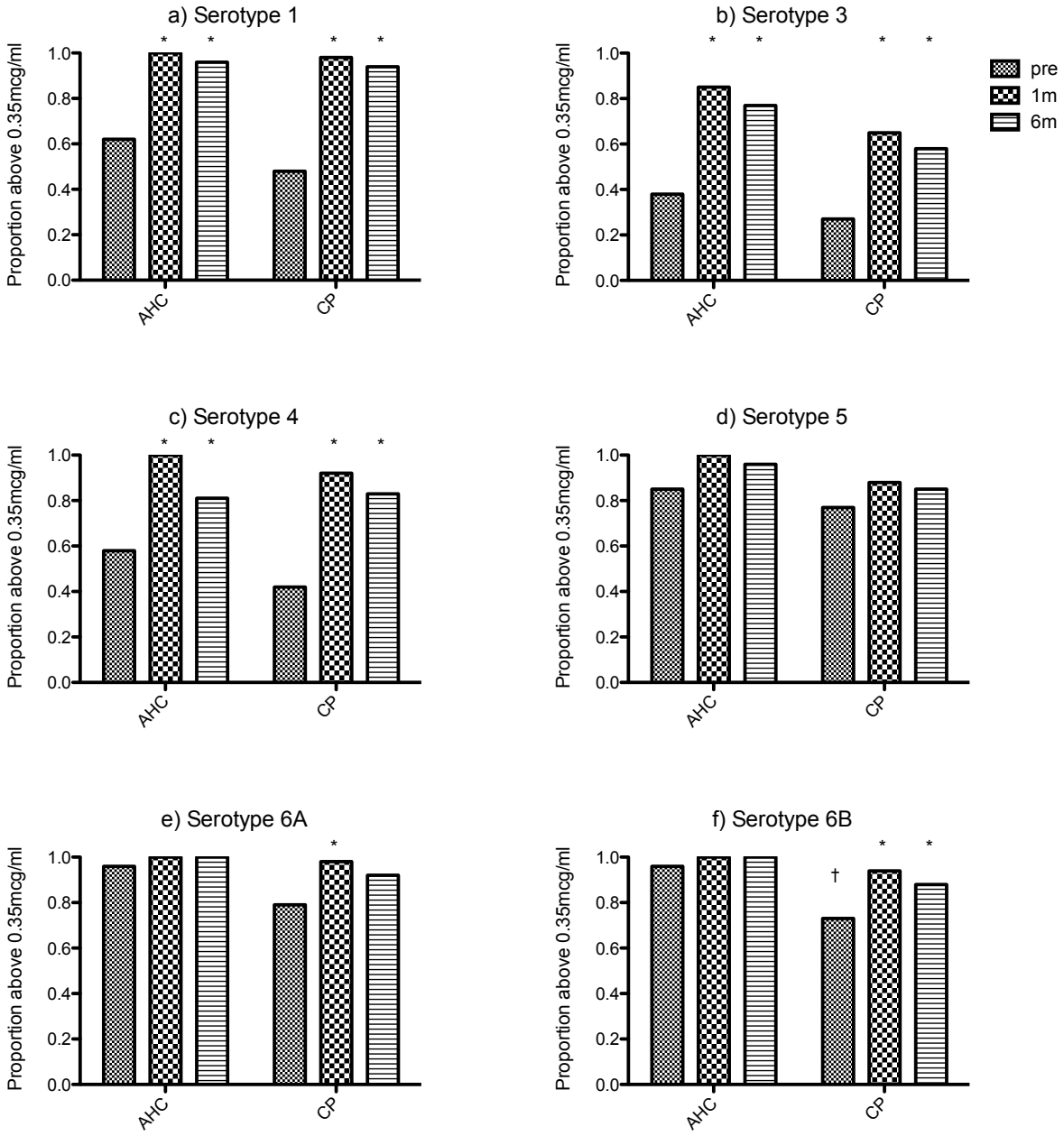


Figure 18 a-m) Bar charts showing the proportion of study subjects with serotype specific serum IgG concentration >0.35μg/ml before immunisation with PCV13 and 1 month and 6 months after immunisation. A higher proportion of AHC were above 0.35μg/ml serotype specific IgG at baseline for 6/13 serotypes: 6B, 7F, 9V, 14, 18C and 23F (p<0.05). When comparing baseline with 1 month results there was a statistically significant increase for 11/13 serotypes for CP and 5/13 serotypes for AHC (p<0.05). When comparing baseline to 6 month, a similar pattern was observed. A high proportion AHC and CP achieved serotype specific concentration greater than 0.35 μg/ml at both 1 month and 6 months post immunisation. The only serotype for which the proportion was higher in AHC than CP was 23F. This was true at 1 month and 6 months post immunisation (p<0.05). (AHC = adult healthy control, CP = child patient, mcg = micrograms, pre = pre immunisation, 1m/6m = 1 month/ 6months post immunisation)

* significant difference in proportion above cut off compared with baseline within group (p<0.05)

† significant difference comparing proportion above cut off, AHC vs. CP (p<0.05)

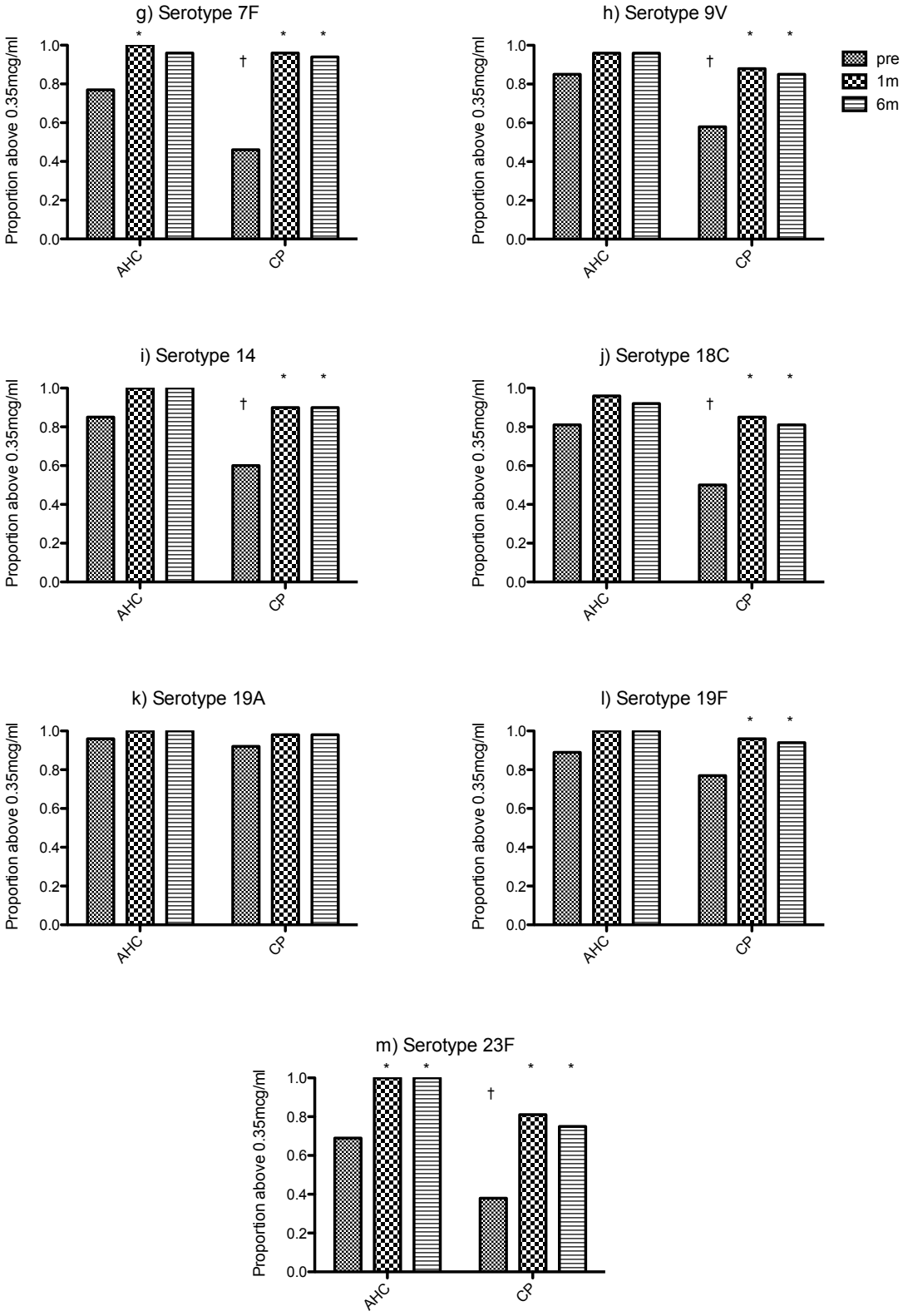


Figure 13. continued

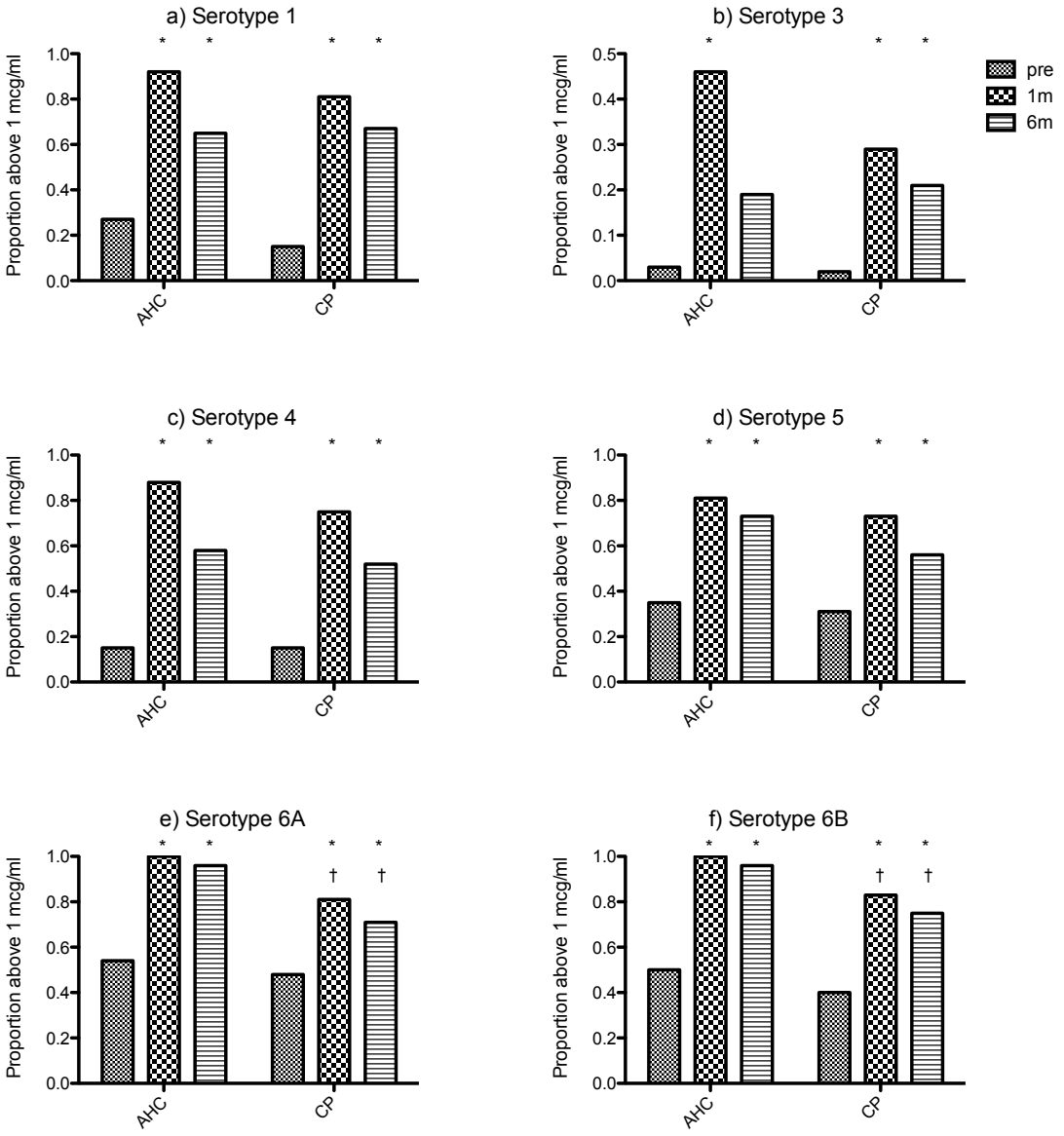


Figure 19 a-m) Bar charts showing the proportion of study subjects with serotype specific serum IgG concentration >1μg/ml before immunisation with PCV13 and 1 month and 6 months after immunisation. A higher proportion of AHC were above 1μg/ml serotype specific IgG at baseline for 4/13 serotypes: 7F, 9V, 18C and 23F. There was a statistically significant increase in the proportion above 1μg/ml for all serotypes in both groups at the 1 month time point (p<0.05). The same was true for the 6 month time point apart from for serotype 3 in the AHC group and for serotype 19A for both groups. The proportions above a cut off of 1 μg/ml were significantly higher for AHC than CP at 1 month post immunisation for 6/13 serotypes: 6A, 6B, 9V, 14, 18C, 23F (p<0.05). The same was true at the 6 month time point apart from for serotype 9V (p<0.05). (AHC = adult healthy control, CP = child patient, mcg = micrograms, pre = pre immunisation, 1m/6m = 1 month/ 6months post immunisation)

* significant difference compared with baseline proportion within group (p<0.05)

† significant difference comparing proportion of AHC with proportion of CP (p<0.05)

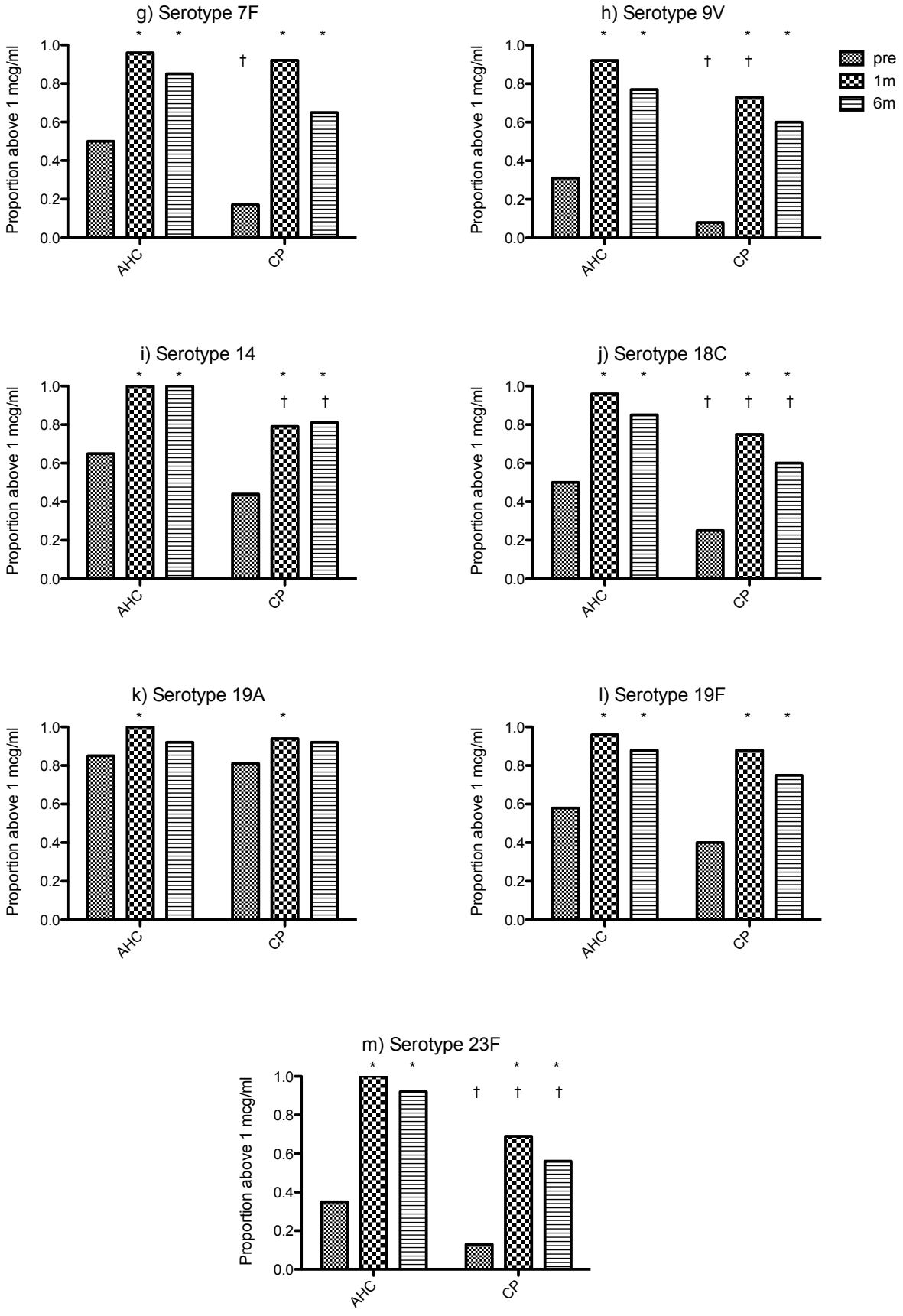


Figure 19. continued

The same was true at the 6 month time point apart from for serotype 9V: **6A** (0.96 vs. 0.71, *p*=0.01), **6B** (0.96 vs. 0.75, *p*= 0.026), **14** (1.0 vs. 0.81, *p*= 0.023), **18C** (0.85 vs. 0.6, *p*=0.032), **23F** (0.92 vs. 0.56, *p*=0.001).

Geometric mean antibody responses to PCV13 are lower in children with paHIV than healthy young adults, an effect that is less pronounced after adjusting for baseline antibody concentration

Repeated measures ANOVA and ANCOVA were used to analyse within group and between group differences at 1 month and 6 months post immunisation for log transformed serotype specific IgG concentrations. Analysis was performed with and without including baseline serum concentrations as a covariate. Where results of the main ANCOVA were significant, simple effects of time and group were then calculated.

Results of this analysis are shown in Table 19. Geometric means are also summarised in Fig. 20.

As can be seen from Table 20 and Fig. 20 antibody responses to all PCV13 serotypes increased significantly 1 month post immunisation and continued to be significantly higher than baseline 6 months post immunisation in both CP and AHC group.

Without adjusting for baseline antibody concentration, serotype specific IgG GMCs were higher in the AHC group than the CP group 1 month post immunisation for 8/13 serotypes: **6A** (10.5 vs. 3.6 μg/ml, *p*<0.001), **6B** (11.3 vs. 3.3 μg/ml, *p*<0.001), **7F** (7.7 vs. 3.0 μg/ml, *p*<0.001), **9V** (4.6 vs. 2.2 μg/ml, *p*=0.017), **14** (18.5 vs. 7.9 μg/ml, *p*=0.04), **18C** (8.6 vs. 2.5 μg/ml, *p*<0.001), **19A** (10.5 vs. 7.1, *p*=0.020), **23F** (17.1 vs. 2.1 μg/ml, *p*<0.001).

These higher antibody levels persisted to 6 months for 5/13 serotypes: **6A** (5.2 vs. 2.1 μg/ml, *p*=0.006), **6B** (5.1 vs. 2.0 μg/ml, *p*=0.004), **7F** (3.4 vs. 1.7 μg/ml, *p*=0.090), **18C** (3.9 vs. 1.3 μg/ml, *p*=0.001), **23F** (7.2 vs. 1.2, μg/ml, *p*<0.001).

After adjusting for baseline antibody concentrations GMCs were significantly higher in AHC than CP for serotypes 6A, 6B and 23F at both the 1 month (*p*<0.01) and 6 month (*p*<0.05) time points.

The paired t-test was used to compare log transformed serotype specific IgG concentrations from baseline compared to 6 months later in the 8 unvaccinated children with paHIV. Although there was a slight downward trend in GMC for all serotypes this was only significant for serotype 4 (0.19 vs. 0.14 μg/ml, *p*=0.04).

	GMC:														
	S1			S3			S4			S5			S6A		
	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m
AHC	0.510	5.434	2.353	0.253	0.996	0.539	0.328	3.287	1.270	0.662	3.216	1.737	1.210	10.536	5.175
CP	0.345	3.315	1.753	0.182	0.598	0.443	0.288	2.533	1.207	0.580	1.756	1.226	0.822	3.563	2.198
p value															
bl vs 1m/6m															
AHC		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001
CP		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001
p value															
AHC vs CP (abl)														0.002	0.024
AHC vs CP (xbl)														<0.001	0.006
	S6B			S7F			S9V			S14			S18C		
	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m	bl	1m	6m
AHC	1.082	11.316	5.117	0.910	7.684	3.448	0.671	4.584	1.933	2.298	18.531	11.359	0.900	8.576	3.930
CP	0.676	3.309	2.043	0.314	3.005	1.656	0.340	2.219	1.176	0.729	7.881	5.435	0.355	2.508	1.314
p value															
bl vs 1m/6m															
AHC		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001
CP		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001
p value															
AHC vs CP (abl)		0.001	0.036												
AHC vs CP (xbl)		<0.001	0.004		<0.001	0.009		0.017	0.099		0.044	0.083		<0.001	0.001
	S19A			S19F			S23F								
	bl	1m	6m	bl	1m	6m	bl	1m	6m						
AHC	2.722	10.492	5.800	1.426	8.562	4.193	0.620	17.094	7.249						
CP	1.968	7.141	4.460	0.661	4.063	2.491	0.290	2.122	1.169						
p value															
bl vs 1m/6m															
AHC		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001						
CP		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001						
p value															
AHC vs CP (abl)								<0.001	<0.001						
AHC vs CP (xbl)		0.022	0.107					<0.001	<0.001						

Table 19. Results of RMANOVA/RMANCOVA comparing baseline serotype specific antibody concentrations to those at 1 month and 6 months post immunisation with PCV13 in the AHC and CP group. Comparison between groups is also reported for both post immunisation timepoints. *p* values are reported for analysis with and without adjusting for baseline antibody concentration (highlighted in dark blue and light blue respectively). (GMC = geometric mean antibody concentration, S = serotype, bl = baseline, 1m/6m = 1 month and 6 months post immunisation, abl = adjusting for baseline, xbl = without adjusting for baseline)

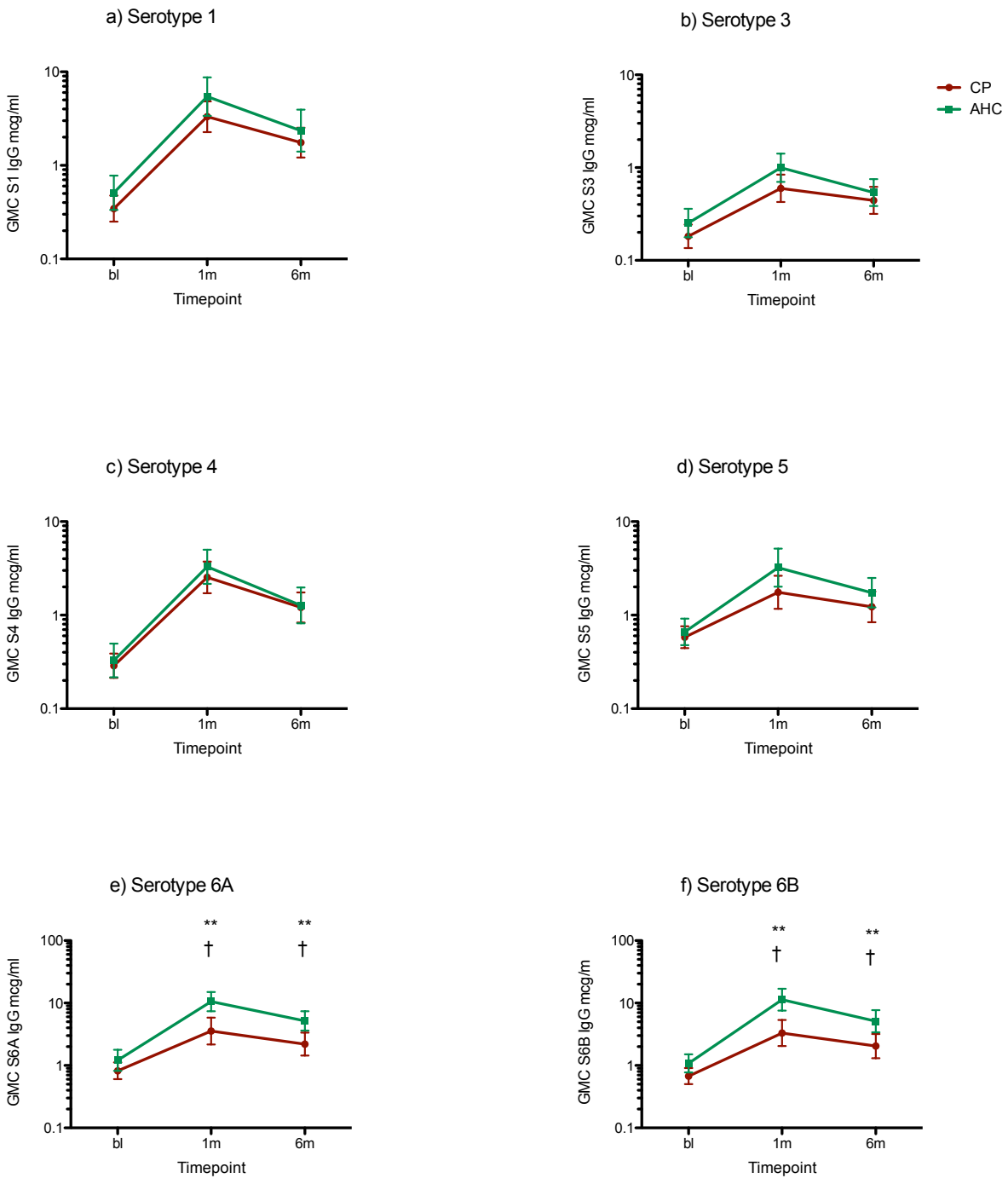


Figure 20 a-m). Geometric mean serotype specific IgG concentrations with 95% confidence intervals for young healthy adults and children with paHIV at baseline, 1 month and 6 months post immunisation with PCV13. Antibody concentrations to all PCV13 serotypes increased significantly 1 month post immunisation and continued to be significantly higher than baseline 6 months post immunisation in both CP and AHC group. Without adjusting for baseline antibody concentration, serotype specific IgGGMCs were higher in the AHC group than the CP group 1 month post immunisation for 8/13 serotypes (p<0.05): 6A, 6B, 7F, 9V, 14, 18C, 19A, 23F. These higher antibody levels persisted to 6 months for 5/13 serotypes (p<0.05): 6A, 6B, 7F, 18C, 23F. After adjusting for baseline antibody concentrations GMCs were significantly higher in AHC than CP for serotypes 6A, 6B and 23F at both the 1 month (p<0.01) and 6 month (p<0.05) time points. (GMC= geometric mean concentration, S = serotype, mcg = μg, bl = baseline, 1m/6m = 1 month and 6 months post immunisation, CP= child patient, AHC = adult healthy control). Continued on next page.

* p<0.05 comparing baseline GMC between groups

** p<0.05 comparing post immunisation GMC between groups

† p<0.05 comparing post immunisation GMC between groups after adjusting for baseline

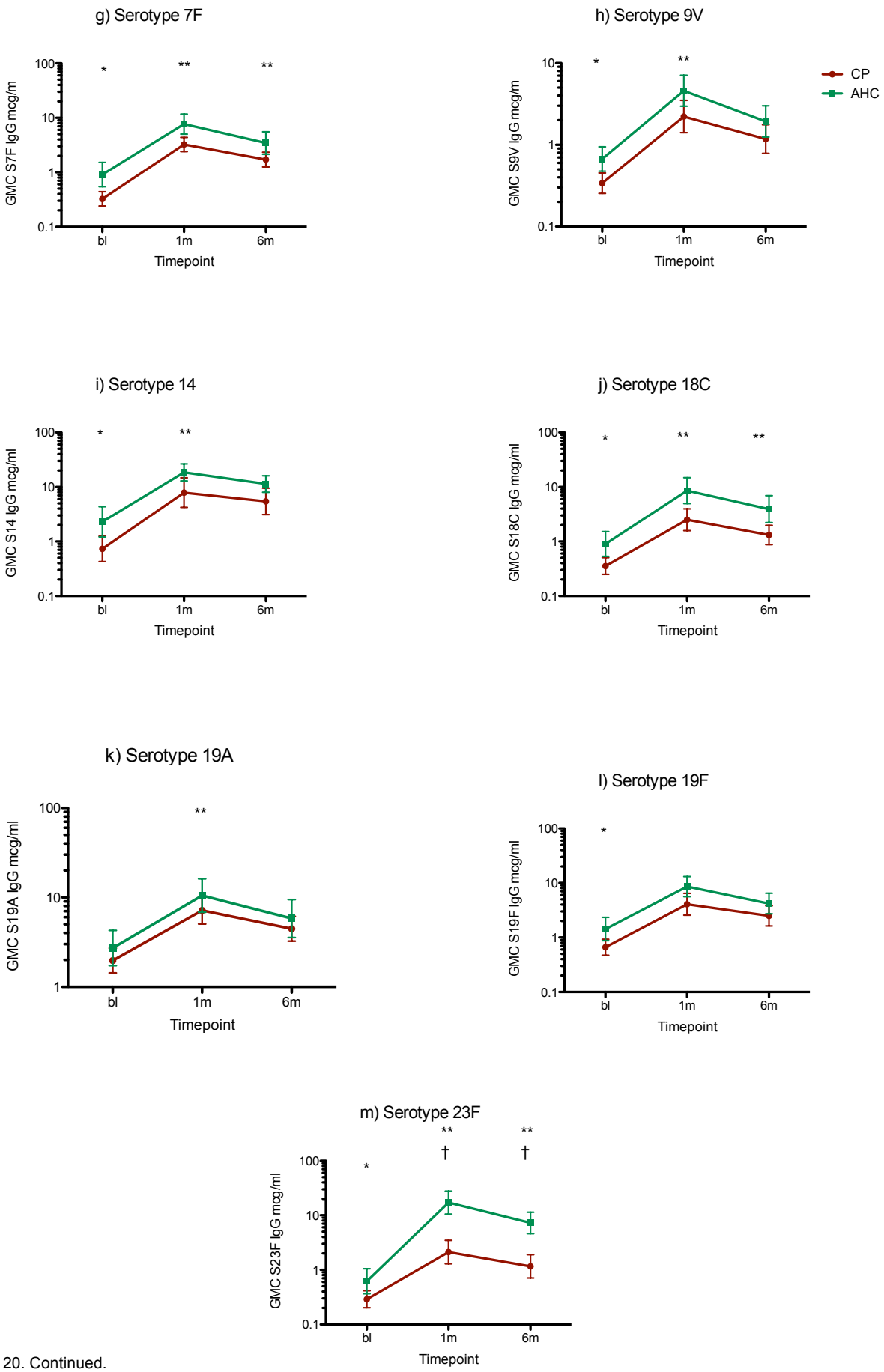


Figure 20. Continued.

5.4.4 *Relating antibody response to immunological status*

Linear regression analysis was used to assess for associations between log transformed antibody response 1 month post immunisation and lymphocyte, B and T cell subsets.

Resting memory B cell percentages are associated with higher antibody responses in children with paHIV for a proportion of serotypes

Having adjusting for baseline antibody concentration, higher CD4 T cell percentage, higher resting memory B cell percentage and lower tissue like/ exhausted memory B cell percentage were most consistently associated with higher antibody response. This was true for 9/13, 9/13 and 8/13 serotypes respectively. After adjusting for detectable viraemia, higher resting memory B cell percentage remained significantly associated with higher antibody responses for 4/13 serotypes (**9V** (p=0.016), **19A** (p=0.019), **19F** (p<0.001), **23F** (p=0.037)) whereas CD4 percentage and exhausted memory B cell were associated with higher antibody response in only 1 serotype each.

No association was found between lymphocyte, B or T cell subsets and magnitude of antibody response in AHC group.

5.4.5 *Relating serological response to clinical parameters*

Linear regression was used to assess for associations between clinical parameters and log transformed baseline serology as well as log transformed antibody response one month post immunisation.

The following factors were assessed for associations with baseline and one month serum IgG concentrations: detectable viraemia (yes/no), treated in the 1st year of life (yes/no), treated in the 1st 2 years of life (yes/no), received PCV7 (yes/no), received PPV (yes/no), proportion of life undetectable, nadir CD4 percent.

For children with paHIV, higher baseline antibody concentrations are associated with previous receipt of PPV for some PCV13 serotypes

After adjusting for age, previous receipt of PPV was the factor most frequently associated with higher baseline serotype specific IgG (6/13 serotypes): **1** (antilog regression coefficient = 2.43 (95%CI: 1.19- 4.96), *p*= 0.016), **4** (antilog regression coefficient = 2.8 (95%CI: 1.66- 4.75), *p*<0.001) , **5** (antilog regression coefficient = 1.71 (95%CI: 1.02- 2.87), *p*= 0.043) , **7F** (antilog regression coefficient = 2.44 (95%CI: 1.19- 4.98), *p*= 0.016) , **9V** (antilog regression coefficient = 2.08 (95%CI: 1.04- 4.15), *p*= 0.039), **14** (antilog regression coefficient = 5.97 (95%CI: 1.74- 20.4), *p*= 0.005).

For children with paHIV, higher antibody concentrations at 1 month were associated with higher baseline antibody concentrations for all vaccine serotypes and undetectable viral load for the majority of vaccine serotypes

Results of analysis of factors predictive of 1 month antibody response are shown in Table 20 (overleaf).

There was a highly significant association between higher baseline antibody levels and higher 1 month antibody levels for all 13 serotypes. Regression coefficients were all less than 1, thus predicting that this effect decreases in magnitude with higher baseline, suggestive of a ceiling to antibody levels. After adjusting for baseline, detectable viraemia was associated with lower 1 month antibody for 11/13 serotypes and older age was associated with lower 1 month antibody response for 6/13 serotypes. After adjusting for age, baseline and viraemia, higher percentage of life with undetectable viral load was associated with higher 1 month antibody responses for 3/13 serotypes.

5.4.6 *Nasopharyngeal carriage*

No carriage was detected in either the adult or child healthy control groups. Results for CP group are reported in Table 21.

Study no.	CP02	CP03	CP05	CP15	CP17	CP27	CP31	CP44	CP51
Age/y	6	1	10	16	6	13	3	11	2
Pre ST	NT		11A	11A	23B		15B		6C
Post ST	38	11A	11A	11A		35B		34	

Table 21. Nasopharyngeal carriage isolates from CP group pre and post immunisation. CP05 and CP17 were not vaccinated. Serotypes in red are a component of PPV. (Pre ST = prevacccination serotype, Post ST = serotype 6 months later)

9/56 (16%) of the children with paHIV were found to be positive for pneumococcal carriage at one or both time points. No child carried a PCV13 serotype, 4/9 children with detectable carriage were carrying serotypes present in PPV (11A and 15B), 11A was the most frequently carried serotype (3/9 children) and persistent carriage of 11A was detected in 2/9 children over the course of 6 months.

Serotype	coeff			exp coef			exp coef			exp coef		
	baseline	95% CI	*p*	viraemia	95% CI	*p*	age/years	95% CI	*p*	prop/%	95% CI	*p*
1	0.47	0.15 - 0.79	0.005	4.31	2.09 - 8.88	0.000	0.92	0.85 - 1.00	0.043			
3	0.72	0.45 - 0.99	0.000	2.21	1.18 - 4.13	0.015	0.92	0.87 - 0.98	0.006			
4	0.43	0.07 - 0.79	0.022	4.58	2.12 - 9.88	0.000				1.02	1.00 - 1.04	0.017
5	0.79	0.41 - 1.18	0.000	3.01	1.37 - 6.61	0.007						
6A	0.70	0.27 - 1.13	0.002									
6B	0.97	0.59 - 1.36	0.000	3.41	1.44 - 8.09	0.006	0.91	0.83 - 0.98	0.021			
7F	0.33	0.02 - 0.63	0.036	3.94	2.05 - 7.57	0.000	0.91	0.85 - 0.97	0.008			
9V	0.91	0.53 - 1.29	0.000	3.95	1.76 - 8.87	0.001						
14	0.72	0.45 - 0.99	0.000	5.31	1.79 - 15.78	0.003						
18C	0.74	0.42 - 1.06	0.000									
19A	0.61	0.34 - 0.88	0.000	2.74	1.44 - 5.19	0.003	0.93	0.87 - 1.00	0.044			
19F	0.71	0.37 - 1.06	0.000	3.62	1.49 - 8.78	0.005	0.88	0.81 - 0.81	0.003	1.02	1.00 - 1.04	0.013
23F	0.53	0.16 - 0.90	0.007	3.99	1.43 - 11.16	0.010				1.04	1.02 - 1.06	0.001

Table 20. Results of regression analysis to determine association between serotype specific IgG concentration at 1 month post immunisation with baseline concentration, detectable viraemia (after adjusting for baseline), age in years (after adjusting for baseline) and proportion of life with undetectable viral load (expressed as a percentage and after adjusting for baseline, detectable viraemia and age). As antibody concentrations were log transformed and for ease of interpretation antilog regression coefficients and 95% confidence intervals are presented for all independent variables except baseline antibody concentration (as this is also log transformed). Only results with a significance level $p<0.05$ are shown. (CI = confidence interval, coef = regression coefficient, exp = antilog).

5.5 Discussion

To our knowledge this is the first report of the use of PCV13 in any HIV infected group. It is also the first to investigate any potential relationship between humoral response to this vaccine and detailed B cell phenotype. We have demonstrated that children with paHIV have pre-existing pneumococcal immunity to a number of PCV13 serotypes, indicating that response, at least in part, reflects a memory response. Baseline antibody concentrations were lower than in healthy controls for some vaccine serotypes and concentrations were correspondingly lower at 1 month and 6 months post immunisation. After adjusting for baseline these differences were less extreme, although impairment was still demonstrated for some serotypes. Viraemia and to a lesser extent, age at vaccination, appear to impact on magnitude of antibody response and there is also a suggestion that a lower percentage of resting memory B cells, a subset shown in the previous chapter to be only partially restored by HAART, may be associated with lower post immunisation antibody concentrations.

Of 13 immunogenicity studies of pneumococcal conjugate vaccines in HIV infected children to date [198, 199, 201, 324-327, 343, 410-414] 7 have examined responses in children older than 2 years of age [198, 199, 201, 324-326, 414]. Of these studies, 1 investigated response to an experimental 5-valent vaccine, 5 investigated PCV7 and 1 investigated an experimental 9-valent vaccine. All reported ELISA responses to at least 3 serotypes (6B, 14 and 23F), though ELISA methods varied, as did timing of blood sampling, dosing schedules, study setting, and immunological and HAART status, thus limiting direct comparison.

We have shown that serotype specific IgG is detectable in a substantial proportion of both healthy control groups and in children with paHIV. A proportion of the children had previously received PCV7 or PPV, which may have partially contributed to this baseline humoral immunity, a suggestion that is supported by the finding of an association between previous receipt of PPV and higher baseline IgG for some serotypes. Nevertheless, the fact that young adult healthy controls, none of whom had ever receive pneumococcal vaccine, had existing serotype specific IgG concentrations of equivalent or higher levels indicates that natural immunity is acquired throughout childhood and early adult life, most likely as a result of natural exposure through nasopharyngeal carriage and non-invasive pneumococcal disease e.g. otitis media [415]. This natural acquisition of anti-pneumococcal IgG with age has been clearly demonstrated by a seroepidemiological study carried out in the UK prior to universal introduction of PCV [416] and numerous studies assessing prevaccination antibody levels outside of the infant period [417].

