Department of Science and Technology/

National Research Foundation: Vaccine Preventable Diseases

Title: Effect of natural colonization by *Streptococcus pneumoniae* on the systemic immune responses to common pneumococcal protein antigens with immune protective potential

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DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science (Dissertation) in the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination in other Universities.

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Signature of candidate

Signed on the 04th day of October 2011

DEDICATION

The Road Not Taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where it bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim
Because it was grassy and wanted wear,
Though as for that the passing there
Had worn them really about the same

And both that morning equally lay
In leaves no step had trodden black.
Oh, I marked the first for another day!
Yet knowing how way leads on to way
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I,
I took the one less traveled by,
And that has made all the difference.

By Robert Frost

This work is dedicated to my parents, Mr. Andrew Ditse and Mrs Tiny Ditse, who have always been my rock and pillar of strength, my sisters; Dudu and Palesa, my niece Andile, my brother, Mpho and Bafana Vumasi. Thank you all for your support, encouragement and always believing in me even when everything seemed impossible. Without you, I would have not gone this far.

ABSTRACT

Background: Due to the high cost and limited serotype coverage of pneumococcal conjugate vaccines (PCV), surface proteins of *Streptococcus pneumoniae* are being investigated for their role as potential vaccine candidates. There are limited data on natural antibody kinetics against pneumococcal surface proteins arising through exposure to pneumococcal nasopharyngeal (NP) colonization in African populations.

Objectives: To characterize the natural antibody kinetics and sero-prevalence to 15 pneumococcal proteins with respect to age, PCV vaccination and HIV status as well as to explore the association between antibody titers and pneumococcal nasopharyngeal colonization in infants, older children and adults.

Methods: We established a 15-plex Luminex assay for the following proteins: PspA, PspC, LytB, IgA1-proteinase, SP 0082, PdB, PcsB, PsaA, SP 0609, SP 0749, PpmA, SlrA, StkP, SP 2027 and SP 2194, and also validated the Luminex assay comparing it to a standard ELISA method for PspA, PspC, PsaA and PdB. We used the Luminex method to characterize the prevalence and dynamics of serum IgG antibodies against the pneumococcal proteins. The study involved 2 166 human subjects which included: i. A longitudinal cohort of children less than 2 years of age, who were vaccinated with the seven-valent pneumococcal conjugate vaccine (PCV-7) and were either a) HIV-exposed infected, b) HIV-exposed uninfected or c) HIV-unexposed uninfected. ii. A longitudinal cohort of PCV-7 unvaccinated children less than 2 years of age who were either: a) HIV-unexposed uninfected or b) HIV-exposed uninfected. The PCV-7 vaccinated and unvaccinated children were followed up from approximately 4 to 24 months of age. In addition, samples were also analyzed from HIV-uninfected and HIV-infected children

aged between 4 to 7 years who received either a primary series of PCV-9 or placebo during infancy. Lastly, we analyzed cross-sectional samples from HIV-uninfected and HIV-infected women.

Results: The multiplex Luminex assay correlated well with singleplex ELISAs for all four analyzed proteins with correlation coefficients of 0.86, 0.90, 0.87 and 0.96 for PspA, PspC, PdB and PsaA respectively. Antibody titers to PspC, PdB, LytB, SP 0082, PcsB and StkP showed increases in titer with respect to increasing age. Prevailing nasopharyngeal pneumococcal colonization in young children was associated with higher antibody titers to PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, PcsB and StkP. Conversely higher antibody titers to PspC, PdB, LytB, SP 0082, PcsB and StkP were associated with lower prevalence of pneumococcal colonization in older children and adults. In children under two years of age, PCV vaccination was associated with lower antibody titers to PspA, PspC, LytB, PdB, IgA1-proteinase, PcsB and StkP as well as higher antibody titers against SP 0082 and PpmA at multiple time-points. In PCV-vaccinated children under two years of age, those who were HIV-unexposed , -uninfected had higher antibody titers to PspA, PspC, SP 0082, IgA1-proteinase, PpmA and StkP compared to HIV-exposed, uninfected children.

Conclusion: There was an age-related increase in antibody titers to PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, PcsB, and StkP in children under two years of age. PCV immunization was, however, associated with lower antibody titers to PspA, PspC, LytB, PdB, IgA1-proteinase, PcsB and StkP in young children which was not attributed to differences in the prevalence of nasopharyngeal colonization. Furthermore, HIV-infection status in young children was associated with higher antibody responses to PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, PcsB and StkP proteins in HIV-unexposed uninfected children compared to HIV-exposed uninfected and HIV-exposed infected children. Higher antibody concentrations to

PspC, PdB, LytB, SP 0082, PcsB and StkP was negatively associated with nasopharyngeal pneumococcal colonization in older children and adults; indicating a protective role against colonization and a potential role as vaccine candidates.

ACKNOWLEDGEMENTS

I would like to thank the Almighty Lord for blessing me every day.

I am grateful to Professor Shabir Ahmed Madhi and Doctor Peter Vincent Adrian for supervision of this project and financial support.

I would like to thank PATH and the Department of Science and Technology / National Research Foundation: Vaccine Preventable Diseases for funding this project.

To Dr. Marta Nunes, thank you for all the helpful discussions.

To Locadiah Kuwanda, thank you for statistical advice and assistance.

I acknowledge the Respiratory and Meningeal Pathogens Research Unit (RMPRU) staff for their assistance.

I would also like to thank the mothers and children who participated in the clinical trials, without whom, my project would not be feasible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

%	percent
<	less than
>	greater than
≤	less than or equal to
≥	greater-than or equal to
±	plus-minus
U	arbitrary units
µl	micro-liter
C	Celsius
°	degree

LIST OF ABBREVIATIONS

°C	degrees Celsius
ABC	ATP-binding cassette
AIDS	acquired immunodeficiency syndrome
AOM	acute otitis media
ART	antiretroviral therapy
CbpA	choline-binding protein A
CI	confidence interval
Clp	caseinolytic protease
CV	coefficient of variation
EDC	1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl
ELISA	enzyme-linked immuno-sorbent assay
FBS	foetal bovine serum
FCMIA	fluorescent covalent microsphere immunoassay
FI	fluorescence intensity
GMT	geometric mean titer
HIV	human immunodeficiency virus
HSP	heat shock protein
IgA1-proteinase	immunoglobulin A1 proteinase
IgG	immunoglobulin G
LLOQ	lower limit of quantification

LM	phospholipid membrane
LTA	lipoteichoic acid
LTFU	loss-to-follow-up
LytB	*Endo-beta-N-acetylglucosamidase*
LPXTG	Leucine-Proline-X-Threonine-Glycine, X is any amino acid
M-/I-	HIV unexposed, uninfected children
M+/I-	HIV exposed, uninfected children
M+/I+	HIV exposed, infected children
MFI	mean fluorescent intensity
NP	nasopharyngeal
OP	oropharyngeal
PBS	phosphate buffer saline
PCho	phosphocholine
PCV	pneumococcal conjugate vaccine
PCV-7	seven-valent pneumococcal conjugate vaccine
PCV-9	nine-valent pneumococcal conjugate vaccine
PdB	pneumolysoid
PG	peptidoglycan
Ply	pneumolysin
Pnc	pneumococci
Pneumococcus	*Streptococcus pneumoniae*
PpmA	putative proteinase maturation protein A
PPV23	23-valent polysaccharide pneumococcal vaccine
PsaA	pneumococcal surface adhesion A

PscB	protein required for cell separation of group B streptococci
PspA	pneumococcal surface protein A
PspC	pneumococcal surface protein C
R-PE	*R*-phycoerythryn
S. pneumoniae	*Streptococcus pneumoniae*
SD	standard deviation
SktP	serine-threonine kinase P
SlrA	streptococcal lipoprotein rotamase A
STGG	skim milk, tryptone, glucose and glycerin
StkP	serine threonine kinase
Sulpho-NHS	*N*-hydroxy-sulphosuccinimide
TA	teichoic acid
TIGR	The Institute for Genomic Research
TIGR4	*Streptococcus pneumoniae* virulent serotype 4
U/ml	arbitrary units per millilitre
ULOQ	upper limit of quantification
Zmp	zinc metalloprotease

Chapter 1: Introduction

Streptococcus pneumoniae (S. pneumoniae) was discovered more than a century ago, yet severe pneumococcal diseases (pneumonia, meningitis, bacteremia and septicaemia) kill approximately 870 000 children per annum, especially in industrializing countries (O'Brien et al., 2009). The most effective strategy for preventing pneumococcal disease is vaccination. The two currently available vaccines that are based on immunity to capsular polysaccharides of the pneumococcus, of which at least 92 serotypes exist (Bratcher et al., 2011), have limitations. The polysaccharide-based vaccine, PPV23, which consists of 23 purified capsular polysaccharide antigens, is not immunogenic in young children for most serotypes (Käyhty et al., 1987). The polysaccharide-protein conjugate vaccine (PCV) has varying potential for reducing pneumococcal disease, due to the limited number of serotypes included in the vaccine, as well geographic and temporal variations in serotypes causing pneumococcal disease in children (Hausdorff et al., 2001, Shinefield et al., 2002a, Siber et al., 2008). In addition, the potential for replacement disease from non-vaccine serotypes increases following immunization with PCV (Singleton et al., 2007). Furthermore, access to PCV is likely to be hampered because of the high costs of these vaccines, which require complex biotechnology to manufacture.