It is perhaps surprising that when comparing healthy children with children with paHIV using GMC or proportion above a cut off of 0.35 μg/ml or 1 μg/ml, HIV infected children had significantly lower baseline antibody levels for only 3-4/13 serotypes. Of all 13 vaccine serotypes only antibodies to serotype 6B and 23F were consistently lower at baseline in children with paHIV using all 3 methods of comparison. These findings are to some extent consistent with other reports comparing pre-vaccination pneumococcal serology in both HIV infected and healthy children. In a study from the pre-HAART era King et al found HIV infected children to have lower GMC serotype specific IgG to serotype 6B, 14 and 23F when compared to age matched

healthy controls. All children were less than 9 years of age and had not previously received PPV or PCV [414]. Spoulou et al found lower antibody to serotype 6B at baseline in a cohort of HIV infected children aged 2-14 years compared to healthy age matched controls. The majority of HIV infected children were on HAART and had received PPV [201]. Abzug et al, although they did not compare results with healthy controls, report similar findings to ours in terms of geometric mean antibody concentrations at baseline and proportions above a cut-off of 1μg/ml in a large cohort of HIV infected children with a median age of 9.6 years. The majority of children were on HAART and had received PPV [198]. Madhi et al compared pre-vaccination antibody concentrations in HIV infected children with healthy controls and found lower GMCs for serotype 9V, 18C, 19F and 23F [326]. The mean age was younger than our cohort (5.8 years) and the majority of children were not on HAART. A recent study of HIV infected children in Thailand compared HIV infected children (median age 97 months) with HIV exposed uninfected children and found no difference in baseline GMCs for any serotype tested. 90% were on HAART and they had not previously received PPV. It should be noted in this study however that the control group were significantly younger than the HIV infected group [325].

Considering these previous findings alongside our own, it appears that children with paHIV acquire serotype specific pneumococcal IgG, most likely through natural exposure, and that steady state levels of antibody are equivalent to healthy control children for a proportion of vaccine serotypes. The persistent increased risk of IPD in children with HIV despite suppressive HAART [270-272] suggests that there may therefore be either some functional impairment measurable circulating antibody or perhaps continued susceptibility to specific serotypes.

We have shown PCV13 to be immunogenic in children with paHIV, albeit less so than in young healthy adults. Vaccination results in a significant increase in the proportion above a cut-off of 1 μg/ml and increased GMC for all vaccine serotypes which persist to the 6-month time point. Without taking baseline antibody levels into consideration, proportions above 1 μg/ml and GMCs were higher at both time points in the young healthy adults than children with paHIV for between 5 and 8 vaccine serotypes. After adjusting for baseline, GMCs were lower in the HIV infected group for serotype 6A, 6B and 23F at 1 month and 6 months post immunisation. These findings are consistent with data from previously published studies of lower valency pneumococcal conjugate vaccines comparing response with healthy controls [201, 324-326, 414].

Using GMC and fold change in antibody 6 weeks post immunisation as a read out of response to vaccination, King et al found a 5-valent vaccine to be less immunogenic in HIV infected children than healthy controls and in particular the proportion of children with ≥ 4 fold rises to serotype 6B and 23F were significantly lower in the HIV infected cohort [414]. Spoulou et al defined “response” to PCV7 vaccination as > 2-fold rise in antibody concentration to >2 of 4 assessed serotypes 1 month post- immunisation and found the proportion of responders to be lower in HIV infected children compared to healthy controls. They also found that 1 month GMC to be significantly lower for serotype 6B in the HIV infected cohort [201]. Madhi et al found significantly higher proportions >0.35 μg/ml and higher GMCs for all 7 vaccine serotypes in healthy controls 1 month post immunisation. However, this study involved younger children than the present study and a very small proportion of HIV infected children were on HAART [326]. Costa Ide et al immunised HIV

infected children aged 2-9 years, 95% of whom were receiving ART, with PCV7. They did not include a healthy control group but did find 6B and 23F to be the least immunogenic serotypes when assessing antibody responses above a cut off of 1.3μg/ml and 4-fold change in antibody concentration [324]. Thanee et al found the ratio of GMCs pre to post immunisation were significantly lower in HIV infected children compared to HIVEU for serotype 9V and 14, and significantly lower proportion with 4 fold rise for serotype 9V. The controls were significantly younger than the patients and for children over 24 months, HIV infected children received 2 doses of PCV7 whereas uninfected children received 1 [325].

As stated earlier, differences in study setting, vaccine formulation, HAART status, immunological status and dosing schedules limit direct comparisons, however taken together with our findings it is clear that pneumococcal conjugate vaccines are immunogenic in HIV infected children but less so than in healthy controls and that HIV may impact upon the immune response to different serotypes to a varying extent.

We found higher serotype specific antibody responses at 1 month to be associated with higher baseline antibody for all vaccine serotypes and with undetectable viraemia for the majority of vaccine serotype. After adjusting for these factors we found, out of all lymphocyte, B cell and T cell subsets assessed, higher resting memory B cell percentages were most frequently associated with higher antibody responses (4/13 serotypes) and that a higher proportion of life spent with undetectable viral load was associated with higher antibody responses for 3/13 serotypes. Although numbers were small we found no evidence of hyporesponsiveness after having received PPV. The phenomenon of hyporesponsiveness following polysaccharide vaccination is well recognised, although clinical implications are unclear [418-420], it may well be the case that the timing of pneumococcal conjugate vaccine following polysaccharide vaccine may be an important factor in determining the degree of serological hyporesponsiveness observed.

Abzug et al investigated predictors of higher antibody response to serotypes 6B, 14, 19F and 23F 8 weeks after a series of 2 doses of PCV7 8 weeks apart followed by a single dose of PPV 8 weeks later. They found higher 24 week antibody levels to be associated with higher entry antibody level and lower HIV viral load (for all serotypes tested), higher immune status based on nadir and current CD4% (for 3/4 serotypes tested) and age <7 years and longer duration of HAART (for 1/4 serotypes tested) [198]. Other groups found no association between immune status (based on CD4 count, CD4 percent or CDC classification) and response to vaccination [199, 326, 414] apart from Thanee at al who found that CDC category C disease was associated with lower response to serotype 23F [325].

There are no published reports relating response to pneumococcal conjugate vaccine to B cell memory phenotype in HIV infected children. Iwajomo et al found that minimally symptomatic HIV infected not on HAART had lower numbers of circulating pneumococcal protein specific B cells [169]. It is tempting to speculate that this might also be the case for polysaccharide specific B cells. A recent report in HIV infected adults found a greater antibody response to PCV7 to be related to class switched memory B cell absolute numbers [164]. Direct comparison is substantially limited by the fact that study subjects received 2 double doses of PCV7 3 months apart, analysis included absolute cell counts rather than percentages, and a combined aggregate readout of immune response to all vaccine serotypes was used. Data from our own

group has shown impaired response to PPV and tetanus to be associated with decreased IgM and class switched memory B cell percentages respectively in HIV infected adults [162]. Our finding of an association of impaired antibody response to PCV13 with decreased resting memory B cell percentages suggests that the mechanisms and cell types involved in the immune response to PCV13 might be different from those in response to “pure” T dependent and T independent vaccines tested in this study. It also might suggest the pattern of impairment in vaccine response might differ in children with paHIV compared to adults with HIV. It is likely that a combination of these two possibilities contributes to the observed differences.

Pneumococcal nasopharyngeal carriage is considered to be a necessary precursor for invasive disease. While pneumococcal conjugate vaccines have been shown to be protective against carriage of vaccine serotypes as well as IPD, replacement with non-vaccine serotypes has been shown to occur [246]. In addition nasopharyngeal carriage around the time of immunisation has been shown to be associated with impaired antibody responses to the carried serotype when infants are vaccinated with PCV7 [421, 422]. Licensure of new pneumococcal vaccine is currently based on non-inferiority to existing vaccines but it is of concern that use of these criteria alone might underestimate effectiveness at the community level especially in relation to herd immunity. For these and a number of other reasons, the inclusion of carriage as an endpoint in pneumococcal vaccine studies is under careful consideration [246].

Pneumococcal carriage rates in healthy individuals are highest in the first 2 years of life then drop to much lower levels in adult life [423]. The finding of no carriage in either of the healthy control groups is possibly lower than expected [259], but may reflect the median age of the child group, the fact that none of the adult healthy controls lived with young children (a risk factor for carriage) as well as small group sizes. This contrasts with the relatively high carriage rates detected in children with paHIV. A recent 3 year epidemiological study of healthy children under 4 years of age in the UK found that since the introduction of PCV7, the proportion of carriage isolates that are vaccine serotypes have decreased and proportions of non-vaccine serotypes (especially 6C, 11A, 19A and 22F) have increased. They found serotype 11A to be present in 6.9% of isolates in the 3rd year of the study whereas we have detected it in 3/6 (50%) of isolates at the 6 month time point. Our numbers are small thus limiting conclusions but higher rates of carriage in children with paHIV than in healthy children, over representation of serotype 11A and occurrence of 15B (both present in PPV) are worthy of note. The efficacy and therefore utility of PPV in children with HIV and other at risk groups is hotly debated [189, 418] and future studies of carriage and invasive disease due to serotypes present in PPV and not in PCV13 post universal introduction of PCV13 will be important in resolving this discussion.

The relationship between replacement of carriage serotypes and pneumococcal disease is not completely clear [424] although evidence suggests that replacement pneumococcal disease (disease due to non-vaccine serotypes after introduction of a conjugate vaccine) may have particular impact upon certain risk groups including adults with HIV and children with underlying medical conditions [425].

The fact that baseline antibody levels are relatively low for certain serotypes and not others and response to vaccination is impaired for some serotypes and not others, raises the possibility that impact of HIV on

serotype specific immunity might vary, both after natural exposure and immunisation. Although possible reasons for this are not immediately obvious, it has been demonstrated in healthy adults that variation in antibody response occur to different polysaccharides following pneumococcal conjugate vaccination despite uniform T cell responses to the carrier protein, suggesting that this variation in response may be a result of variations in B cell repertoire [293]. The authors hypothesised that this may relate to variations in availability of rare antigen specific B cells prior to immunisation, which may vary according to serotype. Irrespective of the underlying mechanism, it is possible that the impact of HIV on various aspects of pneumococcal immunity may exaggerate any existing innate differences in the ability to respond to individual polysaccharides.

When examining the serotype distribution of invasive pneumococcal disease in children and adults with HIV, certain serotypes are more commonly found than in the non-HIV infected population, in particular the "paediatric" serogroups (6, 9, 14, 19 and 23) [426-429]. We found responses to serotypes 6A, 6B and 23F to be particularly impaired in children with paHIV. This finding together with the observation that they are particularly implicated in invasive disease in the context of HIV is further evidence that HIV infection may impact upon both vaccine and naturally acquired immune response to some serotypes more than others. Serotype 6B and 23F have historically been considered to be less immunogenic than other serotypes [417]. In a recent study of comparing 2 different reduced dose schedules of PCV in infancy (dose at 2 and 3 months vs. dose at 2 and 4 months of age), 6B and 23F were the serotypes associated with significantly lower antibody responses in the 2/3 month dosing schedule [430]. It is cause for concern that HIV infected children may respond least well to the vaccine serotypes to which they are most vulnerable.

Are these differences in immunogenicity clinically relevant? There is no available clinical efficacy or effectiveness data for PCV13 for older HIV infected children on HAART. The introduction of PCV7 has had such dramatic effects on the incidence of invasive disease to vaccine serotypes that it would not be feasible or ethical to evaluate clinical efficacy of more recent vaccines such as PCV13 through clinical trials [308, 431]. For this reason immunogenicity is used as a surrogate for clinical protection. Various markers of immunogenicity may be employed including proportion above a predetermined cut-off concentration, GMC, fold change in antibody concentration, avidity, boosting, OPA titre and the proportion of serotypes for which a predetermined level of "response" is achieved [255, 432, 433].

For the purposes of this study, GMC and proportion above a cut-off of 0.35 μg/ml and 1 μg/ml were chosen as a read out of immunogenicity. An antibody concentration of 0.35 μg/ml 1 month post vaccination has been adopted as a cut-off for protection in the evaluation of current pneumococcal conjugate vaccines [255, 308, 309, 431, 433]. This is based on data from 3 large clinical efficacy studies of PCV7. It is mainly of use in demonstrating non-inferiority of next generation conjugate vaccines to PCV7 [308]. It is however, not an indicator of individual protection and has not been validated for non-PCV7 serotypes or children with HIV. A substantial proportion of those in our study, whether HIV infected or not had serotype specific antibody levels greater than 0.35 μg/ml at baseline, thus limiting the utility of this cut-off in examining immune response. Indeed, it has been suggested that a higher threshold may be more appropriate in HIV infected children [271].

It has also been suggested that a more conservative cut-off of 1 μg/ml may be more relevant to long term protection, based on studies of *Haemophilus influenzae* conjugate vaccine [434]. This cut-off has also been used in other studies of vaccine immunogenicity in HIV infected children [198, 412, 413]. We found the proportion of study subjects above this cut-off at baseline to be relatively low, to increase significantly post immunisation and to indicate differences between immune response in the HIV group compared to healthy controls. It is therefore a more useful determinant of differences in immunogenicity in the context of paHIV than the 0.35 μg/ml cut-off. Whether this cut-off is clinically relevant is not known and therefore predictions about clinical efficacy should be made with caution.

Many other definitions of individual protection against pneumococcal disease or successful response to immunisation (both PPV and PCV) have been proposed. These have been described in the context of studies of vaccine immunogenicity and primary immunodeficiency diagnostics. They include various combinations of fold-changes in antibody level, absolute antibody concentration and proportion of serotypes for which there is a "successful response" [417, 432]. With respect to fold-change in antibody concentration, it has been shown that there is likely to be a ceiling to post immunisation antibody concentration. Individuals with high starting concentrations antibody (as was the case particularly with our adult healthy control cohort) may therefore not achieve a given fold-change despite intact immunity [417]. For this reason we have not used this as a read out of immunity in this study.

Using GMCs we have demonstrated significant differences in response to vaccination between HIV infected children and young healthy adults and between responses to individual vaccine serotypes. Again, the clinical relevance in terms of protection from IPD, either short term or long term is not known, but it seems likely that a higher antibody concentration at 1 month post immunisation would be associated with longer term protection.

In summary,we have shown pneumococcal serotype specific serum IgG is detectable at baseline, indicating naturally acquired pneumococcal immunity in both HIV infected and healthy control groups. Lower antibody concentrations to some serotypes and not others in the HIV infected group suggests that HIV may differentially impact upon naturally acquired immunity to individual serotypes. Antibody concentrations one month and 6 month post immunisation were higher in healthy controls for a substantial proportion of vaccine serotypes. After adjusting for baseline antibody concentrations, the responses to serotypes 6A, 6B and 23F were still found to be relatively impaired. For some serotypes lower resting memory B cell percentages were associated with lower post immunisation serotype specific IgG. Detectable viraemia was associated with lower post vaccination antibody concentrations for the majority of serotypes tested and, as with T and B cell phenotype, results indicate a larger proportion of life spent with undetectable viral load to be associated with higher post vaccination antibody levels, at least for some serotypes.

5.6 Conclusion

We have shown that PCV13 is immunogenic in children with paHIV, but that differences exist in the magnitude of the response to some vaccine serotypes. We have demonstrated that baseline antibody concentrations are lower for some serotypes and that higher antibody at baseline is associated with higher antibody levels at 1 month post immunisation. Detectable viraemia is an independent predictor of lower antibody responses for the majority of vaccine serotypes. In the absence of detectable viraemia, at least for some serotypes, lower resting memory B cell proportions are associated with impaired antibody response. The results of the previous chapter demonstrated detectable viraemia to be associated with marked abnormalities of the B cell compartment as well as a relationship between the proportion of life on suppressive HAART and the integrity of the B cell memory subsets.

Taken together with the results of this chapter it is clear that both the phenotype and function of B cells are impaired in the context of detectable viraemia in paHIV. It is also apparent that even when on suppressive HAART, a larger proportion of life spent with undetectable virus can potentially minimise irreversible loss of resting memory B cells, which are also associated with improved response to pneumococcal conjugate vaccine.

It is highly unlikely that the effects of paHIV on B cells alone are solely responsible for observed impairments in IgG response to PCV13. The next chapter will investigate cytokine responses to the CRM197 *in vitro* in order to help delineate to what extent disruption in the normal immune response to vaccine carrier protein might contribute to overall disruption of effective antibody response to PCV13.

Chapter 6: *In vitro* cytokine responses

6.1 Introduction

Protein polysaccharide conjugate vaccines are thought to enlist carrier protein specific T cell help to polysaccharide specific B cells and by doing so transform the antibody response from a T independent one to a T dependent one [155, 288, 435]. Through direct cell-cell contact polysaccharide specific B cells are subject to co-receptor signalling and local T cell cytokine production, which will determine their developmental fate including class-switching, affinity maturation and ultimately, memory cell vs. plasma cell lineage commitment [123, 136, 137, 436].

Dysregulation of circulating cytokines and *in vitro* T cell cytokine production are a well-recognised phenomenon in untreated HIV disease. These abnormalities, observed in both HIV infected adults and children, are not fully corrected by HAART [43, 437-440]. The nature of persistent abnormalities of T cell function and cytokine production, and how this might impact upon the antibody response to conjugate vaccines, remains under-investigated in children with paHIV.

This chapter describes a novel whole blood cytokine release assay used for assessing *in vitro* cytokine responses to CRM197 before and after vaccination with PCV13. By comparing the pattern of cytokine production in children with paHIV with healthy controls and relating it to serotype specific IgG production we aim to gain further insight in to the effects of HIV and HAART on the response to CRM197 and how this may (or may not) impact upon humoral responses to vaccine polysaccharide. By doing so we also hope to shed light on the mechanism of action of conjugate vaccines in this age group.

6.2 Chapter aims

i) To compare CRM197 specific cytokine responses at baseline between children with paHIV and healthy children and young adults using a whole blood cytokine release assay and a high sensitivity multiplex cytokine detection platform.

ii) To assess CRM197 specific cytokine release prior to and 1 month following immunisation compared to baseline in children with paHIV compared to healthy young adults.

iii) To investigate any association between CRM197 specific cytokine response and serotype specific antibody response to immunisation.

iv) To assess serum IL21 levels in children with paHIV compared to healthy children and healthy young adults.

v) To investigate any association between change in serum IL21 1 month post immunisation with serotype specific antibody responses to PCV13.

6.3 Statistical methods

For baseline comparisons, Chi^2 and Fisher's exact test were used to compare proportions. Mann-Whitney U test was used to compare baseline age and for contrasting baseline cell subset data between child and adult groups. Hodges-Lehman median differences with 95% confidence intervals were also calculated. For direct comparison of cell subsets from the two child groups, data was log transformed and compared using linear regression analysis to allow for adjustment for age and to assess age*group interactions. Mann-Whitney U test was used to compare time between vaccination and blood sampling between groups.

A stimulation index (SI) was calculated by dividing cytokine concentration in CRM197 stimulated well by the concentration measured when incubated with medium alone (negative control). Concentrations less than the lower limit of quantification (LLQ) were assigned a value of half the LLQ for the assay. Linear regression was used to investigate correlation between log transformed SI of each cytokine at baseline. One-way ANOVA was used to compare log transformed SI between groups at baseline, while repeated measures ANOVA (RMANOVA) was used to assess differences in cytokine production between vaccinated groups after vaccination. Chi^2 and Fisher's exact test were used to assess differences in proportions responding with different combinations of cytokines between groups at baseline and 1 month following immunisation, while McNemar's test was used to assess for differences in proportions within groups pre and post immunisation. Linear regression was used to investigate association between cytokine response (pre and post immunisation), lymphocyte subsets, clinical parameters, serum serotype specific IgG concentrations.

Linear regression and RMANOVA were similarly employed to compare serum IL21 concentrations within and between groups, before and after vaccination and to investigate associations between serum IL21 concentration and serotype specific IgG concentration.

6.4 Results

There was no loss to follow up. Cytokine release assay failed for one adult healthy control and one child healthy control (no cytokine detected in positive control well). These 2 subjects were therefore excluded from all analysis. 5 child healthy controls were recruited prior to the final modification to the whole blood assay protocol, these children were also therefore excluded from analysis.

6.4.1 *Baseline characteristics*

Baseline characteristics are shown in Table 22.

		CHC (n=24)		AHC (n=26)		CP (n=48)	
Sex	Female	13	(54.2)	15	(57.7)	21	(43.8)
	Male	11	(45.8)	11	(42.3)	27	(56.3)
Ethnicity	White	4	(16.7)	18	(69.2)	2	(4.2)
	Mixed	0	(0)	2	(7.7)	3	(6.3)
	Asian	0	(0)	4	(15.4)	2	(4.17)
	Black Caribbean	1	(4.2)	0	(0)	2	(4.17)
	Black African	10	(41.7)	0	(0)	38	(79.2)
	Other	9	(37.5)	2	(7.7)	1	(2.1)
Age/months		119	(31-159)	319	(245-359)	154	(12-209)
Lymphocyte subsets	lymph cells/μl	2187.5	(1688.5-3389.5)	1807.5	(1377 -2125)	2349.5	(1764-3308.5)
	CD3 cells/μl	1628.5	(1210.5- 2468)	1389	(1094- 1706)	1854.5	(1390.5-2518)
	CD3 %	71.1	(65.95- 76.95)	74.35	(70.1- 79.1)	75.45	(68.9-79.6)
	CD4 cells/μl	849.5	(700.5-1344.5)	794	(694-1061)	855	(525.5-1118)
	CD4 %	42.2	(35.2-46.45)	43.8	(39.8- 50.6)	34.55	(27.2-40.8)
	CD8 cells/μl	560	(398.5- 878.5)	497	(383- 557)	913.5	(659-1074.5)
	CD8 %	23.7	(21.3-27)	26.25	(22.2-28.9)	32.95	(28.9-44.5)
	CD19 cells/μl	471	(310.5-622.5)	257	(153- 326)	403	(266-570.5)
	CD19 %	18.5	(14.6-25.3)	12	(8.9-16.8)	17.3	(12.96-21.4)
	CD56 cells/μl	134.5	(90- 247)	172.5	(96-263)	119	(72-184.5)
	CD56 %	5.8	(2.8- 9.0)	9.4	(6.5-12.7)	4.35	(2.8-5.6)
Prev PCV7		8	(33.3)	0	(0)	7	(14.6)
Prev PPV		0	(0)	0	(0)	12	(25.0)
Nadir CD4%		-	-	-	-	13	(0-48)
Proportion of life undetectable		-	-	-	-	0.49	(0-0.98)
VL <50 copies/ml		-	-	-	-	34	(72.3)
Treated 1st year		-	-	-	-	7	(14.9)
Treated 1st 2 years		-	-	-	-	12	(25.3)

Table 22. Baseline characteristics for all study participants assessed for cytokine responses. Sex, ethnicity, HIV viral load status, treated in the first year of life, treated in the 1st 2 years of life, previous PCV7 and previous PPV are presented as number and percentage. Age, nadir CD4% and proportion of life undetectable are presented as median and range. Lymphocyte subsets are presented as median and interquartile range. (CHC= Child healthy control, AHC= Adult healthy control, CP = Child patient, VL = HIV viral load, PCV7 = 1-valent pneumococcal conjugate vaccine, PPV= pneumococcal polysaccharide vaccine)

In view of exclusion of 1 adult healthy control and 6 healthy children from the analysis, baseline characteristics were again compared using the same methods as in Chapter 4. The pattern of differences were as in Chapter 4 except for baseline CD3 absolute count being higher in the healthy children than healthy young adults (median difference= 279, 95%CI: 6-744, p= 0.04) and for CD8 percentage for which significant group and age effects were demonstrated rather than age*group interaction when comparing

children with paHIV to healthy children. CD8 percentages were higher in older children than younger children and higher in the HIV infected group than the HIV uninfected group.

Prior to this study, 2/24 (8%), 1/24 (4%) and 5/24 (21%) of healthy children had received 1,2 or 3 doses of PCV7 respectively as part of their routine immunisation schedule. Median time since last dose of PCV7 was 29.5 months (range = 18-99 months). 1/48 (2%), 2/48 (4%) and 5/48 (11%) children with paHIV had received 1,2 or 3 doses of PCV7 respectively. Median time since last dose of PCV7 was 16 months (range = 8-28 months). No adult healthy control had received PCV7 at any time and no study subject had received PCV13.

As in Chapter 5 there was no significant difference in time from immunisation with PCV13 to 1 month or 6 month blood sampling between the 2 vaccinated groups.

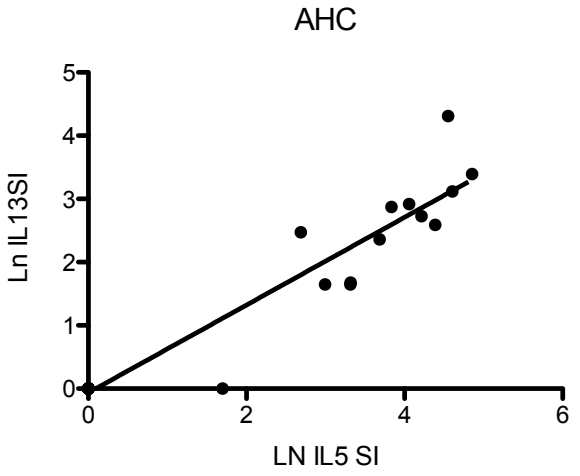
6.4.2 *Baseline CRM197 specific cytokine responses*

In vitro IFNγ, IL1β, IL5, IL10 and IL13 were measured in whole blood culture supernatants after 6 days of incubation with medium alone (negative control), CRM197 or anti-CD3 (positive control). A stimulation index (SI) was calculated by dividing cytokine concentration in CRM197 stimulated well by the concentration when incubated with medium alone. Concentrations less than the LLQ were assigned a value of half the LLQ. There were negligible differences in concentrations of IL1β between CRM stimulated and medium only wells, this cytokine was therefore not included in the analysis. Results for IFNγ, IL5, IL10 and IL13 are reported in Table 23.

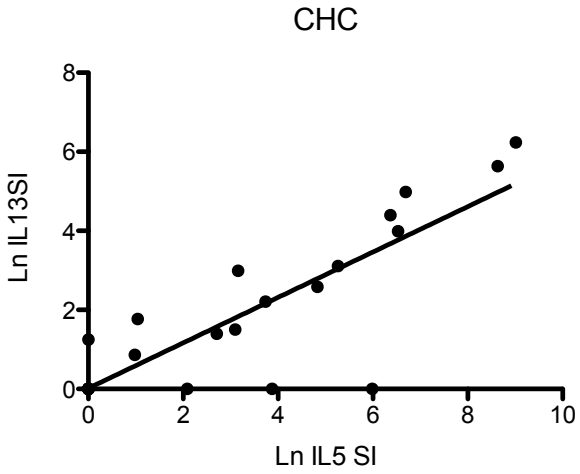
	Geometric mean SI (95% CI)			
	IFNγ	IL5	IL13	IL10
CHC	19.4 (7.9-46.8)	21.8 (6.2- 77.0)	6.0 (2.5-14.0)	2.0 (1.4-2.9)
AHC	11.0 (5.3-22.8)	6.4 (2.9-14.3)	3.4 (1.9-6.1)	1.5 (1.2-2.0)
CP	4.1 (2.4-7.1)	4.7 (2.3-9.5)	2.7 (1.7-4.4)	1.1 (1-1.1)

Table 23. Stimulation index (SI) for CRM197 stimulated cytokine production at baseline for children with paHIV, healthy children and healthy young adults. Results are reported as geometric mean SI (95% confidence interval). (CHC=child healthy controls, AHC= adult

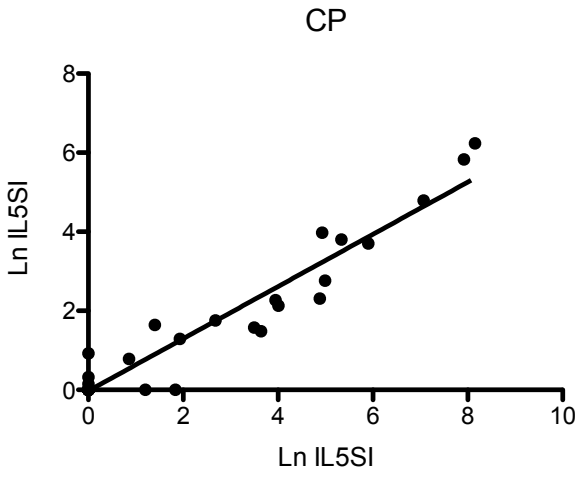
Linear regression was used to assess correlation between log transformed SI for each cytokine. IL13 and IL15 SI were very highly correlated for all 3 groups (AHC (R^2= 0.71, p<0.001), CHC (R^2= 0.91, p<0.001), CP (R^2= 0.94, p<0.001) as shown in Fig. 21. For this reason IL13 results were not analysed further and all subsequent analysis was therefore based on IFNγ, IL5 and IL10 production.



R^2= 0.71, p<0.001



R^2= 0.91, p<0.001



R^2= 0.94, p<0.001

Fig. 21. Correlation between log transformed stimulation index (SI) for each of the 3 studied groups at baseline (AHC= Adult healthy control, CHC = Child healthy control, CP = Child patient, LnSI = log stimulation index)

In vitro whole blood stimulation with CRM197 resulted in mixed cytokine production at baseline, which was lower in children with paHIV than healthy controls with a larger proportion of children being unresponsive for all 3 cytokines

There was a general trend of higher cytokine responses in healthy children compared to healthy adults, which were in turn higher than those in children with paHIV. One way ANOVA with Bonferroni correction was used to compare log transformed baseline SI between the 3 groups. Geometric mean SI were significantly lower in CP than CHC for IFNγ (p=0.005), IL5 (p=0.044) and IL10 (p<0.001). IL10 SI were significantly lower in CP than AHC (p=0.035).

The proportion of subjects classed as responding with a particular cytokine (SI > 2) at baseline is shown in Fig. 22A. Chi^2 or Fisher's exact test were used to assess difference in proportions of responders for each cytokine/cytokine combination. In both healthy groups a substantial proportion of individuals responded with IFNγ either alone or in combination with other cytokines. The proportion of children responding with all 3 cytokines (IFNγ,IL5,IL10) was significantly less for children with paHIV compared to healthy young adults (p=0.013) and healthy children (p <0.001) while the proportion of children unresponsive for all 3 cytokines was significantly more in children with paHIV than healthy young adults (p=0.037) and healthy children (p=0.010).

HAART treatment in the first two years of life is associated with higher baseline in vitro cytokine responses to CRM197 in children with paHIV

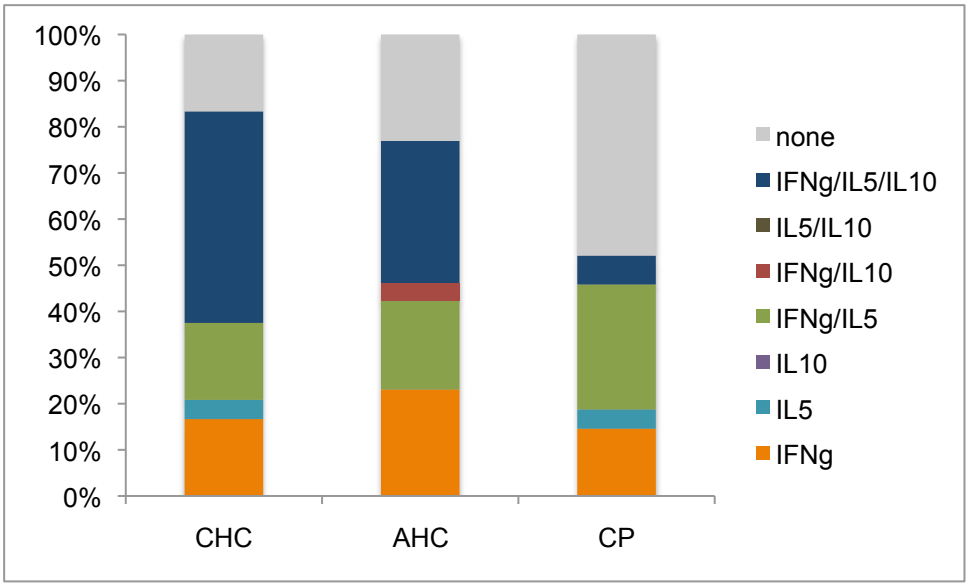
Associations between lymphocyte subsets/clinical parameters and log transformed baseline SI for IFNγ, IL5 and IL10 were assessed using linear regression. Univariate analysis showed younger age, treatment in the 1st year of life, treatment in the 1st two years of life, previous receipt of PCV7 and higher CD4 absolute count to be associated with higher SI (p<0.05). Multivariate analysis showed that after adjusting for age and CD4 count, treatment in the first 2 years of life remained a significant predictor of higher baseline SI for IFNγ and IL5 (p<0.05) with borderline significance for IL10 (p=0.05).

6.4.3 *Response following immunisation*

Results for both vaccinated groups at baseline and 1 month following immunisation are shown in Table 24 and Fig. 23.

Repeated measures ANOVA was used to assess differences in log transformed SI at baseline compared to 1 month post immunisation in the 2 vaccinated groups. Analysis was performed with and without including log transformed CD4 absolute count and log transformed baseline SI's as covariates. Main effects were significant for both group and time for all 3 cytokines. Therefore post hoc contrasts for simple effects were performed in all cases.

A



B

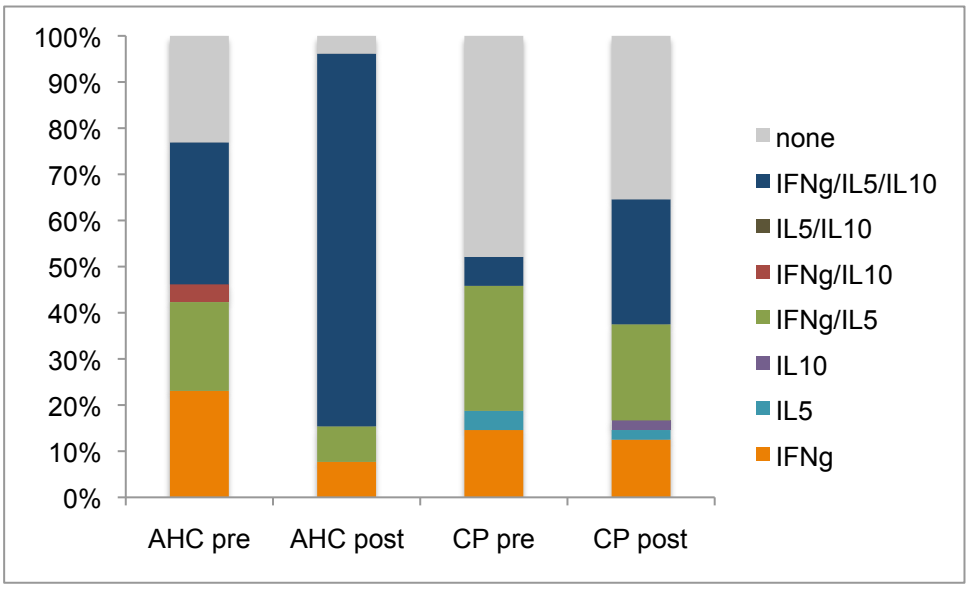


Figure 22. Proportion responding to CRM197 with *in vitro* cytokine SI >2 in children with paHIV and young healthy children and adults at baseline (A) and in children with paHIV and young healthy adults pre and post immunisation (B). At baseline, the proportion of children responding with all 3 cytokines was significantly less for children with paHIV compared to healthy young adults (p=0.013) and healthy children (p<0.001) while the proportion of children unresponsive for all 3 cytokines was significantly more in children with paHIV than healthy young adults (p=0.037) andhealthy children (p=0.010). The proportion responding with all 3 cytokines increased in both young healthy adults and children with paHIV (p< 0.01). The proportion with no response for any cytokine did not change significantly for either group. The proportion responding with all 3 cytokines 1 month post immunisation was significantly higher in young healthy adults than children with paHIV (p<0.001) and the proportion with no response for any cytokine were significantly lower (p=0.003).(CHC = child healthy control, AHC = adult healthy control, CP = child patient, SI = stimulation index, pre = pre immunisation, post = 1 month post immunisation)

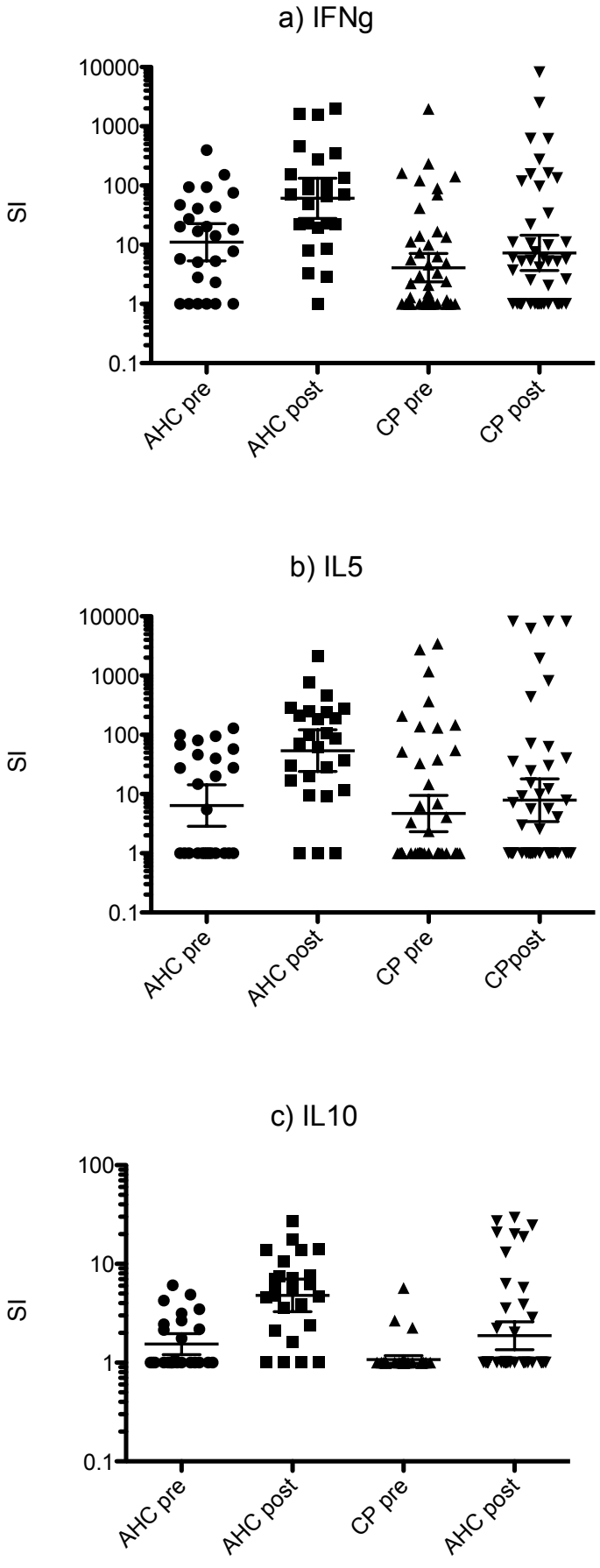


Figure 23. Stimulation index (SI) for a) IFNγ, b) IL5 and c) IL10 following *in vitro* stimulation of whole blood before and 1 month following immunisation with PCV13. Error bars indicate geometric mean SI and 95% confidence interval. Geometric mean SI significantly increased 1-month post immunisation compared to baseline for all 3 cytokines in young healthy adults (p<0.001) but only for IL10 in children with paHIV (p<0.001). Geometric mean SI were higher in young adults than children with paHIV for all 3 cytokines at 1 month post immunisation: IFNγ (p<0.001), IL5 (p=0.002), IL10 (p<0.001). These differences remained significant after adjusting for CD4 absolute count and baseline SI. (AHC = adult healthy control, CHC = child healthy control, pre = pre immunisation, post = 1 month post immunisation)

	IFNγ		IL5		IL10	
	pre	1 month	pre	1 month	pre	1 month
AHC	11.0 (5.3-22.8)	60.7 (27.8-132.6)	6.4 (2.9-14.3)	54.1 (24.0-121.8)	1.5 (1.2-2.0)	4.8 (3.3-7.0)
CP	4.1 (2.4-7.1)	6.5 (3.1-13.4)	4.7 (2.3-9.5)	7.9 (3.4-18.0)	1.1 (1-1.1)	1.9 (1.4-2.6)

Table 24. Stimulation index (SI) for CRM197 stimulated cytokine production at baseline and 1 month following immunisation for children with paHIV and healthy young adults. Results are reported as geometric mean SI (95% confidence interval). (CHC=child healthy controls, AHC= adult healthy controls, CP = child patients, pre = pre vaccination)

Cytokine responses increased post immunisation for all 3 measured cytokines in healthy young adults but only for IL10 in children with paHIV

Geometric mean SI significantly increased 1 month post immunisation compared to baseline for all 3 cytokines in young healthy adults (p<0.001) but only for IL10 in children with paHIV (p<0.001). Geometric mean SI were higher in young adults than children with paHIV for all 3 cytokines at 1 month post immunisation: IFNγ (p<0.001), IL5 (p=0.002), IL10 (p<0.001). These differences remained significant after adjusting for CD4 absolute count and baseline SI.

PCV13 immunisation results in enhanced in vitro cytokine responses 1-month post immunisation in healthy young adults, much more so than is observed in children with paHIV

The proportion of subjects classed as responding with a particular cytokine (SI > 2) at baseline and 1 month post immunisation are shown for both vaccinated groups in Fig. 22B. Chi^2 or Fisher's exact test were used to assess difference in proportions of responders for each cytokine/cytokine combination between groups at 1 month post immunisation and McNemar's test was used to compare proportions at baseline with those at 1 month within group.

The proportion responding with all 3 cytokines increased in both young healthy adults and children with paHIV (p< 0.01). The proportion with no response for any cytokine did not change significantly for either group although it should be noted that it started low and remained low in the young healthy adult group. The proportion responding with all 3 cytokines 1 month post immunisation was significantly higher in young healthy adults than children with paHIV (p<0.001) and the proportion with no response for any cytokine were significantly lower (p=0.003). It can be seen from comparing Fig 22A and Fig 22B that the cytokine response of children with paHIV 1 month post immunisation resemble those of healthy children pre immunisation.

Higher baseline in vitro cytokine responses to CRM197 are predictive of higher in vitro cytokine responses 1 month post immunisation in children with paHIV

Associations between lymphocyte subsets/clinical parameters and log transformed SI one month post immunisation for IFNγ, IL5 and IL10 were assessed using linear regression for children with paHIV. The only

parameter to remain significantly associated with higher post immunisation SI for all 3 cytokines was higher baseline SI (p<0.02).

There is no significant association between magnitude of in vitro cytokine response and serotype specific IgG production for any vaccine serotype in healthy controls or children with paHIV

Linear regression was used to assess association between *in vitro* cytokine responses 1 month post immunisation with serotype specific IgG responses. SI and antibody concentrations were log transformed.

No significant association was found between SI and IgG concentration for any vaccine serotype. This remained true after adjusting for baseline serotype specific IgG, age and CD4 count.

6.4.4 *Serum IL21*

A small proportion of study subjects had insufficient serum for testing. Only those with serum IL21 and pneumococcal ELISA results for baseline and 1 month post immunisation were included in the analysis. (CHC n=28, AHC n=26, CP n=43)

Linear regression was used to assess for correlation between log transformed T helper cell subsets and log transformed serum IL21 for each group and also to compare baseline GMC IL21 concentration, after adjusting for CD4 count. Repeated measures ANOVA was used to assess for change in serum IL21 concentration from baseline to one month post immunisation within group and to compare 1 month results between groups. Results are shown in Fig. 24.

Serum IL21 correlates with CD4 count in children with paHIV, however concentrations do not differ between the HIV infected children and young healthy adults and children and do not change 1 month post immunisation with PCV13

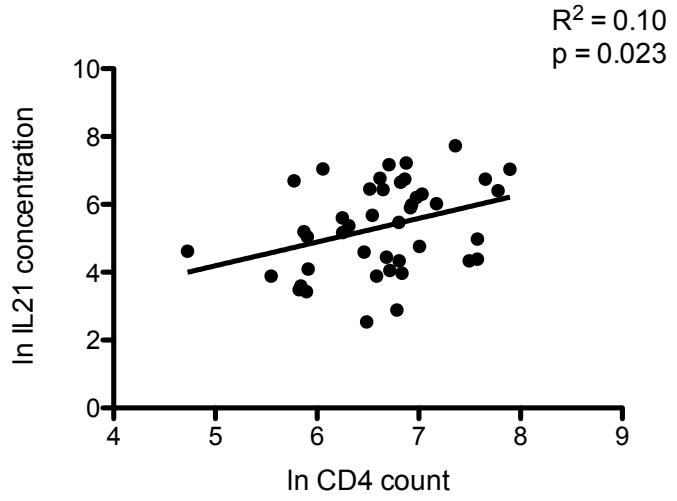
Serum IL21 concentrations significantly correlated with CD4 count for the CP group (R^2 = 0.10, p=0.023) but not for AHC and CHC. There was no difference in GMC IL21 between the 3 groups, which remained true after adjusting for CD4 count (CHC vs. AHC (p=0.317), CHC vs. CP (p=0.378), CP vs. AHC (p=0.818)). There was no significant change in IL21 concentration 1 month following immunisation in either CP or AHC group and no difference between groups at this time point.

Linear regression was used to assess for association between serum IL21 concentrations and serotype specific IgG responses to PCV13

Serum IL21 levels do not correlate with serological response to PCV13

There was no significant association between serum IL21 concentrations (pre or post immunisation) with serotype specific IgG concentrations for any vaccine serotype. This remained true after adjusting for baseline IgG concentrations and CD4 count.

A



B

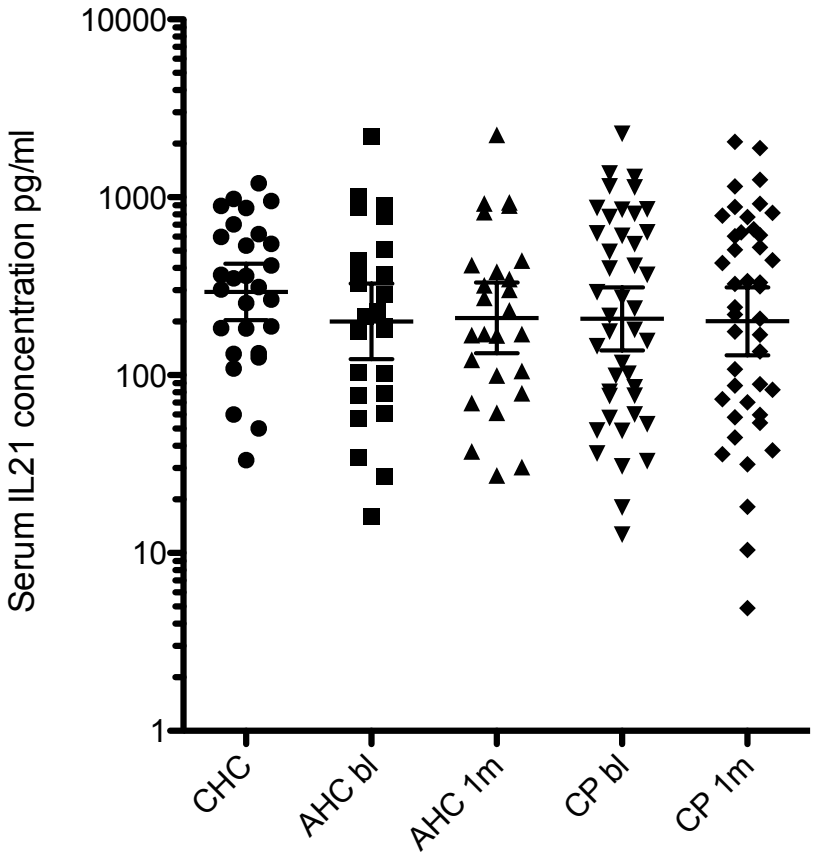


Figure 24. A. Correlation between log transformed CD4 count and serum IL21 concentration in children with paHIV at baseline. B. Serum IL21 concentrations at baseline and 1 month following immunisation (CHC = child healthy controls, AHC = adult healthy controls, CP = child patients, bl = basleine, 1m = 1 month post immunisation)

6.5 Discussion

This is the first report of *in vitro* whole blood cytokine responses to CRM197 before and after vaccination with a CRM197 containing conjugate vaccine. It is also the first to investigate cytokine responses to CRM197 resulting from PCV13 vaccination in healthy adults or children with paHIV.

We have shown that *in vitro* CRM197 stimulation results in mixed production of the cytokines IFNγ, IL10, IL5 and IL13 at baseline in children with paHIV and healthy controls. The magnitude of response and the proportion responding with all 4 cytokines was lower in HIV infected children. HAART treatment commenced in the first 2 years of life was associated with higher baseline cytokine responses.

Vaccination with PCV13 resulted in increased CRM197 specific production of all 4 cytokines in healthy controls but only IL10 in HIV infected children. Levels of cytokine production did not correlate with serotype specific antibody responses in either group. Despite this relatively disordered/impaired cytokine response to CRM197, PCV13 had comparable immunogenicity for the majority of serotypes when comparing HIV infected children with healthy adults (after adjusting for baseline antibody levels).

These results together suggest that children on HAART have relatively impaired T cell responses to CRM197, but that pre-existing immunity to CRM197 and the nature of CRM197 specific T cell responses elicited by PCV13 have relatively little impact on the magnitude of IgG responses to the majority of vaccine serotypes. However, variations in T cell response may well have implications for antibody function, class switching in general (class and subclass) and the establishment and longevity of immune memory, which may in turn impact upon clinical efficacy and the response to booster doses of the vaccine.

A number of studies have assessed CRM197 specific T cell responses to CRM197 containing vaccines in animal models [441], healthy human adults [293, 294, 442], young children [443] and infants [319, 444, 445] and in HIV infected adults [48]. 5 of the human studies involved administration of PCV7 [48, 293, 319, 444, 445] and 1 involved and experimental 9-valent vaccine [443]. All human studies examined *in vitro* CRM197 specific responses of PBMC using a variety of methods including detection of secreted cytokine in culture supernatants, lymphocyte proliferation, T cell ELISPOT, real time polymerase chain reaction (PCR) and microarray. Studies varied in terms of dosing schedule, timing of blood sampling and duration of *in vitro* cell culture.

Our finding of mixed whole blood IFNγ, IL5, IL13 and IL10 responses in healthy control adults and children at baseline are consistent with findings in the human studies which report mixed CRM197 specific cytokine responses in PBMC from healthy individuals without prior exposure to CRM197 [293, 294, 319, 443-445]. These responses were even present in 3 month old infants [319, 444, 445] and are most likely as a result of near universal exposure to multiple doses of diphtheria toxoid containing vaccines in infancy, childhood and adolescence. No study has directly compared CRM197 cytokine responses in healthy adults to those of healthy children. Our finding of a trend toward lower CRM197 specific cytokine responses in healthy adults

compared to healthy children may result from increased time since last exposure to diphtheria toxoid vaccination. A proportion of the healthy children were known to have prior exposure to CRM197 in the form of PCV7, which is likely to have contributed to their observed heightened responsiveness. The healthy children may also have been exposed to CRM197 in the context of other routine conjugate vaccines (although it was not possible to investigate this formally), which could also contribute to baseline responses.

To our knowledge only one study has examined CRM197 specific cytokine responses in the context of HIV infection [48]. As part of a randomised trial comparing prime boost PCV followed by PPV to PPV alone (not including healthy controls), 30 HIV infected adults with mean age of 45 were shown to have CRM197 specific lymphoproliferative responses, and IFNγ and IL2 production at baseline. Levels of IL5 and IL10 were also measured but not reported.

Despite differences in experimental design and methodology, these reports alongside ours clearly demonstrate that memory responses to CRM197 exist at baseline even in individuals unexposed to CRM197 and that a mixed pattern of cytokine production is characteristic. Our results show in addition that children with paHIV, although responding with a similarly mixed pattern of cytokines, do so less vigorously than healthy control children and respond in a way similar to adult healthy controls. One possible reason for this may be lower prior exposure to diphtheria toxoid or CRM197 containing vaccines. Vaccination records were incomplete for many of the HIV infected children so it was not possible to accurately assess this possibility although a previous audit including some of our cohort [446] found rates of vaccination with primary series of diphtheria vaccines to be relatively high with lower rates for preschool booster dose compared to regional reports for the population as a whole [447]. However, our finding that HAART commenced in the first 2 years of life was associated with higher baseline CRM197 specific cytokine responses suggests that HIV associated immune suppression is contributory, and that earlier HAART may preserve antigen specific T cell responses. Studies comparing lymphoproliferative responses to tetanus toxoid in older children with paHIV on HAART with those of healthy adults and children have demonstrated significantly impaired baseline responses to this classic recall antigen [195, 196, 448]. This gives support to the idea that CRM197 acts as a recall antigen reflecting prior exposure to diphtheria toxoid and for this reason, findings of impaired responses in HIV infected children on HAART mirror those observed when assessing *in vitro* cellular response to tetanus.

Reports of *in vitro* responses to CRM197 1 month following vaccination with CRM197 containing conjugate vaccines in both healthy adults and infants have demonstrated enhanced production of IFNγ, IL5 and IL13. Adult studies have also demonstrated enhanced IL10 production whereas infant studies found no increase in IL10 response [293, 294, 319, 445]. Consistent with these reports we have demonstrated significantly enhanced production of IL5, IL13, IL10 and IFNγ in young healthy adults 1 month following PCV13. The only published study of CRM197 specific cytokine responses in HIV infected individuals demonstrated that vaccination with PCV7 resulted in enhanced production of IFNγ but not IL5 or IL10 in HIV infected adults vaccinated with PCV7 [48]. Our finding of significantly increased production of only IL10 in children with paHIV contrasts with findings in HIV infected adults. Although it is possible that these differences may be a result of differences in immune phenotype in children with paHIV, it should be noted that the adult

experiments were performed on PBMC, and culture supernatants were harvested after 2 days rather than 6, which limits direct comparison.

The majority of healthy young adult in our study responded to CRM197 *in vitro* with all 3 cytokines assessed following vaccination. While the proportion responding with all 3 cytokines increased in the paHIV group, it was not to the extent of the healthy controls. This finding is reminiscent of findings of lower polyfunctional T cell cytokine responses to tuberculosis antigens in the context of HIV infection [449, 450], although it is important to note that we have not shown definitively demonstrated the source of cytokine production in our assay.

In their study of healthy neonatal/infant responses to PCV7, Van den Biggelaar et al found that while a significant proportion respond with mixed production of IL5/IL13 and IFNγ, a roughly equal proportion respond with only IL5/IL13 [319, 445]. This is in contrast with our healthy control and HIV infected groups, very few of whom respond with IL5/IL13 in isolation, the majority responding with a combination of IFNγ/IL5/IL13 together or IFNγ alone. This observation may well reflect the general skew of cytokine responses known to occur in infants, favouring production of Th2 cytokines such as IL5, IL13 and IL4, over Th1 cytokines such as IFNγ and IL12 [451-454].

We found no significant correlation between the magnitude of *in vitro* cytokine response to CRM197 either at baseline or 1 month post immunisation and serotype specific IgG responses to PCV13. Despite profound differences in *in vitro* responses to CRM197, for the majority of serotypes, PCV13 is similarly immunogenic in children with paHIV and young healthy adults, as assessed by serotype specific IgG after adjusting for baseline concentrations (reported in Chapter 5). This is at first somewhat surprising in view of the fact that CRM197 specific T cell help is considered to be fundamental to successful polysaccharide specific antibody response to CRM197 containing conjugate vaccines [155, 288, 295, 435].

A substantial proportion of CRM197 specific cytokine production 1 month post immunisation is likely to reflect, at least in part, up-regulation of cytokine production by and/or proliferation of antigen specific T helper cells. The magnitude of this response will be impacted upon by previous antigenic exposure and persistence of resultant immune memory. The effects of carrier priming on response to conjugate vaccines are complex. Previous exposure may enhance, suppress or have minimal effect on subsequent immune response to the polysaccharide depending on various factors including the nature of carrier protein and polysaccharide and antigen dose [455]. Furthermore, observations that the antibody responses to different pneumococcal polysaccharides vary despite uniformly vigorous T cell responses to CRM197 [293, 456] provide additional evidence that the relationship between the CRM197 specific immunity and polysaccharide specific immunity following conjugate vaccination is not a straightforward one.

Patterns of *in vitro* whole blood cytokine response might not directly reflect patterns of response in lymphoid tissue. Cytokine responses at 1 month post immunisation may not reflect T cell responses around the time of vaccination, which may be more likely to impact upon the development of the antibody secreting cells responsible for peak antibody concentrations one month post immunisation. They give little information on

direct cell/cell contact and co-receptor signalling. The pattern of T cell cytokine response may not impact upon the magnitude of cytokine response, but may still affect class switch recombination (IgG1, IgG2, IgG3, IgG4, IgA, IgE), B cell maturation (memory vs. plasma cell) and antibody function [138, 139, 457-459].

Taking the above in to consideration alongside recent reports that carbohydrate epitopes may be processed and presented alongside carrier protein in the context of MHCII and that carbohydrate specific T cells may also be playing a role, it is perhaps less surprising that we have not observed a direct relationship between CRM197 specific cytokine responses in whole blood and serotype specific IgG responses.

Van Den Biggelaar et al found there to be a correlation between CRM197 specific IFNγ production in PBMC and pneumococcal antibody responses in immunised infants [445]. This, alongside our finding of no correlation in young healthy adults and HIV infected children, raises the possibility that the response to conjugate vaccine in older children and adults may not be as dependent on CRM197 specific T cell help as it is in infants. This possibility is supported by findings in healthy and HIV infected adults suggesting that pneumococcal conjugate vaccines may not prime as well for memory responses in adults and offer little advantage, at least in terms of serotype specific IgG concentrations, over PPV [271, 460].

What then may we hypothesise from the results of the whole blood assay about the mechanism of action of CRM197 conjugated vaccines, how HIV impacts on this process and potential clinical implications of our findings?

Firstly it is worth considering the possible differences in the read out from our whole blood cytokine release assay as compared to that of published PBMC based assays. One advantage of the use of PBMC assays is the ability to standardise concentrations of PBMC in culture, thus facilitating direct comparison within and between study subjects. It can be argued that it only partially achieves this aim and that use of PBMC is a less accurate reflection of *in vivo* antigen response than whole blood. PBMC isolation, storage and processing can alter the ratio of monocytes to lymphocytes and affect cell function. Although allowing standardisation of mononuclear cell concentrations, variations in lymphocyte/monocyte proportions are often not taken into account. The preparation process removes many other cells and soluble factors, both stimulatory and inhibitory that may be influential in the immune response to a given antigen. For these reasons, whole blood stimulation, although it does not allow standardisation for mononuclear cell frequency, may be a more accurate reflection of *in vivo* immune responses [341, 461]. Indeed whole blood cytokine release assays may be highly standardised and have great clinical value as demonstrated by the QuantiFERON-TB® Gold In-Tube test used for diagnosis of latent TB infection [462]. A recent publication in the field of visceral leishmaniasis research has reported striking differences in the readout from whole blood assays compared to PBMC, which has shed interesting new light on disease immunopathogenesis [463].

It is clear that *in vitro* response to CRM197 in healthy controls, whether using PBMC or whole blood, involves mixed cytokine production including IFNγ, IL5, IL13 and IL10. Kamboj et al demonstrated that CRM197 specific proliferative and cytokine responses in PBMC are abrogated by depletion of $CD4^+$ T cells, suggesting that the PBMC assay results reflect predominately CRM197 specific $CD4^+$ T cell responses [293,

294]. It is therefore likely that our own findings in whole blood similarly reflect $CD4^+$ T cell responses in the main although we have not shown this to be the case experimentally and other potential sources of these cytokines should be taken in to consideration.

IFNγ is a pleiotropic cytokine, mainly produced by T lymphocytes and NK cells, but also known to be produced by APCs and B cells [171, 172, 464]. It is known for its role as a classical Th1 cytokine, promoting immune responses to certain viral and intracellular pathogens. It also impacts upon humoral immunity, affecting B cell development, antibody production and class switch recombination (both class and subclass) [250, 464-470].

Of the two cytokines IL5 and IL13, IL13 has a more established role in humoral immunity in humans, Il5 being more associated with eosinophil immunity. IL13 is mainly produced by T lymphocytes and NK cells, though it is also produced by basophils, eosinophils, dendritic cells and B cells [172, 471]. Its influence on B cell development, class switch and antibody production hold much in common with IL4 and it is classically considered an important Th2 cytokine involved in parasite immunity and allergic disease [471]. Our finding of highly correlated production of IL5 and IL13 in both healthy controls and children with paHIV, pre and post immunisation, is unsurprising. IL4/IL5/IL13 co-expression is a well-documented phenomenon, the genes for these cytokines are found clustered on chromosome 5 and regulation of their transcription has been shown to share many common elements (e.g. GATA3) [471-474].

IL10 is considered to be a cytokine with a generally more regulatory role. It is produced by activated T cells, B cells and monocytes [475] and, as with the other cytokines mentioned above, contributes to B cell development, class switch and antibody production [476-478]. It is being increasingly studied in the context of B cell immunity in relation to regulatory B cell function in health and disease [171, 172].

The balance of production of these cytokines resulting from exposure to CRM197 is therefore likely to contribute to the overall pattern of humoral response following conjugate vaccination. We have shown that despite dramatically different *in vitro* cytokine responses to CRM197, children with paHIV produce significant amounts of serotype specific IgG. In view of the altered cytokine milieu at the time of vaccination it is probable that there are differences in IgG subclass, IgA and IgM responses, antibody avidity/function and in the development of memory cells and long lived plasma cells, all of which warrant further investigation. Lower baseline immunity was associated with lower antibody concentrations at 1-month post immunisation. This, together with the factors discussed above may explain the lower efficacy of PCV observed in HIV infected children and will have implications with regards to the need for booster doses [271, 312, 327, 343].

IL21 is a relatively recently characterised member of the family of cytokines signalling via the common γ chain, which include IL2, IL4, IL7, IL9 and IL15. It is produced by T cells, in particular CD4+ T_{FH} and Th17 cells, as well as NKT cells [320, 479]. The IL21 receptor (IL21R) is expressed on a number of immune cell types including T cells, B cells, NK cells and dendritic cells resulting in pleiotropic effects. Of particular relevance to this study are its critical role in T_{FH} development, T_{FH} signalling to B cells, B cell maturation, class switch recombination and affinity maturation [458, 459, 479-489]. Furthermore, over the past 4 years

evidence has accumulated implicating IL21 in many aspects of HIV immunology. *In vitro* work in HIV infected adults has found IL21 to enhance and/or modulate cytotoxic $CD8^+$ and NK cell function and its potential immunotherapeutic properties are currently under investigation in animal models [479]. To our knowledge, there are no reports regarding IL21 in children with paHIV to date.

Studies in HIV infected adults have shown serum IL21 to be reduced, that levels correlate with $CD4^+$ T cell count and that HAART only partially restores levels to those observed in healthy controls [321, 322]. Ruffin et al demonstrated IL21R upregulation on B cells from HIV infected patients and showed an association with measures of immune activation, increased B cell apoptosis and reduced proportions of resting memory B cells [377]. A recent investigation of response to H1N1/09 vaccination in HIV infected adults and healthy controls, found successful antibody response to the vaccine to be associated with an increase in serum IL21 1 month post immunisation, expansion of circulating IL21 producing T_{FH}-like cells as well as HIV specific plasmablasts and memory B cells [47, 323]. This led us to investigate serum levels IL21 in children with paHIV pre and post immunisation with PCV13 and any association with antibody response to immunisation.

Consistent with adult studies described above, we found a moderate positive correlation between $CD4^+$ T cell count and serum IL21 concentration in the HIV infected cohort. In contrast with these studies, we found no difference in serum IL21 concentrations between children with paHIV and healthy young adults and children. This suggests either that HIV has less of an impact on circulating IL21 in children than in adults, or considering the fact that the majority of our cohort were on suppressive HAART, antiretroviral therapy may be more effective in restoring IL21 levels to normal in children with paHIV than in adults. A formal study comparing IL21 levels in untreated children to those on HAART and a longitudinal study of children commencing HAART therapy would serve to clarify this further.

We also found there to be no significant increase in serum IL21 1 month post immunisation with PCV13 either in healthy controls or children with paHIV and no correlation between IL21 concentrations and serotype specific IgG concentrations. This indicates that the mechanism of action of PCV13 in older children and adults may differ from that of H1N1/09 influenza vaccine. PCV13 consists of bacterial polysaccharide bound to CRM197 carrier protein with aluminium phosphate as an adjuvant [332], whereas the H1N1/09 influenza vaccine is a sub-unit (purified surface antigen) vaccine with a squalene based MF59 adjuvant [490]. Differences in the mechanism of action are therefore likely. Robust IgG responses to PCV13 in our vaccinated cohorts, in the absence of significant increase in serum Il21, lead us tentatively to suggest once more that the IgG response to PCV13 in adults and older children may not be entirely T dependent.

6.6 Conclusion

The resounding success of protein polysaccharide conjugate vaccines in protecting against invasive bacterial disease has transformed the epidemiology of severe infection in vaccinated infants and young children. In the case of pneumococcal conjugate vaccines, additional protection against nasopharyngeal carriage has, through the process of herd immunity, provided the additional benefit of reducing vaccine serotype invasive disease in older unvaccinated populations. Yet it is still debated whether vaccination with

PCV offers additional protection over and above that provided by PPV in at risk individuals outside of early childhood. PCV has proven immunogenicity and clinical efficacy in HIV adults and infants and young children [312, 327, 330], yet efficacy data in older HIV infected children on HAART is lacking. As universal access to HAART is achieved and the global paediatric population enters adolescence, it will be increasingly important to study vaccine immunogenicity in this unique cohort in order to ensure protection against vaccine preventable disease that lasts into adulthood.

We have demonstrated PCV13 to be immunogenic in a cohort of HIV infected children and adolescents despite altered *in vitro* T cell cytokine responses. As well as providing much needed vaccine immunogenicity data, we have shown persistent alterations in T cell function in older children on HAART, which has shed light on the possible mechanism of action of CRM197 conjugated vaccines in both HIV infected and uninfected older age groups.

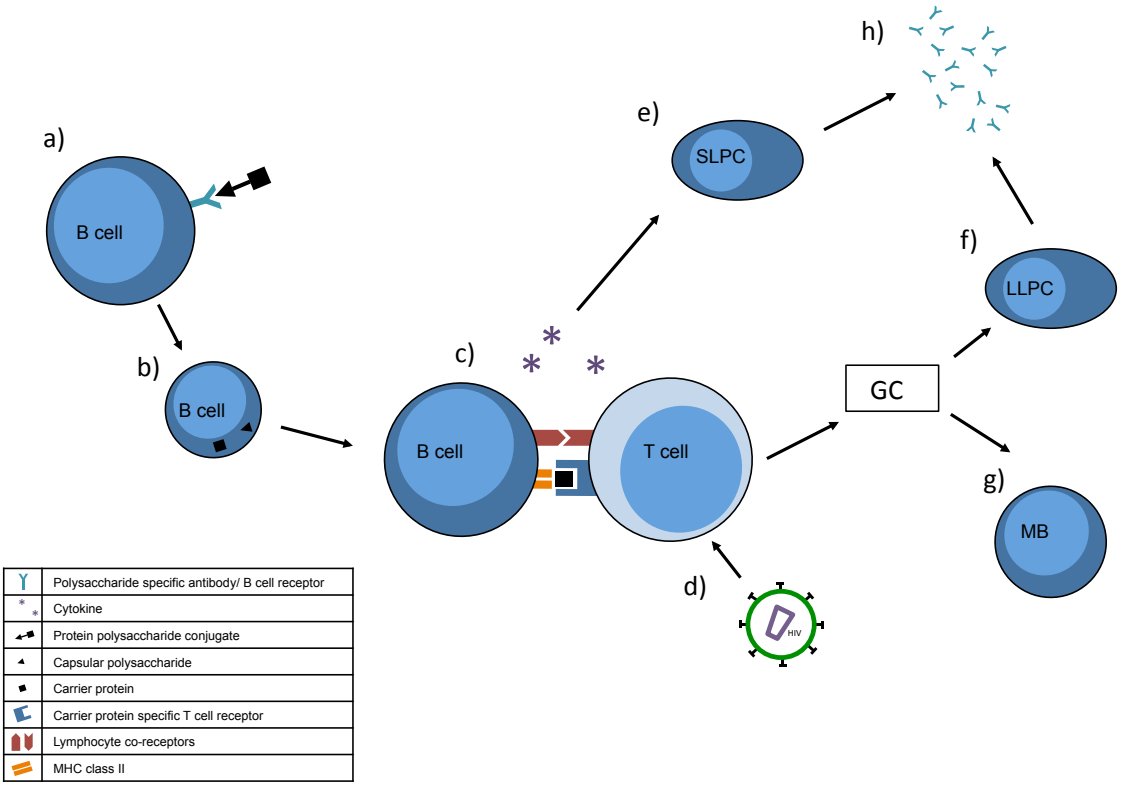


Figure 25. Illustration of the presumed mechanism of action of CRM-polysaccharide conjugate vaccine and the way in which HIV infection might influence antibody response with respect to T cell involvement. a) Vaccine polysaccharide is recognised by polysaccharide specific B cell receptor.b) Vaccine is processed by the B cell and c) the CRM protein epitopes are presented in the context of MHC II to CRM specific T cells. d) We have shown that HIV infection impacts upon the magnitude and balance of the mixed CRM specific T cell cytokine production. Depending on the nature of T cell help, polysaccharide specific B cells can either develop e) into short lived plasma cells, producing low affinity, unswitched antibody, or via the germinal centre reaction, resulting in f) long lived plasma cells, producing class switched, higher affinity antibody, or g) memory B cells. In chapter 4 we have demonstrated differences in memory B cell phenotype between children with HIV and healthy controls. In chapter 5 we have shown that quantitative antibody responses are relatively in tact for the majority of vaccine serotypes in children with paHIV on HAART. This leads us to hypothesise that impairments in T cell cytokine production described in this chapter, may have more of an impact on antibody function and subsequent B cell development, than on concentration of antibody in serum directly after immunisation.

Chapter 7: Overall discussion and final conclusion

7.1 Summary of study

This work set out to characterise circulating T and B cell phenotype in a cohort of children with paHIV in comparison to healthy controls and to relate any observed abnormalities in phenotype to clinical parameters such as HAART treatment history and detectable viraemia. Baseline pneumococcal serology was quantified alongside short and long term antibody responses to the newly licensed 13-valent pneumococcal conjugate vaccine and associations between the magnitude of serological response and lymphocyte phenotype were investigated. A novel whole blood assay was used to measure *in vitro* cytokine responses to vaccine carrier protein, before and after vaccination, so as to determine the possible contribution of altered antigen specific cytokine production to any observed impairment in serological response and to provide further information on the mechanism of action of conjugate vaccines outside of the infant period. Serum levels of IL21, a key cytokine involved in T cell help to B cells, were measured before and after immunisation, to evaluate any association between cytokine concentration and antibody response to immunisation. Finally, pneumococcal nasopharyngeal colonisation was assessed before and after vaccination to provide data on carriage serotypes in HIV infected children 4 years after introduction of universal infant immunisation in the UK as well as the potential interaction between carriage and vaccine responses.

7.2 Summary of findings

We have found significant abnormalities of B cell and T cell phenotype in children with paHIV when compared to healthy controls, specifically reduced proportions of memory B cell subsets and T_{FH}-like cells. These differences were present despite fully suppressive HAART in the majority of patients. Viraemia was associated with higher proportions of $CD21^-$ B cell populations including transitional cells, activated mature and tissue like/exhausted memory B cells. In relation to treatment history, results suggest that a greater proportion of life spent with undetectable viral load is associated with a more preserved lymphocyte phenotype.

Pneumococcal serotype specific serum IgG was detected at baseline, indicating naturally acquired pneumococcal immunity in both HIV infected and healthy control groups. Lower antibody concentrations to some serotypes and not others in the HIV infected group suggests that HIV may differentially impact upon naturally acquired immunity to individual serotypes. Antibody concentrations one month and 6 month post immunisation were higher in healthy controls for a substantial proportion of vaccine serotypes. After adjusting for baseline antibody concentrations, the responses to serotypes 6A, 6B and 23F were still found to be relatively impaired. For some serotypes lower resting memory B cell percentages were associated with lower post immunisation serotype specific IgG. Detectable viraemia was associated with lower post vaccination antibody concentrations for the majority of serotypes tested and, as with T and B cell phenotype, results indicate a larger proportion of life spent with undetectable viral load to be associated with higher post vaccination antibody levels, at least for some serotypes.

Nasopharyngeal colonisation was only detectable in the HIV infected group. Carried serotypes were not those included in PCV13, but a proportion of them are covered by the currently available PPV formulation. Although numbers were small, serotype 11A was isolated most frequently, at a higher rate than observed in recently reported UK carriage studies [215]. Lower than expected frequency of carriage prevented meaningful analysis of the interaction between carriage and serological response to vaccination.

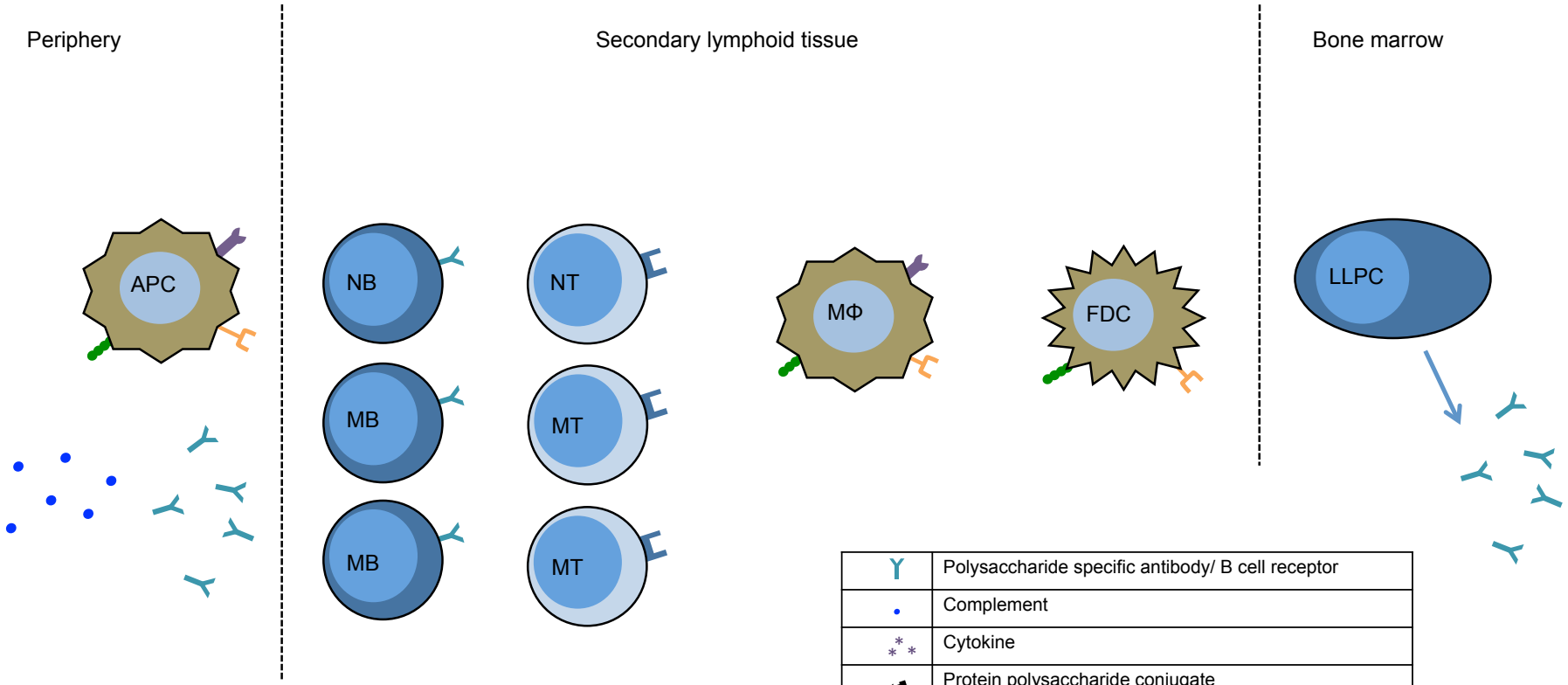
CRM197 specific cytokine production was present at baseline in both children with paHIV and healthy controls, most likely as a result of previous exposure to diphtheria toxoid vaccination. A higher proportion responding with all 4 measured cytokines and a greater magnitude of cytokine response was observed at baseline in healthy control groups. Immunisation resulted in enhanced *in vitro* cytokine responses in healthy controls, the majority responding with a combination of all 4 measured cytokines. Although an increase in the proportion responding with all 4 cytokines was also observed in the paHIV group, this proportion was significantly lower than that of the healthy control group. A substantial proportion remained unresponsive for all measured cytokines, and the increase in magnitude of cytokine response was significant for IL10 alone. *In vitro* cytokine production was not found to correlate with antibody response in either vaccinated group. These dramatic differences in *in vitro* cytokine production were in contrast to the relatively well-preserved serotype specific IgG responses described above.

As in recent studies in HIV infected adults [321, 322], serum IL21 was found to correlate with CD4 count in children with paHIV. However, in contrast to adult studies, there was no significant difference between IL21 concentrations in the HIV infected group and healthy controls, there was no detectable increase in serum IL21 1 month post immunisation and there was no correlation between serum IL21 and post vaccination antibody concentrations. Taken together with the *in vitro* cytokine data described above, it seems possible that the response to conjugate vaccines outside of the infant period may not be as dependent on T cell help as in the infant period when the response to both polysaccharide and carrier protein is predominately a primary rather than memory response.

7.3 Model of conjugate vaccine response outside of infant period and impact of paHIV

Considering our findings alongside existing published data and current opinion on T and B cell responses to T dependent, T independent and protein-polysaccharide conjugate antigens and current theories of HIV pathogenesis [40, 120, 123, 136, 137, 347, 491], I propose a summary model of the response to conjugate vaccines in antigen experienced children and adults (Figs. 26 and 27). The ways in which paHIV infection may impact on the various aspects of this immune response are highlighted.

Fig. 26 illustrates elements of the immune system likely to be involved in antibody responses to pneumococcal conjugate vaccine outside of the infant period.