Consequently, surface proteins of *S. pneumoniae* are being investigated for their role in pneumococcal pathogenicity and as candidate antigens for protein-based vaccines (Bogaert et al., 2004a). Thus far, proteins associated with pneumococcal virulence which have shown the greatest potential as vaccine-antigen candidates are the two choline-binding surface proteins,

pneumococcal surface protein A (PspA) (Crain et al., 1990, McDaniel et al., 1987) and choline-binding protein A (CbpA) (Brooks-Walter et al., 1999) and a metal-binding lipoprotein pneumococcal surface antigen A (PsaA) (Dintilhac et al., 1997, Sampson et al., 1994) and the thiol-activated toxin pneumolysin, Ply (Paton and Ferrante, 1983).

The natural immune responses and interaction thereof, with pneumococcal nasopharyngeal colonization were evaluated for fifteen pneumococcal protein candidates in this study (see Table 1). The antigens were PspA, PspC, PsaA and pneumolysoid (PdB), the non-toxic variant of Ply, serine-threonine kinase P (SktP), protein required for separation of group B *streptococci* (PscB), putative proteinase maturation protein A (PpmA), IgA1-proteinase, streptococcal lipoprotein rotamase A (SlrA), *endo-beta-N-acetylglucosamidase* (LytB), and some exploratory proteins obtained from Intercell AG, which were only described according to their *Streptococcus pneumoniae* virulent serotype 4 (TIGR4) annotation i.e. SP 0609, SP 0082, SP 0749, SP 2194 and SP 2027.

The advantages of pneumococcal proteins include: i.) proteins elicit a T-cell dependent immune response and consequently are likely to be immunogenic and induce anamnestic immune responses even when administered during early infancy; ii.) they could potentially protect against all pneumococcal serotypes; and iii) they are likely to be less expensive in production and cost than PCV. Previous studies measured antibodies to PspA in healthy adults and children, in children with invasive infection due to pneumococci and in children with invasive infection caused by other bacteria (Virolanein et al., 2000). Antibodies to Ply have been evaluated in elderly adults infected with *S. pneumoniae* among whom antibody responses were detected in 92 % of serum samples (Musher et al., 2001). In children with acute otitis media, antibodies to Ply

were detected from early infancy but there was no association between the concentration of anti-Ply antibodies and future risk of developing pneumococcal AOM (Simell et al., 2001).

Previous studies in European and Filipo children also evaluated the prevalence and the natural development of antibodies to PspA, PspC, PsaA and Ply in young infants and their association to pneumococcal colonization and carriage (Holmlund et al., 2006, Rapola et al., 2000, Simell et al., 2009). In addition, there have been studies in European children on the natural development of antibodies to PpmA, SlrA and IgA1-proteinase in young infants in response to colonization(Adrian et al., 2004). No such studies have however been undertaken in an African population, among whom the dynamics of pneumococcal exposure and nasopharyngeal colonization is more intense compared to children from developed countries (O'Brien et al., 2003). In addition, to our knowledge there has been no other single study which has evaluated the natural kinetics of antibody responses to all 15 proteins evaluated in our study.

The objective of this study was to characterize the natural development of antibodies to 15 pneumococcal proteins and determine whether age, PCV vaccination and HIV status have an impact on naturally acquired antibodies to these proteins. The study also explored the association of development of antibodies to the prevalence of pneumococcal colonization in children and adults.

Chapter 2: Literature Review

2.1 Identification and classification of the pneumococcus

S. pneumoniae or pneumococcus is a Gram positive, bile soluble, alpha-hemolytic diplococcus. It is a member of the human commensal flora and the genus *Streptococcus*. It is one of the most important bacterial pathogens that cause both mucosal and invasive disease. *S. pneumoniae* is responsible for approximately 1.5 million deaths worldwide and is the most common cause, from any single pathogen, of childhood deaths in the developing world. In addition, it kills more people than any other vaccine-preventable disease (Centers for Disease and Prevention, 2007).

2.2 Pneumococcal colonization

Mucosal colonization by pneumococcus is a crucial step in the pathogenesis of all pneumococcal diseases (Musher et al., 2000). *S. pneumoniae* is known to be associated with asymptomatic colonization of the mucosal surface of the human nasopharynx in up to 40-90 % of healthy children and 10-30 % of healthy adults (Bridy-Pappas et al., 2005, Hill et al., 2010). The survival of pneumococci in the nasopharynx is dependent on the ability of these organisms to compete or interact synergistically with other organisms in the nasopharynx, as well as engage with innate and acquired immune effectors. The prevalence of pneumococcal colonization is greater in

settings with over-crowding including in hospitals, and day-care centers (Kellner and Ford-Jones, 1999, Principi et al., 1999).

Nasopharyngeal pneumococcal colonization in children from developing countries may be established within days after birth. Up to 90 % of children from Gambia are found to be colonized by 5 years of age (Hill et al., 2010). Whilst the prevalence of nasopharyngeal colonization declines to 10% and remains constant through adulthood in developed countries, (Goldblatt et al., 2005, Gray and Dillon Jr, 1988), a high prevalence of colonization may persist in African settings among children (97%) and in adults (85%) (Hill et al., 2010). Children, may experience a number of carriage episodes with different serotypes and the duration of carriage varies with the age of the carrier and the type of serotype (Dagan et al., 2004). The duration of colonization in young children lasts for one to two months, whereas duration of colonization in older individuals is shorter, suggesting development of natural immunity against colonization (Gray et al., 1980, Hill et al., 2010).

High prevalence of pneumococcal nasopharyngeal colonization in children in the developing world are frequently associated with the carriage of pneumococci of more than one serotype (Gratten et al., 1989). The distribution of pneumococcal serotypes associated with nasopharyngeal colonization may vary with age with sero-groups 6, 19 and 23 being the frequent colonizers in children. These sero-groups are not only found in the asymptomatic carriage state in children but also are common causes of invasive pneumococcal disease in this age group (Bogaert et al., 2004b, Gray and Dillon Jr, 1988, O'Brien et al., 2008).

Pneumococcal colonization induces a variety of immune responses in humans, including production of antibodies directed at surface proteins antigens (Adrian et al., 2004, Bogaert et al., 2006, Cao et al., 2007, Giefing et al., 2008, Gosink et al., 2000, Hermans et al., 2006, Holmlund et al., 2006, McCool et al., 2002, Ogunniyi et al., 2007b, Rapola et al., 2000) and polysaccharide capsule epitopes (Soininen et al., 2009). This process requires direct interaction between the pnemococcal cell surface proteins and mucosal immune cell mediators. There is evidence which suggests that pneumococcal colonization in human infants can stimulate immunity, and prevent re-colonization by the same or different serotypes of pneumococci, although the mechanism for such protection is yet to be established (Granat et al., 2009).

2.3 Pneumococcal infections

The spectrum of pneumococcal infection and disease differs with age and ethnic groups. Several risk factors for pneumococcal infection, such as age, race, immunodeficiency, antibiotic resistance, socio-economic status and day care attendance have been reported (O'Brien and Santosham, 2004). Disease rates are particularly high in young children, the elderly, and patients with predisposing conditions such as, chronic illnesses and/or immunosuppressive illnesses including AIDS (Gray and Dillon Jr, 1988, Johnston, 1991, Musher, 1992). The relationship of carriage to the development of natural immunity is poorly understood. Children acquire a variety of serotypes early in life (Gray et al., 1980, Leach et al., 1994) and are considered to be the most important sources of pneumococcus transmission in communities (Gray and Dillon Jr, 1988).