	Polysaccharide specific antibody/ B cell receptor
	Complement
	Cytokine
	Protein polysaccharide conjugate
	Capsular polysaccharide
	Carrier protein
	Complement receptor
	Carrier protein specific T cell receptor
	Pattern recognition receptor
	Fc receptor
	Lymphocyte co-receptors
	MHC class II

Figure 26. Elements of immune system thought to be involved in the antibody responses to pneumococcal conjugate vaccine outside of the infant period. (APC= antigen presenting cell, MB = memory B cell, NB= naïve B cell, MT = memory T cell, NT = naïve T cell, FDC = follicular dendritic cell, LLPC = long lived plasma cell, mϕ= subcapsular sinus macrophape)

Prior to immunisation, baseline immunity to both carrier and polysaccharide will have been affected by paHIV infection. Our findings indicate that baseline polysaccharide antibody concentrations are lower for some serotypes and circulating memory B cell and T_{FH}-like cell proportions are reduced. The pattern of carrier protein specific T cell cytokine production is also altered. Published data indicates that FDC and lymphoid tissue architecture are also disrupted by HIV infection and that lymphoid tissue may actually be a sanctuary site for latent HIV or even ongoing viral replication [390, 397, 398, 406]. Therefore the stage is already set for suboptimal antibody responses. A proportion of these abnormalities may be prevented/corrected by HAART, with younger age at HAART commencement and sustained viral suppression possibly associated with a more intact immune armoury.

Fig. 27 summarises the immune mechanisms likely to be of importance in response to pneumococcal conjugate vaccine outside of the infant period and highlights points at which HIV may have a detrimental effect.

Following immunisation, lower circulating antibody levels may impact upon the efficiency of immune complex formation and consequently the efficiency by which professional APC bind vaccine antigen as well as antigen presentation to B cells by subcapsular sinus macrophages. Differences in the proportion and numbers of naive and memory carrier specific T cells would influence the rate and magnitude of T cell activation via APC. Similarly, a reduction in memory B cell proportions might affect the rate and likelihood of extrafollicular short-lived plasma cell development and the nature and efficiency of extrafollicular T/B cell interaction. An alteration in the balance of cytokine expression by extrafollicular T cells would impact upon short lived plasma cell development and also developmental programming of B cells destined for the germinal centre reaction. Once the germinal centre is established, dysregulated T_{FH} function is likely to affect the process of B cell affinity maturation and class switch recombination.

We (and others [120, 165, 169]) have found numbers of $CD21^{lo/-}$ B cells to be increased in untreated HIV. Interestingly, in studies of germinal centre dynamics in mice, it has been demonstrated that B cells play a key role in shuttling of antigen to FDC in GC via non-cognate binding of immune complexes. This binding is most likely mediated via CD21 (also known as CR2) [491-494]. These findings in mice, although not yet demonstrated in humans lead us to speculate that reduced CD21 expression on circulating B cells, and reduced antigen shuttling capacity, might be an important mechanism by which GC reaction and antibody responses are particularly impaired in HIV infected individuals.

Reduced antigen delivery, altered lymphoid architecture and altered FDC function are all likely to contribute to disruption of GC dynamics and the efficient production of class switched, affinity matured antibody. Developmental programming of B cells (into memory B cells or long lived plasma cells) and T cells (into memory T cell subsets) will also be influenced by altered B cell, T cell and FDC function and phenotype. Long term survival and effective functioning of memory B and T cells are also impaired while viral replication is ongoing, possibly even after viral suppression by HAART.

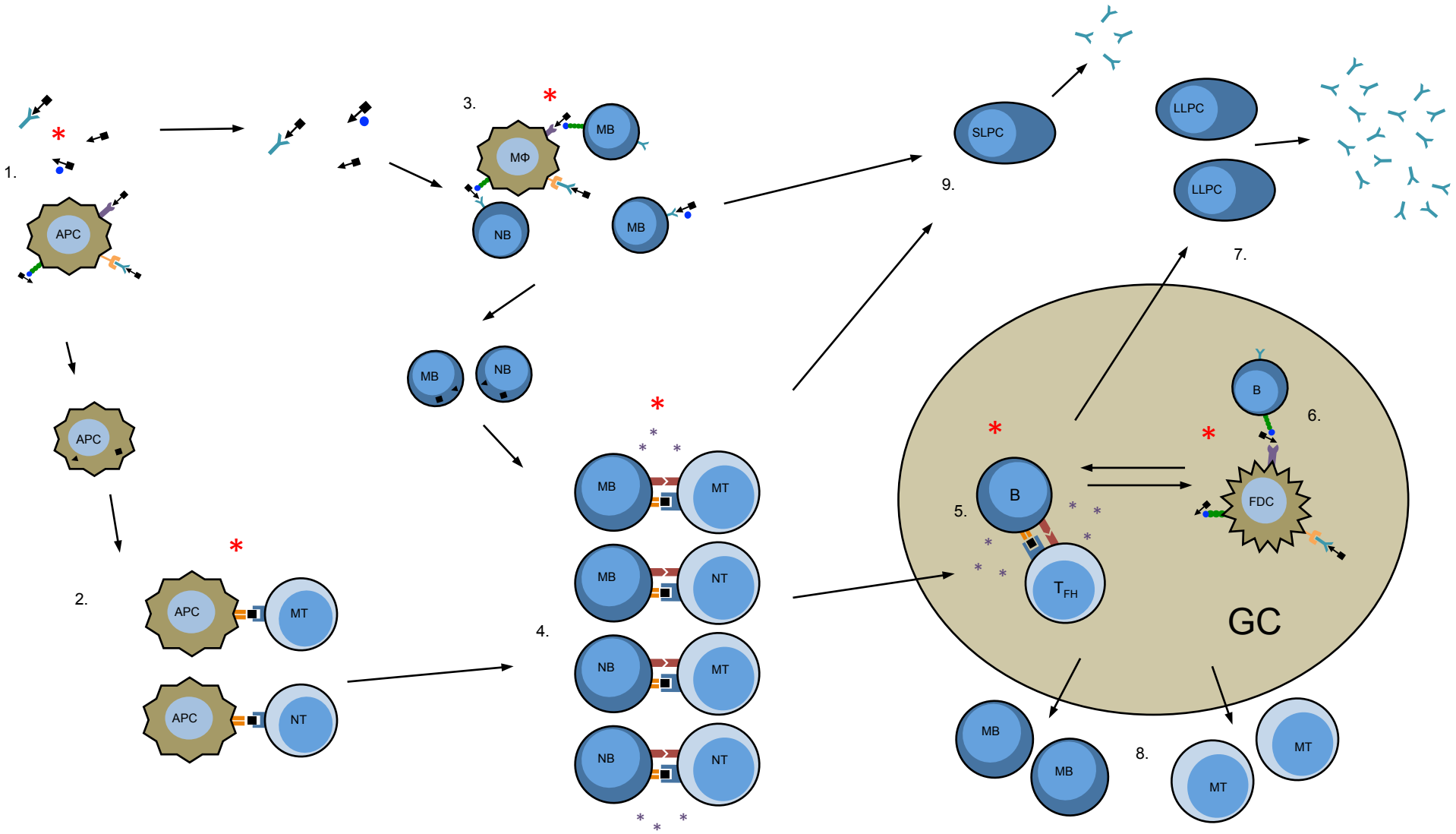


Figure 27. Summary of immune mechanisms likely to be of importance in response to pneumococcal conjugate vaccine outside of the infant period. **1.** Free vaccine is bound by PS specific antibody and complement and captured by APC via FcR, CR and PRR. **2.** Internalised vaccine is processed and carrier protein epitopes are presented in context of MHCII to carrier specific naïve and memory T cells. **3.** Free vaccine and immune complexes are transported to secondary lymphoid tissue via lymphatics where polysaccharide specific naïve and memory B cells can bind cognate antigen, facilitated by CR and SCS macrophages. **4.** Naïve and memory B cells internalise and process vaccine and present carrier protein epitopes to activated carrier protein specific naïve and memory T cells. Two way communication via coreceptors and cytokine signalling determines further developmental fate of lymphocytes. **5.** GC reaction is initiated. Carrier specific T_{FH} and PS specific B cells interact via TCR, coreceptors and cytokine signalling. B cells undergo affinity maturation and class switch through dynamic interaction with T_{FH} and FDC bound antigen. **6.** Antigen is transported to FDC via complement receptor on B cells. GC reaction results in **7.** long lived plasma cell development and **8.** memory B cell and possibly memory T_{FH} cell development. **9.** Short lived plasma cells may arise via

7.4 Clinical implications

What are the clinical implications of our findings in the context of published data on responses to conjugate vaccines and the immunology of paHIV?

When considering the implications for planning pneumococcal conjugate vaccination for children outside the infant period, our results suggest that the magnitude of IgG responses is related to pre-immunisation antibody concentrations, that the immunogenicity of each serotype varies and that viraemia has a negative effect. The latter finding would lead us to recommend that in order to optimise antibody response to PCV13, vaccination should be delayed until after viral load is undetectable on HAART. If HAART is not indicated then PCV13 may be given but the prospect of waning immunity and the potential benefit of booster doses once HAART is indicated and initiated should be considered. The fact that certain serotypes appear less immunogenic in HIV infected children, lead us to suggest that vaccine formulations with altered dosing of these particular serotypes be considered especially when planning and developing lower cost alternatives to existing conjugate vaccines specifically for resource poor countries, where the incidence of paHIV might be relatively high. This, however, is unlikely to be a feasible option and consideration should also be given to the possibility of providing booster doses in HIV infected children to increase response to less immunogenic serotypes, or even doubling the dose of PCV, as has been shown to increase immunogenicity in older adults [495].

Our finding that earlier HAART initiation and sustained viral suppression is associated with a more preserved immune phenotype and potentially enhanced immune response to vaccination lends support to current guidelines to commence HAART in all HIV infected infants irrespective of immune status [181, 186]. The possibility of raising the CD4 threshold for HAART initiation in older children is also supported. Alongside findings in adults with HIV infection [165, 182], our results do not support the suggestion of prolonged treatment interruption in later childhood or adolescence, at least in terms of preservation of the B cell compartment and humoral immunity.

Detectable pneumococcal carriage of non-PCV13 serotypes in a proportion of children with paHIV might be an early indicator of serotype replacement in the UK and highlights the importance of carriage studies in risk groups now and following introduction of future pneumococcal vaccines into national schedules [246].

7.5 Limitations

When considering the limitations of this study, it is worth considering those specific to individual aspects of the research and then some more general limitations.

As discussed in Section 4.3 the T and B cell flow panels were designed after consideration of existing published literature and current quality assurance measures. This resulted in reliable and accurate determination of cell surface phenotype and quantification of some relatively rare cell populations. The

inclusion of further markers of exhaustion, apoptosis and proliferation to both the T and B cell panel would have enabled further characterisation of identified subsets, although addition of further colours to each panel would bring its own limitations in terms of cost, fluorescence spillover effects and complexity of data analysis. The addition of further antibody isotypes to the B cell panel would have allowed more detailed phenotyping of B cell subsets in line with the most recent published data on memory B cells subsets in humans [146]. We assessed immune phenotype at baseline and 6 months post immunisation, allowing us to confirm that measured subsets were relatively stable over time and not significantly influenced in the long term by immunisation. Assessing phenotype at 1 month post immunisation may have revealed more short-term impacts of vaccination on T and B cell subsets as observed, for example, in HIV infected adults following immunisation with flu vaccine [47, 323]. As discussed in Section 4.5 measurement of circulating cell phenotype might not reflect cell populations in secondary lymphoid tissue. The ability to examine parallel samples from tissues such as lymph nodes, tonsils, spleen, MALT and bone marrow would provide valuable extra information to that effect. However, access to these sites is extremely limited, especially in children, therefore it is unlikely that studies of these compartments will be included in paediatric HIV research in the near future. Nevertheless, research in HIV infected adults and animal models of HIV are providing tissue data [46, 347, 387], which are of relevance to studies of peripheral blood in paediatric disease.

We employed current WHO reference lab methodology to assess serotype specific IgG for all 13 vaccine serotypes using relatively low sample volumes. This method is extremely well validated and guaranteed highly accurate ELISA results which can be directly compared with published data produced using the same protocols. As discussed in Sections 1.3.3 and 5.5, the IgG cut off for protection are not definitively established in HIV infected and uninfected older children and adults, which limits interpretation of our data. Bearing in mind the many other aspects of pneumococcal immunity that may be affected by HIV, it is unlikely that an absolute cut off antibody concentration will be established in immunologically heterogeneous HIV infected groups. *In vitro* assays such as the OPA are somewhat more likely to provide an accurate prediction in the context HIV, however it is still not a perfect model of *in vivo* pneumococcal immunity. In the future, in depth analysis using systems biology methods to integrate gene expression data, flow cytometry derived phenotypic information and read outs of quantitative and functional antigen specific cellular and humoral vaccine response, or “vaccinomics” will hopefully allow us to more accurately predict clinical protection in healthy and diseased groups [496]. We measured only serotype specific IgG in serum, measurement of additional immunoglobulin isotypes and subclass in serum and at mucosal sites might reveal additional differences in serological response to vaccine. We have not measured antibody avidity or response to a booster dose of the conjugate and/or polysaccharide vaccine, which would provide further knowledge of the impact of HIV on functional immunity following vaccination.

The relative merits of whole blood cytokine release assays compared to those using PBMC have been discussed in Section 6.5. It stands that information on cytokine release from PBMC may have yielded additional information, however one of the aims of this research was to perform multiple assays on minimal blood volume and we maintain our view that results of whole blood assays can yield useful, informative and replicable results which may reflect *in vivo* cellular responses more accurately. Concentrations of 5 cytokines were measured in culture supernatants on day 6 of incubation at baseline and one month following

immunisation. Early optimisation experiments indicated that cytokine concentrations were highest by day 6. Nevertheless, increasing the number of relevant measured cytokines (in particular IL21) and analysis of samples from earlier time points during incubation, while increasing the complexity of data analysis, may well have revealed further differences in the dynamics and balance of *in vitro* cytokine production. We measured antigen specific cytokine production in culture supernatants. The addition of further methods for assessment of antigen specific cellular cytokine responses including ELISPOT, flow cytometry and lymphocyte proliferation assays would also have provided further insights into the cellular sources of measured cytokine.

We have used standard WHO and HPA methodology to detect single pneumococcal carriage isolates. Coincident carriage of more than 1 serotype is a well-recognised phenomenon and the complex interplay between pneumococcus and other pathogenic viruses and bacteria in the nasopharynx is being increasingly investigated. Larger study numbers and the use of multiplexed carriage techniques and even determination of the nasopharyngeal microbiome as a whole in healthy and HIV infected individuals, before and after immunisation would be a useful addition to our carriage data.

One obvious general limitation is the use of young adult healthy controls rather than age matched healthy controls to compare vaccine responses. While an age matched group would have been desirable, we did not deem it either ethically or logistically feasible to recruit a large enough group of children of an appropriate age to receive a vaccine outside of the national schedule and licensed indication and willing to have blood sampling on 3 separate occasions. Bearing in mind that the most dramatic changes in immune phenotype and development occur in the first 2 years of life, the comparison between young adults and adolescents with paHIV is a valid one, yet we acknowledge that observed differences due to age can not be completely excluded.

The large amount of data acquired through the techniques employed in this study, while providing a wealth of information, has inevitably also resulted in multiple statistical comparisons. This can lead to an increase in the likelihood of Type 1 error (false positive). To minimise the impact of this, we have adhered strictly to a pre-formulated analysis plan. Where possible we have used statistical techniques that integrate corrections for multiple comparisons. We have maintained reporting of results at the $p<0.05$ significance level, while fully acknowledging that a more conservative cut-off might be considered more appropriate. We have reported confidence intervals as much as possible in order to allow assessment of the nature of observed differences and have been cautious in drawing strong conclusions when p values are closer to 0.05. Whenever possible we have used parametric tests and only used non-parametric tests when data violate parametric test assumptions.

We have assessed responses to pneumococcal conjugate vaccine in a cohort of older children with paHIV in the UK. The prevalence of paHIV in the UK is thankfully decreasing. Secondary to the phenomenal success of universal infant vaccination in reducing vaccine serotype pneumococcal disease at the population level through herd immunity, vaccine serotype disease is also disappearing in the UK. It may then be argued that knowledge of response to this vaccine is no longer relevant in this setting. However our findings are of relevance when considering the response to other conjugate vaccines, future higher valency pneumococcal

conjugate vaccines and other routine vaccines. Although disease due to vaccine serotypes is dwindling in the UK, our patient cohort frequently travel to countries that unfortunately do not have well established infant pneumococcal conjugate vaccination programmes and still have high levels of invasive disease due to vaccine serotypes. PCV13 in this respect might almost be therefore considered a “travel vaccine” in this context. We are fortunate to have had access to HAART and pneumococcal vaccines for children for a number of years in the UK. This is by no means the case in resource poor countries with much higher prevalence of paHIV and IPD. Our findings are still therefore very relevant for vaccination and treatment practice in these settings.

Due to logistic considerations we were unable to include a follow up time longer than 6 months post immunisation. Blood sampling extending to 1 year following immunisation may have revealed differences in the persistence of antibody response that were not apparent at the 6 month time-point. Finally, it is worth considering the ongoing debate surrounding the use of PPV vs. PCV in HIV infected older children and adults [271]. Our data suggests that the response to PCV in older children may be more polysaccharide dependent rather than carrier dependent. It would have been very interesting to include a cohort of children and adults immunised with PPV in the study to interrogate this possibility further and provide further evidence to guide practice.

7.6 Future directions

There are many avenues for further research based on findings from this and other published studies to date. With the recommendation of HAART for all HIV infected infants it would be of great interest to perform longitudinal studies combining B and T cell phenotypic analysis and information on B cell repertoire with readouts of vaccine induced immunity in HIV infected children on HAART compared to HIV exposed uninfected children (as controls). In doing so, the persistent effects of HIV on the developing immune system in the absence of detectable viraemia in peripheral blood could be clearly delineated. Furthermore it now seems clear that studies of B cell phenotype and function should be included in longitudinal studies on treatment interruption in older children with paHIV as well as in randomised trials investigating the possible benefits of lower thresholds for HAART initiation.

Higher valency pneumococcal conjugate vaccines are in the pipeline as are candidate protein vaccines. Early involvement of HIV infected individuals (and other risk groups) in immunogenicity studies such as ours would hopefully serve to expedite guideline and policy decision making for these groups allowing more rapid access to potentially life saving vaccines. Multiplex technology, such as the MSD technique used for cytokine detection in this study, can also be applied to antibody detection techniques and their validation for this purpose is currently under investigation [497]. The low sample volumes required for these assays make them very useful in paediatric studies and it is hoped that they will facilitate rapid, thorough and accurate analysis of serological response to vaccines in future immunogenicity studies in the paediatric age range. Moreover they allow analysis of cytokines and antibody in low volume specimens such as those obtained from mucosal sites including the nasopharynx. The importance of immune responses at mucosal sites to protection against pneumococcal carriage and invasive and non-invasive pneumococcal disease is clear and

analysis of the immune response at these sites may be an important aspect of future pneumococcal vaccine efficacy studies.

The dynamics of the immune response to vaccine antigens in the days, weeks and months following immunisation in HIV infected children compared to healthy controls could be assessed through blood sampling at earlier and later time points and by the inclusion of methods for detection of antigen specific T and B cells, including T and B cell ELISPOT and flow cytometry based methods employing fluorescently labelled MHC tetramers and antigen. It would be of interest to compare HIV infected groups with others that have increased susceptibility to pneumococcal disease including children with sickle cell disease, those with bone marrow transplant and chemotherapy induced immunosuppression, CVID patients and children with monogenic mutations associated with invasive pneumococcal disease (MyD88/IRAK4 deficiency). Indeed, the susceptibility of MyD88/IRAK4 deficient children to invasive pneumococcal disease and abnormalities in B cell phenotype of a similar nature to those observed in HIV [498], suggests that these innate pathways might also be of relevance in paHIV, it will therefore be important to include analysis of these and other innate signalling pathways in future work. Studies of pneumococcal immunity (including gene expression data) in otherwise healthy children with no known immune deficiency who nevertheless develop invasive pneumococcal disease, warrants further investigation in the hope that insights into the failings of an apparently intact immune system would allow more focussed investigation of the more generalised immunological disorder characteristic of HIV infection.

The lack of an appropriate murine model for HIV infection has significantly hampered research efforts to date. Humanised murine models have recently allowed murine HIV infection that mirrors many aspects of human HIV infection [499]. It is hoped that these will provide new insights into the HIV disease process that will mirror the wealth of information on T and B cell immunity and vaccine immunogenicity that studies in mice have provided to date.

Immunological research often involves the study of the T cell or B cell immunity in isolation. This project set out to try and integrate analysis of both T cell and B cell immunity in children with paHIV. Although the importance of B cells in HIV immunology has been somewhat sidelined by research into T cell immunology, it is now increasingly recognised that a knowledge of both T and B cell immunity is of importance when investigating the response to not only vaccine antigens, but also other potential infectious pathogens and HIV itself. In addition and in light of observations consistent with ongoing immune activation with wide ranging consequences in HIV infected patients despite suppressive HAART, the potential immune regulatory role of B cells seems likely to be a key area for future research [407].

7.7 Final conclusion

It is now 100 years since the first trial of a candidate pneumococcal vaccine [280]. Since that time, progressive improvements in vaccine technology and a greater understanding of vaccine induced protection have allowed the almost complete elimination of vaccine serotypes invasive disease in populations with high levels of vaccination coverage. Conjugate vaccines have been a resounding success in protecting vulnerable

infants against invasive bacterial disease and much is now known about immune mechanisms of conjugate vaccine response in this age group. Despite these advances, invasive pneumococcal disease continues to be a problem for other risk groups such as the elderly and HIV infected older children and adults. The potential role for conjugate vaccines, and the characteristics of the immune response in these groups is subject to ongoing research, but it is likely that they may provide additional benefits over and above that provided by existing polysaccharide vaccines. The establishment of *in vitro* correlates of vaccine-induced protection is an urgent priority and will hopefully expedite development and licensing of novel and improved vaccine candidates. Studies of immunogenicity in individual risk groups may also contribute to directed vaccine discovery and development aimed at protecting those with differing reasons for increased disease susceptibility.

It is 30 years since the HIV pandemic started to take hold. The development of HAART, the success of PMTCT strategies and the success of basic methods in preventing horizontal onward transmission have made dramatic differences in the epidemiology of the infection and it is now possible to almost guarantee that the infant of an HIV infected mother will not be infected (at least in the resource rich setting). Nevertheless, it is still the case that approximately 1000 HIV infected children are born per day, predominately in sub-Saharan Africa, an area where rates of invasive pneumococcal disease are also at their highest. With access to HAART these children have the potential to survive in to adulthood. We hope that this research will provide a piece of the jigsaw that will inform HAART and vaccination practices in this particularly vulnerable group and that optimal treatment in childhood might prevent the immune system being potentially scarred for life. Knowledge of the impact of HIV on the developing immunity will be key to ensuring that children with paHIV can live a long and healthy life, protected from vaccine preventable disease.

References

1. Ammann AJ, Wara DW, Cowan MJ. Pediatric acquired immunodeficiency syndrome. *Ann N Y Acad Sci* 1984;**437**:340-9.
2. Scott GB, Buck BE, Leterman JG, Bloom FL, Parks WP. Acquired immunodeficiency syndrome in infants. *N Engl J Med* 1984;**310**:76-81.
3. UNAIDS. Together we will end AIDS. 2012 [Accessed October 2012]; Available from: http://www.unaids.org/en/resources/campaigns/togetherwewillendaids/unaidsreport/.
4. Kourtis AP, Lee FK, Abrams EJ, Jamieson DJ, Bulterys M. Mother-to-child transmission of HIV-1: timing and implications for prevention. *Lancet Infect Dis* 2006;**6**:726-32.
5. Townsend CL, Cortina-Borja M, Peckham CS, et al. Low rates of mother-to-child transmission of HIV following effective pregnancy interventions in the United Kingdom and Ireland, 2000-2006. *AIDS* 2008;**22**:973-81.
6. Paintsil E, Andiman WA. Update on successes and challenges regarding mother-to-child transmission of HIV. *Curr Opin Pediatr* 2009;**21**:94-101.
7. Goetghebuer T, Haelterman E, Le Chenadec J, et al. Effect of early antiretroviral therapy on the risk of AIDS/death in HIV-infected infants. *AIDS* 2009;**23**:597-604.
8. Patel K, Hernan MA, Williams PL, et al. Long-term effectiveness of highly active antiretroviral therapy on the survival of children and adolescents with HIV infection: a 10-year follow-up study. *Clin Infect Dis* 2008;**46**:507-15.
9. Brady MT, Oleske JM, Williams PL, et al. Declines in mortality rates and changes in causes of death in HIV-1-infected children during the HAART era. *J Acquir Immune Defic Syndr* 2010;**53**:86-94.
10. Judd A, Doerholt K, Tookey PA, et al. Morbidity, mortality, and response to treatment by children in the United Kingdom and Ireland with perinatally acquired HIV infection during 1996-2006: planning for teenage and adult care. *Clin Infect Dis* 2007;**45**:918-24.
11. Violari A, Cotton MF, Gibb DM, et al. Early antiretroviral therapy and mortality among HIV-infected infants. *N Engl J Med* 2008;**359**:2233-44.
12. Walker AS, Mulenga V, Ford D, et al. The impact of daily cotrimoxazole prophylaxis and antiretroviral therapy on mortality and hospital admissions in HIV-infected Zambian children. *Clin Infect Dis* 2007;**44**:1361-7.
13. Song R, Jelagat J, Dzombo D, et al. Efficacy of highly active antiretroviral therapy in HIV-1 infected children in Kenya. *Pediatrics* 2007;**120**:e856-61.
14. Puthanakit T, Aurpibul L, Oberdorfer P, et al. Sustained immunologic and virologic efficacy after four years of highly active antiretroviral therapy in human immunodeficiency virus infected children in Thailand. *Pediatr Infect Dis J* 2007;**26**:953-6.
15. Prendergast A, Tudor-Williams G, Jeena P, Burchett S, Goulder P. International perspectives, progress, and future challenges of paediatric HIV infection. *Lancet* 2007;**370**:68-80.
16. Hall SM, Glickman M. The British Paediatric Surveillance Unit. *Arch Dis Child* 1988;**63**:344-6.
17. NSHPC. NSHPC website. [Accessed October 2012]; Available from: http://www.nshpc.ucl.ac.uk/.
18. CHIPS website. [Accessed October 2012]; Available from: http://www.chipscohort.ac.uk/default.asp.
19. Gibb DM, Duong T, Tookey PA, et al. Decline in mortality, AIDS, and hospital admissions in perinatally HIV-1 infected children in the United Kingdom and Ireland. *BMJ* 2003;**327**:1019.
20. Foster C, Judd A, Tookey P, et al. Young people in the United Kingdom and Ireland with perinatally acquired HIV: the pediatric legacy for adult services. *AIDS Patient Care STDS* 2009;**23**:159-66.
21. Doerholt K, Duong T, Tookey P, et al. Outcomes for human immunodeficiency virus-1-infected infants in the United kingdom and Republic of Ireland in the era of effective antiretroviral therapy. *Pediatr Infect Dis J* 2006;**25**:420-6.
22. ICTV website. ICTV 2011 taxonomy. 2011 [Accessed October 2012]; Available from: http://ictvonline.org/virusTaxonomy.asp?version=2011.
23. Barre-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983;**220**:868-71.
24. Levy JA, Hoffman AD, Kramer SM, et al. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science* 1984;**225**:840-2.
25. Gallo RC, Salahuddin SZ, Popovic M, et al. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* 1984;**224**:500-3.
26. Coffin J, Haase A, Levy JA, et al. Human immunodeficiency viruses. *Science* 1986;**232**:697.
27. de Silva TI, Cotten M, Rowland-Jones SL. HIV-2: the forgotten AIDS virus. *Trends Microbiol* 2008;**16**:588-95.
28. Valadas E, Franca L, Sousa S, Antunes F. 20 years of HIV-2 infection in Portugal: trends and changes in epidemiology. *Clin Infect Dis* 2009;**48**:1166-7.

29. HPA. United Kingdom new HIV diagnoses to the end of December 2011. [Accessed October 2012]; Available from: http://www.hpa.org.uk/webc/HPAwebFile/HPAweb_C/1237970242135.
30. Haile-Selassie H, Masters J, Tookey P. HIV-2 infection in pregnant women living in the UK/ Ireland: data from national surveillance 1997–2010. Poster presentation. 17th Annual Conference of the British HIV Association. *HIV Medicine* 2011;**12**:30.
31. Sattentau QJ, Moore JP. The role of CD4 in HIV binding and entry. *Philos Trans R Soc Lond B Biol Sci* 1993;**342**:59-66.
32. Deng H, Liu R, Ellmeier W, et al. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 1996;**381**:661-6.
33. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 1996;**272**:872-7.
34. Noursadeghi M, Katz DR, Miller RF. HIV-1 infection of mononuclear phagocytic cells: the case for bacterial innate immune deficiency in AIDS. *Lancet Infect Dis* 2006;**6**:794-804.
35. Coleman CM, Wu L. HIV interactions with monocytes and dendritic cells: viral latency and reservoirs. *Retrovirology* 2009;**6**:51.
36. Levy JA. HIV pathogenesis: 25 years of progress and persistent challenges. *AIDS* 2009;**23**:147-60.
37. Gringhuis SI, van der Vlist M, van den Berg LM, et al. HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells. *Nat Immunol* 2010;**11**:419-26.
38. Murphy K, Travers P, Walport M. Janeway's Immunobiology. 7th ed. New York: Garland Science; 2008. Chapter 1: Basic Concepts in Immunology. The effector mechanisms of adaptive immunity p. 30-4.
39. Sallusto F, Lanzavecchia A. Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity. *Eur J Immunol* 2009;**39**:2076-82.
40. Moir S, Chun TW, Fauci AS. Pathogenic mechanisms of HIV disease. *Annu Rev Pathol* 2011;**6**:223-48.
41. Gougeon ML. Apoptosis as an HIV strategy to escape immune attack. *Nat Rev Immunol* 2003;**3**:392-404.
42. Sodora DL, Silvestri G. Immune activation and AIDS pathogenesis. *AIDS* 2008;**22**:439-46.
43. Mosmann TR. Cytokine patterns during the progression to AIDS. *Science* 1994;**265**:193-4.
44. Clerici M, Shearer GM. The Th1-Th2 hypothesis of HIV infection: new insights. *Immunol Today* 1994;**15**:575-81.
45. Clerici M, Shearer GM. A TH1-->TH2 switch is a critical step in the etiology of HIV infection. *Immunol Today* 1993;**14**:107-11.
46. Lindqvist M, van Lunzen J, Soghoian DZ, et al. Expansion of HIV-specific T follicular helper cells in chronic HIV infection. *J Clin Invest* 2012;**122**:3271-80.
47. Pallikkuth S, Parmigiani A, Silva SY, et al. Impaired peripheral blood T-follicular helper cell function in HIV-infected nonresponders to the 2009 H1N1/09 vaccine. *Blood* 2012;**120**:985-93.
48. Rabian C, Tschope I, Lesprit P, et al. Cellular CD4 T Cell Responses to the Diphtheria-Derived Carrier Protein of Conjugated Pneumococcal Vaccine and Antibody Response to Pneumococcal Vaccination in HIV-Infected Adults. *Clin Infect Dis* 2010;**50**:1174-83.
49. Prendergast A, Prado JG, Kang YH, et al. HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells. *AIDS* 2010;**24**:491-502.
50. ElHed A, Unutmaz D. Th17 cells and HIV infection. 2010.
51. Murphy K, Travers P, Walport M. Janeway's Immunobiology. 7th ed. New York: Garland Science; 2008. Chapter 1: Basic Concepts in Immunology. Principles of innate and adaptive immunity p. 9-10.
52. Pantaleo G, Fauci AS. Tracking HIV during disease progression. *Curr Opin Immunol* 1994;**6**:600-4.
53. Dion ML, Poulin JF, Bordi R, et al. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. *Immunity* 2004;**21**:757-68.
54. Su L, Kaneshima H, Bonyhadi M, et al. HIV-1-induced thymocyte depletion is associated with indirect cytopathogenicity and infection of progenitor cells in vivo. *Immunity* 1995;**2**:25-36.
55. Wilkins BS, Davis Z, Lucas SB, Delsol G, Jones DB. Splenic marginal zone atrophy and progressive CD8+ T-cell lymphocytosis in HIV infection: a study of adult post-mortem spleens from Cote d'Ivoire. *Histopathology* 2003;**42**:173-85.
56. Lederman MM, Margolis L. The lymph node in HIV pathogenesis. *Semin Immunol* 2008;**20**:187-95.
57. Brenchley JM, Schacker TW, Ruff LE, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 2004;**200**:749-59.
58. van Grevenynghe J, Halwani R, Chomont N, et al. Lymph node architecture collapse and consequent modulation of FOXO3a pathway on memory T- and B-cells during HIV infection. *Semin Immunol* 2008;**20**:196-203.
59. Hong JJ, Amancha PK, Rogers K, Ansari AA, Villinger F. Spatial alterations between CD4(+) T follicular helper, B, and CD8(+) T cells during simian immunodeficiency virus infection: T/B cell homeostasis, activation, and potential mechanism for viral escape. *J Immunol* 2012;**188**:3247-56.

60. Mattapallil JJ, Douek DC, Hill B, et al. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 2005;**434**:1093-7.
61. Mueller BU, Sei S, Anderson B, et al. Comparison of virus burden in blood and sequential lymph node biopsy specimens from children infected with human immunodeficiency virus. *J Pediatr* 1996;**129**:410-8.
62. Stiehm ER, Ochs HD, Winkelstein JA. Immunologic Disorders of Infants and Children. In: J F, editor. 5th ed. Philadelphia: Elsevier; 2004. Chapter 22: The physiologic immunodeficiency of immaturity. 687-740.
63. Prendergast AJ, Klenerman P, Goulder PJ. The impact of differential antiviral immunity in children and adults. *Nat Rev Immunol* 2012;**12**:636-48.
64. Goulder PJ, Prendergast AJ. Approaches towards avoiding lifelong antiretroviral therapy in paediatric HIV infection. *Adv Exp Med Biol* 2011;**719**:25-37.
65. Chakraborty R. HIV-1 infection in children: a clinical and immunologic overview. *Curr HIV Res* 2005;**3**:31-41.
66. Clerici M, Saresella M, Colombo F, et al. T-lymphocyte maturation abnormalities in uninfected newborns and children with vertical exposure to HIV. *Blood* 2000;**96**:3866-71.
67. Nielsen SD, Jeppesen DL, Kolte L, et al. Impaired progenitor cell function in HIV-negative infants of HIV-positive mothers results in decreased thymic output and low CD4 counts. *Blood* 2001;**98**:398-404.
68. Embree J, Bwayo J, Nagelkerke N, et al. Lymphocyte subsets in human immunodeficiency virus type 1-infected and uninfected children in Nairobi. *Pediatr Infect Dis J* 2001;**20**:397-403.
69. Gesner M, Papaevangelou V, Kim M, et al. Alteration in the proportion of CD4 T lymphocytes in a subgroup of human immunodeficiency virus-exposed-uninfected children. *Pediatrics* 1994;**93**:624-30.
70. Bunders M, Thorne C, Newell ML, European Collaborative S. Maternal and infant factors and lymphocyte, CD4 and CD8 cell counts in uninfected children of HIV-1-infected mothers. *AIDS* 2005;**19**:1071-9.
71. Bunders MJ, Bekker V, Scherpbier HJ, et al. Haematological parameters of HIV-1-uninfected infants born to HIV-1-infected mothers. *Acta Paediatr* 2005;**94**:1571-7.
72. Rich KC, Siegel JN, Jennings C, Rydman RJ, Landay AL. Function and phenotype of immature CD4+ lymphocytes in healthy infants and early lymphocyte activation in uninfected infants of human immunodeficiency virus-infected mothers. *Clin Diagn Lab Immunol* 1997;**4**:358-61.
73. Kuhn L, Coutsoudis A, Moodley D, et al. Interferon-gamma and interleukin-10 production among HIV-1-infected and uninfected infants of HIV-1-infected mothers. *Pediatr Res* 2001;**50**:412-6.
74. Hygino J, Lima PG, Filho RG, et al. Altered immunological reactivity in HIV-1-exposed uninfected neonates. *Clin Immunol* 2008;**127**:340-7.
75. Van Rie A, Madhi SA, Heera JR, et al. Gamma interferon production in response to Mycobacterium bovis BCG and Mycobacterium tuberculosis antigens in infants born to human immunodeficiency virus-infected mothers. *Clin Vaccine Immunol* 2006;**13**:246-52.
76. Ono E, Nunes dos Santos AM, de Menezes Succi RC, et al. Imbalance of naive and memory T lymphocytes with sustained high cellular activation during the first year of life from uninfected children born to HIV-1-infected mothers on HAART. *Braz J Med Biol Res* 2008;**41**:700-8.
77. Economides A, Schmid I, Anisman-Posner DJ, et al. Apoptosis in cord blood T lymphocytes from infants of human immunodeficiency virus-infected mothers. *Clin Diagn Lab Immunol* 1998;**5**:230-4.
78. Epalza C, Goetghebuer T, Hainaut M, et al. High incidence of invasive group B streptococcal infections in uninfected infants born to HIV-1-infected mothers. *Retrovirology* 2009;**6**:O22.
79. Slogrove AL, Cotton MF, Esser MM. Severe infections in HIV-exposed uninfected infants: clinical evidence of immunodeficiency. *J Trop Pediatr* 2010;**56**:75-81.
80. Mussi-Pinhata MM, Freimanis L, Yamamoto AY, et al. Infectious disease morbidity among young HIV-1-exposed but uninfected infants in Latin American and Caribbean countries: the National Institute of Child Health and Human Development International Site Development Initiative Perinatal Study. *Pediatrics* 2007;**119**:e694-704.
81. Filteau S. The HIV-exposed, uninfected African child. *Trop Med Int Health* 2009;**14**:276-87.
82. Jones CE, Naidoo S, De Beer C, et al. Maternal HIV infection and antibody responses against vaccine-preventable diseases in uninfected infants. *JAMA* 2011;**305**:576-84.
83. Mofenson LM, Korelitz J, Meyer WA, 3rd, et al. The relationship between serum human immunodeficiency virus type 1 (HIV-1) RNA level, CD4 lymphocyte percent, and long-term mortality risk in HIV-1-infected children. National Institute of Child Health and Human Development Intravenous Immunoglobulin Clinical Trial Study Group. *J Infect Dis* 1997;**175**:1029-38.
84. van Gent R, van Tilburg CM, Nibbelke EE, et al. Refined characterization and reference values of the pediatric T- and B-cell compartments. *Clin Immunol* 2009;**133**:95-107.

85. Shearer WT, Rosenblatt HM, Gelman RS, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: The pediatric AIDS clinical trials group P1009 study. *Journal of Allergy and Clinical Immunology* 2003;**112**:973-80.
86. Rich KC, Brambilla D, Pitt J, et al. Lymphocyte phenotyping in infants: maturation of lymphocyte subpopulations and the effects of HIV infection. *Clin Immunol Immunopathol* 1997;**85**:273-81.
87. Shearer WT, Quinn TC, LaRussa P, et al. Viral load and disease progression in infants infected with human immunodeficiency virus type 1. Women and Infants Transmission Study Group. *N Engl J Med* 1997;**336**:1337-42.
88. McIntosh K, Shevitz A, Zaknun D, et al. Age- and time-related changes in extracellular viral load in children vertically infected by human immunodeficiency virus. *Pediatr Infect Dis J* 1996;**15**:1087-91.
89. Abrams EJ, Weedon J, Steketee RW, et al. Association of human immunodeficiency virus (HIV) load early in life with disease progression among HIV-infected infants. New York City Perinatal HIV Transmission Collaborative Study Group. *J Infect Dis* 1998;**178**:101-8.
90. Koup RA, Safrit JT, Cao Y, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 1994;**68**:4650-5.
91. Streeck H, Jolin JS, Qi Y, et al. Human immunodeficiency virus type 1-specific CD8+ T-cell responses during primary infection are major determinants of the viral set point and loss of CD4+ T cells. *J Virol* 2009;**83**:7641-8.
92. Goonetilleke N, Liu MK, Salazar-Gonzalez JF, et al. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med* 2009;**206**:1253-72.
93. Yang H, Wu H, Hancock G, et al. Antiviral inhibitory capacity of CD8+ T cells predicts the rate of CD4+ T-cell decline in HIV-1 infection. *J Infect Dis* 2012;**206**:552-61.
94. Virgin HW, Walker BD. Immunology and the elusive AIDS vaccine. *Nature* 2010;**464**:224-31.
95. Weinberg A, Dickover R, Britto P, et al. Continuous improvement in the immune system of HIV-infected children on prolonged antiretroviral therapy. *AIDS* 2008;**22**:2267-77.
96. Huang KH, Bonsall D, Katzourakis A, et al. B-cell depletion reveals a role for antibodies in the control of chronic HIV-1 infection. *Nat Commun* 2010;**1**:102.
97. De Rossi A. Virus-host interactions in paediatric HIV-1 infection. *Curr Opin HIV AIDS* 2007;**2**:399-404.
98. Natural history of vertically acquired human immunodeficiency virus-1 infection. The European Collaborative Study. *Pediatrics* 1994;**94**:815-9.
99. Biggar RJ. AIDS incubation in 1891 HIV seroconverters from different exposure groups. International Registry of Seroconverters. *AIDS* 1990;**4**:1059-66.
100. Siegrist CA. The challenges of vaccine responses in early life: selected examples. *J Comp Pathol* 2007;**137 Suppl 1**:S4-9.
101. Siegrist CA. Neonatal and early life vaccinology. *Vaccine* 2001;**19**:3331-46.
102. Douek DC, Koup RA, McFarland RD, Sullivan JL, Luzuriaga K. Effect of HIV on thymic function before and after antiretroviral therapy in children. *J Infect Dis* 2000;**181**:1479-82.
103. Douek DC, McFarland RD, Keiser PH, et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 1998;**396**:690-5.
104. Feeney ME, Tang Y, Pfafferott K, et al. HIV-1 viral escape in infancy followed by emergence of a variant-specific CTL response. *J Immunol* 2005;**174**:7524-30.
105. Kourtis AP, Ibegbu C, Nahmias AJ, et al. Early progression of disease in HIV-infected infants with thymus dysfunction. *N Engl J Med* 1996;**335**:1431-6.
106. Pelak K, Goldstein DB, Walley NM, et al. Host determinants of HIV-1 control in African Americans. *J Infect Dis* 2010;**201**:1141-9.
107. Tiemessen CT, Kuhn L. Immune pathogenesis of pediatric HIV-1 infection. *Curr HIV/AIDS Rep* 2006;**3**:13-9.
108. Kiepiela P, Leslie AJ, Honeyborne I, et al. Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* 2004;**432**:769-75.
109. Pillay T, Zhang HT, Drijfhout JW, et al. Unique acquisition of cytotoxic T-lymphocyte escape mutants in infant human immunodeficiency virus type 1 infection. *J Virol* 2005;**79**:12100-5.
110. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004;**22**:745-63.
111. Gibb DM, Newberry A, Klein N, et al. Immune repopulation after HAART in previously untreated HIV-1-infected children. Paediatric European Network for Treatment of AIDS (PENTA) Steering Committee. *Lancet* 2000;**355**:1331-2.
112. Pakker NG, Notermans DW, de Boer RJ, et al. Biphasic kinetics of peripheral blood T cells after triple combination therapy in HIV-1 infection: a composite of redistribution and proliferation. *Nat Med* 1998;**4**:208-14.

113. Tena-Coki NG, Scriba TJ, Peteni N, et al. CD4 and CD8 T Cell Responses to Mycobacterial Antigens in African Children. *Am J Respir Crit Care Med* 2010.
114. Resino S, Seoane E, Perez A, et al. Different profiles of immune reconstitution in children and adults with HIV-infection after highly active antiretroviral therapy. *BMC Infect Dis* 2006;**6**:112.
115. Clerici M, Saresella M, Trabattoni D, et al. Thymic volume predicts long-term immune reconstitution in HIV-infected children treated with highly active antiretroviral therapy. *AIDS* 2002;**16**:2219-21.
116. De Rossi A, Walker AS, Klein N, et al. Increased thymic output after initiation of antiretroviral therapy in human immunodeficiency virus type 1-infected children in the Paediatric European Network for Treatment of AIDS (PENTA) 5 Trial. *J Infect Dis* 2002;**186**:312-20.
117. Correa R, Harari A, Vallelian F, et al. Functional patterns of HIV-1-specific CD4 T-cell responses in children are influenced by the extent of virus suppression and exposure. *AIDS* 2007;**21**:23-30.
118. Luzuriaga K, Wu H, McManus M, et al. Dynamics of human immunodeficiency virus type 1 replication in vertically infected infants. *J Virol* 1999;**73**:362-7.
119. Palumbo P, Wu H, Chadwick E, et al. Virologic response to potent antiretroviral therapy and modeling of HIV dynamics in early pediatric infection. *J Infect Dis* 2007;**196**:23-9.
120. Moir S, Fauci AS. B cells in HIV infection and disease. *Nat Rev Immunol* 2009;**9**:235-45.
121. Murphy K, Travers P, Walport M. Janeway's Immunobiology. New York: Garland Science; 2008. Chapter 7: The development and survival of lymphocytes. Development of B lymphocytes p. 257-73.
122. Suryani S, Fulcher DA, Santner-Nanan B, et al. Differential expression of CD21 identifies developmentally and functionally distinct subsets of human transitional B cells. *Blood* 2009.
123. McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L. Molecular programming of B cell memory. *Nat Rev Immunol* 2012;**12**:24-34.
124. Stuart G. Tangye, David M. Tarlinton. Memory B cells: Effectors of long-lived immune responses. *European Journal of Immunology* 2009;**39**:2065-75.
125. Carsetti R, Rosado MM, Wardmann H. Peripheral development of B cells in mouse and man. *Immunol Rev* 2004;**197**:179-91.
126. Benson MJ, Erickson LD, Gleeson MW, Noelle RJ. Affinity of antigen encounter and other early B-cell signals determine B-cell fate. *Curr Opin Immunol* 2007;**19**:275-80.
127. McHeyzer-Williams LJ, Malherbe LP, McHeyzer-Williams MG. Helper T cell-regulated B cell immunity. *Curr Top Microbiol Immunol* 2006;**311**:59-83.
128. Fairfax KA, Kallies A, Nutt SL, Tarlinton DM. Plasma cell development: from B-cell subsets to long-term survival niches. *Semin Immunol* 2008;**20**:49-58.
129. Tarlinton D, Radbruch A, Hiepe F, Dorner T. Plasma cell differentiation and survival. *Curr Opin Immunol* 2008;**20**:162-9.
130. MacLennan IC, Toellner KM, Cunningham AF, et al. Extrafollicular antibody responses. *Immunol Rev* 2003;**194**:8-18.
131. Reynaud CA, Descatoire M, Dogan I, et al. IgM memory B cells: a mouse/human paradox. *Cell Mol Life Sci* 2012;**69**:1625-34.
132. Steiniger B, Timphus EM, Barth PJ. The splenic marginal zone in humans and rodents: an enigmatic compartment and its inhabitants. *Histochem Cell Biol* 2006;**126**:641-8.
133. Pillai S, Cariappa A. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat Rev Immunol* 2009;**9**:767-77.
134. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. *J Exp Med* 1998;**188**:1679-89.
135. Murphy K, Travers P, Walport M. Janeway's Immunobiology. 7th ed. New York: Garland Science; 2008. Chapter 9: The humoral immune response. B-cell responses to bacterial polysaccharides do not require peptide-specific T-cell help p. 397-8.
136. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 2011;**29**:621-63.
137. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med* 2012;**209**:1241-53.
138. King C, Tangye SG, Mackay CR. T Follicular Helper (TFH) Cells in Normal and Dysregulated Immune Responses. *Annual Review of Immunology* 2008;**26**:741-66.
139. McHeyzer-Williams LJ, Pelletier N, Mark L, Fazilleau N, McHeyzer-Williams MG. Follicular helper T cells as cognate regulators of B cell immunity. *Curr Opin Immunol* 2009;**21**:266-73.
140. Rasheed AU, Rahn HP, Sallusto F, Lipp M, Muller G. Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. *Eur J Immunol* 2006;**36**:1892-903.
141. Warnatz K. Dissecting CXCR5+ T cell populations- on the quest for a better understanding of B cell help during T dependent antibody responses. *Eur J Immunol* 2006;**36**:1662-4.
142. Breitfeld D, Ohl L, Kremmer E, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* 2000;**192**:1545-52.

143. Campbell DJ, Kim CH, Butcher EC. Separable effector T cell populations specialized for B cell help or tissue inflammation. *Nat Immunol* 2001;**2**:876-81.
144. Mackay CR. Follicular homing T helper (Th) cells and the Th1/Th2 paradigm. *J Exp Med* 2000;**192**:F31-4.
145. Shi Y, Agematsu K, Ochs HD, Sugane K. Functional analysis of human memory B-cell subpopulations: IgD+CD27+ B cells are crucial in secondary immune response by producing high affinity IgM. *Clin Immunol* 2003;**108**:128-37.
146. Berkowska MA, Driessen GJ, Bikos V, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. *Blood* 2011;**118**:2150-8.
147. Tangye SG, Good KL. Human IgM+CD27+ B cells: memory B cells or "memory" B cells? *J Immunol* 2007;**179**:13-9.
148. Weill JC, Weller S, Reynaud CA. Human marginal zone B cells. *Annu Rev Immunol* 2009;**27**:267-85.
149. Griffin DO, Holodick NE, Rothstein TL. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. *J Exp Med* 2011;**208**:67-80.
150. Descatoire M, Weill JC, Reynaud CA, Weller S. A human equivalent of mouse B-1 cells? *J Exp Med* 2011;**208**:2563-4
151. Perez-Andres M, Grosserichter-Wagener C, Teodosio C, et al. The nature of circulating CD27+CD43+ B cells. *J Exp Med* 2011;**208**:2565-6
152. Reynaud CA, Weill JC. Gene profiling of CD11b(+) and CD11b(-) B1 cell subsets reveals potential cell sorting artifacts. *J Exp Med* 2012;**209**:433-4.
153. Siegrist C-A, Aspinall R. B-cell responses to vaccination at the extremes of age. *Nat Rev Immunol* 2009;**9**:185-94.
154. Huck K, Feyen O, Ghosh S, et al. Memory B-cells in healthy and antibody-deficient children. *Clin Immunol* 2009;**131**:50-9.
155. Pollard AJ, Perrett KP, Beverley PC. Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. *Nat Rev Immunol* 2009;**9**:213-20.
156. Lane HC, Masur H, Edgar LC, et al. Abnormalities of B-cell activation and immunoregulation in patients with the acquired immunodeficiency syndrome. *N Engl J Med* 1983;**309**:453-8.
157. Bernstein LJ, Ochs HD, Wedgwood RJ, Rubinstein A. Defective humoral immunity in pediatric acquired immune deficiency syndrome. *J Pediatr* 1985;**107**:352-7.
158. De Milito A, Morch C, Sonnerborg A, Chiodi F. Loss of memory (CD27) B lymphocytes in HIV-1 infection. *AIDS* 2001;**15**:957-64.
159. Chong Y, Ikematsu H, Kikuchi K, et al. Selective CD27+ (memory) B cell reduction and characteristic B cell alteration in drug-naive and HAART-treated HIV type 1-infected patients. *AIDS Res Hum Retroviruses* 2004;**20**:219-26.
160. Titanji K, Chiodi F, Bellocco R, et al. Primary HIV-1 infection sets the stage for important B lymphocyte dysfunctions. *AIDS* 2005;**19**:1947-55.
161. D'Orsogna LJ, Krueger RG, McKinnon EJ, French MA. Circulating memory B-cell subpopulations are affected differently by HIV infection and antiretroviral therapy. *AIDS* 2007;**21**:1747-52.
162. Hart M, Steel A, Clark SA, et al. Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease. *J Immunol* 2007;**178**:8212-20.
163. van Grevenynghe J, Cubas RA, Noto A, et al. Loss of memory B cells during chronic HIV infection is driven by Foxo3a- and TRAIL-mediated apoptosis. *J Clin Invest* 2011;**121**:3877-88.
164. Johannesson TG, Sogaard OS, Tolstrup M, et al. The Impact of B-Cell Perturbations on Pneumococcal Conjugate Vaccine Response in HIV-Infected Adults. *PLoS ONE* 2012;**7**:e42307.
165. Moir S, Buckner CM, Ho J, et al. B cells in early and chronic HIV infection: evidence for preservation of immune function associated with early initiation of antiretroviral therapy. *Blood* 2010;**116**:5571-9.
166. Jacobsen MC, Thiebaut R, Fisher C, et al. Pediatric human immunodeficiency virus infection and circulating IgD+ memory B cells. *J Infect Dis* 2008;**198**:481-5.
167. Ghosh S, Feyen O, Jebran AF, et al. Memory B-cell function in HIV-infected children - decreased memory B cells despite ART. *Pediatr Res* 2009.
168. Pensieroso S, Cagigi A, Palma P, et al. Timing of HAART defines the integrity of memory B cells and the longevity of humoral responses in HIV-1 vertically-infected children. *Proc Natl Acad Sci U S A* 2009;**106**:7939-44.
169. Iwajomo OH, Finn A, Moons P, et al. Deteriorating pneumococcal-specific B-cell memory in minimally symptomatic African children with HIV infection. *J Infect Dis* 2011;**204**:534-43.
170. Plotkin SA. Correlates of protection induced by vaccination. *Clin Vaccine Immunol* 2010;**17**:1055-65.
171. Mauri C, Bosma A. Immune regulatory function of B cells. *Annu Rev Immunol* 2012;**30**:221-41.
172. Lund FE, Randall TD. Effector and regulatory B cells: modulators of CD4(+) T cell immunity. *Nat Rev Immunol* 2010;**10**:236-47.

173. Usuga X, Montoya CJ, Landay AL, Rugeles MT. Characterization of quantitative and functional innate immune parameters in HIV-1-infected Colombian children receiving stable highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* 2008;**49**:348-57.
174. Zhang Z, Fu J, Zhao Q, et al. Differential restoration of myeloid and plasmacytoid dendritic cells in HIV-1-infected children after treatment with highly active antiretroviral therapy. *J Immunol* 2006;**176**:5644-51.
175. Azzoni L, Rutstein RM, Chehimi J, et al. Dendritic and natural killer cell subsets associated with stable or declining CD4+ cell counts in treated HIV-1-infected children. *J Infect Dis* 2005;**191**:1451-9.
176. Meddows-Taylor S, Kuhn L, Meyers TM, Sherman G, Tiemessen CT. Defective neutrophil degranulation induced by interleukin-8 and complement 5a and down-regulation of associated receptors in children vertically infected with human immunodeficiency virus type 1. *Clin Diagn Lab Immunol* 2001;**8**:21-30.
177. Roilides E, Mertins S, Eddy J, et al. Impairment of neutrophil chemotactic and bactericidal function in children infected with human immunodeficiency virus type 1 and partial reversal after in vitro exposure to granulocyte-macrophage colony-stimulating factor. *J Pediatr* 1990;**117**:531-40.
178. European Collaborative S. Levels and patterns of neutrophil cell counts over the first 8 years of life in children of HIV-1-infected mothers. *AIDS* 2004;**18**:2009-17.
179. Lian YC, Della-Negra M, Rutz R, et al. Immunological analysis in paediatric HIV patients at different stages of the disease. *Scand J Immunol* 2004;**60**:615-24.
180. Hyams C, Tam JCH, Brown JS, Gordon SB. C3b/iC3b Deposition on Streptococcus pneumoniae Is Not Affected by HIV Infection. *PLoS ONE* 2010;**5**:e8902.
181. Welch S, Sharland M, et al. PENTA 2009 guidelines for the use of antiretroviral therapy in paediatric HIV-1 infection. *HIV Med* 2009;**10**:591-613.
182. El-Sadr WM, Lundgren JD, et al. CD4+ count-guided interruption of antiretroviral treatment. *N Engl J Med* 2006;**355**:2283-96.
183. PENTA. Response to planned treatment interruptions in HIV infection varies across childhood. *AIDS* 2010;**24**:231-41.
184. Harrison L, Ananworanich J, Hamadache D, et al. Adherence to Antiretroviral Therapy and Acceptability of Planned Treatment Interruptions in HIV-Infected Children. *AIDS Behav* 2012.
185. Sefe D, Klein N, Mosconi I, et al. Immunologic and viral dynamics among HIV-infected children after planned treatment interruption: a substudy of the PENTA 11 trial: poster presentation. 17th Conference on Retroviruses and Opportunistic Infections (CROI); San Fransisco, USA 2010.
186. CDC. Panel on Antiretroviral Therapy and Medical Management of HIV-Infected Children. Guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection. 2011 [Accessed October 2012]; Available from: http://aidsinfo.nih.gov/contentfiles/lvguidelines/PediatricGuidelines.pdf.
187. CDC. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. 2012 [Accessed October 2012]; Available from: http://aidsinfo.nih.gov/contentfiles/lvguidelines/AdultandAdolescentGL.pdf.
188. Cagigi A, Cotugno N, Giaquinto C, et al. Immune reconstitution and vaccination outcome in HIV-1 infected children: Present knowledge and future directions. *Hum Vaccin Immunother* 2012;**8**.
189. Menson EN, Mellado MJ, Bamford A, et al. Guidance on vaccination of HIV-infected children in Europe. *HIV Med* 2012;**13**:333-6; e1-14.
190. Sutcliffe CG, Moss WJ. Do children infected with HIV receiving HAART need to be revaccinated? *The Lancet Infectious Diseases* 2010;**10**:630-42.
191. Obaro SK, Pugatch D, Luzuriaga K. Immunogenicity and efficacy of childhood vaccines in HIV-1-infected children. *Lancet Infect Dis* 2004;**4**:510-8.
192. Bekker V, Scherpbier H, Pajkrt D, et al. Persistent humoral immune defect in highly active antiretroviral therapy-treated children with HIV-1 infection: loss of specific antibodies against attenuated vaccine strains and natural viral infection. *Pediatrics* 2006;**118**:e315-22.
193. Farquhar C, Wamalwa D, Selig S, et al. Immune responses to measles and tetanus vaccines among Kenyan human immunodeficiency virus type 1 (HIV-1)-infected children pre- and post-highly active antiretroviral therapy and revaccination. *Pediatr Infect Dis J* 2009;**28**:295-9.
194. Abzug MJ, Warshaw M, Rosenblatt HM, et al. Immunogenicity and Immunologic Memory after Hepatitis B Virus Booster Vaccination in HIV-1 Infected Children Receiving Highly Active Antiretroviral Therapy. *The Journal of Infectious Diseases* 2009;**200**:935-46.
195. Rigaud M, Borkowsky W, Muresan P, et al. Impaired immunity to recall antigens and neoantigens in severely immunocompromised children and adolescents during the first year of effective highly active antiretroviral therapy. *J Infect Dis* 2008;**198**:1123-30.
196. Ching N, Deville JG, Nielsen KA, et al. Cellular and humoral immune responses to a tetanus toxoid booster in perinatally HIV-1-infected children and adolescents receiving highly active antiretroviral therapy (HAART). *Eur J Pediatr* 2007;**166**:51-6.

197. Montoya CJ, Toro MF, Aguirre C, et al. Abnormal humoral immune response to influenza vaccination in pediatric type-1 human immunodeficiency virus infected patients receiving highly active antiretroviral therapy. *Mem Inst Oswaldo Cruz* 2007;**102**:501-8.
198. Abzug MJ, Pelton SI, Song LY, et al. Immunogenicity, safety, and predictors of response after a pneumococcal conjugate and pneumococcal polysaccharide vaccine series in human immunodeficiency virus-infected children receiving highly active antiretroviral therapy. *Pediatr Infect Dis J* 2006;**25**:920-9.
199. Tarrago D, Casal J, Ruiz-Contreras J, et al. Assessment of antibody response elicited by a 7-valent pneumococcal conjugate vaccine in pediatric human immunodeficiency virus infection. *Clin Diagn Lab Immunol* 2005;**12**:165-70.
200. Rosenblatt HM, Song LY, Nachman SA, et al. Tetanus immunity after diphtheria, tetanus toxoids, and acellular pertussis vaccination in children with clinically stable HIV infection. *J Allergy Clin Immunol* 2005;**116**:698-703.
201. Spoulou VI, Tsoumas DL, Papaevangelou VG, Mostrou GI, Theodoridou MC. Immunogenicity and immunological memory induced by a 7-valent pneumococcal CRM197 conjugate vaccine in symptomatic HIV-1 infected children. *Vaccine* 2005;**23**:5289-93.
202. Melvin AJ, Mohan KM. Response to immunization with measles, tetanus, and Haemophilus influenzae type b vaccines in children who have human immunodeficiency virus type 1 infection and are treated with highly active antiretroviral therapy. *Pediatrics* 2003;**111**:e641-4.
203. Cotugno N, Douagi I, Rossi P, Palma P. Suboptimal immune reconstitution in vertically HIV infected children: a view on how HIV replication and timing of HAART initiation can impact on T and B-cell compartment. *Clin Dev Immunol* 2012;**2012**:805151.
204. Cagigi A, Nilsson A, Pensieroso S, Chiodi F. Dysfunctional B-cell responses during HIV-1 infection: implication for influenza vaccination and highly active antiretroviral therapy. *Lancet Infect Dis* 2010;**10**:499-503.
205. Kosalaraksa P, Srirompotong U, Newman RW, Lumbiganon P, Wood JM. Serological response to trivalent inactive influenza vaccine in HIV-infected children with different immunologic status. *Vaccine* 2011;**29**:3055-60.
206. Forster R, Emrich T, Kremmer E, Lipp M. Expression of the G-protein--coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood* 1994;**84**:830-40.
207. Chevalier N, Jarrossay D, Ho E, et al. CXCR5 expressing human central memory CD4 T cells and their relevance for humoral immune responses. *J Immunol* 2011;**186**:5556-68.
208. Morita R, Schmitt N, Bentebibel SE, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 2011;**34**:108-21.
209. Simpson N, Gatenby PA, Wilson A, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis & Rheumatism* 2010;**62**:234-44.
210. Forster R, Schweigard G, Johann S, et al. Abnormal Expression of the B-Cell Homing Chemokine Receptor BLR1 During the Progression of Acquired Immunodeficiency Syndrome. *Blood* 1997;**90**:520-5.
211. Malito E, Bursulaya B, Chen C, et al. Structural basis for lack of toxicity of the diphtheria toxin mutant CRM197. *Proc Natl Acad Sci U S A* 2012;**109**:5229-34.
212. Uchida T, Pappenheimer AM, Jr., Harper AA. Reconstitution of Diphtheria Toxin from Two Nontoxic Cross-Reacting Mutant Proteins. *Science* 1972;**175**:901-3.
213. Gray BM, Converse GM, 3rd, Dillon HC, Jr. Epidemiologic studies of Streptococcus pneumoniae in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* 1980;**142**:923-33.
214. Bogaert D, De Groot R, Hermans PW. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *Lancet Infect Dis* 2004;**4**:144-54.
215. Gladstone RA, Jefferies JM, Faust SN, Clarke SC. Continued control of pneumococcal disease in the UK - the impact of vaccination. *J Med Microbiol* 2011;**60**:1-8.
216. Hausdorff WP, Bryant J, Paradiso PR, Siber GR. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin Infect Dis* 2000;**30**:100-21.
217. Scott JA. The preventable burden of pneumococcal disease in the developing world. *Vaccine* 2007;**25**:2398-405.
218. O'Brien KL, Wolfson LJ, Watt JP, et al. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet* 2009;**374**:893-902.
219. van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet* 2009;**374**:1543-56.

220. Ladhani S, Pebody RG, Ramsay ME, et al. Continuing impact of infectious diseases on childhood deaths in England and Wales, 2003-2005. *Pediatr Infect Dis J* 2010;**29**:310-3.
221. Trotter CL, Waight P, Andrews NJ, et al. Epidemiology of invasive pneumococcal disease in the pre-conjugate vaccine era: England and Wales, 1996-2006. *Journal of Infection* 2010;**60**:200-8.
222. Miller E, Andrews NJ, Waight PA, Slack MP, George RC. Herd immunity and serotype replacement 4 years after seven-valent pneumococcal conjugate vaccination in England and Wales: an observational cohort study. *Lancet Infect Dis* 2011;**11**:760-8.
223. Miller E, Andrews NJ, Waight PA, Slack MP, George RC. Effectiveness of the new serotypes in the 13-valent pneumococcal conjugate vaccine. *Vaccine* 2011;**29**:9127-31.
224. HPA: pneumococcal disease website. 2012 [Accessed October 2012]; Available from: http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/Pneumococcal/.
225. Mitchell AM, Mitchell TJ. Streptococcus pneumoniae: virulence factors and variation. *Clin Microbiol Infect* 2010;**16**:411-8.
226. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol* 2008;**6**:288-301.
227. Felgentreff K, Beisswenger C, Griese M, et al. The antimicrobial peptide cathelicidin interacts with airway mucus. *Peptides* 2006;**27**:3100-6.
228. Lee HY, Andalibi A, Webster P, et al. Antimicrobial activity of innate immune molecules against Streptococcus pneumoniae, Moraxella catarrhalis and nontypeable Haemophilus influenzae. *BMC Infect Dis* 2004;**4**:12.
229. Murphy K, P T, Walport M. Janeway's Immunobiology. New York: Garland Science; 2008. Chapter 2: Innate immunity. Pattern recognition in the innate immune system p. 53-60.
230. Mitchell TJ. Streptococcus pneumoniae: infection, inflammation and disease. *Adv Exp Med Biol* 2006;**582**:111-24.
231. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;**124**:783-801.
232. Lee MS, Kim YJ. Pattern-recognition receptor signaling initiated from extracellular, membrane, and cytoplasmic space. *Mol Cells* 2007;**23**:1-10.
233. Picard C, von Bernuth H, Ghandil P, et al. Clinical features and outcome of patients with IRAK-4 and MyD88 deficiency. *Medicine (Baltimore)* 2010;**89**:403-25.
234. Ku CL, von Bernuth H, Picard C, et al. Selective predisposition to bacterial infections in IRAK-4-deficient children: IRAK-4-dependent TLRs are otherwise redundant in protective immunity. *J Exp Med* 2007;**204**:2407-22.
235. Kadioglu A, Andrew PW. The innate immune response to pneumococcal lung infection: the untold story. *Trends Immunol* 2004;**25**:143-9.
236. Winkelstein JA. The Role of Complement in the Host's Defense against Streptococcus pneumoniae [with Discussion]. *Reviews of Infectious Diseases* 1981;**3**:289-98.
237. Johnston RB, Jr. The Host Response to Invasion by Streptococcus pneumoniae: Protection and the Pathogenesis of Tissue Damage. *Reviews of Infectious Diseases* 1981;**3**:282-8.
238. Catterall JR. Streptococcus pneumoniae. *Thorax* 1999;**54**:929-37.
239. Dallaire F, Ouellet N, Bergeron Y, et al. Microbiological and inflammatory factors associated with the development of pneumococcal pneumonia. *J Infect Dis* 2001;**184**:292-300.
240. Standish AJ, Weiser JN. Human neutrophils kill Streptococcus pneumoniae via serine proteases. *J Immunol* 2009;**183**:2602-9.
241. Romero-Steiner S, Frasch CE, Carlone G, et al. Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. *Clin Vaccine Immunol* 2006;**13**:165-9.
242. Gordon SB, Jarman ER, Kanyanda S, et al. Reduced interleukin-8 response to Streptococcus pneumoniae by alveolar macrophages from adults with HIV/AIDS. *AIDS* 2005;**19**:1197-200.
243. Nakamatsu M, Yamamoto N, Hatta M, et al. Role of interferon-gamma in V alpha14+ natural killer T cell-mediated host defense against Streptococcus pneumoniae infection in murine lungs. *Microbes Infect* 2007;**9**:364-74.
244. Nakasone C, Yamamoto N, Nakamatsu M, et al. Accumulation of gamma/delta T cells in the lungs and their roles in neutrophil-mediated host defense against pneumococcal infection. *Microbes Infect* 2007;**9**:251-8.
245. Gonzalez-Fernandez A, Faro J, Fernandez C. Immune responses to polysaccharides: lessons from humans and mice. *Vaccine* 2008;**26**:292-300.
246. Simell B, Auranen K, Kayhty H, et al. The fundamental link between pneumococcal carriage and disease. *Expert Rev Vaccines* 2012;**11**:841-55.
247. Austrian R. Some aspects of the pneumococcal carrier state. *J Antimicrob Chemother* 1986;**18 Suppl A**:35-45.
248. Kadioglu A, Gingles NA, Grattan K, et al. Host cellular immune response to pneumococcal lung infection in mice. *Infect Immun* 2000;**68**:492-501.

249. Lipsitch M, Whitney CG, Zell E, et al. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? *PLoS Med* 2005;**2**:e15.
250. Zhang Q, Bernatoniene J, Bagrade L, et al. Regulation of production of mucosal antibody to pneumococcal protein antigens by T-cell-derived gamma interferon and interleukin-10 in children. *Infect Immun* 2006;**74**:4735-43.
251. Malley R. Antibody and cell-mediated immunity to Streptococcus pneumoniae: implications for vaccine development. *J Mol Med* 2010.
252. Weber SE, Tian H, Pirofski LA. CD8+ cells enhance resistance to pulmonary serotype 3 Streptococcus pneumoniae infection in mice. *J Immunol* 2011;**186**:432-42.
253. Oksenhendler E, Gerard L, Fieschi C, et al. Infections in 252 patients with common variable immunodeficiency. *Clin Infect Dis* 2008;**46**:1547-54.
254. Cecil RL. Present Status of Serum Therapy in Pneumonia. *Bull N Y Acad Med* 1939;**15**:104-15.
255. Jodar L, Butler J, Carlone G, et al. Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. *Vaccine* 2003;**21**:3265-72.
256. Zhou ZH, Zhang Y, Hu YF, et al. The broad antibacterial activity of the natural antibody repertoire is due to polyreactive antibodies. *Cell Host Microbe* 2007;**1**:51-61.
257. Ochsenbein AF, Zinkernagel RM. Natural antibodies and complement link innate and acquired immunity. *Immunology Today* 2000;**21**:624-30.
258. Baxendale HE, Johnson M, Stephens RCM, et al. Natural human antibodies to pneumococcus have distinctive molecular characteristics and protect against pneumococcal disease. *Clinical & Experimental Immunology* 2008;**151**:51-60.
259. Goldblatt D, Hussain M, Andrews N, et al. Antibody Responses to Nasopharyngeal Carriage of Streptococcus pneumoniae in Adults: A Longitudinal Household Study. *The Journal of Infectious Diseases* 2005;**192**:387-93.
260. Simell B, Kilpi TM, Kayhty H. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal capsular polysaccharides in children. *J Infect Dis* 2002;**186**:1106-14.
261. Rennels MB, Edwards KM, Keyserling HL, et al. Safety and immunogenicity of heptavalent pneumococcal vaccine conjugated to CRM197 in United States infants. *Pediatrics* 1998;**101**:604-11.
262. Regev-Yochay G, Raz M, Dagan R, et al. Nasopharyngeal carriage of Streptococcus pneumoniae by adults and children in community and family settings. *Clin Infect Dis* 2004;**38**:632-9.
263. Uddin S, Borrow R, Haeney MR, et al. Total and serotype-specific pneumococcal antibody titres in children with normal and abnormal humoral immunity. *Vaccine* 2006;**24**:5637-44.
264. Barrett DJ, Ayoub EM. IgG2 subclass restriction of antibody to pneumococcal polysaccharides. *Clin Exp Immunol* 1986;**63**:127-34.
265. Douglas RM, Paton JC, Duncan SJ, Hansman DJ. Antibody response to pneumococcal vaccination in children younger than five years of age. *J Infect Dis* 1983;**148**:131-7.
266. Timens W, Boes A, Rozeboom-Uiterwijk T, Poppema S. Immaturity of the human splenic marginal zone in infancy. Possible contribution to the deficient infant immune response. *J Immunol* 1989;**143**:3200-6.
267. Wara DW. Host defense against Streptococcus pneumoniae: the role of the spleen. *Rev Infect Dis* 1981;**3**:299-309.
268. Moffitt KL, Malley R. Next generation pneumococcal vaccines. *Curr Opin Immunol* 2011;**23**:407-13.
269. Grau I, Ardanuy C, Linares J, et al. Trends in mortality and antibiotic resistance among HIV-infected patients with invasive pneumococcal disease. *HIV Med* 2009;**10**:488-95.
270. Bliss SJ, O'Brien KL, Janoff EN, et al. The evidence for using conjugate vaccines to protect HIV-infected children against pneumococcal disease. *Lancet Infect Dis* 2007.
271. Nunes MC, Madhi SA. Safety, immunogenicity and efficacy of pneumococcal conjugate vaccine in HIV-infected individuals. *Hum Vaccin Immunother* 2012;**8**:161-73.
272. Nunes MC, von Gottberg A, de Gouveia L, et al. Persistent high burden of invasive pneumococcal disease in South African HIV-infected adults in the era of an antiretroviral treatment program. *PLoS ONE* 2011;**6**:e27929.
273. Anthony L, Meehan A, Amos B, et al. Nasopharyngeal carriage of Streptococcus pneumoniae: prevalence and risk factors in HIV-positive children in Tanzania. *Int J Infect Dis* 2012;**16**:e753-7.
274. Cardoso VC, Cervi MC, Cintra OA, Salathiel AS, Gomes AC. Nasopharyngeal colonization with Streptococcus pneumoniae in children infected with human immunodeficiency virus. *J Pediatr (Rio J)* 2006;**82**:51-7.
275. Pugliese A, Vidotto V, Beltramo T, Torre D. Phagocytic activity in human immunodeficiency virus type 1 infection. *Clin Diagn Lab Immunol* 2005;**12**:889-95.
276. Poles MA, Barsoum S, Yu W, et al. Human immunodeficiency virus type 1 induces persistent changes in mucosal and blood gammadelta T cells despite suppressive therapy. *J Virol* 2003;**77**:10456-67.

277. Leeansyah E, Zhou J, Paukovics G, et al. Decreased NK Cell FcRgamma in HIV-1 infected individuals receiving combination antiretroviral therapy: a cross sectional study. *PLoS ONE* 2010;**5**:e9643.
278. Heggelund L, Mollnes TE, Ueland T, et al. Mannose-binding lectin in HIV infection: relation to disease progression and highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* 2003;**32**:354-61.
279. Austrian R. Pneumococcus: The First One Hundred Years [with Discussion]. *Reviews of Infectious Diseases* 1981;**3**:183-9.
280. Grabenstein JD, Klugman KP. A century of pneumococcal vaccination research in humans. *Clin Microbiol Infect* 2012.
281. Obituary. *Br Med J* 1952;**1**:871-3.
282. Obituary. *Br Med J* 1947;**1**:657-e-60.
283. Obituary. *Br Med J* 1941;**2**:69-71.
284. Department of Heath: Immunisation against infectious disease -'The Green Book'. [Accessed October 2012]; Available from: http://immunisation.dh.gov.uk/category/the-green-book/.
285. Andrews R, Moberley SA. The controversy over the efficacy of pneumococcal vaccine. *CMAJ* 2009;**180**:18-9.
286. Scott P, Huss A, Stuck AE, Trotter C, Egger M. Efficacy of pneumococcal polysaccharide vaccine. *CMAJ* 2009;**180**:1134-5.
287. Huss A, Scott P, Stuck AE, Trotter C, Egger M. Efficacy of pneumococcal vaccination in adults: a meta-analysis. *CMAJ* 2009;**180**:48-58.
288. Goldblatt D. Conjugate vaccines. *Clin Exp Immunol* 2000;**119**:1-3.
289. Avery OT, Goebel WF. Chemo-immunological studies on conjugated carbohydrate-proteins : II. Inmmunolgical specificity of synthetic sugar-protein antigens. *J Exp Med* 1929;**50**:533-50.
290. Kaye P, Malkani R, Martin S, et al. Invasive pneumococcal disease (IPD) in England and Wales after 7-valent conjugate vaccine (PCV7); potential impact of 10 and 13-valent vaccines: poster presentation. The 27th Annual Meeting of the European Society for Paediatric Infectious Diseases; Brussels, Belgium 2009.
291. Choi YH, Jit M, Gay N, et al. 7-Valent pneumococcal conjugate vaccination in England and Wales: is it still beneficial despite high levels of serotype replacement? *PLoS ONE* 2011;**6**:e26190.
292. Mond JJ, Lees A, Snapper CM. T cell-independent antigens type 2. *Annu Rev Immunol* 1995;**13**:655-92.
293. Kamboj KK, Kirchner HL, Kimmel R, Greenspan NS, Schreiber JR. Significant variation in serotype-specific immunogenicity of the seven-valent Streptococcus pneumoniae capsular polysaccharide-CRM197 conjugate vaccine occurs despite vigorous T cell help induced by the carrier protein. *J Infect Dis* 2003;**187**:1629-38.
294. Kamboj KK, King CL, Greenspan NS, Kirchner HL, Schreiber JR. Immunization with Haemophilus influenzae type b-CRM(197) conjugate vaccine elicits a mixed Th1 and Th2 CD(4+) T cell cytokine response that correlates with the isotype of antipolysaccharide antibody. *J Infect Dis* 2001;**184**:931-5.
295. Avci FY, Li X, Tsuji M, Kasper DL. A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. *Nat Med* 2011;**17**:1602-9.
296. Lai Z, Schreiber JR. Antigen processing of glycoconjugate vaccines; the polysaccharide portion of the pneumococcal CRM(197) conjugate vaccine co-localizes with MHC II on the antigen processing cell surface. *Vaccine* 2009;**27**:3137-44.
297. Defrance T, Taillardet M, Genestier L. T cell-independent B cell memory. *Curr Opin Immunol* 2011;**23**:330-6.
298. Taillardet M, Haffar G, Mondiere P, et al. The thymus-independent immunity conferred by a pneumococcal polysaccharide is mediated by long-lived plasma cells. *Blood* 2009;**114**:4432-40.
299. Hadler SC, Dietz V, Okwo-Bele JM, Cutts FT. Immunization in developing countries. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines: Expert Consult. London: Saunders; 2008. 1541-71.
300. Baylor NW, Midthun K, Falk LA. The role of the food and drug administration in vaccine testing and licensure. In: Levine MM, Kaper JB, Rappuoli R, Liu MA, Good MF, editors. New generation vaccines. New York: Marcel Dekker; 2004. 117-25.
301. Baylor NW, Midthun K. Regulation and testing of vaccines. In: Plotkin SA, Orenstein WA, Offit PA, editors. Vaccines: Expert Consult. 5th ed. London: Saunders; 2008. 1611-27.
302. Loughlin AM, Strathdee SA. Vaccines: past, present and future. In: Nelson KE, Williams CM, editors. Infectious disease epidemiology: theory and practice. London: Jones and Bartlett; 2007. 345-82.
303. Andrews N, Borrow R, Miller E. Validation of serological correlate of protection for meningococcal C conjugate vaccine by using efficacy estimates from postlicensure surveillance in England. *Clin Diagn Lab Immunol* 2003;**10**:780-6.