2.4 Bacterial transformation

Pneumococci may be both un-encapsulated and encapsulated. Un-encapsulated pneumococci are present in 0.5 -2 % of invasive isolates from normally sterile sites (Broome and Facklam, 1981, Carvalho et al., 2003), in 20 % of conjunctival isolates (Finland and Barnes, 1977) and in up to 10 % of isolates identified in the sputum and nasopharynx (Carvalho et al., 2003). Pneumococci are able to acquire new phenotypic traits through natural transformation from a "smooth" encapsulated form to a "rough" un-encapsulated form, so that they are genetically flexible (Avery et al., 1944).

Frequent recombination between individual strains allows gene mosaicism (Bruckner et al., 2004, Musher, 1992). Recombination at the capsular level may enable the bacteria to escape the immune system, by serotype switching into phylogenetically related strains with a different capsule type (Jefferies et al., 2004). Genetic exchanges of virulence factors among streptococcal species as well as the emergence and spread of antibiotic resistant strains of diverse serotypes have been widely documented (Klugman, 1990, Lister, 1995, Nissinen et al., 1995, Whitney et al., 2000).

2.5 The capsular polysaccharide and serotypes

S. pneumoniae, like most human pathogenic bacteria, consists of a polysaccharide capsule that surrounds the bacterial cell wall. The antiphagocytic capsule forms the basis for pneumococcal classification into at least 92 serotypes, which are further divided into 46 serogroups. The

stratification of these serotypes is based on the structural and chemical composition of polysaccharides, and on the basis of antigenic properties of the capsular polysaccharides (Bratcher et al., 2010, Henrichsen, 1995). There is varying invasive potential between the serotypes (Pantosti et al., 2003). The thick capsular polysaccharide is one of the most important virulence factors of pneumococci. It protects bacterial cells from phagocytosis, inhibits complement activation and plays a major role in the competition with other bacteria to enable colonization of the human nasopharynx (Gehre et al., 2008, Werner, 2000, Jedrzejas, 2004). The polysaccharide capsule is immunogenic and may induce immune responses (Jedrzejas, 2001).

2.6 Pneumococcal vaccination strategies

2.6.1 Polysaccharide- and protein-polysaccharide based vaccines

Pneumococcal vaccines have been developed to prevent pneumococcal infections. The first pneumococcal vaccines were the polysaccharide vaccines, however, they have been shown to be protective in adults, but not in the elderly, immuno-compromised patients and children under 2 years of age (Musher et al., 2001). The lack of protection in these at-risk groups relates to the poor immunogenicity of polysaccharide antigen and failure thereof to induce immunologic memory or maturation of the immune response (Käyhty et al., 1987). The polysaccharide-protein conjugate vaccines (PCV), however, are immunogenic and protective in infants. Nevertheless, protection is largely specific to serotypes included in the vaccine (Eskola et al., 2001, Shinefield et al., 2002b). Other limitations associated with PCV are the risk for natural serotype switching (Coffey et al., 1998), serotype replacement by non-vaccine type pneumococci in both

colonization (Eskola et al., 2001, Mbelle et al., 1999) and invasive diseases (Singleton et al., 2007) as well as geographic and temporal variations in serotype distribution (Hausdorff et al., 2000).

2.6.2 Protein-based vaccines

A promising alternative approach for new-generation pneumococcal vaccines is the use of pneumococcal proteins that are highly conserved among most pneumococcal strains (Bergmann and Hammerschmidt, 2006, Bogaert et al., 2004a). Antibody production against pneumococcal proteins begins in infancy, whereas the production of antibodies directed to capsular polysaccharides does not begin until the second or third year of life (Bogaert et al., 2004a). The relevance of this naturally acquired immunity to proteins in modifying colonization may identify potential vaccine candidate targets (Bogaert et al., 2004a). Immunological response against proteins may provide protection against nasopharyngeal colonization and/or pneumococcal infections independent of capsular antibodies.

Previous studies in animal models targeted single candidate proteins such as PspA, PsaA and Ply based on their roles in pneumococcal pathogenicity (McDaniel et al., 1991, Paton and Ferrante, 1983, Rosenow et al., 1997). These proteins are common to all pneumococcal isolates and have been shown to be immunogenic and protective against subsequent challenge with different strains of pneumococci in animal model studies (Russell et al., 1990, Tart et al., 1996, White et al., 1999, Wu et al., 1997). Reverse vaccinology and systemic analysis of multiple genomes together with thorough understanding of the molecular epidemiology of the disease plays a

crucial role in the development of a vaccine based on the combination of antigens from a representative species (Barocchi et al., 2007). Identification of immunogenic surface proteins and assessment of their role against pneumococcal colonization and/or disease is crucial in the development of a vaccine with the ability to protect independent of serotype (Barocchi et al., 2007).

2.7 Pneumococcal protein vaccine candidates

The identification of virulence determinants can facilitate the development of new vaccines. The major virulence factor required for all pneumococcal diseases is the extracellular capsular polysaccharide. In addition to the capsule, many surface-exposed proteins and toxins that are released after the autolysis of pneumococci, contribute significantly to the pathogenesis of *S. pneumoniae* disease as indicated in Table 1.

Table 1: Table of the 15 pneumococcal proteins studied in this project as potential vaccine candidates and their role in pneumococcal pathogenicity.

Classification	Antigen	Role and function in pathogenicity	Model	References
Choline-binding proteins	Pneumococcal surface protein A (PspA)	Inhibits complement activation, cross-protective, colonization	Humans and mice	(Briles et al., 2000, Briles et al., 1996, Hammerschmidt et al., 2000, Wu et al., 1997)
	Pneumococcal surface protein C (PspC/ CbpA)	Adherence, colonization	Rat, mouse, humans	(Balachandran et al., 2002, Cundell and Tuomanen, 1994, Hammerschmidt et al., 2000, McCool et al., 2002, Rosenow et al., 1997)
	Endo-beta-*N*-acetylglucosamidase (LytB)	Colonization	Rats, mice	(Gosink et al., 2000, Lopez et al., 2000)
Sortase-dependent proteins (LPXTG motif)	IgA1-proteinase	Colonization, adherence	Mice	(Kilian et al., 1996, Ogunniyi et al., 2000, Weiser et al., 2003)
	*SP 0082	Cell wall surface anchor family protein	**N/A	(Tettelin et al., 2001)
Lipoproteins:				
ABC-transporters	Pneumococcal surface adhesion A (PsaA)	ABC-transporter, adherence, colonization	Mice, humans	(Dintilhac et al., 1997, Johnson et al., 2002, Rapola et al., 2000, Romero-Steiner et al., 2003)
	*SP 0749	Amino acid transport and metabolism	**N/A	(Tettelin et al., 2001)
	*SP 0609	Amino acid transport and metabolism	**N/A	(Tettelin et al., 2001)
Non-ABC transporters	Streptococcal lipoprotein rotamase A (SlrA)	Colonization, adherence	Mouse, humans	(Adrian et al., 2004, Hermans et al., 2006)
	Putative proteinase maturation protein A (PpmA)	Adherence, colonization	Mice, humans	(Cron et al., 2009, Hermans et al., 2006, Overweg et al., 2000)
Integral membrane lipoproteins	Serine-threonine kinase P (StkP)	Growth, competence and colonization	Humans	(Giefing et al., 2008, Rajagopal et al., 2003, Rajagopal et al., 2006)
Secreted proteins	Pneumolysoid (PdB)	Aids penetration of host tissues, inhibits immune responses, colonization	Mice, humans	(Ferrante et al., 1984, Holmlund et al., 2006, Musher et al., 2001, Paton and Ferrante, 1983, Rapola et al., 2000)
	PcsB	Cell division, colonization, bacterial survival and growth	Humans	(Giefing et al., 2008, Mills et al., 2007, Ng et al., 2004)
Other pneumococcal proteins	Caseinolytic protease (Clp)	Virulence gene expression, adherence	Mice	(Kwon et al., 2003, Kwon et al., 2004)
	*SP 2027	Conserved hypothetical protein	**N/A	**N/A

* Pneumococcal proteins from Intercell AG that were described according to their TIGR4 annotation. **N/A: their function(s) are unknown, as they have not been characterized yet.