304. Borrow R, Andrews N, Goldblatt D, Miller E. Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: reevaluation of correlates of protection. *Infect Immun* 2001;**69**:1568-73.
305. Miller E, Salisbury D, Ramsay M. Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story. *Vaccine* 2001;**20 Suppl 1**:S58-67.
306. Goldblatt D, Southern J, Ashton L, et al. Immunogenicity and boosting after a reduced number of doses of a pneumococcal conjugate vaccine in infants and toddlers. *Pediatr Infect Dis J* 2006;**25**:312-9.
307. Lee LH, Frasch CE, Falk LA, Klein DL, Deal CD. Correlates of immunity for pneumococcal conjugate vaccines. *Vaccine* 2003;**21**:2190-6.
308. Siber GR, Chang I, Baker S, et al. Estimating the protective concentration of anti-pneumococcal capsular polysaccharide antibodies. *Vaccine* 2007;**25**:3816-26.
309. Feavers I, Knezevic I, Powell M, Griffiths E. Challenges in the evaluation and licensing of new pneumococcal vaccines, 7-8 July 2008, Ottawa, Canada. *Vaccine* 2009;**27**:3681-8.
310. Moss SJ, Fenton AC, Toomey J, et al. Immunogenicity of a Heptavalent Conjugate Pneumococcal Vaccine Administered Concurrently with a Combination Diphtheria, Tetanus, Five-Component Acellular Pertussis, Inactivated Polio, and Haemophilus influenzae Type b Vaccine and a Meningococcal Group C Conjugate Vaccine at 2, 3, and 4 Months of Age. *Clin Vaccine Immunol* 2010;**17**:311-6.
311. Madhi SA, Petersen K, Madhi A, Khoosal M, Klugman KP. Increased disease burden and antibiotic resistance of bacteria causing severe community-acquired lower respiratory tract infections in human immunodeficiency virus type 1-infected children. *Clin Infect Dis* 2000;**31**:170-6.
312. Klugman KP, Madhi SA, Huebner RE, et al. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. *N Engl J Med* 2003;**349**:1341-8.
313. Whitney CG, Pilishvili T, Farley MM, et al. Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. *The Lancet* 2006;**368**:1495-502.
314. O'Brien KL, Levine OS. Effectiveness of pneumococcal conjugate vaccine. *Lancet* 2006;**368**:1469-70.
315. Black S, Shinefield H, Fireman B, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. *Pediatr Infect Dis J* 2000;**19**:187-95.
316. Madhi SA, Whitney CG, Nohynek H. Lessons learned from clinical trials evaluating pneumococcal conjugate vaccine efficacy against pneumonia and invasive disease. *Vaccine* 2008;**26**:B9-B15.
317. Lucero MG, Dulalia VE, Nillos LT, et al. Pneumococcal conjugate vaccines for preventing vaccine-type invasive pneumococcal disease and X-ray defined pneumonia in children less than two years of age. *Cochrane Database Syst Rev* 2009:CD004977.
318. EMA. Assessment report for Prevenar 13. [Accessed April 2010]; Available from: http://www.ema.europa.eu/humandocs/PDFs/EPAR/Prevenar13/H-1104-en6.pdf.
319. van den Biggelaar AHJ, Richmond PC, Pomat WS, et al. Neonatal pneumococcal conjugate vaccine immunization primes T cells for preferential Th2 cytokine expression: A randomized controlled trial in Papua New Guinea. *Vaccine* 2009;**27**:1340-7.
320. Spolski R, Leonard WJ. Interleukin-21: Basic Biology and Implications for Cancer and Autoimmunity. *Annual Review of Immunology* 2008;**26**:57-79.
321. Iannello A, Tremblay C, Routy J-P, et al. Decreased Levels of Circulating IL-21 in HIV-Infected AIDS Patients: Correlation with CD4+ T-Cell Counts. *Viral Immunology* 2008;**21**:385-8.
322. Iannello A, Boulassel MR, Samarani S, et al. Dynamics and consequences of IL-21 production in HIV-infected individuals: a longitudinal and cross-sectional study. *J Immunol* 2010;**184**:114-26.
323. Pallikkuth S, Pilakka Kanthikeel S, Silva SY, et al. Upregulation of IL-21 receptor on B cells and IL-21 secretion distinguishes novel 2009 H1N1 vaccine responders from nonresponders among HIV-infected persons on combination antiretroviral therapy. *J Immunol* 2011;**186**:6173-81.
324. Costa Ide C, Guilardi F, Kmiliauskis MA, Arslanian C, Baldacci ER. Evaluation of humoral response to heptavalent pneumococcal conjugate vaccine in HIV-infected children. *Rev Saude Publica* 2008;**42**:844-50.
325. Thanee C, Pancharoen C, Likitnukul S, et al. The immunogenicity and safety of pneumococcal conjugate vaccine in human immunodeficiency virus-infected Thai children. *Vaccine* 2011;**29**:5886-91.
326. Madhi SA, Klugman KP, Kuwanda L, et al. Quantitative and qualitative anamnestic immune responses to pneumococcal conjugate vaccine in HIV-infected and HIV-uninfected children 5 years after vaccination. *J Infect Dis* 2009;**199**:1168-76.

327. Madhi SA, Adrian P, Kuwanda L, et al. Long-term immunogenicity and efficacy of a 9-valent conjugate pneumococcal vaccine in human immunodeficient virus infected and non-infected children in the absence of a booster dose of vaccine. *Vaccine* 2007;**25**:2451-7.
328. Steenhoff AP, Wood SM, Rutstein RM, et al. Invasive Pneumococcal Disease Among Human Immunodeficiency Virus-Infected Children, 1989–2006. *The Pediatric Infectious Diesease Journal* 2008;**27**:886-91.
329. Flannery B, Heffernan RT, Harrison LH, et al. Changes in invasive Pneumococcal disease among HIV-infected adults living in the era of childhood pneumococcal immunization. *Ann Intern Med* 2006;**144**:1-9.
330. French N, Gordon SB, Mwalukomo T, et al. A trial of a 7-valent pneumococcal conjugate vaccine in HIV-infected adults. *N Engl J Med* 2010;**362**:812-22.
331. EMA.Prevenar:Summary of product characteristics.2009[Accessed October 2012];Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000323/WC500041563.pdf.
332. EMA.Prevenar 13:Summary of product characteristics.2010[Accessed October 2012];Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/001104/WC500057247.pdf.
333. Maecker HT, Trotter J. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry A* 2006;**69**:1037-42.
334. Hulspas R, O'Gorman MR, Wood BL, Gratama JW, Sutherland DR. Considerations for the control of background fluorescence in clinical flow cytometry. *Cytometry B Clin Cytom* 2009;**76**:355-64.
335. Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol* 2004;**4**:648-55.
336. McCormack RT, Nelson RD, LeBien TW. Structure/function studies of the common acute lymphoblastic leukemia antigen (CALLA/CD10) expressed on human neutrophils. *J Immunol* 1986;**137**:1075-82.
337. Perfetto SP, Chattopadhyay PK, Lamoreaux L, et al. Amine reactive dyes: an effective tool to discriminate live and dead cells in polychromatic flow cytometry. *J Immunol Methods* 2006;**313**:199-208.
338. Perfetto SP, Chattopadhyay PK, Lamoreaux L, et al. Amine-reactive dyes for dead cell discrimination in fixed samples. *Curr Protoc Cytom* 2010;**Chapter 9**:Unit 9 34.
339. Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Methods* 2000;**243**:77-97.
340. Hughes OR, Stewart R, Dimmick I, Jones EA. A critical appraisal of factors affecting the accuracy of results obtained when using flow cytometry in stem cell investigations: where do you put your gates? *Cytometry A* 2009;**75**:803-10.
341. De Groote D, Zangerle PF, Gevaert Y, et al. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. *Cytokine* 1992;**4**:239-48.
342. O'Brien KL, Nohynek H. Report from a WHO working group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae. *Pediatr Infect Dis J* 2003;**22**:133-40.
343. Madhi SA, Kuwanda L, Cutland C, et al. Quantitative and qualitative antibody response to pneumococcal conjugate vaccine among African human immunodeficiency virus-infected and uninfected children. *Pediatr Infect Dis J* 2005;**24**:410-6.
344. Cagigi A, Palma P, Nilsson A, et al. The impact of active HIV-1 replication on the physiological age-related decline of immature-transitional B-cells in HIV-1 infected children. *AIDS* 2010;**24**:2075-80.
345. Schatorje EJ, Gemen EF, Driessen GJ, et al. Paediatric reference values for the peripheral T cell compartment. *Scand J Immunol* 2012;**75**:436-44.
346. Vinuesa CG, Cook MC. Blood relatives of follicular helper T cells. *Immunity* 2011;**34**:10-2.
347. Vinuesa CG. HIV and T follicular helper cells: a dangerous relationship. *J Clin Invest* 2012;**122**:3059-62.
348. Yue FY, Lo C, Sakhdari A, et al. HIV-specific IL-21 producing CD4+ T cells are induced in acute and chronic progressive HIV infection and are associated with relative viral control. *J Immunol* 2010;**185**:498-506.
349. Perez-Andres M, Paiva B, Nieto WG, et al. Human peripheral blood B-cell compartments: a crossroad in B-cell traffic. *Cytometry B Clin Cytom* 2010;**78 Suppl 1**:S47-60.
350. Sanz I, Wei C, Lee FE, Anolik J. Phenotypic and functional heterogeneity of human memory B cells. *Semin Immunol* 2008;**20**:67-82.
351. Jackson SM, Wilson PC, James JA, Capra JD. Human B cell subsets. *Adv Immunol* 2008;**98**:151-224.
352. Warnatz K, Schlesier M. Flowcytometric phenotyping of common variable immunodeficiency. *Cytometry B Clin Cytom* 2008;**74**:261-71.

353. Wu YC, Kipling D, Dunn-Walters DK. The relationship between CD27 negative and positive B cell populations in human peripheral blood. *Front Immunol* 2011;**2**:81.
354. Sims GP, Ettinger R, Shirota Y, et al. Identification and characterization of circulating human transitional B cells. *Blood* 2005;**105**:4390-8.
355. Malaspina A, Moir S, Ho J, et al. Appearance of immature/transitional B cells in HIV-infected individuals with advanced disease: correlation with increased IL-7. *Proc Natl Acad Sci USA* 2006;**103**:2262-7.
356. Ibegbu C, Spira TJ, Nesheim S, et al. Subpopulations of T and B cells in perinatally HIV-infected and noninfected age-matched children compared with those in adults. *Clin Immunol Immunopathol* 1994;**71**:27-32.
357. Rodriguez C, Thomas JK, O'Rourke S, Stiehm ER, Plaeger S. HIV disease in children is associated with a selective decrease in CD23+ and CD62L+ B cells. *Clin Immunol Immunopathol* 1996;**81**:191-9.
358. Bohnhorst JO, Bjorgan MB, Thoen JE, Natvig JB, Thompson KM. Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjogren's syndrome. *J Immunol* 2001;**167**:3610-8.
359. Moir S, Ho J, Malaspina A, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med* 2008;**205**:1797-805.
360. Schatorje EJ, Gemen EF, Driessen GJ, et al. Age-matched reference values for B-lymphocyte subpopulations and CVID classifications in children. *Scand J Immunol* 2011;**74**:502-10.
361. Comans-Bitter WM, de Groot R, van den Beemd R, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* 1997;**130**:388-93.
362. Zaccarelli-Filho CA, Ono E, Machado DM, et al. HIV-1-infected children on HAART: immunologic features of three different levels of viral suppression. *Cytometry B Clin Cytom* 2007;**72**:14-21.
363. Anselmi A, Vendrame D, Rampon O, et al. Immune reconstitution in human immunodeficiency virus type 1-infected children with different virological responses to anti-retroviral therapy. *Clin Exp Immunol* 2007;**150**:442-50.
364. Resino S, Galan I, Bellon JM, et al. Characterizing the immune system after long-term undetectable viral load in HIV-1-infected children. *J Clin Immunol* 2003;**23**:279-89.
365. Resino S, Bellon JM, Gurbindo D, Leon JA, Munoz-Fernandez MA. Recovery of T-cell subsets after antiretroviral therapy in HIV-infected children. *Eur J Clin Invest* 2003;**33**:619-27.
366. Chun TW, Justement JS, Pandya P, et al. Relationship between the size of the human immunodeficiency virus type 1 (HIV-1) reservoir in peripheral blood CD4+ T cells and CD4+:CD8+ T cell ratios in aviremic HIV-1-infected individuals receiving long-term highly active antiretroviral therapy. *J Infect Dis* 2002;**185**:1672-6.
367. Lewis J, Walker AS, Castro H, et al. Age and CD4 count at initiation of antiretroviral therapy in HIV-infected children: effects on long-term T-cell reconstitution. *J Infect Dis* 2012;**205**:548-56.
368. Morbach H, Eichhorn EM, Liese JG, Girschick HJ. Reference values for B cell subpopulations from infancy to adulthood. *Clin Exp Immunol* 2010;**162**:271-9.
369. Piatosa B, Wolska-Kusnierz B, Pac M, et al. B cell subsets in healthy children: reference values for evaluation of B cell maturation process in peripheral blood. *Cytometry B Clin Cytom* 2010;**78**:372-81.
370. Malaspina A, Moir S, Chaitt DG, et al. Idiopathic CD4+ T lymphocytopenia is associated with increases in immature/transitional B cells and serum levels of IL-7. *Blood* 2007;**109**:2086-8.
371. Froebel KS, Doherty KV, Whitelaw JA, et al. Increased expression of the CD45RO (memory) antigen on T cells in HIV-infected children. *AIDS* 1991;**5**:97-9.
372. Brugnoni D, Soresina A, Airo P, et al. Effect of HIV vertical transmission on the ontogeny of T cell antigens involved in the regulation of humoral immune response. *Clin Exp Immunol* 1995;**102**:238-42.
373. McCloskey TW, Kohn N, Lesser M, Bakshi S, Pahwa S. Immunophenotypic analysis of HIV-infected children: alterations within the first year of life, changes with disease progression, and longitudinal analyses of lymphocyte subsets. *Cytometry* 2001;**46**:157-65.
374. Kelley CF, Kitchen CM, Hunt PW, et al. Incomplete peripheral CD4+ cell count restoration in HIV-infected patients receiving long-term antiretroviral treatment. *Clin Infect Dis* 2009;**48**:787-94.
375. Thompson MA, Aberg JA, Hoy JF, et al. Antiretroviral treatment of adult HIV infection: 2012 recommendations of the International Antiviral Society-USA panel. *JAMA* 2012;**308**:387-402.
376. Moir S, Malaspina A, Pickeral OK, et al. Decreased Survival of B Cells of HIV-viremic Patients Mediated by Altered Expression of Receptors of the TNF Superfamily. *J Exp Med* 2004;**200**:587-600.
377. Ruffin N, Lantto R, Pensieroso S, et al. Immune activation and increased IL-21R expression are associated with the loss of memory B cells during HIV-1 infection. *J Intern Med* 2012.

378. Ho J, Moir S, Malaspina A, et al. Two overrepresented B cell populations in HIV-infected individuals undergo apoptosis by different mechanisms. *Proc Natl Acad Sci U S A* 2006;**103**:19436-41.
379. Chess Q, Daniels J, North E, Macris NT. Serum immunoglobulin elevations in the acquired immunodeficiency syndrome (AIDS): IgG, IgA, IgM, and IgD. *Diagn Immunol* 1984;**2**:148-53.
380. Goedert JJ. The epidemiology of acquired immunodeficiency syndrome malignancies. *Semin Oncol* 2000;**27**:390-401.
381. Zandman-Goddard G, Shoenfeld Y. HIV and autoimmunity. *Autoimmun Rev* 2002;**1**:329-37.
382. Stratton R, Slapak G, Mahungu T, Kinloch-de Loes S. Autoimmunity and HIV. *Curr Opin Infect Dis* 2009;**22**:49-56.
383. Haas A, Zimmermann K, Oxenius A. Antigen-dependent and -independent mechanisms of T and B cell hyperactivation during chronic HIV-1 infection. *J Virol* 2011;**85**:12102-13.
384. Ruffin N, Thang PH, Rethi B, Nilsson A, Chiodi F. The impact of inflammation and immune activation on B cell differentiation during HIV-1 infection. *Front Immunol* 2011;**2**:90.
385. Cagigi A, Nilsson A, De Milito A, Chiodi F. B cell immunopathology during HIV-1 infection: Lessons to learn for HIV-1 vaccine design. *Vaccine* 2008;**26**:3016-25.
386. Das A, Veazey RS, Wang X, et al. Simian immunodeficiency virus infection in rhesus macaques induces selective tissue specific B cell defects in double positive CD21+CD27+ memory B cells. *Clin Immunol* 2011;**140**:223-8.
387. Petrovas C, Yamamoto T, Gerner MY, et al. CD4 T follicular helper cell dynamics during SIV infection. *J Clin Invest* 2012;**122**:3281-94.
388. Peruchon S, Chaoul N, Burelout C, et al. Tissue-specific B-cell dysfunction and generalized memory B-cell loss during acute SIV infection. *PLoS ONE* 2009;**4**:e5966.
389. Titanji K, Velu V, Chennareddi L, et al. Acute depletion of activated memory B cells involves the PD-1 pathway in rapidly progressing SIV-infected macaques. *J Clin Invest* 2010;**120**:3878-90.
390. Zeng M, Smith AJ, Wietgrefe SW, et al. Cumulative mechanisms of lymphoid tissue fibrosis and T cell depletion in HIV-1 and SIV infections. *J Clin Invest* 2011;**121**:998-1008.
391. Schnittman SM, Lane HC, Higgins SE, Folks T, Fauci AS. Direct polyclonal activation of human B lymphocytes by the acquired immune deficiency syndrome virus. *Science* 1986;**233**:1084-6.
392. Moir S, Malaspina A, Li Y, et al. B cells of HIV-1-infected patients bind virions through CD21-complement interactions and transmit infectious virus to activated T cells. *J Exp Med* 2000;**192**:637-46.
393. Qiao X, He B, Chiu A, et al. Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells. *Nat Immunol* 2006;**7**:302-10.
394. Swingler S, Zhou J, Swingler C, et al. Evidence for a pathogenic determinant in HIV-1 Nef involved in B cell dysfunction in HIV/AIDS. *Cell Host Microbe* 2008;**4**:63-76.
395. Xu W, Santini PA, Sullivan JS, et al. HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits. *Nat Immunol* 2009;**10**:1008-17.
396. Rieckmann P, Poli G, Fox CH, Kehrl JH, Fauci AS. Recombinant gp120 specifically enhances tumor necrosis factor-alpha production and Ig secretion in B lymphocytes from HIV-infected individuals but not from seronegative donors. *J Immunol* 1991;**147**:2922-7.
397. Schacker T. The role of secondary lymphatic tissue in immune deficiency of HIV infection. *AIDS* 2008;**22 Suppl 3**:S13-8.
398. Cohen J. HIV/AIDS research. Tissue says blood is misleading, confusing HIV cure efforts. *Science* 2011;**334**:1614.
399. Cagigi A, Mowafi F, Phuong Dang LV, et al. Altered expression of the receptor-ligand pair CXCR5/CXCL13 in B cells during chronic HIV-1 infection. *Blood* 2008;**112**:4401-10.
400. Prendergast A, O'Callaghan M, Menson E, et al. Factors influencing T cell activation and programmed death 1 expression in HIV-infected children. *AIDS Res Hum Retroviruses* 2012;**28**:465-8.
401. Imbeault M, Ouellet M, Giguere K, et al. Acquisition of host-derived CD40L by HIV-1 in vivo and its functional consequences in the B-cell compartment. *J Virol* 2011;**85**:2189-200.
402. Perise-Barrios AJ, Munoz-Fernandez MA, Pion M. Direct phenotypical and functional dysregulation of primary human B cells by human immunodeficiency virus (HIV) type 1 in vitro. *PLoS ONE* 2012;**7**:e39472.
403. Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* 2006;**12**:1365-71.
404. Qi H. From SAP-less T cells to helpless B cells and back: dynamic T-B cell interactions underlie germinal center development and function. *Immunol Rev* 2012;**247**:24-35.
405. Patakas A, Platt AM, Butcher JP, et al. Putative existence of reciprocal dialogue between Tfh and B cells and its impact on infectious and autoimmune disease. *Immunol Lett* 2011;**138**:38-46.

406. Zhang ZQ, Schuler T, Cavert W, et al. Reversibility of the pathological changes in the follicular dendritic cell network with treatment of HIV-1 infection. *Proc Natl Acad Sci U S A* 1999;**96**:5169-72.
407. Siewe B, Landay A. Effector and regulatory roles for B cells in HIV infection? *Autoimmunity* 2012;**45**:348-52.
408. Linterman MA, Rigby RJ, Wong RK, et al. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* 2009;**206**:561-76.
409. Connick E, Schlichtemeier R, Pham M, Folkvord J. T Follicular Helper Cells from Naive Hosts Are Highly Permissive to HIV ex vivo. Poster #299 CROI 2012; Seattle2012.
410. Madhi SA, Adrian P, Cotton MF, et al. Effect of HIV infection status and anti-retroviral treatment on quantitative and qualitative antibody responses to pneumococcal conjugate vaccine in infants. *J Infect Dis* 2010;**202**:355-61.
411. Nachman S, Kim S, King J, et al. Safety and immunogenicity of a heptavalent pneumococcal conjugate vaccine in infants with human immunodeficiency virus type 1 infection. *Pediatrics* 2003;**112**:66-73.
412. King JC, Jr., Vink PE, Chang I, et al. Antibody titers eight months after three doses of a five-valent pneumococcal conjugate vaccine in HIV and non-HIV-infected children less than two years of age. *Vaccine* 1998;**16**:361-5.
413. King JC, Jr., Vink PE, Farley JJ, et al. Safety and immunogenicity of three doses of a five-valent pneumococcal conjugate vaccine in children younger than two years with and without human immunodeficiency virus infection. *Pediatrics* 1997;**99**:575-80.
414. King JC, Jr., Vink PE, Farley JJ, et al. Comparison of the safety and immunogenicity of a pneumococcal conjugate with a licensed polysaccharide vaccine in human immunodeficiency virus and non-human immunodeficiency virus-infected children. *Pediatr Infect Dis J* 1996;**15**:192-6.
415. Soininen A, Pursiainen H, Kilpi T, Kayhty H. Natural development of antibodies to pneumococcal capsular polysaccharides depends on the serotype: association with pneumococcal carriage and acute otitis media in young children. *J Infect Dis* 2001;**184**:569-76.
416. Balmer P, Borrow R, Findlow J, et al. Age-stratified prevalences of pneumococcal-serotype-specific immunoglobulin G in England and their relationship to the serotype-specific incidence of invasive pneumococcal disease prior to the introduction of the pneumococcal 7-valent conjugate vaccine. *Clin Vaccine Immunol* 2007;**14**:1442-50.
417. Orange JS, Ballow M, Stiehm ER, et al. Use and interpretation of diagnostic vaccination in primary immunodeficiency: A working group report of the Basic and Clinical Immunology Interest Section of the American Academy of Allergy, Asthma & Immunology. *J Allergy Clin Immunol* 2012;**130**:S1-S24.
418. Borrow R, Heath PT, Siegrist CA. Use of pneumococcal polysaccharide vaccine in children: what is the evidence? *Curr Opin Infect Dis* 2012;**25**:292-303.
419. O'Brien KL, Hochman M, Goldblatt D. Combined schedules of pneumococcal conjugate and polysaccharide vaccines: is hyporesponsiveness an issue? *Lancet Infect Dis* 2007;**7**:597-606.
420. Clutterbuck EA, Lazarus R, Yu LM, et al. Pneumococcal conjugate and plain polysaccharide vaccines have divergent effects on antigen-specific B cells. *J Infect Dis* 2012;**205**:1408-16.
421. Dagan R, Givon-Lavi N, Greenberg D, Fritzell B, Siegrist CA. Nasopharyngeal carriage of Streptococcus pneumoniae shortly before vaccination with a pneumococcal conjugate vaccine causes serotype-specific hyporesponsiveness in early infancy. *J Infect Dis* 2010;**201**:1570-9.
422. Madhi SA, Violari A, Klugman KP, et al. Inferior quantitative and qualitative immune responses to pneumococcal conjugate vaccine in infants with nasopharyngeal colonization by Streptococcus pneumoniae during the primary series of immunization. *Vaccine* 2011;**29**:6994-7001.
423. Hendley JO, Sande MA, Stewart PM, Gwaltney JM, Jr. Spread of Streptococcus pneumoniae in families. I. Carriage rates and distribution of types. *J Infect Dis* 1975;**132**:55-61.
424. Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after pneumococcal vaccination. *Lancet* 2011;**378**:1962-73.
425. Dagan R. Serotype replacement in perspective. *Vaccine* 2009;**27**:C22-C4.
426. Madhi SA, Petersen K, Madhi A, Wasas A, Klugman KP. Impact of human immunodeficiency virus type 1 on the disease spectrum of Streptococcus pneumoniae in South African children. *Pediatr Infect Dis J* 2000;**19**:1141-7.
427. Crewe-Brown HH, Karstaedt AS, Saunders GL, et al. Streptococcus pneumoniae blood culture isolates from patients with and without human immunodeficiency virus infection: alterations in penicillin susceptibilities and in serogroups or serotypes. *Clin Infect Dis* 1997;**25**:1165-72.
428. Jones N, Huebner R, Khoosal M, Crewe-Brown H, Klugman K. The impact of HIV on Streptococcus pneumoniae bacteraemia in a South African population. *AIDS* 1998;**12**:2177-84.
429. Fry AM, Facklam RR, Whitney CG, Plikaytis BD, Schuchat A. Multistate evaluation of invasive pneumococcal diseases in adults with human immunodeficiency virus infection: serotype and antimicrobial resistance patterns in the United States. *J Infect Dis* 2003;**188**:643-52.

430. Goldblatt D, Southern J, Ashton L, et al. Immunogenicity of a reduced schedule of pneumococcal conjugate vaccine in healthy infants and correlates of protection for serotype 6B in the United Kingdom. *Pediatr Infect Dis J* 2010;**29**:401-5.
431. Paradiso P. Essential criteria for evaluation of pneumococcal conjugate vaccine candidates. *Vaccine* 2009;**27**:C15-C8.
432. Pedrono G, Thiebaut R, Alioum A, et al. A new endpoint definition improved clinical relevance and statistical power in a vaccine trial. *J Clin Epidemiol* 2009;**62**:1054-61.
433. WHO. Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines. 2010 [Accessed October 2012]; Available from: http://www.who.int/biologicals/areas/vaccines/pneumo/Pneumo_final_23APRIL_2010.pdf.
434. Barzilay EJ, O'Brien KL, Kwok YS, et al. Could a single dose of pneumococcal conjugate vaccine in children be effective? Modeling the optimal age of vaccination. *Vaccine* 2006;**24**:904-13.
435. Guttormsen HK, Sharpe AH, Chandraker AK, et al. Cognate stimulatory B-cell-T-cell interactions are critical for T-cell help recruited by glycoconjugate vaccines. *Infect Immun* 1999;**67**:6375-84.
436. Reinhardt RL, Liang H-E, Locksley RM. Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat Immunol* 2009;**10**:385-93.
437. Reuter MA, Pombo C, Betts MR. Cytokine production and dysregulation in HIV pathogenesis: Lessons for development of therapeutics and vaccines. *Cytokine Growth Factor Rev* 2012;**23**:181-91.
438. Hainaut M, Verscheure V, Ducarme M, et al. Cellular immune responses in human immunodeficiency virus (HIV)-1-infected children: is immune restoration by highly active anti-retroviral therapy comparable to non-progression? *Clin Exp Immunol* 2011;**165**:77-84.
439. Kamat A, Misra V, Cassol E, et al. A plasma biomarker signature of immune activation in HIV patients on antiretroviral therapy. *PLoS ONE* 2012;**7**:e30881.
440. Keating SM, Jacobs ES, Norris PJ. Soluble mediators of inflammation in HIV and their implications for therapeutics and vaccine development. *Cytokine Growth Factor Rev* 2012;**23**:193-206.
441. Mawas F, Feavers IM, Corbel MJ. Serotype of Streptococcus pneumoniae capsular polysaccharide can modify the Th1/Th2 cytokine profile and IgG subclass response to pneumococcal-CRM(197) conjugate vaccines in a murine model. *Vaccine* 2000;**19**:1159-66.
442. McNeela EA, Jabbal-Gill I, Illum L, et al. Intranasal immunization with genetically detoxified diphtheria toxin induces T cell responses in humans: enhancement of Th2 responses and toxin-neutralizing antibodies by formulation with chitosan. *Vaccine* 2004;**22**:909-14.
443. Vernacchio L, Bernstein H, Pelton S, et al. Effect of monophosphoryl lipid A on T-helper cells when administered as an adjuvant with pneumococcoccal-CRM197 conjugate vaccine in healthy toddlers. *Vaccine* 2002;**20**:3658-67.
444. van den Biggelaar AH, Pomat WS, Phuanukoonnon S, et al. Effect of early carriage of Streptococcus pneumoniae on the development of pneumococcal protein-specific cellular immune responses in infancy. *Pediatr Infect Dis J* 2012;**31**:243-8.
445. van den Biggelaar AH, Pomat W, Bosco A, et al. Pneumococcal conjugate vaccination at birth in a high-risk setting: no evidence for neonatal T-cell tolerance. *Vaccine* 2011;**29**:5414-20.
446. Zinna S, Bamford A, Cunnington A, et al. Immunization status of children with HIV: failure to protect a vulnerable population. *HIV Med* 2011;**12**:447-8.
447. HPA. Vaccine coverage and COVER (Cover of Vaccination Evaluated Rapidly). 2012 [Accessed October 2012]; Available from: http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/VaccineCoverageAndCOVER/.
448. Blazevic V, Jankelevich S, Steinberg SM, et al. Highly active antiretroviral therapy in human immunodeficiency virus type 1-infected children: analysis of cellular immune responses. *Clin Diagn Lab Immunol* 2001;**8**:943-8.
449. Mansoor N, Scriba TJ, de Kock M, et al. HIV-1 infection in infants severely impairs the immune response induced by Bacille Calmette-Guerin vaccine. *J Infect Dis* 2009;**199**:982-90.
450. Sutherland JS, Young JM, Peterson KL, et al. Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment. *J Immunol* 2010;**184**:6537-44.
451. Ota MO, Vekemans J, Schlegel-Haueter SE, et al. Hepatitis B immunisation induces higher antibody and memory Th2 responses in new-borns than in adults. *Vaccine* 2004;**22**:511-9.
452. Ota MO, Vekemans J, Schlegel-Haueter SE, et al. Influence of Mycobacterium bovis bacillus Calmette-Guerin on antibody and cytokine responses to human neonatal vaccination. *J Immunol* 2002;**168**:919-25.
453. Wood N, Siegrist CA. Neonatal immunization: where do we stand? *Curr Opin Infect Dis* 2011;**24**:190-5.
454. PrabhuDas M, Adkins B, Gans H, et al. Challenges in infant immunity: implications for responses to infection and vaccines. *Nat Immunol* 2011;**12**:189-94.

455. Peeters CC, Tenbergen-Meekes AM, Poolman JT, et al. Effect of carrier priming on immunogenicity of saccharide-protein conjugate vaccines. *Infect Immun* 1991;**59**:3504-10.
456. McCool TL, Harding CV, Greenspan NS, Schreiber JR. B- and T-cell immune responses to pneumococcal conjugate vaccines: divergence between carrier- and polysaccharide-specific immunogenicity. *Infect Immun* 1999;**67**:4862-9.
457. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG. Follicular helper T cells: lineage and location. *Immunity* 2009;**30**:324-35.
458. Good KL, Bryant VL, Tangye SG. Kinetics of human B cell behavior and amplification of proliferative responses following stimulation with IL-21. *J Immunol* 2006;**177**:5236-47.
459. Avery DT, Bryant VL, Ma CS, de Waal Malefyt R, Tangye SG. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J Immunol* 2008;**181**:1767-79.
460. Goldblatt D, Southern J, Andrews N, et al. The immunogenicity of 7-valent pneumococcal conjugate vaccine versus 23-valent polysaccharide vaccine in adults aged 50-80 years. *Clin Infect Dis* 2009;**49**:1318-25.
461. Thurm CW, Halsey JF. Measurement of cytokine production using whole blood. *Curr Protoc Immunol* 2005;**Chapter 7**:Unit 7 18B.
462. Diel R, Goletti D, Ferrara G, et al. Interferon-gamma release assays for the diagnosis of latent Mycobacterium tuberculosis infection: a systematic review and meta-analysis. *Eur Respir J* 2011;**37**:88-99.
463. Singh OP, Gidwani K, Kumar R, et al. Reassessment of immune correlates in human visceral leishmaniasis as defined by cytokine release in whole blood. *Clin Vaccine Immunol* 2012;**19**:961-6.
464. Schreiber GH, Schreiber RD. Interferon-gamma. In: Thomson AW, Lotze MT, editors. The Cytokine Handbook 4th ed: Academic Press; 2003. 24. 567-601.
465. Kawano Y, Noma T. Role of interleukin-2 and interferon-gamma in inducing production of IgG subclasses in lymphocytes of human newborns. *Immunology* 1996;**88**:40-8.
466. Snapper CM, Paul WE. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 1987;**236**:944-7.
467. Kawano Y, Noma T, Kou K, Yoshizawa I, Yata J. Regulation of human IgG subclass production by cytokines: human IgG subclass production enhanced differentially by interleukin-6. *Immunology* 1995;**84**:278-84.
468. Jelinek DF, Splawski JB, Lipsky PE. The roles of interleukin 2 and interferon-gamma in human B cell activation, growth and differentiation. *Eur J Immunol* 1986;**16**:925-32.
469. Snapper CM, McIntyre TM, Mandler R, et al. Induction of IgG3 secretion by interferon gamma: a model for T cell-independent class switching in response to T cell-independent type 2 antigens. *J Exp Med* 1992;**175**:1367-71.
470. Khan AQ, Shen Y, Wu ZQ, Wynn TA, Snapper CM. Endogenous pro- and anti-inflammatory cytokines differentially regulate an in vivo humoral response to Streptococcus pneumoniae. *Infect Immun* 2002;**70**:749-61.
471. Grunig G, de Vries JE, de Waal Malefyt R. Interleukin-13. In: Thomson AW, Lotze MT, editors. The Cytokine Handbook: Academic Press; 2003. 17. 409-29.
472. Loots GG, Locksley RM, Blankespoor CM, et al. Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science* 2000;**288**:136-40.
473. Verheyen J, Bonig H, Kim YM, et al. Regulation of interleukin-2 induced interleukin-5 and interleukin-13 production in human peripheral blood mononuclear cells. *Scand J Immunol* 2000;**51**:45-53.
474. Miyatake S, Arai N, Arai K. Chromatin remodeling and T helper subset differentiation. *IUBMB Life* 2000;**49**:473-8.
475. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. *Annu Rev Immunol* 1993;**11**:165-90.
476. Rousset F, Garcia E, Defrance T, et al. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A* 1992;**89**:1890-3.
477. Defrance T, Vanbervliet B, Briere F, et al. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J Exp Med* 1992;**175**:671-82.
478. Levy Y, Brouet JC. Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the bcl-2 protein. *J Clin Invest* 1994;**93**:424-8.
479. Pallikkuth S, Parmigiani A, Pahwa S. The role of interleukin-21 in HIV infection. *Cytokine Growth Factor Rev* 2012;**23**:173-80.
480. Karnell JL, Ettinger R. The Interplay of IL-21 and BAFF in the Formation and Maintenance of Human B Cell Memory. *Front Immunol* 2012;**3**:2.
481. Bryant VL, Ma CS, Avery DT, et al. Cytokine-Mediated Regulation of Human B Cell Differentiation into Ig-Secreting Cells: Predominant Role of IL-21 Produced by CXCR5+ T Follicular Helper Cells. *J Immunol* 2007;**179**:8180-90.

482. Ettinger R, Kuchen S, Lipsky PE. The role of IL-21 in regulating B-cell function in health and disease. *Immunol Rev* 2008;**223**:60-86.
483. Konforte D, Simard N, Paige CJ. IL-21: An Executor of B Cell Fate. *J Immunol* 2009;**182**:1781-7.
484. Spolski R, Leonard WJ. IL-21 and T follicular helper cells. *Int Immunol* 2009:dxp112.
485. Avery DT, Deenick EK, Ma CS, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *J Exp Med* 2010.
486. Zotos D, Coquet JM, Zhang Y, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. *J Exp Med* 2010;**207**:365-78.
487. Rodriguez-Bayona B, Ramos-Amaya A, Bernal J, Campos-Caro A, Brieva JA. Cutting edge: IL-21 derived from human follicular helper T cells acts as a survival factor for secondary lymphoid organ, but not for bone marrow, plasma cells. *J Immunol* 2012;**188**:1578-81.
488. Recher M, Berglund LJ, Avery DT, et al. IL-21 is the primary common gamma chain-binding cytokine required for human B-cell differentiation in vivo. *Blood* 2011;**118**:6824-35.
489. Rankin AL, MacLeod H, Keegan S, et al. IL-21 receptor is critical for the development of memory B cell responses. *J Immunol* 2011;**186**:667-74.
490. EMA. Focetria: Summary of Product Characteristics. 2012 [Accessed October 2012]; Available from: http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000710/WC500023749.pdf.
491. Batista FD, Harwood NE. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* 2009;**9**:15-27.
492. Heinen E, Braun M, Coulie PG, et al. Transfer of immune complexes from lymphocytes to follicular dendritic cells. *Eur J Immunol* 1986;**16**:167-72.
493. Cinamon G, Zachariah MA, Lam OM, Foss FW, Jr., Cyster JG. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat Immunol* 2008;**9**:54-62.
494. Phan TG, Green JA, Gray EE, Xu Y, Cyster JG. Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nat Immunol* 2009;**10**:786-93.
495. Jackson LA, Neuzil KM, Nahm MH, et al. Immunogenicity of varying dosages of 7-valent pneumococcal polysaccharide-protein conjugate vaccine in seniors previously vaccinated with 23-valent pneumococcal polysaccharide vaccine. *Vaccine* 2007;**25**:4029-37.
496. Poland GA, Kennedy RB, Ovsyannikova IG. Vaccinomics and personalized vaccinology: is science leading us toward a new path of directed vaccine development and discovery? *PLoS Pathog* 2011;**7**:e1002344.
497. Goldblatt D, Ashton L, Zhang Y, Antonello J, Marchese RD. Comparison of a new multiplex binding assay versus the enzyme-linked immunosorbent assay for measurement of serotype-specific pneumococcal capsular polysaccharide IgG. *Clin Vaccine Immunol* 2011;**18**:1744-51.
498. Weller S, Bonnet M, Delagreverie H, et al. IgM+IgD+CD27+ B cells are markedly reduced in IRAK-4-, MyD88- and TIRAP- but not UNC-93B-deficient patients. *Blood* 2012.
499. Denton PW, Olesen R, Choudhary SK, et al. Generation of HIV latency in humanized BLT mice. *J Virol* 2012;**86**:630-4.
500. Broder S. The development of antiretroviral therapy and its impact on the HIV-1/AIDS pandemic. *Antiviral Res.* 2010;**85**:1-18.
501. Esté JA, Cihlar T. Current status and challenges of antiretroviral research and therapy *Antiviral Res.* 2010;**85:**25-33.
502. Kuller LH, Tracy R, Belloso W, et al. Inflammatory and coagulation biomarkers and mortality in patients with HIV infection. *PLoS Med*. 2008;**5**:e203.
503. Rodger AJ, Fox Z, Lundgren JD et al. Activation and coagulation biomarkers are independent predictors for the development of opportunistic disease in patients with HIV infection. *J Infect Dis*. 2009;**200**:973-983.
504. Neuhaus J, Jacobs DR, Baker JV et al. Markers of inflammation, coagulation and renal function are elevated in adults with HIV infection. *J Infect Dis.* 2010;**201**:1788-1795.
505. Duprez DA, Neuhaus J, Kuller LH et al. Inflammation, coagulation and cardiovascular disease in HIV-infected individuals. *PLoS One.* 2012;**7**:e44454.
506. Bunupuradah T, Duong T, Compagnucci A et al. Outcomes after reinitiating antiretroviral therapy in children randomized to planned treatment interruptions. *AIDS.* 2013;**27**:579-589.