2.7.1 Pneumococcal proteins

It is estimated that the pneumococcal genome encodes over 100 surface proteins, most of which play a role in pathogenicity and virulence (Wizemann et al., 2001). Bacterial surface proteins are essential for the interaction of bacterial pathogens with their environment during infection and contribute to the disease pathogenesis. Three clusters of surface proteins can be distinguished by genome analysis: the choline-binding proteins, the lipoproteins and sortase-dependent surface proteins that are anchored in the cell wall (Figure 1). Non-classical surface proteins that lack a leader peptide and membrane-anchoring motifs have also been identified on the pneumococcal surface.

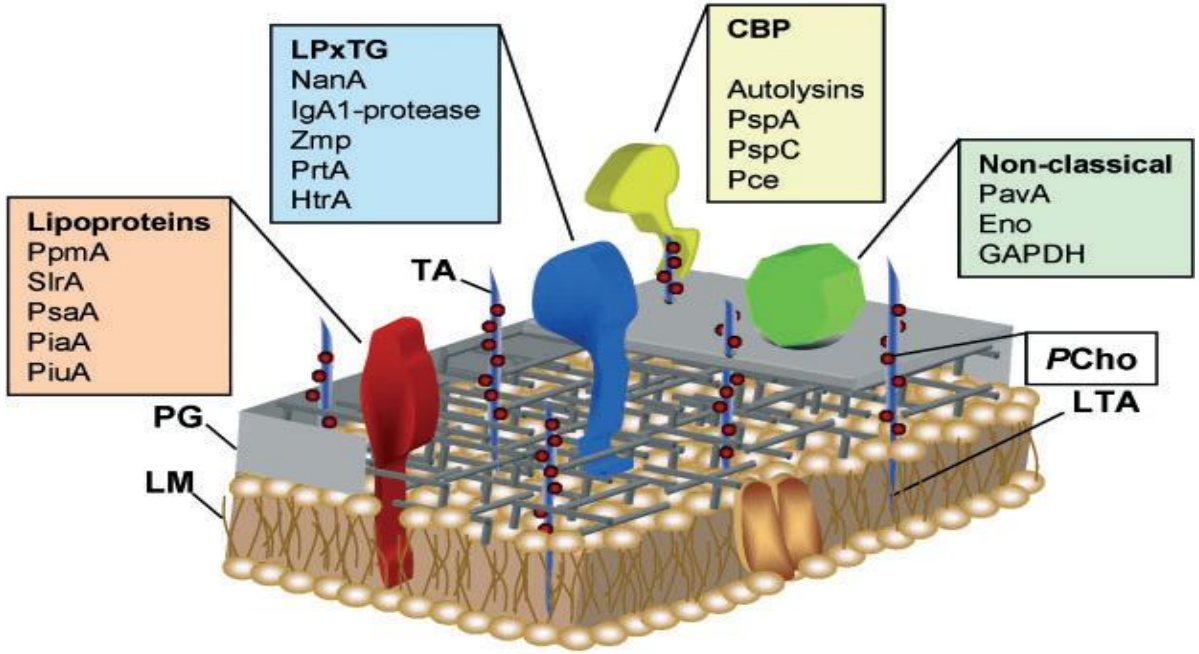


Figure 1: Diagram showing pneumococcal cell-wall and surface-exposed proteins; the choline-binding proteins, lipoproteins and the sortase-dependent proteins. The pneumococcal cell wall consists of a phospholipid membrane (LM) containing peptidoglycan (PG), teichoic acid (TA) and lipoteichoic acid (LTA). Phosphocholine (PCho) anchors choline-binding proteins to the cell wall, as indicated in the diagram (Bergmann and Hammerschmidt, 2006). CBP = choline-binding proteins and LPXTG = Leucine-Proline-X-Threonine-Glycine sequence, where X is any amino acid. This motif is shared by a majority of sortase-dependent proteins as depicted in the diagram.

2.7.1.1 Choline-binding proteins (CBPs)

Choline-binding proteins (CBPs) are a diverse super family of proteins which are attached to the pneumococcal surface via non-covalent interactions with phosphoryl-choline properties on cell wall teichoic acid and membrane lipoteichoic acid. In vaccine development studies, CBPs are of particular interest since a number of proteins interact with the cell wall non-covalently through a choline-binding domain consisting of 2 - 10 sequence repeats (Fernandez-Tornero et al., 2001). Pneumococci can produce 13 to 16 different CBPs; including four cell wall hydrolases that are important for virulence: autolysin LytA, LytB, LytC and a phosphorylcholine esterase. CBPs have diverse functions, including cell wall modification, adherence to host cell surface molecules and the modulation of complement activation. In spite of the similarity of the choline-binding repeat domains, the CBP molecules are structurally and functionally different (Swiatlo et al., 2004). The following CBPs: PspA, PspC and LytB (SP 0498) were evaluated in this project.

2.7.1.1.1 Pneumococcal surface protein A (PspA)

Pneumococcal surface protein A (PspA) is one of the most studied pneumococcal proteins. It is a highly variable protein found on all strains of pneumococci (Crain et al., 1990). PspA is divided into three families, which are further stratified into clades based upon relatedness of DNA and protein sequences. PspA family 1 is composed of clades 1 and 2, family 2 is composed of clades 3 – 5 and family 3 is equivalent to clade 6, which is very distant from all of the other clades. The

biological properties of PspA reside in the N-terminal of the protein, which forms a highly charged, alpha-helical anti-parallel coiled-coil structure (Hollingshead et al., 2000, Jedrzejas et al., 2000). The alpha-helical region of PspA is exposed on the surface of the pneumococcus, however, the presence of the capsule does not prevent accessibility to antibodies, suggesting that antibodies against PspA could be opsonophagocytic (Gor et al., 2005). The proline-rich region has been found to be effective in eliciting cross-protective immunity across different serotypes (McDaniel et al., 1994, Nabors et al., 2000).

PspA plays a pivotal role in preventing complement-mediated opsonization, is capable of binding to and preventing killing by lactoferrin and is involved in iron uptake and thus contributes to pneumococcal growth (Hammerschmidt et al., 1999, Ogunniyi et al., 2007b, Tu et al., 1999). Studies with PspA deletion mutants have shown that PspA is a virulence factor (Crain et al., 1990, McDaniel et al., 1987). Although this antigen is structurally variable, antibody responses raised to it are cross-reactive to heterologous PspA proteins, and immunization with one PspA protein can protect mice against pneumoccoci that contain a heterologous PspA clade and capsule type. PspA has been used for intranasal, intraperitoneal and subcutaneous immunization in mice, which could elicit significant protection against pneumococcal infection, making it a promising candidate vaccine (Nguyen et al., 2011, Ogunniyi et al., 2007a, Ogunniyi et al., 2007b). Previous studies in humans have demonstrated that antibodies against PspA increase during colonization and pre-existing low antibody titers against this protein were associated with pneumococcal colonization (McCool et al., 2002). In addition to its promise as a potential vaccine antigen capable of preventing systemic disease, PspA exhibits promise as a mucosal vaccine antigen for the prevention of nasopharyngeal carriage (Wu et al., 1997). A

vaccine capable of preventing carriage is likely to prevent disease in vaccinated individuals and contribute to indirect protection by reducing transmission of pneumococcus.

2.7.1.1.2 Pneumococcal surface protein C

Pneumococcal surface protein C (PspC) also known as choline-binding protein A (CbpA), is one of the 15 proteins identified in the genome of TIGR4 strains that exhibit multiple C-terminal repeats of an approximately 19-amino-acid motif that binds choline moieties present on the bacterial cell wall. PspC is structurally related to PspA and is present in 75 - 100 % of all pneumococcal strains (Ianelli et al., 2002, Ogunniyi et al., 2000). Sequence analyses have shown that there are many variants of the PspC protein, and different functions have given these variants different names (eg. PspC, Hic, PbcA). PspC proteins can be classified into 11 groups, their common features being an N-terminal signal peptide, followed by an alpha-helical region, a proline-rich region and a C-terminal anchor. The N-terminal region consists of an alpha-helical structure with its size varying between 118 - 589 amino acids resulting in the size variation of PspC across different pneumococcal strains (Ianelli et al., 2002).

PspC mediates adherence to cytokine-activated lung cells via a human-specific interaction with the polymeric immunoglobulin receptor, pIgR (Hammerschmidt et al., 2000). It plays a major role in colonization of the nasopharynx, adherence to the nasopharyngeal and lung epithelium and the brain microvascular endothelium (Cundell and Tuomanen, 1994). It also mediates the invasion of host cells at these locations. PspC allows bacteria to cross through the blood-brain barrier, implying that it plays an essential role in pneumococcal meningitis (Cao et al., 2007,

Linder et al., 2007, Ogunniyi et al., 2007b, Brooks-Walter et al., 1999). Furthermore, PspC has been suggested to protect pneumococci from opsonization with the components of the alternative pathway by binding to factor H, which accelerates the degradation of C3b by factor I (Janulczyk et al., 2000, Jarva et al., 2002).

Antibodies directed against PspC are able to elicit protection against nasopharyngeal colonization in a mouse model (Balachandran et al., 2002), and conferred protection against death when mice were challenged with the highly virulent pneumococcal strain D39 (Ogunniyi et al., 2001). Studies have also shown that rabbits immunized with recombinant PspC elicited cross-protective antibodies to PspA and provided protection against pneumococcal bacteraemia and sepsis (Brooks-Walter et al., 1999, Ogunniyi et al., 2000). Antibody responses, in an experimental human pneumococcal colonization model, indicated that PspC is exposed and immunogenic (McCool et al., 2002, Overweg et al., 2000). There are currently no published data on whether vaccination with PspC elicits protection against heterologous PspC type strains.

2.7.1.1.3 Endo-beta-N-acetylglucosamidase (LytB)

Endo-beta-N-acetylglucosamidase (LytB) is a glucosaminidase that belongs to a family of murein hydrolases. The lysis of pneumococci during stationary growth or antibiotic treatment is the result of murein hydrolases that require choline for activity (Hollingshead and Briles, 2001). It is highly expressed in the early exponential growth phase and has been shown to be important for cell separation of pneumococci at the end of cell division (Garcia et al., 1999a, Garcia et al., 1999b)(Garcia *et al.* 1999a; Garcia *et al.* 1999b). LytB and LytC are unusual in that their

choline-binding domains are located in the N-terminal parts of the molecule, while the C-terminal portions have murein hydrolase activity (Lopez et al., 2000). LytB plays a significant role in colonization and the significant loss of ability to colonize the nasopharynx in the LytB mutant with slight changes in adherence *in vitro*, could possibly suggests that LtyB could also play a role in toxin release. Loss of function of LytB or LytC was associated with reduced nasopharyngeal colonization in rats (Gosink et al., 2000). Studies by Wizeman *et al.* demonstrated that immunization with LytB conferred protection against disseminated *S. pneumoniae* infection, in mouse models (Wizemann et al., 2001).

2.7.1.2 Sortase-dependent proteins

Sortase-dependent surface proteins of gram-positive bacteria are characterized by the presence of a C-terminal motif, which consists of a conserved Leucine-Proline-X-Threonine-Glycine, LPXTG sequence, where X is any amino acid. This motif is recognized by a sortase, which is a protease that cleaves between the T and G residues and covalently links the protein to the peptidoglycan cross-bridges (Schneewind et al., 1993). Pneumococcal strains produce up to four zinc metalloproteases, including IgA1-protease, ZmpB, ZmpC and ZmpD, which are anchored to the cell wall by an N-terminal LPXTG motif.

2.7.1.2.1 Immunoglobulin A1 (IgA1) proteinase

Immunoglobulin A1 (IgA1) is the major immunoglobulin isotype involved in immunity of the mucosal membrane of the respiratory tract in humans. IgA1-proteinases are extracellular bacterial enzymes that cleave human IgA1. All streptococcal IgA1-proteinases cleave the same Proline-Threonine (Pro-Thr) bond at position 227 and 228 in the region of human IgA1, thus preventing opsonophagocytosis. Cleavage of surface-bound serotype specific IgA1 by the IgA1-proteinase enhances adherence of pneumococci to host cells (Weiser et al., 2003).

IgA1-proteinase is produced by virtually all strains of pneumococci. The N-terminal region of this protein is essential for proper function and surface localization (Bender and Weiser, 2006). Most human pathogens, including *S. pneumoniae,* which invade mucosal membranes, possess IgA1-cleaving activity. Studies by Polissi *et al*. have shown that IgA1-proteinase plays a role in pneumococcal lung infections and bacteraemia (Polissi et al., 1998). Previous studies have also demonstrated that IgA1 protease is diverse and heterologous among *Haemophilus influenzae* strains and is involved in immune escape by allowing a number of clones of the same species to colonize one host (Lomholt et al., 1993). There is some evidence that suggests that IgA1-proteinase enables bacteria to evade the mucosal immune barrier and therefore plays a role in colonization (Kilian et al., 1996). Previous studies in humans demonstrated that IgA1-proteinase is highly immunogenic early in life (Adrian et al., 2004).

2.7.1.2.2 SP 0082

SP 0082 is a cell wall surface anchor protein. This protein has not been characterized yet, therefore, it is only represented by its TIGR4 annotation gene symbol. It consists of the LPXTG motif at the C-terminal region and is homologous to IgA1-proteinase (Tettelin et al., 2001). Due to its surface localization and homogeneity to IgA1-proteinase, SP 0082 is predicted to be involved in adherence and pneumococcal colonization.

2.7.1.3 Lipoproteins

S. pneumoniae have over 30 putative lipoproteins, with pro-lipoprotein signal peptidase recognition sequences. They are located beneath the cell wall and the capsule, which suggests that they are not exposed on the cell surface. This implies that they do not elicit opsonic antibodies (Siber et al., 2008). Lipoproteins are anchored into the membrane by the N terminus Leucine-X-X-Cysteine motif (LXXC, where X represents any amino acid) that is cleaved and covalently attached to palmitic acid in the membrane (Gilson et al., 1988, Pearce et al., 1994). Cell surface lipoproteins are important for the full virulence of a number of both Gram -negative and –positive bacterial pathogens. The most important role of lipoproteins is to serve as substrate-binding components of *A*TP-*b*inding *c*assette (ABC) transport systems. ABC transporters contribute in many bacterial processes such as acquisition of vital nutrients, stress responses and intracellular signalling, which is vital for bacterial growth and survival *in vivo* and

in vitro. Lipoproteins studied in this project include the pneumococcal surface adhesion A (PsaA), SP 0749, SP 0609, PpmA, SlrA and StkP.

2.7.1.3.1 ABC transporters

2.7.1.3.1.1 Pneumococcal surface adhesion A (PsaA)

Pneumococcal surface adhesion A (PsaA) is a member of the metal-binding lipoproteins and a highly conserved 37 kDa lipoprotein that is produced by all strains of pneumococci. It is the substrate-binding lipoprotein of an ABC-type manganese-transport system (Dintilhac et al., 1997). Antibodies against PsaA are associated with reduction in adherence of pneumococci to nasopharyngeal epithelial cells (Romero-Steiner et al., 2003). This is consistent with the finding that mucosal immunization of mice with PsaA is highly protective against pneumococcal carriage (Johnson et al., 2002).

Mutations in the *PsaA* gene have been reported to have pleiotropic effects on various pneumococcal functions, including adherence, autolysis virulence and increased sensitivity to oxidative stress (Novak *et al.* 1998; Berry & Paton 1996; Claverys *et al.* 1999). PsaA is highly immunogenic and antibodies directed against PsaA are protective against pneumococcal carriage and invasive infection in animal models (Briles *et al.* 2000a; Talkington *et al.* 1996). Oral vaccination with PsaA also elicited significant protection against colonization, pneumonia as

well as septicemia in mice (Seo *et al.* 2002). Studies have shown that PsaA and PspA have different functions in virulence and promising results have been reported for the combination of PsaA and PspA in prevention of colonization and otitis media in animal models (Briles *et al.* 2000b; Ogunniyi *et al.* 2000).

2.7.1.3.1.2 SP 0749

SP 0749 is a branched-chain amino acid ABC transporter. This protein has also not been characterized, and therefore, it is only represented by its TIGR4 annotation gene symbol (Tettelin et al., 2001).

2.7.1.3.1.3 SP 0609

SP 0609 is an amino acid ABC transporter. This protein has also not been characterized, and therefore, it is only represented by its TIGR4 annotation gene symbol (Tettelin et al., 2001). Like other ABC-transporters, SP 0749 and SP 0609 are predicted to play a role in transportation of amino acids, metabolism and bacterial growth (Dintilhac et al., 1997).