Appendix 1

WHO clinical staging of HIV/AIDS for children with confirmed HIV infection

World Health Organization. WHO Case Definitions of HIV for Surveillance, and Revised Clinical Staging and Immunological Classification of HIV Related Disease in Adults and Children. Geneva, World Health Organization, 2006

Clinical Stage 1

- Asymptomatic
- Persistent generalized lymphadenopathy

Clinical Stage 2

- Unexplained persistent hepatosplenomegaly
- Papular pruritic eruptions
- Extensive wart virus infection
- Extensive molluscum contagiosum
- Fungal nail infections
- Recurrent oral ulcerations
- Unexplained persistent parotid enlargement
- Lineal gingival erythema
- Herpes zoster Recurrent or chronic upper respiratory tract infections (otitis media, otorrhoea, sinusitis, tonsillitis)

Clinical Stage 3

- Unexplained* moderate malnutrition not adequately responding to standard therapy
- Unexplained persistent diarrhoea (14 days or more)
- Unexplained persistent fever (above 37.51C intermittent or constant, for longer than one month)
- Persistent oral candidiasis (after first 6–8 weeks of life)
- Oral hairy leukoplakia
- Acute necrotizing ulcerative gingivitis/periodontitis
- Lymph node TB
- Pulmonary TB
- Severe recurrent bacterial pneumonia
- Symptomatic lymphoid interstitial pneumonitis
- Chronic HIV-associated lung disease including bronchiectasis
- Unexplained anaemia (<8 g/dL), neutropenia (<0.5 x 10^9/l) or chronic thrombocytopenia (<50 x 10^9/l)

Clinical Stage 4

- Unexplained severe wasting, stunting or severe malnutrition not responding to standard therapy
- Pneumocystis pneumonia
- Recurrent severe bacterial infections (e.g. empyema, pyomyositis, bone or joint infection, meningitis, but excluding pneumonia)
- Chronic herpes simplex infection; (orolabial or cutaneous of more than one month's duration or visceral at any site)
- Extrapulmonary/disseminated tuberculosis
- Kaposi's sarcoma
- Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs)
- Central nervous system toxoplasmosis (after one month of life)
- HIV encephalopathy
- Cytomegalovirus infection retinitis or CMV infection affecting another organ, with onset at age over 1 month
- Extrapulmonary cryptococcosis (including meningitis)
- Disseminated endemic mycosis (extrapulmonary histoplasmosis, coccidiomycosis)
- Chronic cryptosporidiosis
- Chronic isosporiasis
- Disseminated non-tuberculous mycobacteria infection
- HIV associated tumours including cerebral or B cell non-Hodgkin lymphoma
- Progressive multifocal leukoencephalopathy
- Symptomatic HIV-associated nephropathy or HIV-associated cardiomyopathy

*Unexplained refers to where the condition is not explained by other conditions
**Some additional specific conditions can also be included in regional classifications (e.g. reactivation of American trypanosomiasis)

Appendix 2

CDC 1994 revised classification system for HIV infection in children less than 13 years of age Centers for Disease Control and Prevention. 1994 revised classification system for human immunodeficiency virus infection in children less than 13 years of age. Morbid Mortal Wkly Rep 1994; 43 (RR-12): 1–10.

Clinical categories for children with HIV infection

Category N: Not symptomatic

Children who have no signs or symptoms considered to be the result of HIV infection or who have only one of the conditions listed in Category A

Category A: Mildly symptomatic

Children with two or more of the conditions listed below but none of the conditions listed in Categories B and C:

- Lymphadenopathy (≥0.5 cm at more than two sites; bilateral = one site)
- Hepatomegaly
- Splenomegaly
- Dermatitis
- Parotitis
- Recurrent or persistent upper respiratory infection, sinusitis, or otitis media

Category B: Moderately symptomatic

Children who have symptomatic conditions other than those listed for Category A or C that are attributed to HIV infection. Examples of conditions in clinical Category B include but are not limited to:

- Anaemia (<8 g/dL), neutropenia (<1.0 $x10^9$/L), or thrombocytopenia (<100 $x10^9$/L) persisting ≥30 days
- Bacterial meningitis, pneumonia, or sepsis (single episode)
- Candidiasis, oropharyngeal (thrush), persisting (>2 months) in children >6 months of age
- Cardiomyopathy
- Cytomegalovirus infection, with onset before 1 month of age
- Diarrhoea, recurrent or chronic
- Hepatitis
- Herpes simplex virus (HSV) stomatitis, recurrent (more than two episodes within 1 year)
- HSV bronchitis, pneumonitis, oroesophagitis with onset before1 month of age
- Herpes zoster (shingles) involving at least two distinct episodes or more than one dermatome
- Leimyosarcoma
- Lymphoid interstitial pneumonia (LIP) or pulmonary lymphoid hyperplasia complex
- Nephropathy
- Nocardiosis
- Persistent fever (lasting >1 month)
- Toxoplasmosis, onset before 1 month of age
- Varicella, disseminated (complicated chickenpox)

Category C: Severely symptomatic

- Serious bacterial infections, multiple or recurrent (i.e. any combination of at least two culture-confirmed infections within a 2-year period), of the following types: septicaemia, pneumonia, meningitis, bone or joint infection, or abscess of an internal organ or body cavity (excluding otitis media, superficial skin or mucosal abscesses, and indwelling catheter-related infections)
- Candidiasis, oesophageal or pulmonary (bronchi, trachea, lungs)
- Coccidioidomycosis, disseminated (at site other than or in addition to lungs or cervical or hilar lymph nodes)
- Cryptococcosis, extrapulmonary
- Cryptosporidiosis or isosporiasis with diarrhoea persisting > 1 month
- Cytomegalovirus disease with onset of symptoms at age > 1 month (at a site other than liver, spleen, or lymph nodes)
- Encephalopathy (at least one of the following progressive findings present for at least 2 months in the absence of a concurrent illness other than HIV infection that could explain the findings):
    - failure to attain or loss of developmental milestones or loss of intellectual ability, verified by standard developmental scale or neuropsychological tests;
    - impaired brain growth or acquired microcephaly demonstrated by head circumference measurements or brain atrophy demonstrated by computerised tomography or magnetic resonance imaging (serial imaging is required for children >2 years of age);
    - acquired symmetric motor deficit manifested by two or more of the following: paresis, pathological reflexes, ataxia, or gait disturbance
- Herpes simplex virus infection causing a mucocutaneous ulcer that persists for >1 month; or bronchitis, pneumonitis, or oesophagitis for any duration affecting a child >1 month of age
- Histoplasmosis, disseminated (at a site other than or in addition to lungs or cervical or hilar lymph nodes)

- Kaposi's sarcoma
- Lymphoma, primary, in brain
- Lymphoma, small, noncleaved cell (Burkitt's), or immunoblastic or large cell lymphoma of B-cell or unknown immunological phenotype
- Mycobacterium tuberculosis, disseminated or extrapulmonary
- Mycobacterium, other species or unidentified species, disseminated (at a site other than or in addition to lungs, skin, or cervical or hilar lymph nodes)
- Mycobacterium avium complex or Mycobacterium kansasii, disseminated (at site other than or in addition to lungs, skin, or cervical or hilar lymph nodes)
- Pneumocystis jiroveci pneumonia (formerly carinii)
- Progressive multifocal leukoencephalopathy
- Salmonella (nontyphoid) septicaemia, recurrent
- Toxoplasmosis of the brain with onset at >1 month of age
- Wasting syndrome in the absence of a concurrent illness other than HIV infection that could explain the following findings:
    - persistent weight loss 410% of baseline OR
    - downward crossing of at least two of the following percentile lines on the weight-for-age chart (e.g., 95th, 75th, 50th, 25th, 5th) in a child ≥1 year of age OR
    - <5th percentile on weight-for-height chart on two consecutive measurements, ≥30 days apart PLUS a) chronic diarrhoea (i.e., at least two loose stools per day for ≥30 days) OR b) documented fever (for ≥30 days, intermittent or constant)

Appendix 3

CHIVA treatment grid for children with HIV
First and second line antiretroviral choices – 2009

This table summarises the basic principles of first and second line treatment choices. For complete notes and guidance see reference 181 (Welch et al)

First line antiretroviral therapy		
<3 years	>3 years <40kg	>40kg
Nevirapine + lamividune + abacavir + (zidovudine) or lopinavir/ritonavir + lamivudine + abacavir + (zidovudine	Efavirenz + lamivudine + abacavir or lopinavir/ritonavir + lamivudine + abacavir	Efavirenz + (emtricitabine + tenofovir)* or (lamivudine + abacavir)** or lopinavir/ritonavir + (emtricitabine + tenofovir) or (lamivudine + abacavir)
Most likely second-line ART when first line NNRTI based		
<30kg	>30kg	>40kg
Lopinavir/ritonavir + zidovudine + didanosine	Lopinavir/ritonavir + zidovudine + tenofovir	Previous (emtricitabine + tenofovir): Lopinavir/ritonavir + didanosine + abacavir) Previous (lamivudine + abacavir): Lopinavir/ritonavir + zidovudine + tenofovir

Most likely second-line ART when first line PI based			
<3 years	>3 years <30kg	>30kg	>40kg
Nevirapine + zidovudine + didanosine	Efavirenz + zidovudine + didanosine	Efavirenz + zidovudine + tenofovir	Previous (emtricitabine + tenofovir): efavirenz + didanosine + abacavir Previous (lamivudine + abacavir): Efavirenz + zidovudine + tenofovir

*"Truvada", **"Kivexa"

Appendix 4: Ethical approval

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Dr Alasdair R J Bamford
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28 May 2010

Dear Dr Bamford

Study title: **The impact of impaired T cell function on B cell memory responses in HIV infected children**
REC reference: **09/H0706/23**
Protocol number: **CRO1262**
Amendment number: **1**
Amendment date: **30 April 2010**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

Favourable Opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

I would also like to inform you that you will need to amend all documentation to correctly state that West London REC 3 has reviewed the application.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Letter to GP: Healthy Control	3	28 April 2010
Participant Consent Form: Healthy Control: Consent Form	4	28 April 2010
Participant Consent Form: Healthy Control - Patient Consent Form: Child Control	4	28 April 2010
Participant Consent Form: Healthy Control - Assent Form: Child Control	3	28 April 2010
Participant Consent Form: Healthy Adults	3	28 April 2010
Participant Information Sheet: Information Sheet: Child Healthy Control	4	28 April 2010
Participant Information Sheet: Teenage Information Sheet: (na)	3	28 April 2010
Participant Information Sheet: Teen Information Sheet: Child Control	4	28 April 2010

Participant Information Sheet: Information Sheet: Healthy Adult	4	28 April 2010
Participant Information Sheet: Child Information Sheet: Child Control	4	28 April 2010
Protocol	6	28 April 2010
Notice of Substantial Amendment (non-CTIMPs)	1	30 April 2010
Covering Letter		30 April 2010
Letter to GP	2	28 April 2010
Participant Consent Form: Assent Form: (a)	3	28 April 2010
Participant Consent Form: Assent Form: (na)	3	28 April 2010
Participant Consent Form: Patient Consent Form: Family Clinic	3	28 April 2010
Participant Information Sheet: Child Information Sheet: (a)	3	28 April 2010
Participant Information Sheet: Child Information Sheet: (na)	3	28 April 2010
Participant Information Sheet: Information Sheet: Family Clinic	3	28 April 2010
Participant Information Sheet: Teenage Information Sheet: (a)	3	28 April 2010
Participant Consent Form: Consent Form: Family Clinic	3	28 April 2010

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

09/H0706/23:	**Please quote this number on all correspondence**

Yours sincerely

Louise Moran
Committee Co-ordinator
E-mail: louise.moran2@imperial.nhs.uk

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Mr Gary Roper, Research Service*

West London REC 3

Attendance at Sub-Committee of the REC meeting on 28 May 2010

Name	*Profession*	*Capacity*
Dr Matthew Hyde	Research Scientist	Lay
Dr Sabita Uthaya	Consultant Neonatologist	Expert

Appendix 5: Substantial amendment approval letter

NHS
National Research Ethics Service

Riverside Research Ethics Committee
Room 4W/12, 4th Floor West
Charing Cross Hospital
Fulham Palace Road
London W6 8RF
Telephone: 020 8846 7282
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Dr Alasdair R J Bamford
Imperial College London
Academic Department of Paediatrics
2nd Floor Wright-Fleming Building
Norfolk Place, Paddington, London
W2 1PG

31 March 2009

Dear Dr Bamford

Full title of study: **The impact of impaired T cell function on B cell memory responses in HIV infected children**
REC reference number: **09/H0706/23**

Thank you for your letter of 19 March 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to the research sites listed on the attached form.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission at NHS sites ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Information Sent to MHRA		
E-mail to MHRA		10 November 2008
Sample Size Calculation		23 November 2007
NIHR Award		17 July 2008
Educational Supervisor CV		
Summary/Synopsis	1	16 January 2009
Covering Letter		11 February 2009
Investigator CV		
Application	12674/25418/1/1	12 February 2009
Peer Review		
Letter from Sponsor		21 January 2009
Participant Consent Form: Family Clinic - Consent	1	16 January 2009
Participant Consent Form: Family Clinic - Assent, Not Aware	1	16 January 2009
Participant Consent Form: Family Clinic - Assent, Aware	1	16 January 2009
Participant Information Sheet: Family Clinic - Teenage, Aware	1	16 January 2009
Participant Information Sheet: Family Clinic - Child, Not Aware	1	16 January 2009
Participant Information Sheet: Family Clinic - Child, Aware	1	16 January 2009
Participant Information Sheet: Family Clinic - Main	1	16 January 2009
GP/Consultant Information Sheets	1 (Family Clinic)	16 January 2009
Letter of invitation to participant	1	16 January 2009
Participant Information Sheet: Family Clinic - Teenage, Not Aware	1	16 January 2009
Participant Consent Form: Healthy Adult	1	16 January 2009
GP/Consultant Information Sheets	1 (Healthy Adult)	15 January 2009
Letter of invitation to participant	1 (Healthy Adult)	16 January 2009
Participant Consent Form: Child Control - Assent	1	16 January 2009
Participant Consent Form: Child Control - Consent	1	16 January 2009
GP/Consultant Information Sheets	1 (Child Control)	16 January 2009
Key Investigator CV		15 December 2008
Response to Request for Further Information		19 March 2009
Participant Consent Form: Child Control - Patient Consent	1	18 March 2009
Participant Consent Form: Family Clinic - Patient Consent	1	18 March 2009
Protocol	2	18 March 2009
Participant Information Sheet: Healthy Adult	2	18 March 2009
Participant Information Sheet: Child Control - Child	2	18 March 2009
Participant Information Sheet: Child Control - Teen	2	18 March 2009
Participant Information Sheet: Child Control - Main	2	18 March 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document "After ethical review –guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0706/23	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

pp

Dr Sabita Uthaya
Chair

Email: atul.patel@imperial.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Mr Gary Roper, Research Service*

Riverside Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

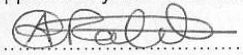
For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	09/H0706/23	**Issue number:**	1	**Date of issue:**	31 March 2009
Chief Investigator:	Dr Alasdair R J Bamford				
Full title of study:	The impact of impaired T cell function on B cell memory responses in HIV infected children				

This study was given a favourable ethical opinion by Riverside Research Ethics Committee on 24 March 2009. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes (1)*
Dr Alasdair R J Bamford	Paediatric SpR	Imperial College Healthcare NHS Trust	St Mary's REC	31/03/2009	

Approved by the Chair on behalf of the REC:



..... (Signature of ~~Chair~~/Co-ordinator)

(delete as applicable)

ATUL PATEL ..... (Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

This Research Ethics Committee is an advisory committee to London Strategic Health Authority
The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

Appendix 6: WHO Reference laboratory ELISA protocol

Training manual for Enzyme linked immunosorbent assay for the quantitation of *Streptococcus pneumoniae* serotype specific IgG (Pn PS ELISA).

(007sp Version)

A guide to procedures for qualification of materials and analysis of assay performance.

This manual describes procedures utilizing 007sp as a standard. Click here for the manual utilizing 89SF as a standard.

Prepared by the World Health Organization Pneumococcal Serology Reference Laboratories at the Institute of Child Health, University College London, London, England and the Department of Pathology at the University of Alabama at Birmingham, Birmingham Alabama, USA.

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Introduction

To develop and evaluate the efficacy of pneumococcal vaccines it is important to have an accurate method for measuring the concentration of human antibodies directed against pneumococcal capsular polysaccharides (Pn PS). In 2000, representatives from academia, government, and industry met at the WHO in Geneva, Switzerland, and selected an enzyme-linked immunosorbent assay protocol for quantitation of human IgG antibodies specific for *Streptococcus pneumoniae* capsular polysaccharides (Pn PS ELISA). The protocol was selected to guide assay development within individual laboratories.

The selected protocol is as follows. Human serum samples are mixed before analysis with an absorbent containing C-polysaccharide (C-PS) and 22F capsular PS to neutralize antibody binding to C-PS and other common contaminants present in the Pn PS coating antigens. 22F PS is used because it is a rare serotype, not present in any conjugate vaccine, and is thought to contain contaminating non-Pn PS epitopes. ELISA plates are coated with Pn PS by adsorbing individual Pn PS serotype antigens to micro-titer plates. Dilutions of absorbed human sera are then added to the ELISA plates. The serotype specific antibody bound to the ELISA plate is detected with anti-human IgG antibody conjugated with alkaline phosphatase, followed by addition of the substrate, *p*-nitrophenyl phosphate. The optical density of each well is measured at 405 nm and 690 nm (reference) using an ELISA plate reader. By comparing the optical density of the sample wells to that of the standard (human anti-pneumococcal reference serum, 007sp) the level of antibody in the human serum can be calculated.

To facilitate the development of Pn PS ELISA with uniform assay performance throughout the world, we have prepared this training document describing the selected assay in detail and have explained how the assay can be set up in a laboratory. General good laboratory practices are described as SOP 1. In addition, this training document provides a set of standard operating procedures (SOP's) that describe the procedures for the basic Pn PS ELISA assay (SOP 2), selecting the lot of ELISA plates (SOP 3), determining the optimal antigen coating concentration (SOP 4), selecting the enzyme conjugated polyclonal anti-human antisera with proper specificity, sensitivity and high enzyme activity (SOP 5) and determining the titer of the enzyme conjugated polyclonal anti-human antisera (SOP 6). This document and additional information can be found in a website (http://www.vaccine.uab.edu) and a review article (CM Wernette et al, Clinical and Diagnostic Laboratory Immunology 10(4): pages 514-519).

This manual was prepared with the financial support of the Vaccines, Immunization and Biologicals Department of WHO in Geneva Switzerland; by Dr. David Goldblatt and Ms. Lindsay Ashton of University College, London, England; and Drs. Moon H. Nahm and William H. Benjamin at the University of Alabama at Birmingham, Birmingham, Alabama, USA with technical assistance from Wyeth Lederle Vaccines, Rochester, NY, USA. We are indebted to Mr. Keith Friedman, Dr. Dan Sikkema and Dr. Dace Madore of Wyeth Lederle Vaccines, Rochester, NY and Dr. Luis Jodar and Dr. Elwyn Griffiths at WHO, Geneva.

Comments and queries should be addressed to:

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SOP 1: Good Laboratory Practice Guidelines

Prepared by the laboratories of:	Dr. Moon Nahm and Dr. David Goldblatt
History of Revision:	July 31, 2002; November 26, 2002; June 9, 2004
Referenced SOP:	SOP 1

Safety first: All serum samples are potentially infectious and all chemicals are potentially toxic. Wear gloves, protective clothing, and/or eye protection when handling human sera or chemicals. Prepare only the minimum amounts necessary and discard any remaining solutions or samples properly. Any potentially contaminated materials should be disinfected and discarded properly. Disinfect laboratory benches after work.

Water: Use a water purification system such as a Synergy 185 from Millipore (Bedford, MA). This water is defined as reagent grade water type 1. Avoid prolonged storage since microorganisms can grow and contaminate the water. In some critical cases (e.g., preparation of antigen coating solution), use commercially available, bottled, pyrogen-free water (for example, sterile water for irrigation distributed by Abbott Laboratories, North Chicago, IL).

Equipment: Equipment must be in good working order (e.g., pH meter, single and multi-channel micropipettors).

Supplies: Store supplies properly. ELISA plates should not be stored in areas exposed to heat, direct sunlight or excessive humidity. Also, minimize dust, particulates or fingerprints on plates. Solution storage containers must be free of contaminants as residual amounts of detergent on glassware can inhibit the binding of antibody to antigen or could strip adsorbed polysaccharides from the microtiter plates. All glassware/plastic ware used for preparing buffers/solutions must be depyrogenated by heating in an oven at 180°C for a minimum of 2 hours to remove endotoxin. The decontaminated glassware/plastic ware must be stored aseptically. For critical applications, use brand new magnetic stir bars, glassware or plastic ware.

Labels: All containers for solutions prepared in-house should be labeled with reagent name, date prepared, name of technician, and expiration date. Discard all solutions after the expiration date. When necessary, the preparation date can be used as the lot number.

Contamination: Before use, check buffers/solutions for signs of contamination, which may include flocculence or cloudiness. Discard the solution if there are signs of contamination. Also, all reagents and antigen-coated plates should be equilibrated to room temperature prior to use to reduce variability in daily assay performance.

Temperature and humidity: Conditions in the laboratory can affect day-to-day performance of ELISA methods. Very low humidity (often found in winter) can cause evaporation of components during incubation steps, which can result in an increase in blank values or overestimation of the titer for unknown or control sera. Care should be taken to minimize evaporation of assay components during incubation steps.

SOP 2: Quantitation of human IgG antibodies specific for *Streptococcus pneumoniae* capsular polysaccharides by enzyme linked immunosorbent assay (Pn PS ELISA)

Prepared by the laboratories of:	Dr. Moon Nahm and Dr. David Goldblatt
History of revision dates:	June 30, 2002; November 26, 2002; June 9, 2004; March 31, 2011
Referenced SOP:	SOP 1

Purpose

To quantitate human IgG antibody to *S. pneumoniae* capsular polysaccharide in human serum using a standardized ELISA assay.

Principle of the assay

The ELISA measures type specific IgG anti-*S. pneumoniae* capsular polysaccharide (PS) antibodies present in human serum. When dilutions of human sera are added to type-specific capsular PS-coated microtiter plates, antibodies specific for that capsular PS bind to the microtiter plates. The antibodies bound to the plates are detected using a goat anti-human IgG alkaline phosphatase-labeled antibody followed by a *p*-nitrophenyl phosphate substrate. The optical density of the colored end product is proportional to the amount of anti-capsular PS antibody present in the serum.

Materials

- Microtiter plates for ELISA: 96 well, flat bottom, polystyrene, medium binding plate (Greiner 655001/Costar 9017, or equivalent)
- Plate lids (Greiner 656161/Costar 3931, or equivalent)
- Serum dilution plates: Deep well, large capacity microtiter plates, 96 well conical bottom (BD Falcon 353966, or equivalent) or cluster tube strips (Costar 4408, or equivalent)
- Disposable polypropylene containers: 50 ml, 110 ml (Falcon 354014, or equivalent)
- Tissue culture media bottles with screw cap and pouring ring: 250 ml, 500 ml, 1000 ml (Pyrex or Duran, brands that can be heated to 200°C, or equivalent)
- Plastic beakers: 1000 ml, 2000 ml
- Weighing boats
- Pipette tips for pipettors
- Sterile 0.2 µm filter units (Millipore, SCGPT05RE, or equivalent)
- Sterile disposable serological pipettes: 10 ml, 20 ml, 50 ml
- Freezer pen (Sanford Sharpie, extra fine point permanent markers or equivalent)
- Graduated cylinders: 100 ml, 250 ml, 1000 ml, 2000 ml
- Micropipettors : Gilson P20, P200, P1000 or equivalent
- Multichannel pipettors (12 channel): 50 µl-200 µl, 50 µl-300 µl
- Microtiter 12 well washing device (Nunc ImmunoWash 12 or Automated ELISA microtiter plate washer or equivalent). (Note 1)
- Mixer (Tube Rotator, Scientific Equipment Products, Cat. No: 60448 or equivalent)
- Magnetic stirrer (Corning, PC- 353, or equivalent)
- Bench top vortex mixer (Scientific products, S-8220, or equivalent)
- pH meter (Tris-compatible) (Orion Research, model- 601A/analyzer, or equivalent)
- Analytical balances (Ohaus, Galaxy-400 and Galaxy-110 or equivalent)
- ELISA microtiter plate reader with 405 nm and 690 nm filters
- Cryovials, 1.5 ml (Sarstedt, 72.694.005; Corning, 430489, or equivalent)
- 37°C incubator
- 2°C to 8°C refrigerator for storage of sera and reagents
- -70°C freezer for storage of sera and reagents
- 180 °C oven

Reagents and Chemicals

- Pneumococcal capsular polysaccharide (Pn PS) – American Type Culture Collection (ATCC)

  Detailed instructions on re-suspending lyophilized materials
  Remove the vial of lyophilized material from the freezer and allow it to come to room temperature (approximately one hour). Remove the septum and take care not to let any lyophilized material escape. Reconstitute the lyophilized antigen to 1 mg/ml by adding the necessary volume of sterile reagent grade water (type 1) to the vial and replace the septum. Gently shake the vial to moisten any lyophilized material along the top of the vial and place the vial in a mixer (rotator) at 4° C overnight. The mixer turns the vials slowly and helps the polysaccharide dissolve. Aliquot the polysaccharide into 1.5 ml cryovials, label with reagent name, date, lot number, initials and store at –70°C.

Ordering information for capsular Pn PS from ATCC

Danish Pn PS Serotype	Catalogue #	American Serotype #	Order size
1	164-X	Type 1	200 mg
2	168-X	Type 2	200 mg
3	172-X	Type 3	200 mg
4	176-X	Type 4	200 mg
5	180-X	Type 5	200 mg
6B	228-X	Type 26	200 mg
7F	240-X	Type 51	200 mg
8	188-X	Type 8	200 mg
9V	256-X	Type 68	200 mg
9N	192-X	Type 9	200 mg
10A	232-X	Type 34	200 mg
11A	236-X	Type 43	200 mg
12F	196-X	Type 12	200 mg
14	200-X	Type 14	200 mg
15B	244-X	Type 54	200 mg
17F	204-X	Type 17	200 mg
18C	248-X	Type 56	200 mg
19A	252-X	Type 57	200 mg
19F	208-X	Type 19	200 mg
20	212-X	Type 20	200 mg
22F	216-X	Type 22	200 mg
23F	220-X	Type-23	200 mg
33F	260-X	Type 70	200 mg

Note: A typical 9 valent conjugate vaccine may have capsular PS of serotypes 1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F.

- Pneumococcal cell wall polysaccharide (C-PS, Statens Serum Institut, 3459; Distributed in the US by MiraVista Diagnostics, Indianapolis, IN). Reconstitute lyophilized antigen to 1 mg/ml with sterile reagent grade water (type 1) and store in aliquots at -70°C as described above for capsular polysaccharide.
- Sterile reagent grade water (type 1) (See Note 1)
- Alkaline phosphatase-conjugated goat anti-human IgG (binding all 4 IgG subclasses, Southern Biotech, Birmingham, AL, catalog number 2040-04, or equivalent), store at 4°C.
- US Reference Pneumococcal antiserum (007sp). To obtain this, contact Dr. Mustafa Akkoyunlu at US FDA (Mustafa.Akkoyunlu@fda.hhs.gov).
- Quality control (QC) sera: (QC sera, which are often referred to as "in house control sera", are human sera with known ranges of acceptable results. Store in aliquots at – 70°C. (Note 2)
- Diethanolamine (Fisher D45-500)
- *p*-nitrophenyl phosphate powder (Sigma 104-0)
- Brij-35 solution (30% w/v, Polyoxyethylene 23 lauryl ether, Sigma, 430AG-6)
- Disodium hydrogen phosphate, hydrate, ($Na_2HPO_4 \cdot 7H_2O$; Sigma S-9390 or Fisher S373)
- Sodium azide (NaN_3; Sigma, S-2002)

- Sodium chloride (NaCl; BDH,10241AP or Sigma, S-9625)
- Potassium phosphate, monobasic, (KH_2PO_4; BDH 102034B or Sigma P-0662)
- Potassium chloride (KCl; BDH 101984L or Fisher P-217)
- Tween-20 (Polyoxyethylenesorbitan monolaurate; Sigma P-5927)
- Magnesium chloride, hydrate, ($MgCl_2.6H_2O$; Sigma M-9272 or Fisher BP-214-500)
- Concentrated hydrochloric acid (HCl , Fisher A-144-500)
- Sodium hydroxide (NaOH; BDH 102524X or Sigma S-0899)
- Trizma HCl (Sigma T-3253)
- Trizma Base (Sigma T-1503)

Solutions

Instructions herein are for commonly used volumes as indicated in square brackets. The solution volume can be adjusted depending on specific needs. Use only reagent grade water (type 1) or equivalent. DO NOT USE OTHER TYPES OF WATER (Note 1). Use disposable plastic ware for all containers or dedicate glassware to use with these solutions. Never use glassware that has been washed with detergent. See Note 9 for more information on containers. Use sterile technique whenever possible to prolong the shelf life of the solutions.

Substrate stop solution: 3M NaOH [1 liter]

Preparation of this solution generates extreme heat and can be dangerous. Detailed steps are provided here.

Place 800 ml of water and a magnetic stir bar in a new 2000 ml plastic beaker. Place the beaker on top of a magnetic stirrer. Weigh 120 gm of NaOH pellets and slowly add NaOH pellets. If the water becomes too hot, stop adding the NaOH pellets and wait for it to cool. After adding all the NaOH pellets, mix the solution thoroughly, let it cool and bring the solution to 1 liter with water using a 1000 ml graduated cylinder. Mix well and store in a bottle at room temperature for up to 12 months.

6M HCl [200 ml]

Place 100 ml of water in a beaker in a fume hood. Add 100 ml of concentrated HCl slowly with stirring. Extreme care should be taken when handling concentrated HCl.

Coating Buffer: 10 X PBS /0.2% NaN_3 [1L] (Note 3)

Dry chemical	Weight (grams)
NaCl	80.00
KH_2PO_4	3.14
$Na_2HPO_4{\cdot}7H_2O$	20.61
KCl	1.60
NaN_3	2.00

Add 800 ml of **Abbott Laboratories** water to a 2-liter beaker placed on a magnetic stirrer. Weigh out the dry chemicals listed above and add them to the water. Dissolve the chemicals and bring the volume to 1000 ml with **Abbott Laboratories** water. Do not adjust pH (see instructions for preparing 1X coating buffer below). Sterilize the solution by filtering it with a 0.22 µm filter and store it in a sterile container at room temperature.

Coating Buffer: 1X PBS / 0.02% NaN_3 [1L]

Add 900 ml of sterile **Abbott Laboratories** water to 100 ml 10X PBS/0.2% NaN_3 prepared above. Check the pH of a small aliquot (do not insert the pH probe into the entire solution to avoid possible contamination). The pH should be 7.2 ± 0.2. If the pH is not within this range, discard the solution, and prepare new 10X buffer.

Antibody Buffer: 10 X PBS /0.2% NaN_3/0.5% Tween-20 [1L] (Note 3)

Dry chemical	Weight (grams)
NaCl	80.00
KH_2PO_4	3.14
$Na_2HPO_4{\cdot}7H_2O$	20.61
KCl	1.60
NaN_3	2.00

Add 800 ml of reagent grade water type 1 to a 2-liter beaker placed on a magnetic stirrer. Weigh out the dry chemicals listed above and add them to the water. Dissolve the chemicals and bring the volume to 1000 ml with reagent grade water type 1. Add 5 ml Tween-20. Do not adjust pH (see instructions for preparing 1X antibody buffer below).

Antibody Buffer: 1X PBS /0.02% NaN_3/0.05% Tween-20 [1L]

Add 900 ml of reagent grade water type 1 to a 1000 ml graduated cylinder and add 100 ml 10X PBS/0.2% NaN_3/0.5% Tween-20. Check the pH of a small aliquot (do not insert the pH probe into the entire solution to avoid possible contamination). The pH should be 7.2 ± 0.2. If the pH is not within this range, discard the solution, and prepare new 10X buffer.

10X TBS/1% Brij solution [1 liter]

Dry chemical	Weight (grams)
NaCl	80
KCl	1.60
Trizma Base	0.94
Trizma HCl	14.56

Weigh out dry chemicals and dissolve in approximately 800 ml of reagent grade water (type 1) in the appropriate size container. Mix the solution thoroughly using a magnetic stir plate and a stir bar. Add 33 ml of Brij-35 (30% w/v) and mix thoroughly. Bring the solution to 1 liter with reagent grade water (type 1). Store at room temperature for up to 12 months.

Wash buffer: 1X TBS /0.1% Brij solution

Mix one part of 10X TBS/1.0% Brij-35 with 9 parts of Reagent grade water type 1 in an appropriate size container. The pH should be 7.2 ± 0.2.

Substrate Buffer: 1M diethanolamine, 0.5 mM $MgCl_2$ [1 liter]

Calibrate the pH meter before making this solution. Details for preparing the solution are given below because the pH of the substrate buffer is very important.

1. Place a 2000 ml plastic beaker with a magnetic stir bar on a magnetic stirrer in a fume hood. (Use a beaker with 1liter calibration marker.)
2. Add about 800 ml of water.
3. Add 97 ml of diethanolamine to the water using a 100 ml graduated cylinder and mix the solution well with the magnetic stirrer.
4. Add 0.1 gm of $MgCl_2.6H_2O$ to the solution.
5. While thoroughly mixing with a magnetic stirrer, adjust the pH to 9.8 ± 0.05 by slowly adding 6M HCl. (~60 ml.)
6. Allow the solution to cool to room temperature.
7. Check the pH again and adjust to pH 9.8 ± 0.05 with 6M HCl if necessary.

8. Bring the solution to 1 liter with water and a 1000 ml graduated cylinder.
9. Since pH is critical, recheck the pH. If the pH is not 9.8 ± 0.05, discard the diethanolamine buffer.
10. This buffer may be stored sealed in a light protected container at room temperature for up to 6 months.

SOP 2A: Adsorption of type-specific capsular polysaccharide antigen to microtiter plates ("Coating" the plates with the antigen)

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of revision dates: June 30, 2002; November 26, 2002; March 31, 2011
Referenced SOP: SOP 2

1. Retrieve the appropriate frozen aliquot of capsular PS that was stored at –70°C at 1 mg/ml. Thaw the aliquot at room temperature.
2. Dilute the capsular PS to a predetermined concentration (generally 1-10 µg/ml) with sterile antigen-coating buffer (1X PBS/0.02%NaN_3). (See SOP 4 for additional information).
3. Pipette 100 µl of capsular PS antigen into each well of the microtiter plate(s) and cover with a lid.
4. Incubate the plates at 37°C for 5 hours in an appropriate tissue culture grade incubator. Plates should not be stacked more than 2 layers high, and no more than 18 plates per shelf (using a standard tissue-culture grade incubator of 5.7 cu ft). This precaution is taken in order to maintain the air circulation in the incubator, to allow the heat penetration to microtiter plates, and to warm the plates evenly. Stacking plates higher or overloading an incubator can lead to 'edging' or poor quality coating of pneumococcal polysaccharide antigens.
5. Store the plates in a humidified chamber kept at 4°C. If sodium azide is used, coated plates may be stored for several months at 4°C, provided that storage conditions are suitable to prevent evaporation and contamination of the coating solution.

SOP 2B: Procedure for testing human sera with unknown anti-Pn PS antibody concentrations

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of revision dates: June 30, 2002; November 26, 2002; June 9, 2004; August 2010; March 31, 2011
Referenced SOP: SOP 2

This protocol assumes that each serum sample is analyzed for 9 Pn PS serotypes. If this is not the case, adapt this protocol for the particular situation.

1. Plan the sample layout:

The example of a plate layout (shown below) has 4 samples with unknowns (Unknowns 1-4), one QC, and one standard (007sp). All samples are analyzed in duplicate (e.g. columns 1 and 2 for Unknown 1, etc). Place the least diluted samples in row A and the most diluted in row H. The reference serum, 007sp, is used as the standard and is placed in rows A through G of columns 7 and 8. Wells H7 and H8 (marked Blank) are used as "blank wells". Serial dilutions of a QC are placed in columns 11 and 12. See Note 2 for additional description of QC.

Typical microtiter plate layout for Pn PS ELISA

	Unknown 1		Unknown 2		Unknown 3		Standard 007sp		Unknown 4		QC	
Serum dilutions	1	2	3	4	5	6	7	8	9	10	11	12
A 1:50												
B 1:125												
C ↓etc.												
D												
E												
F												
G												
H							Blank	Blank				

2. Retrieve all serum samples, controls and reference sera stored frozen and place them at room temperature to thaw (~30 minutes). Samples should not be freeze/thawed more than 3 times and markings on the lid can be used to track the number of freeze/thaw cycles. After using the samples, mark the remainder on the lid with a freezer pen and immediately return them to the freezer.
3. Retrieve all the required antigen-coated plates from the refrigerator. Place them on the bench at room temperature. Prepare wash and antibody buffers.
4. Prepare the absorption solution by adding C-PS and 22F capsular PS to antibody buffer to make 5 µg/ml of C-PS and 5 µg/ml of 22F capsular PS. Typically, 65 ml of the absorption solution is needed for a 9-microtiter plate assay. (Note 11)
5. Mix the thawed samples, QC, and the standard (007sp) thoroughly. It is convenient to dilute the samples in the "serum dilution plates" (i.e., deep-well 96-well plates or 1.1 ml tubes arranged in a 96-well plate format). Remove appropriate amounts of the samples (or QC) and mix them with the appropriate amounts of the absorption solution in Row A. For instance, to prepare a 50-fold diluted serum sample for 9 serotypes, one may mix 20 µl of the sample with 980 µl of the absorption solution. It is convenient to dilute the samples in the "serum dilution plates".
6. Perform serial dilutions (usually 2- to 3-fold; see Note 10) as necessary with absorption solution. A 2.5-fold serial dilution can be performed as follows. First, add 600 µl of absorption solution to all columns of rows B through H in the "serum dilution plates". Then, mix the wells in row A and transfer 400 µl to Row B. Next, mix the well in Row B and transfer 400 µl to Row C. Repeat these steps through Row H. **When transferring the 400 ul from Row G to Row H, make sure that no serum is transferred for columns 7 and 8—these are the "blank wells".**
7. Incubate the diluted samples at room temperature for 30 minutes.
8. Wash the antigen-coated microtiter plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells. (Note 4) (Note 5) (Note 6)
9. Using a multichannel pipettor, transfer 50 µl of each serum dilution from the dilution plates to the washed antigen coated plates. Add only antibody buffer to two wells (H7 and H8) in each plate to serve as blanks.
10. Cover plates with lids and incubate at room temperature for 2 to 18 hours (see Note 12). For the longer incubations or in low humidity situations, use a humidified sealed box (with an anti-microbial agent), to prevent excessive evaporation.
11. Prepare the appropriate dilution of goat anti-human IgG conjugate in antibody buffer 15 minutes before its use (SOP 6).
12. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
13. Add 100 µl of diluted enzyme conjugate to all microtiter plate wells. Cover plates with lids and incubate for 2 hours at room temperature.
14. Prepare a 1 mg/ml solution of *p*-nitrophenyl phosphate in the diethanolamine substrate buffer 15 minutes before it is required. Mix the substrate solution on the shaker while wrapped in a paper towel to protect it from light.

15. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
16. Add 100 µl of substrate solution to all microtiter plate wells. Put lids on plates and incubate for 2 hours at room temperature.
17. Add 50 µl of 3M NaOH to all wells to stop the enzyme reaction.
18. Wait at least 5 minutes before reading the optical density of the plates on a microtiter plate reader at 405 nm and 690 nm (Note 7).

Data analysis

1. Optical density data is converted to antibody concentrations with a computer program like Genesis, Softmax PRO, Revelations, or "ELISA". Acceptable results are obtained with computer programs using a standardized curve-fitting four parameter logistic method, a weighted log-logit method, or a logistic/log linear regression analysis. See Note 8 for obtaining the programs mentioned here.
2. The program calls for a "calculation template", which tells the calculation-program the location of samples, standards, QC, the initial dilutions and serial dilutions. Wells H7 and H8 should be labeled as "blank wells" in the template. The program should subtract the average OD of the two "blank wells" from the OD of other wells.
3. The anti-Pneumococcal capsular PS antibody concentration for each sample is found by calculating the mean of the serial dilutions of the sample. The following data inspection rules should be followed to ensure that the assay results are valid.

Data inspection rules

1. Blanks -The optical density of blank wells should be less than 0.1 OD units for all assay plates. In general, the optical density will be less than 0.05.

2. Duplicates - If the absorbance values of replicates at one dilution vary widely and have a coefficient of variation (CV) >15%, the concentrations obtained with the absorbance data at that dilution should be excluded from the calculation of the antibody concentration of the sample.

3. Standards - The slope of the linear portion of the reference standard curve (e.g., OD 0.1 to 2.0) should be very near 1.0 (0.9-1.1) when the log of the OD signal is graphed against the log of the standard concentration.

4. Sensitivity - Calculate the lower detection limit for the assay and confirm that the detection limit is within the established range.

5. Quality control sera - Control sample values must be within the established range (which is 3 SD), or the plate is rejected and samples are reanalyzed.

6. Rules for the samples with unknown concentrations:
   a. If a sample has OD readings greater than 2.0 at the highest dilution used in the assay, the sample should be reassayed after additional dilution.
   b. If the antibody concentration of the sample was calculated by averaging the data from multiple dilutions and the CV of the concentration exceeds 30%, then the data should be examined for inappropriate results (e.g., poor duplications, and/or non-linearity) and should be reassayed if no apparent causes are found.
   c. If upon reassay, the %CV value is greater than 30% due to the fact that the titration slope is not parallel to the assay reference standard sera, the median predicted value should be reported. For additional information on data analysis see CM Wernette et al, Clinical and Diagnostic Laboratory Immunology 10(4): pages 514-519.
   d. If the lower limit of detection is equal to or less than the established value and a sample has undetectable antibody concentration, report one half of the established assay lower limit as the concentration for the sample. If the lower limit of detection is more than the established value and a sample has undetectable antibody concentration, do not report the result for the sample and reanalyze the sample.

Assay notes

Note 1: Water is extremely important. We use fresh water from Synergy 185 water purification system from Millipore (Bedford, MA). If the water is stored for a prolonged period, microorganisms grow and contaminate the water. To prepare solutions used in coating the plates with Pn PS, we use pyrogen-free water from Abbott (North Chicago, IL, catalog# NDC 0074-7139-36).

Note 2: QC (Quality control) refers to in-house controls used to monitor inter-assay variation. Human serum pools are commonly used as QC after their antibody levels have been very well characterized. Some control sera should have high values and some should have low values. All QC samples should have known ranges of acceptable results. There should be one QC per plate and at least one QC with high values for every 3 assay runs. A set of serum samples with known concentrations (referred to as "calibration sera") is available from Dr. David Goldblatt (consult http://www.vaccine.uab.edu/refer/qc3.pdf for additional information on the sera). The calibration sera are to validate a procedure and each laboratory will need to obtain their own standard sera for routine QC checks. These can be made from Red Cross plasma or from sera collected from subjects immunized with a vaccine. Usually screening 30 plasmas from expired units high and low titer sera can be found. If plasma is used, it needs to be converted to serum.

Note 3: To simplify making PBS buffers, there are PBS tablets, which contain pre-measured amounts of all the required chemicals. However, these PBS tablets often include stabilizers or other components, which can affect ELISA results. Instead of tablets, use of ACS grade chemicals in preparation of buffers improves assay performance.

Note 4: ELISA plates can be washed manually by repeatedly filling the wells with the buffer and removing the buffer. Alternatively, one can wash the plates with a 12-well washing device or a machine that washes 96 wells simultaneously. A popular model of 96-well washing machine is ELx 405 Microplate washer from Bio-Tek (Princeton, NJ).

Note 5: Follow the manufacturer's recommendations to remove proteins from plate washers. Alternate use of HCl and NaOH, following with several rinses with deionized water. Use of alcohol for the cleaning or decontamination of the plate washers should only be considered once proteins have been removed from the system as alcohol can further cement the proteins to the hardware.

Note 6: Do not allow microtiter plates to dry between wash steps and reagent addition; they should not be left empty for more than 10 minutes.

Note 7: Signal for each well = $OD_{405} - OD_{690}$. The wavelength for the reference may vary from 600 to 690 nm.

Note 8: "ELISA" is a computer program developed by Mr. Brian Plikaytis at The Centers for Disease Control and Prevention in Atlanta, Georgia. To obtain this free program, obtain the necessary contact information from: http://www.cdc.gov/ncidod/dbmd/bimb/elisa.htm. Softmax PRO can be purchased from Molecular Devices Corp. (Sunnyvale CA).

Note 9: Glassware that is detoxified by heating at 180ºC can be used. Glassware should never be contaminated with detergent, as this will affect binding of some of the Ps antigens more than others (type 14 is particularly sensitive).

Note 10: Some high titer sera will not be sufficiently diluted in 7 wells to use 2-fold dilutions, but by turning the plate the other way, 11 dilutions may allow sufficient dilution. Some low titer sera may not give enough usable points with 3-fold dilutions. Thus, 2.5-fold dilutions have been found to be the best except under special circumstances.

Note 11: Some use 10 µg/ml instead of 5 µg/ml for absorption.

Note 12: If measuring antibodies to serotype 3, this incubation should be done for only 2 hours. For the other serotypes tested (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F), incubation times of 2 to 18 hours are acceptable.

SOP 3: ELISA plate lot selection

Prepared by the laboratories of:	Dr. Moon Nahm and Dr. David Goldblatt
History of Revision dates:	July 31, 2002; November 26, 2002; June 9, 2004; June 12, 2006; March 31, 2011
Referenced SOP:	SOP 1, SOP 2

To qualify a new lot of plates, establish that:

1) The optimal antigen coating concentration is within the acceptable limit by determining the optimal concentration for Pn PS serotypes (eg, 9 serotypes) using an antigen titration experiment.
2) The "plate CV" is within the acceptable limit by testing 3 or more plates from different locations in one box with Pn PS ELISA
3) The plates produce comparable results by performing a side-by-side comparison with a minimum of 10 control sera and at least 4 QC sera for all serotypes with old and new plates and show that the new plates yield similar values and lower limits of detection.

Tests designed to establish these aspects are described below as SOP3A, SOP 3B, and SOP 3C.

SOP 3A: Determine the optimal Pn PS antigen coating concentration for a lot of ELISA plates

Prepared by the laboratories of:	Dr. Moon Nahm and Dr. David Goldblatt
History of Revision:	July 31, 2002; November 26, 2002; June 12, 2006; March 31, 2011
Referenced SOP:	SOP1, SOP 2

Purpose

To determine whether the optimal antigen-coating concentrations of the Pn PS for the new lot of ELISA plates are similar to those for the old lot.

Principles of the test

Antigen is adsorbed on to the surface of a microtiter plate in increasing concentrations. Reference serum is added at one dilution across the plate and the ELISA is completed using the type specific Pn PS ELISA (SOP 2). The optimal coating concentration of an antigen lot is determined by inspecting optical density values vs. antigen concentration. Try to achieve the maximum signal without increasing the OD of the blank wells. The antigen concentration should not be greater than 10 µg/ml.

Materials and reagents

Materials and reagents are outlined in type specific Pn PS ELISA (SOP 2).

Methods

1. Dilute the Pn PS to 20 µg/ml in coating buffer in polypropylene tubes. Add 200 µl to the wells in the first column (8 wells).

ANTIGEN CONCENTRATION (µg/ml)

	20	10	5	2.5	1.25	0.625	0.312	0.156	0.078	0.039	0.020	Buffer
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Rows A, B, C and D: optimum dilution of the reference serum (007sp).
Rows E, F, G, and H: buffer ("background wells")
Column 12 contains "blank wells"

2. Fill the wells in columns 2-12 with 100 µl of the coating buffer. Perform 2-fold serial dilutions from column 1 to column 11 by repeatedly mixing and transferring 100 µl to the wells in the next column. After the 11th column, discard 100 µl from the 11th column (so that the wells in column 11 have only 100 µl). Leave column 12 undisturbed. Put a lid on each plate.
3. Incubate the plate at 37°C for 5 hours and then keep the plates at 4°C for storage (See SOP 2A).
4. Dilute the human anti-pneumococcal PS standard reference serum, 007sp, in the absorption solution (antibody buffer with C-PS and 22F PS, each at 5 micrograms/ml) so it is expected to give an OD of 1.0 under conditions explained in the Pn PS ELISA (SOP 2).
5. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
6. Add the diluted reference serum to all wells in rows A, B, C and D and the antibody buffer to all wells in rows E, F, G, and H. Incubate as specified in the Pn PS ELISA (SOP 2).
7. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
8. Add goat anti-human alkaline phosphatase-labeled secondary antibody at the optimal dilution to each well and incubate, as specified in the Pn PS ELISA (SOP 2).
9. Wash the plates 5 times with wash buffer. Allow the wash buffer to soak on the plate 30 seconds to 1 minute after the first filling.
10. Add substrate, incubate, and read the optical density of the plate as specified in the type specific Pn PS ELISA (SOP 2).

Data analysis

1. All blank wells must have optical density values lower than 0.1. If any blank wells are ≥0.1, the assay must be repeated (Note 1). All background wells should have optical densities less than 0.1.
2. Obtain average OD of rows A, B, C and D, and E, F, G, and H for each column.
3. For each antigen concentration, obtain the "signal" by subtracting the average OD value of the background wells (antigen and 2° antibody) from the average OD value of the corresponding wells that contain the reference serum (007sp).
4. Plot the signal on the Y-axis and the logarithm of antigen concentration on the X-axis.
5. Possible outcomes:
   a) a sigmoid curve with a plateau occurring at high antigen concentrations,
   b) a sigmoid curve where the plateau falls to base-line (X-axis, sometimes the signal decreases when the antigen coating concentration is too high).
   c) an increasing semi-linear plot with no observable plateau or peak in the range of antigen concentrations.

6. For outcomes a) and b), pick the antigen concentration yielding the maximum signal because any higher concentrations of antigen will not yield a higher signal.
7. For outcome c), pick a coating concentration yielding the maximum possible signal with a low background.
8. The optimal coating concentration chosen in this manner provides maximum sensitivity, consistent ELISA performance, and acceptably low signals from blank wells (Note 2).

Assay notes

Note 1: If optical density values are repeatedly higher in the blank wells in the new lot of plates but not in the old lot of plates, there may be a problem with the new lot of microtiter plates.

Note 2: Antigen titration should yield similar optimal coating concentrations. If marked differences in optimal coating concentrations are found, a different lot of plates should be requested, and the testing procedure should be repeated.

SOP 3B: Determine the variability of results between different lots of ELISA plates

Prepared by the laboratories of:	Dr. Moon Nahm and Dr. David Goldblatt
History of Revision:	July 31, 2002; November 26, 2002; June 9, 2004; June 12, 2006; March 31, 2011
Referenced SOP:	SOP 1, SOP 2

Purpose

To select a new lot of microtiter plates producing results with minimal well-to-well variation.

Principle of the assay

A single dilution of serum is added to 92 out of 96 wells of an antigen-coated plate with 4 wells acting as blank controls with buffer only. Mean absorbance values and their variation from the 92 wells are calculated between wells on a single plate and between each of the plates. Standard deviation and % CV are assessed where the inter-plate CV should be ≤20% and the average intra-plate CV should be ≤10% for acceptability. Although this procedure can be performed using only one Pn serotype, it is desirable to test all serotypes that will be used in the ELISA. If select Pn serotypes are used, choose ones that give consistent results with controls (e.g., serotype 1).

Materials and reagents

- 3 microtiter plates (for each serotype) from a new lot number from different positions in the box. (See Note 1)
- 3 microtiter plates (for each serotype) from a previously qualified lot number
- Materials and reagents listed in SOP 2

Methods

1. Obtain 3 plates of the qualified lot and 3 plates of a new lot.
2. Using the optimal antigen coating concentration determined in SOPA 3A, add 100 µl of the Pn PS antigen to each of the 6 microtiter plates (see SOP 2A).
3. Prepare a dilution of reference serum 007sp in absorption solution (antibody buffer with C-PS and 22F PS, each at 5 micrograms/ml) that will give an OD of approximately 1.0 within 2 hours of addition of substrate.
4. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
5. Add the serum dilution to all wells of the plates, excluding 4 blank wells where diluent alone is added.

The blank wells are typically B2, B9, F4 and F11 (see template below).

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Buffer							Buffer			
C												
D												
E												
F				Buffer							Buffer	
G												
H												

6. Incubate according to the type-specific Pn PS ELISA procedure (SOP 2).
7. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
8. Add goat anti-human IgG alkaline phosphatase labeled secondary antibody at the optimal dilution (SOP 6) to each well and incubate as specified in the Pn PS ELISA procedure (SOP 2).
9. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
10. Add substrate to each well and incubate until OD is approximately 1.0 (check OD frequently). After OD has reached approximately 1.0, stop the reaction, and read optical density in the ELISA plate reader as in SOP 2.

Data analysis

1. Calculate the mean optical density of the 4 blank wells (i.e. buffer only wells) and subtract this from the optical density of all 92 other wells. Average optical densities for the test wells must be between 0.8 and 1.5 units.
2. Calculate the intra-plate mean, standard deviation, and %CV of the absorbance for the 92 wells of all 6 plates to determine well-to-well variation. If the CV of each of the previously qualified plates is not within the specification for acceptance, the assay must be repeated.
3. Calculate the inter-plate mean, standard deviation, and %CV of the absorbance for the three plates in each group. If the CV of the previously qualified plates is not within the specification for acceptance, the assay must be repeated.
4. *Evaluation criteria for intra-plate variation*:
    a. OD values of blank wells must be consistently ≤ 0.1 (better if they are ≤ 0.05).
    b. Intra-plate well-to-well CV must be ≤ 10%.
    c. Trends should not be observed in a single plate (e.g., spotting, edge effects). Note any trends in locations of the wells with standard deviations greater than 20% from the intra-plate mean OD.
5. *Evaluation criteria for inter-plate variation:*
    a. OD values of blank wells must be consistent and ≤ 0.1 (better if they are ≤ 0.05).
    b. Inter-plate CV for the 3 new plates must be less than 20%.
    c. No significant inter-plate trends should be observed from the intra-plate mean OD.

Assay notes

Note 1: Medium binding ELISA-grade microtiter plates should be used for the Pn PS ELISA (NUNC, Greiner, Costar, Dynatech etc.).

SOP 3C: Side by side comparison of antibody concentrations and lower limits of antibody detection between old and new lots of ELISA plates

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002, November 26, 2002; March 31, 2011
Referenced SOP: SOP1, SOP 2

Purpose

To demonstrate that the new lot of ELISA plates produces comparable results and comparable lower limits of detection as the old lot of ELISA plates using real serum samples.

Principles of the assay

Perform the assay for anti-Pn PS antibody with a set of serum samples and compare the results.

Materials and reagents

- ELISA plates: new and old lots.
- Test serum samples: Use a minimum of 12 unknown samples (testing 24-32 samples is better) and at least 4 QC sera or any well-established samples. They should have antibody levels in all ranges (high and low).
- QC serum samples: Use different sera with high and low antibody levels for each of the 9 serotypes.
- All other materials and reagents as described in SOP 2.

Methods

1. Perform the Pn PS ELISA for all 9 serotypes with the 10 (or more) serum samples and also for QC serum samples on both the new and old ELISA plates as described in SOP 2.

2. Plot the concentrations obtained with the new plates (Y-axis) against those obtained with the old plates (X-axis). The axes should be in log scale. Obtain the best-fit line using the least squares method and log-transformed results. The slopes of the best-fit lines should be 1 ± 0.1 and the intercept must be <0.1 and the r^2 should be >0.95.

3. Calculate the lower limits of detection for new and old ELISA plates. It should be <0.01 for both lots of plates.

Data analysis

1. Perform a separate linear regression analysis on the optical density values from the 7 dilutions of reference serum (007sp) for both new and old ELISA plates.

2. Calculate the 'absolute detection limit' as determined by the antibody concentration present in the dilution of reference serum yielding two times the assay background.

3. Calculate the 'lower limit of detection' by multiplying the 'absolute detection limit' by the lowest dilution of serum tested in the assay (i.e., 1:50 for human serum).

4. The 'lower limit of detection' should be comparable (±20%) between old and new lots of ELISA plates. To be practical, more than 99% of the samples should be above the 'lower limit of detection'. This is about 0.01 µg/ml in the case of the Pn PS ELISA.

SOP 4: Procedures for selecting a new lot of Pn PS antigen

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002, November 26, 2002; March 31, 2011
Referenced SOP: SOP 1, SOP 2, SOP 3

When new lots of antigen are purchased from the ATCC, they must be tested as discussed earlier for new lots of ELISA plates. The tests are:

1. *To determine the antigen titration curve with all 9 serotypes.*
2. *To determine uniformity of antigen coating by calculating %CV.*
3. *To determine lower limit of detection (LLQ), and to perform a side-by-side comparisons for acceptability of results using at least 10 serum samples representing the test population.*

Methods for testing these aspects are described in SOP 4A, SOP4B, and SOP4C.

SOP 4A: Determine the optimal antigen coating concentration for a new lot of Pn PS antigen

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
History of Revision: July 31, 2002, November 26, 2002; March 31, 2011
Referenced SOP: SOP 1, SOP 2, SOP 3, SOP 4

Purpose

To determine the optimal antigen-coating concentration for a new Pn PS antigen lot.

Principle of the assay

Antigen is adsorbed on to the surface of a microtiter plate in increasing concentrations. Reference serum is added at one dilution across the plate and the ELISA is completed using the Pn PS ELISA (SOP 2). The optimal coating concentration of an antigen lot is determined by inspecting optical density values vs. antigen concentrations.

Materials and reagents

- Materials and reagents are outlined in type specific Pn PS ELISA (SOP 2).

Methods

1. Dilute the new Pn PS to 20 µg/ml in coating buffer in polypropylene tubes. Add 200 µl to the wells in the first column. If the old, established lot of antigen is available, wells A1, B1, C1, and D1 are filled with the old qualified antigen, and wells E1, F1, G1 and H1 are coated with the new antigen (see the plate layout figure below). If the old, qualified antigen is not available, all 8 wells are filled with the new antigen.
2. Fill the wells in columns 2-12 with 100 µl of the coating buffer. Perform 2-fold serial dilutions from column 1 to column 10 by repeatedly mixing and transferring 100 µl to the wells in the next column. After the 10th column, discard 100 µl from the 10th column (so that the wells in column 10 have only 100 µl). Leave columns 11 and 12 undisturbed. Put a lid on each plate.
3. Incubate the plate at 37°C for 5 hours and then keep the plates at 4 °C for storage (see SOP 2A).
4. Dilute the human anti-pneumococcal standard reference serum, 007sp in the absorption solution (antibody buffer with C-PS and 22F PS, each at 5 micrograms/ml) to give an OD of 1.0 under conditions explained in the Pn PS ELISA (SOP 2).
5. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.

ANTIGEN CONCENTRATION (µg/ml)

	20	10	5	2.5	1.25	0.625	0.312	0.156	0.078	0.039	Buffer	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Rows A, B, C, D: Old qualified capsular Ps antigen (if old antigen is not available, these rows are coated with new antigen)
Rows E, F, G, H: New capsular Ps antigen
Columns 11 and 12 are "blank wells".

Rows A and B: optimum dilution of the reference serum (007sp).
Rows C and D: Buffer ("background wells")
Rows E and F: optimum dilution of the reference serum (007sp).
Rows G and H: Buffer ("background wells")

6. Add the diluted reference serum to all wells in rows A, B, E and F and the diluent buffer to all wells in rows C, D, G and H. Incubate as specified in the Pn PS ELISA (SOP 2).
7. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
8. Add goat anti-human IgG alkaline phosphatase-labeled secondary antibody at the optimal dilution to each well and incubate, as specified in the Pn PS ELISA (SOP 2).
9. Wash the plates 5 times with wash buffer. Allow the wash buffer to soak on the plate 30 seconds to 1 minute after the first filling.
10. Add substrate, incubate, and read the absorbance of the plate as specified in the Pn PS ELISA (SOP 2).

Data analysis

1. All blank wells must have OD values lower than 0.1. If blank wells are ≥ 0.1 the assay must be repeated (Note 1). (If new antigen-coated background wells have high OD, then the new antigen lot is not acceptable. Old antigen was tested and should give low OD.)
2. Obtain average OD of rows A and B, C and D, E and F, and G and H for each column.
3. For each antigen concentration, obtain the "signal" by subtracting the average OD value of the background wells (antigen and 2° antibody) from the average OD value of the corresponding wells that contain the reference serum.
4. Plot the signal on the Y-axis and the logarithm of antigen concentration on the X-axis.
5. Possible outcomes:
   a. a sigmoid curve with a plateau occurring at high antigen concentrations,
   b. a sigmoid curve where the plateau falls to base-line (X-axis, sometimes the signal decreases when the antigen coating concentration is too high),
   c. an increasing semi-linear plot with no observable plateau or peak in the range of antigens.
6. For outcomes a) and b), pick the antigen concentration yielding the maximum signal because higher concentrations of antigen do not yield a higher signal.
7. For outcome c), pick a coating concentration yielding the maximum possible signal with low background.

8. The optimal coating concentration chosen in this manner provides maximum sensitivity, consistent ELISA performance, and acceptably low signals of blank wells (Note 2).

Assay notes

Note 1: If OD values are repeatedly higher in the blank wells, there may be a problem with the microtiter plate. However, this should not occur since the plates that were tested in the past are being used in this test.

Note 2: Antigen titration should yield similar (to the previous) optimal coating concentrations. If marked differences in optimal coating concentrations are found, the new lot of the antigen should be evaluated for differences in purity and for differences in composition with other analytical techniques (e.g., NMR, chemical analysis) or with serological analysis (e.g., competition ELISA using homologous and heterologous antigens).

SOP 4B: Determine the variability of antigen coating between old and new lots of Pn PS antigen

Prepared by the laboratories of:	Dr. Moon Nahm and Dr. David Goldblatt
History of Revision:	July 31, 2002, November 26, 2002; March 31, 2011
Referenced SOPs:	SOP 1, SOP 2

Purpose

To determine whether the new lot of Pn PS antigen coats the ELISA plates uniformly.

Principle of the assay

A single dilution of serum is added to 92 out of 96 wells of an antigen-coated plate with 4 wells acting as blank controls with buffer only. Mean absorbance values and their variation from the 92 wells are calculated between wells on a single plate and between each plate. Standard deviation and % CV are assessed where an average CV should be equal to or less than 10% for acceptability.

Materials and reagents

- Pn PS of old and new lot numbers
- Materials and reagents listed in SOP 2

Methods

1. Using the optimal antigen coating concentration determined in SOP 4A, coat three ELISA plates with the new antigen, and three ELISA plates with the old antigen (at the previously determined antigen coating concentration). See SOP2A.
2. Prepare a dilution of reference serum 007sp in absorption solution (antibody buffer with C-PS and 22F PS, each at 5 micrograms/ml) that will give an OD of approximately 1.0 with the particular Pn PS serotype.
3. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
4. Add the serum dilution to all wells of the plates, excluding 4 blank wells where diluent alone is added. The 4 blank wells are typically B2, B9, F4 and F11 (see template below).
5. Incubate according to the Pn PS ELISA procedure (SOP 2).
6. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
7. Add goat anti-human IgG alkaline phosphatase labeled secondary antibody at the optimal dilution (SOP 6) to each well and incubate as specified in the ELISA procedure (SOP 2).

8. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.
9. Add substrate to each well and incubate, stop the reaction, and read absorbance in the ELISA plate reader as in SOP 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Buffer							Buffer			
C												
D												
E												
F				Buffer							Buffer	
G												
H												

Data analysis

1. Find the mean optical density of the 4 blank wells (i.e., buffer only wells) and subtract this from the optical density values of all 92 other wells. The average optical density for the test wells must be between 0.8 and 1.5 units.

2. Calculate the intra-plate mean, standard deviation, and %CV for the 92 wells to determine well-to-well variation. If the CV of the previously qualified Pn PS lot is not within the specification for acceptance, the assay must be repeated.

3. *Evaluation criteria for intra-plate variation*:
   OD values of blank wells must be "consistent" and ≤ 0.1 (preferably < 0.05).
   Intra-plate well-to-well CV must be ≤ 10%. Trends should not be observed in a single plate (e.g., spotting, edge effect). Note any trends in locations of the wells with deviations greater than 20% from the intra-plate mean optical density.

SOP 4C: Side by side comparison of the antibody concentrations and the lower limits of antibody detection (LLQ) between old and new lots of Pn PS antigen

Prepared by the laboratories of:	Dr. Moon Nahm and Dr. David Goldblatt
History of Revision:	July 31, 2002, November 26, 2002; March 31, 2011
Referenced SOPs:	SOP 1, SOP 2

Purpose

To demonstrate that the new lot of Pn PS produces comparable results and comparable lower limits of detection as the old lot of Pn PS using real serum samples.

Principle of the assay

Perform the assay for anti-Pn PS antibody with a set of serum samples using new and old lots of Pn PS antigens and compare the results.

Materials and reagents

- Pn PS: new and old lots.
- Test serum samples: Use at least 10 QC samples or any well established samples (testing 24-32 samples is better). They should have antibody levels in all ranges (high and low).
- All other materials and reagents are described in SOP 2.

Methods

1. Analyze at least 10 samples with new and old Pn PS for anti-Pn PS IgG antibody as described in SOP 2.

2. Plot the results with new and old Pn PS. Plot the concentrations with the new lot (Y-axis) against those with old lot (X-axis). The axes should be in log scale. Obtain the best-fit line using the least squares method. The slope of the best-fit line should be 1 ± 0.1. The scatter among the data points should be within 3-fold.

3. Calculate the lower limits of detection for new and old lots of Pn PS.

Data analysis

1. Perform a separate linear regression analysis on the OD values from the 7 dilutions of reference serum (007sp) for both new and old Pn PS lots.
2. Calculate the 'absolute detection limit' as determined by the units of antibodies present in the dilution of reference serum yielding two times the assay background.
3. Calculate the 'lower limit of detection' by multiplying the 'absolute detection limit' by the lowest dilution of serum tested in the assay (i.e., 1:50 for human serum). The 'lower limit of detection' should be comparable ($\pm$20%) between old and new lots of PnPS. To be practical, more than 99% of the samples should be above the 'lower limit of detection'. This is about 0.01 µg/ml in the case of the anti-Pn PS assay.

SOP 5: Selection of a new lot of enzyme-labeled secondary antibody specific for all human IgG subclasses.

Prepared by the laboratories of:	Dr. Moon Nahm and Dr. David Goldblatt
Effective date:	June 30, 2002, November 26, 2002; March 31, 2011
Applicable to:	The Pn PS assay, SOP2

Purpose

Polyclonal secondary antibodies (e.g., goat anti-human IgG) are more sensitive than monoclonal antibodies in the Pn PS ELISA. However, properties of polyclonal antisera vary from lot to lot, and each lot of polyclonal antisera must be tested for its binding specificity. The polyclonal antisera for human IgG should bind to all human IgG subclasses (IgG1, IgG2, IgG3, and IgG4) equivalently with minimal cross-reactivity to IgA or IgM (less than 5%). This SOP describes how to evaluate the isotype specificity of the secondary antibody specific for human IgG.

Materials and reagents

- Purified human IgG1 (Sigma I-5154, or equivalent)
- Purified human IgG2 (Sigma I-4264, or equivalent)
- Purified human IgG3 (Sigma I-4389, or equivalent)
- Purified human IgG4 (Sigma I-4639, or equivalent)
- Bovine serum albumin (RIA grade, Sigma A2934, or equivalent)
- Enzyme labeled secondary antibody to be tested ("new secondary antibody")
- Enzyme labeled secondary antibody currently in use ("old secondary antibody")
- Sodium carbonate (Na_2CO_3, BDH #10240, or equivalent)
- Sodium bicarbonate ($NaHCO_3$, BDH #102474V, or equivalent)

All other reagents and materials are itemized in the Pn PS ELISA (SOP 2).

Solutions

- **Sterile carbonate/bicarbonate buffer pH 9.6:**

  0.76g Na_2CO_3
  1.50g $NaHCO_3$
  0.10g NaN_3

Dissolve the dry chemicals in 400 ml reagent grade water (type 1) and bring up to 500 ml with reagent grade water (type 1). Adjust the pH to 9.55-9.65 with 6M HCl/6 M NaOH and sterile filter the solution using a 0.2 µm filter. Remove aliquots from this stock solution using sterile technique. Store at 4°C for a maximum of 1 week.

- **2% BSA in carbonate/bicarbonate buffer**

Dissolve 2.0 g of RIA grade bovine serum albumin (BSA) in 100 ml of sterile carbonate/bicarbonate. Must be made fresh on day of use. All other solutions are described in SOP 2.

Procedure

Conditions used for all steps below should conform to those used in the Pn PS ELISA as defined in SOP 2. This procedure is used for testing one lot of polyclonal antiserum.

1. Dilute each of the human proteins (IgG, IgA, IgM, IgG1, IgG2, IgG3, and IgG4) to 1 µg/ml in carbonate/bicarbonate buffer. Add 100 µl of diluted human proteins to appropriate wells of the microtiter

plates as shown in Diagram 1 (to show specificity for IgG) and Diagram 2 (to analyze Ig isotypes). Cover the plate with a lid and incubate for 90 minutes at 37°C. (NOTE THE SHORT INCUBATION TIME.)

Diagram 1

	Old qualified 2º antibody						New candidate 2º antibody					
	1	2	3	4	5	6	7	8	9	10	11	12
A 1:250	IgG	IgG	IgA	IgA	IgM	IgM	IgG	IgG	IgA	IgA	IgM	IgM
B 1:500	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
C ↓ etc.												
D												
E												
F												
G												
H												

Diagram 2

	1	2	3	4	5	6	7	8	
A 1:250	IgG1	IgG1	IgG2	IgG2	IgG3	IgG3	IgG4	IgG4	Old qualified 2º antibody
B 1:500	↓	↓	↓	↓	↓	↓	↓	↓	
C 1:1000									
D 1:2000									
E 1:250									New candidate 2º antibody
F 1:500									
G 1:1000									
H 1:2000									

2. Without removing the above antigens, add 100 µl of 2% BSA in carbonate/bicarbonate buffer to all wells. Cover the plates with lids and incubate for 60 minutes at room temperature.

3. Prepare serial 2-fold dilutions for the new and old secondary antibodies in antibody buffer (SOP2), in 50 ml polystyrene tubes, starting at 1:250 and ending at 1:16000 as shown in Diagram 2. Typically prepare about 20 ml for each dilution.

4. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.

5. Add 100 µl of the dilutions of the secondary antibody to the appropriate wells (rows A through G) of the plate, and antibody buffer as a blank (see Diagram 2). Incubate at room temperature for 1 hour.

6. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.

7. Add 100 µl of *p*-nitrophenyl phosphate solution to all wells of the plate. Incubate for 15 minutes at room temperature and then add 50 µl 3M NaOH to stop the reaction (SOP 2).

8. After 5 minutes, read optical density values at 405 nm and 690 nm (SOP 2).

Data analysis

1. Average the duplicate optical density values.
2. Subtract the average background (OD of blank wells) from each of the wells for each of the immunoglobulin antigens. Background should be less than 0.1. If not, reject the secondary antibody.

3. To test for IgG specificity, select the dilution that produces an optical density of roughly 1 in the wells coated with IgG. Obtain the optical density of IgG, IgA and IgM isotype coated wells at the dilution of the secondary antibody. Calculate the % cross reactivity as shown below. A specific reagent should have cross reactivity less than 3%.

   [% cross reactivity=(Average OD of IgA wells (X100) / (average OD of IgG wells)]

4. To test for equivalent binding of all IgG subclasses, select the dilution of secondary antibody that produces OD of approximately 1 for IgG2. Obtain the optical density for IgG1, IgG2, IgG3 and IgG4 wells at the dilution. Calculate the binding ratio for all IgG subclasses as shown below by using OD of different IgG subclass in the numerator. A balanced reagent should have a binding ratio close to 1 for all IgG subclass combinations.

   [binding ratio of IgG1= (Average OD of IgG1 wells)/(Average OD of IgG2 wells)]

SOP 6: Determine the optimum working dilution for the enzyme labeled secondary antibody

Prepared by the laboratories of: Dr. Moon Nahm and Dr. David Goldblatt
Effective date: June 30, 2002, November 26, 2002; March 31, 2011
Applicable to: Pn Ps ELISA (SOP 2)

Purpose

The purpose is to determine the optimal working dilution of the enzyme-labeled secondary antibody, which is used for the Pn PS ELISA.

Materials and reagents

- Reagents and materials specified in the Pn PS ELISA for which the enzyme-labeled secondary antibody will be used.
- Enzyme-labeled secondary antibody to be tested ("new secondary antibody")
- Enzyme-labeled secondary antibody currently used ("old secondary antibody")

All other reagents and materials are specified in SOP 2.

Procedure

Conditions for all steps below should conform to those used in the antigen specific Pn PS ELISA as defined in SOP 2. Also it is assumed that one new secondary antibody is being tested with one old secondary antibody. Depending on the situation, the reagent volumes can be changed.

1. Coat 4 microtiter plates with Pn PS (one plate each of serotypes 4, 6B, 14 and 18C) at the optimal antigen concentration (SOP 4A) for each lot of enzyme-labeled secondary antibody to be tested.

2. Prepare 4 ml of "absorption solution" (i.e. antibody buffer containing C-PS and 22F PS, each at 5 micrograms/ml). Dilute the standard serum (007sp) with 4 ml of the "absorption buffer" such that the optical density value at the end of the assay would be about 2.0. Although the exact dilutions are serotype specific, this dilution is usually about 1:1500 for 007sp (the reference standard). Transfer 200 µl of the diluted serum to each well in row A of the "dilution plate".

3. Add 100 µl of the antibody buffer to wells in rows B through H in the "dilution plate". Prepare six 2-fold serial dilutions of the standard serum in the "dilution plate" by transferring 100 µl from the wells in row A to those in row B etc. These dilutions should yield a linear range of optical density values between 0.1 and 2.0. Incubate for 30 minutes.

4. Wash the antigen-coated assay plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.

5. Transfer 50 µl from each well of the "dilution plate" to that of the assay plate. Start transferring from row G then move up the next row etc. Add only the antibody buffer to the wells in row H. Incubate the plate for 2 hours (SOP 2).

Diagram 1

Dilution of 007sp ⇓		New candidate 2° antibody										Old qualified 2° antibody at in use dilution	
		D1 1:500		D2 1:1000		D3 1:2000		D4 1:4000		D5 1:8000			
		1	2	3	4	5	6	7	8	9	10	11	12
1:500	A												
1:1000	B												
1:2000	C												
1:4000	D												
1:8000	E												
1:16000	F												
1:32000	G												
Antibody buffer	H												

6. In 15-ml polystyrene tubes, prepare five 2-fold serial dilutions (dilutions D1, D2, D3, D4, D5 and D6) of the new secondary antibody. Generally, 2 ml of the new antibody dilutions are prepared at 1:500, 1:1000, 1:2000, 1:4000, 1:8000 and 1:16000 dilutions, but the exact dilutions may vary with the new secondary antibody type, source, and lot. If the old secondary antibody is available, prepare the working dilution of the old secondary antibody and use it instead of D6.

7. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.

8. Add serial dilutions of enzyme-labeled secondary antibodies in duplicates (100 µl/well) to appropriate columns as shown in Diagram 1. For instance, D1 dilution of the new antibody will be added to wells in columns 1 and 2 (including rows G and H), D2 to columns 3 and 4, etc. The old antibody (or D6) will be added to wells in columns 11 and 12. Incubate the plates according to antigen specific Pn PS ELISA (SOP 2).

9. Wash the plates 5 times with wash buffer. During the first wash, allow the wash buffer to soak on the plate 30 seconds to 1 minute after filling the wells.

10. Add substrate to the plates, incubate the plates, and obtain the OD of the plates using an ELISA reader as described in SOP 2.

Data analysis

Choose the dilution of new secondary antibody that satisfies all 5 requirements described below.

1. Signal strength: Examine OD values of the wells in row A and determine the dilution of the new secondary antibody with OD values close to 2. Usually, 1:1000 or higher dilutions would provide the high OD values. Occasionally, a working dilution as low as 1:250 may be required for a very poor secondary antibody to achieve the required high OD. At these low dilutions, the secondary antibody may have high background binding.

2. Background binding: Examine OD values of the wells in rows G and H (antigen + enzyme labeled secondary antibody) and determine the dilutions of the new secondary antibody with OD values less than 0.1. If a new secondary antibody has a working dilution 1:1000 or higher, it usually has low background binding. Occasionally, the secondary antibody with high working dilution may bind the antigen and may have high background binding. The overall performance of the secondary antibody is determined by the signal to noise ratio. Therefore, select the optimal working dilution, which maximizes the signal and minimizes the noise.

3. Lower limit of detection: Perform a separate linear regression analysis on the absorbance from the 6 dilutions of reference serum for each dilution of enzyme labeled secondary antibody. Calculate the 'lower limit of detection' as determined by the units of antibodies present in the dilution of reference serum yielding two times the assay background. Calculate the units/ml detectable at the lowest dilution of serum tested in the assay (i.e., 1:50 for human serum). The optimal dilution chosen must yield values on more than 99% of cases. In the case of the Pn PS ELISA assay, the sensitivity limit is about 0.01 µg/ml.

4. Comparability: Select the dilution of new secondary antibody that yields equivalent OD (i.e., ± 10% of the current lot) while meeting the lower limit of assay sensitivity. A side-by-side analysis of a panel of sera should confirm that results obtained using the 'new' lot of the secondary antibody is equivalent to those attained with the old, previously qualified antibody.

5. Enzyme development time: If robotic applications of the ELISA are planned, the substrate development time for the alkaline phosphatase enzyme is often made similar to typical ELISA antibody incubation times (e.g. 1-2 hours). Incubation periods less than one hour may severely limit the number of plates the robot can process.

Appendix 7

CDC Skim-milk tryptone glucose glycerol (STGG) transport and storage medium recipe

STGG transport medium

Skim milk powder 2 g
Tryptone Soy Broth 3 g
Glucose 0.5 g
Glycerol 10 ml
Distilled water 100 ml

- Mix to dissolve all ingredients.
- Dispense in 1.0 ml amounts in screw-capped 1.5-ml vials.
- Loosen the screw-cap tops and autoclave for 10 minutes (at 15 pounds).
- Tighten caps after autoclaving.
- Store STGG frozen at -20°C or refrigerate until use.

STGG storage medium

Skim milk powder 10g
Glycerol 10ml
Distilled water 90ml

-10g of Skim milk in 90 mL of distilled water in flask A
-10 mL of glycerol (autoclave in separate flask B)
-Autoclave at 115 C for 10 min, and exhaust the pressure carefully.
-Mix flasks A and B after autoclaving while still hot
-Use 1.0 mL of skim milk solution for each isolate in a cryotube
-Freeze at -70C