2.7.1.3.2 Non ABC transporters

Pneumococci produce two conserved, non-ABC transporter, surface-exposed lipoproteins belonging to a family of chaperones, the peptidyl-prolyl isomerases (PPIases). These proteins are the putative proteinase maturation protein A (PpmA) and streptococcal lipoprotein rotamase A

(SlrA) (Cron et al., 2009). PPIases are ubiquitous foldases, which accelerate the cis–trans conformational changes at the Xaa-Pro bonds (where X represents any amino acid and Pro = Proline residue) during protein folding in eukaryotes and prokaryotes (Hermans et al., 2006). They are involved in secretion and activation of cell surface molecules. There is evidence that PPIases contribute to bacterial virulence (Hermans et al., 2006). There are three discrete classes of PPIases: the cyclophilins, which bind the immunosuppressant cyclosporin A, the FK506-binding proteins and the parvulins (Cron et al., 2009).

2.7.1.3.2.1 Putative proteinase maturation protein A (PpmA)

Putative proteinase maturation protein A (PpmA) is a 35 kDa lipoprotein that contains an N-terminal sequence which is essential for translocation and cell membrane anchoring. PpmA shares sequence homology with the parvulins, which belongs to the family of peptidyl-prolyl *cis-trans* isomerases. However, to date, no detectable PPIase activity in PpmA has been found (Hermans et al., 2006, Overweg et al., 2000). PpmA is identified frequently in nasopharynx isolates of the transparent phenotype, suggesting that this protein plays a role in pneumococcal adherence through maturation of surface components (Cron et al., 2009). It induces antibodies with opsonophagocytic activity which are species-specific and cross-reactive among heterologous pneumococcal strains (Overweg et al., 2000). Studies also demonstrated that PpmA plays a role in pneumococcal virulence, elicits protective antibodies that are species-specific and antibodies against this protein are induced early in life (Adrian et al., 2004).

2.7.1.3.2.1 Streptococcal lipoprotein rotamase A (SlrA)

Streptococcal lipoprotein rotamase A (SlrA) is a cyclophilin lipoprotein that catalyzes cis/trans isomerization of proline containing peptides (Cron et al., 2009). Previous studies have shown that SlrA is involved in colonization, by modulating the biological function of important virulence proteins, but does not contribute significantly to invasive pneumococcal disease (Hermans et al., 2006). Previous studies demonstrated that SlrA and PpmA are both immunogenic and elicit antibody responses early in life (Adrian et al., 2004) contribute to pneumococcal colonization, avoidance of phagocytosis and pulmonary infections (Hermans et al., 2006).

2.7.1.3.3 Integral membrane protein(s)

2.7.1.3.3.1 *Serine-threonine kinase P (StkP)*

Serine- threonine kinases play an important role in signal transduction and control various cellular functions i.e. cellular adaptation to different environments. Serine-threonine phosphorylation plays an important role in the regulation of growth and competence in *S. pneumoniae* (Osaki et al., 2009). StkP shares amino acid sequence with serine/threonine kinases with an important role in cell-cell signalling and competence signalling as well as stress conditions by acting as a transcriptional regulator (Bogaert et al., 2006, Novakova et al., 2005).

Immuno-precipitation and cellular organization data suggests that StkP is associated with the lipid membrane (Osaki et al., 2009). The N-terminal region of StkP contains the eukaryotic-type serine threonine kinase domain with 35 % identity to the corresponding human protein; therefore the C-terminal region is used for immunogenicity studies. They also contribute to regulatory and developmental processes (Adler et al., 1997).

Previous studies on *Streptococcus agalactiae*, demonstrated that StkP plays a role in virulence by modifying cytotoxin production and purine metabolism (Rajagopal et al., 2003, Rajagopal et al., 2005, Rajagopal et al., 2006). Previous studies have demonstrated that StkP is immunogenic in both the elderly and very young children, is expressed during invasive disease as well as colonization and induces opsonophagocytic antibodies suggesting that they it might be a good candidate for a protein-based pneumococcal vaccines (Giefing et al., 2008).

2.7.1.4 Secreted proteins

2.7.1.4.1 Pneumolysin (Ply)

Pneumolysin (Ply) is a 53-kDa thiol-activated hemolysin produced by all strains of pneumococci. Unlike other pneumococcal antigens, it is not surface-exposed. Ply has both direct cytotoxic and complement activation properties, mediated by different domains within the toxin (Boulnois et al., 1991). The cytotoxic property is essential for inhibition of specific and non-specific immune responses, (Ferrante et al., 1984, Paton and Ferrante, 1983) and the stimulation

of inflammatory cytokine release from host cells (Houldsworth et al., 1994). Direct activation of the classical complement pathway is the result of the binding of Ply to the Fc region of immunoglobulin G, which also contributes to inflammation and depletes serum opsonic activity (Mitchell et al., 1991, Paton et al., 1984).

In vitro studies using purified antigen have demonstrated that Ply functions in pneumococcal pathogenesis by interfering with ciliary clearance of pneumococci, blocking humoral immune responses and by aiding the penetration of host tissues (Cundell and Tuomanen, 1994, Gray and Dillon Jr, 1988). Antibodies against Ply are produced early in life in response to pneumococcal carriage and infection (Musher et al., 2001). In humans, colonized patients with non-bacteraemic pneumococcal pneumonia had higher levels of antibodies to Ply compared to patients with bacteraemic pneumococcal pneumonia, suggesting that antibodies against Ply may protect the host against bacteraemic pneumococcal disease(Musher et al., 2001). Antibody titers to Ply rise in humans following pneumococcal infection, suggesting that the protein is synthesized by the bacteria while they are growing in the host (Jalonen et al., 1989). Wild-type Ply is not suitable as a human vaccine antigen because of its toxicity. In this study, pneumolysoid (PdB), which is a non-toxic variant carrying a mutation of tryptophan residue to phenylalanine at position 433 (Trp_{433} – Phe), of the wild-type Ply protein, was used. Previous studies have indicated that Ply may also be suitable as a vaccine adjuvant (Ogunniyi et al., 2001).

2.7.1.4.2 Protein required for cell separation in group B streptococci (PcsB)

Protein required for cell separation in group B streptococci (PcsB) is one of the essential hydrolases identified in *S. pneumoniae.* It is involved in important bacterial mechanisms, maintenance of cell morphology and bacterial growth (Ng et al., 2004, Reinscheid et al., 2001). Recent studies by Mills *et al* showed that PcsB is secreted and is associated with the plasma membrane (Mills et al., 2007). PcsB expression increases as a result of oxidative stress, high temperatures and the presence of salts. This suggests that PcsB is involved in the response to changes of environment incurred in pathogenesis of illness. Previous studies showed that PscB protected mice against death caused by *S. pneumoniae* serotype 1 strain in an intranasal sepsis model. These studies also showed that this protein is highly immunogenic in both children and the elderly, and is expressed during invasive diseases, colonization and exposure (Giefing et al., 2008).

2.7.1.5 Other pneumococcal proteins

2.7.1.5.1 Heat shock proteins

S. pneumoniae may encounter heat stress after penetration from the nasal mucosa (30 to 34°C) into the blood and/or meninges (37°C) during colonization (Lindemann et al., 2002). Changes in temperature induce the production of highly conserved proteins, referred to as heat shock

proteins (HSPs). The induction of HSPs protects bacteria against stress, therefore increasing their prevalence (Neidhardt and Van Bogelen, 1987). HSPs can be classified into different families depending on molecular weight and are present in both eukaryotes and prokaryotes. They are induced during infection and have been shown to play a role in adherence and invasion, and have a major role in protein folding (Charpentier et al., 2000, Nair et al., 2000). One of the HSPs, caseinolytic protease (Clp), contains two ATP-binding regions and functions as a chaperone and modulates virulence gene expression (Kwon et al., 2004).

Studies performed on this antigen demonstrated that immunization of mice with pneumococcal caseinolytic protease P, ClpP, elicited protective immunity against systemic challenge with pneumococcal strain D39 (an encapsulated and virulent strain of pneumococcus) to a level comparable to that of well-studied vaccine candidates PspA and PdB. Antigen-specific antibody responses elicited in immunized mice prior to challenge suggests that protection by this antigen could be antibody-mediated (Kwon et al., 2004). Other studies have also shown that the HSP, ClpP, is highly immunogenic in *S. pneumoniae* and modulates virulence gene regulation (Kwon et al., 2003). Although Clp has been extensively studied in Gram-negative bacteria, there are only limited data for this antigen in Gram-positive bacteria. There are no published data on the natural immune response to Clp in humans. HSP, SP 2194, which is predicted to be an ATP dependant Clp protease, was evaluated in this study.

2.7.1.5.2 SP 2027

SP 2027 is a conserved hypothetical protein found in all strains of *S. pneumoniae*. There are no characteristic data on this protein; therefore, it is only represented by its TIGR4 annotation gene symbol.

2.8 Effect of HIV infection on the antibody response

Human immunodeficiency virus (HIV) positive patients have a significantly higher morbidity from influenza virus infection and invasive pneumococcal disease compared to HIV-negative individuals (Horster et al., 2010). Previous studies have demonstrated that HIV-infected, exposed infants with low CD4+ T-cells have an impaired immune response upon T-cell dependent antigens like the influenza vaccine, tetanus toxoid, diphtheria toxoid and the conjugated *Haemophilus infuenzae* type b (Kroon et al., 1994, Kroon et al., 1997). Previous studies have also shown the impact of HIV on the antibody responses to PCV. These studies demonstrated that children with HIV have significantly increased risk of pneumococcal disease compared to HIV uninfected children, and that the serotypes included in currently licensed vaccines include most serotypes that cause invasive pneumococcal disease (IPD) in HIV-infected children and adults (Bliss et al., 2008). Our previous study showed that HIV-uninfected, unexposed infants had superior OPA responses, compared with those in HIV+/ART+ infants, who in turn had better OPA responses, compared with those in HIV+/ART- infants. These findings suggest that

changes in the immune response in HIV-uninfected, exposed infants could contribute to increased morbidity and mortality in these children (Madhi et al., 2010).

2.9 Summary

Previous studied in European children looked at the prevalence and the natural development of antibodies to PspA, PsaA, PdB, PpmA, SlrA and IgA1-proteinase and the association between antibody titers and pneumococcal colonization in young infants and adults(Adrian et al., 2004, Holmlund et al., 2006, Rapola et al., 2000, Simell et al., 2009).

Chapter 3: Aims and Objectives

The aims of this study were to describe the kinetics of antibodies against select pneumococcal proteins with regards to age, HIV infection status and PCV vaccination status. In addition, we explored the association between antibody titers and pneumococcal nasopharyngeal colonization in infants, older children, and adults. Proteins that were evaluated were PcsB, StkP, PsaA, LytB, SP 0082, SP 2027, SP 0609, SP 0749, SP 2194, IgA1-proteinase, SlrA, PpmA, PspA, PspC and PdB. To date, no data are available on the natural development of antibodies in African children and adults, against these proteins.

Specific objectives of this study were to:

1. Compare the correlation between a multiplex Luminex-based assay (Bio-Plex, Bio-Rad Laboratories, USA) to a singleplex ELISA assay for four of the proteins, namely; PspA, PspC, PsaA and PdB.
2. Define whether there are any differences in natural development of antibody titers against various proteins among PCV-vaccinated children under two years of age who were either HIV-unexposed, uninfected (M-/I-), HIV-exposed, uninfected (M+/I-) or HIV-exposed, infected (M+/I+).
3. Define whether there are any differences in the serum antibody titers against various proteins between PCV unvaccinated M+/I- and M-/I- children under two years of age.

4. Evaluate the effect of PCV vaccination on the dynamics of serum IgG antibody titers against various proteins in M+/I- and M-/I- children under two years of age.

5. Cross-sectional determination of the concentration of serum IgG antibodies to the studied pneumococcal proteins and their association with pneumococcal colonization in HIV-infected and -uninfected children between 4 to 7 years of age, stratified by previous PCV immunization status during infancy.
6. Determine the association of HIV-infection status and serum antibody titers against various proteins in women.
7. Explore the association between antibody titers to the proteins and prevalence of nasopharyngeal colonization in HIV-infected and -uninfected women.

Chapter 4: Materials and Methods

4.1 Study population

The study on natural kinetics of antibody responses to the proteins involved 2166 samples from 1917 subjects. This included M-/I-, M+/I- and M+/I+ children aged between 4 and 24 months who had been vaccinated with the 7-valent vaccine, Prevnar® (Wyeth) containing 2 µg each of serotype polysaccharides 4, 9V, 14, 19F and 23F; 4 µg of serotype 6B polysaccharide and 2 µg of serotype 18C oligosaccharide, each conjugated individually to a protein carrier CRM_{197}, at 6, 10 and 14 weeks of age (Madhi et al., 2007a). Two groups of M+/I+ children were co-enrolled from the Children with HIV Early Antiretroviral (CHER) Study in South Africa (Violari *et al.* 2008) with a $CD4^+$ T lymphocyte cell percentage $\geq$ 25%. These children were randomized either to initiate antiretroviral therapy (ART) immediately, or to initiate ART when immunologically indicated as per prevailing WHO recommendations (World Health Organization 2002). The ART regimen included zidovudine, lamivudine and lopinavir-ritonavir. A parallel cohort of M-/I- and M+/I- children aged between 4 and 24 months not vaccinated with PCV were enrolled during a similar period as the CHER cohort. Nasopharyngeal swabs, for identification of pneumococcal colonization, were collected at multiple time- points in these two longitudinal cohorts including at 4, 10, 18 and 24 months of age.

In addition, the mothers of children who had participated in the above mentioned cohorts were also enrolled into a study which explored the dynamics of nasopharyngeal colonization between PCV-vaccinated and PCV-unvaccinated mother-child (HIV-uninfected) dyads (Madhi et al., 2010) None of the mothers received any pneumococcal vaccine formulation. A fourth study group were samples from HIV-infected and –uninfected children previously enrolled into a 9-valent PCV efficacy trial who were subsequently sampled at a single time-point, between 4 and 7 years of age (Madhi et al., 2007b). These children had been previously randomized to receive 3 doses of either 9-valent PCV containing 2 µg of capsular polysaccharide serotypes 1, 4, 5, 9V, 14, 19F and 23F; 4 µg of serotype 6B polysaccharide and 2 µg of serotype 18C oligosaccharide, each conjugated individually to a protein carrier or placebo at 5.5, 111.2 and 15.8 weeks of age. Figure 2 summarizes the study population (the number of children and women), stratified by HIV exposure and PCV-vaccination status.

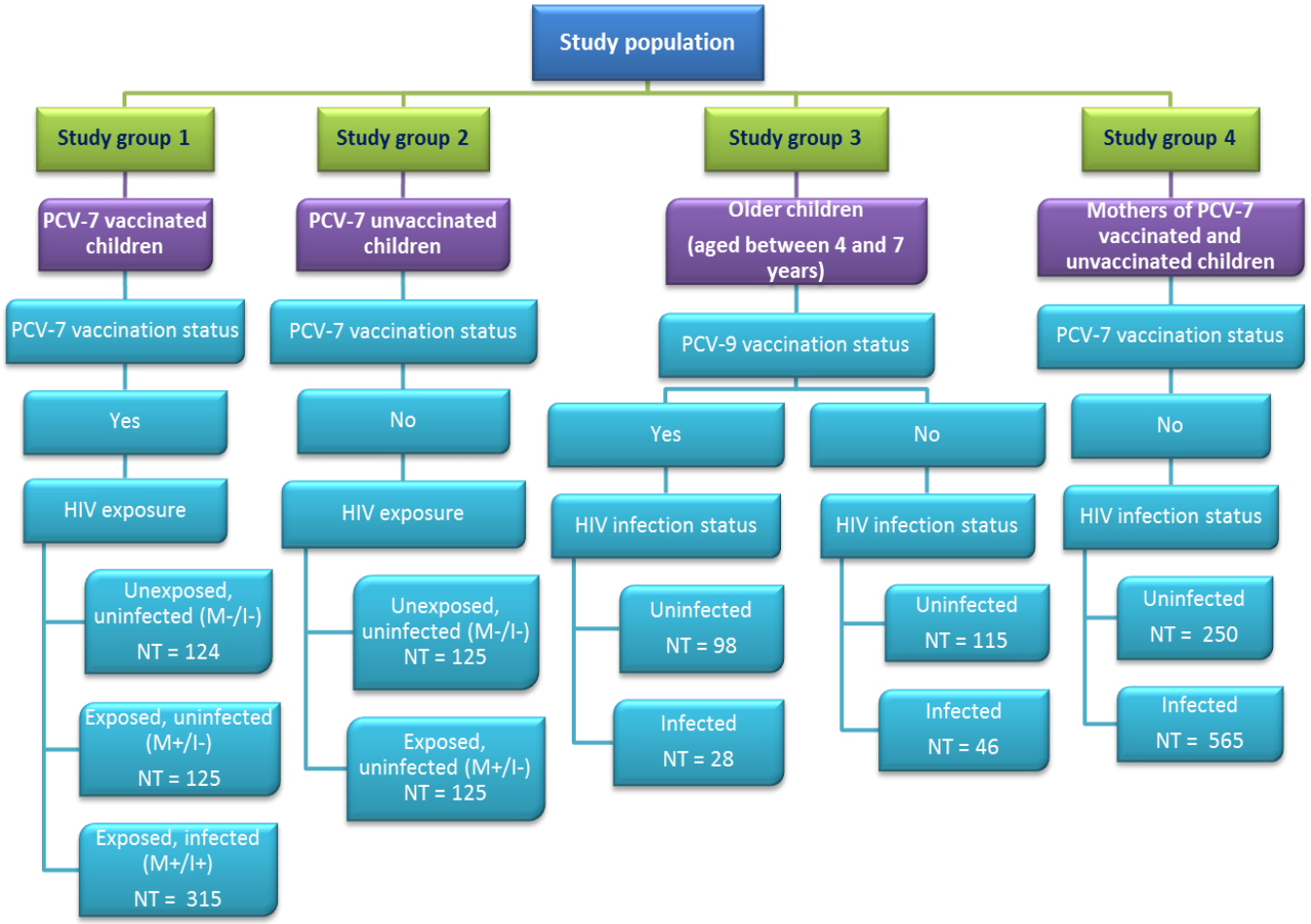


Figure 2: Flow diagram showing all the study participants. PCV-vaccinated children received three doses of the 7-valent pneumococcal conjugate vaccine; Prevnar® (Wyeth), at 6, 10 and 15 weeks of age, containing 2 µg of each of serotype polysaccharides 4, 9V, 14, 19F and 23F; 4 µg of serotype 6B polysaccharide and 2 µg of serotype 18C oligosaccharide, each conjugated individually to a protein carrier CRM197 (Madhi et al., 2007b) and the PCV-unvaccinated children received placebo. Only children were recruited in the PCV-9 efficacy trial and they received three doses of the PCV-9 vaccine, at 6.6, 11.2 and 15.8 weeks of age, containing 2 µg of capsular polysaccharide serotypes 1, 4, 5, 9V, 14, 19F and 23F; 4 µg of serotype 6B polysaccharide and 2 µg of serotype 18C oligosaccharide, each conjugated individually to a protein carrier CRM_{197} (Madhi et al., 2007b). NT = total number recruited, but there may have been missing samples at later visits.

4.2 Materials

Recombinant PspA, CbpA and PdB proteins were obtained from St. Jude Children's Hospital (Memphis, USA). Recombinant IgA1-proteinase, SlrA, PpmA proteins were expressed and purified by Mucosis (Netherlands) and PcsB, StkP, PsaA, SP 0082, SP 2027, SP 0609, SP 0749, SP 0498 and SP 2194 proteins were expressed and purified by Intercell AG (Austria). *R*-phycoerythryn (*R-PE*) conjugated goat anti-human IgG (gamma-chain specific) was obtained from Jackson ImmunoResearch Laboratories Inc. (Westgrove, USA). Carboxylated microsphere beads were obtained from Bio-Rad (Bio-Rad Laboratories, Hercules, USA). Alkaline-phosphatase-conjugated goat anti-human IgG (Cat # A3188), 4- nitrophenyl phosphate disodium salt hexadydrate (Cat # 71768) substrate and *N*-hydroxy-sulphosuccinimide, Sulpho-NHS, (Cat # 56485), were purchased from Sigma (Sigma-Aldrich, Germany). 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl, EDC, (Cat # 77149) was obtained from Thermo Scientific (Thermo Scientif, Rockford IL, USA). 96 well ELISA plates Maxisorb (Cat # 442404) and Costar (Cat # 9017) were obtained from Nunc, Denmark and Costar, USA, respectively. AcroPrep multi-well filter-plates, 1.2 µm, were purchased from Pall (Pall Corporation, USA).

4.3 Reference and serum samples

Purified pooled human immunoglobulin (Polygam, National Bioproducts Institute, Pinetown South Africa) was used as an in-house standard/ reference sera for determining titers of natural antibodies against the 15 pneumococcal proteins studied. The reference serum was given an arbitrary titer of 100 units/ml (U/ml) for each protein. In-house high and low controls consisting of pooled adult serum were used on each Luminex and ELISA run. The low control was adsorbed with whole pneumococci to reduce the titer of the serum. Serum samples, for comparison of the ELISA to multiplex Luminex assay were undertaken on archived samples of 100 HIV-uninfected participants. These included children who had been enrolled into an immunogenicity study of PCV and included samples from 10, 18 and 28 months of age(Madhi et al., 2010), HIV uninfected adults and children between 4 and 7 years of age (Madhi et al., 2007a, Madhi et al., 2007b). For further details of cohorts refer to section 4.3.

The natural antibody responses to the 15 pneumococcal proteins involved analysis of archived samples from different cohorts of children and adults previously enrolled into studies as detailed in section 4.3. Consent for collection, storage, and further testing of these samples was included in initial trial consent forms. Serum samples were stored at -80 °C.

4.4 ELISA for anti-PspA, -PspC, -PdB and –PsaA

ELISA assays for anti-PspA, -PspC, -PdB and –PsaA were performed as previously reported (Quataert et al., 2001), with some modifications. Antigens were diluted in phosphate buffered saline (PBS) containing 0.05% Tween-20 and 0.05% sodium azide (PBS-T-N), pH 7.3. Nunc Maxisorb microtiter plates were coated with 0.5 µg/ ml PspA antigen. PspC, PdB and PsaA antigens were coated onto Costar Polysorb microtiter plates with coating concentrations of 0.25, 4 and 1 µg/ml at 4 °C overnight. Plates were washed with PBS-T buffer and blocked with PBS buffer containing 10% foetal bovine serum (PBS-F) pH 7.3, for an hour, at 37 °C. Pre-dilutions of the reference, controls and unknown sera were done in PBS-F. Samples were then serially diluted (100 µl/well) in the pre-coated microtiter plates. Following 2 hours incubation at 37 °C, plates were washed three times with PBS-T and the alkaline-phosphatase conjugated goat anti-human IgG was added across all wells, and incubated for 2 hours at 37 °C. After incubation, the plates were washed three times with PBS-T and twice with distilled water and the colour was developed using p-nitrophenyl phosphate substrate. Plates were read at 405 nm using the RS-232C Labsystem Multiskan RC plate reader (Labsystems, Finland).

4.5 Coupling of antigens to Luminex carboxylated microspheres

Pneumococcal proteins were coupled to Luminex carboxylated microspheres using a two-step carbodiimide reaction (Grabarek and Gergely, 1990). The carboxylated Luminex microspheres

were activated by the addition of 50 mg/ml 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl) and 50 mg/ml N-hydroxysulfosuccinimide (Sulpho-NHS) diluted in PBS-F buffer, pH 6.1. The activated microspheres were then washed with PBS buffer, pH 7.3 and incubated with 15 µg/ml of each antigen. The microspheres were incubated in a shaker, at room temperature, overnight to allow the proteins to attach. The conjugated beads were then washed and stored in PBS-F buffer, containing 0.05% sodium azide, (PBS-F-N) pH 7.3, at 4°C in the dark (Pickering et al., 2002b). Beads coated with PdB were stored at -20°C in PBS-F with 10% glycerol.

4.6 Multiplexed fluorescent covalent microsphere immunoassay (FCMIA) for quantification of antibodies against pneumococcal proteins

A standard curve was prepared from seven 4-fold dilutions (1:50 to 1: 204800) of the reference serum in PBS-F. Controls and unknown sera were diluted in PBS-F at 1:100. Samples that were above the detection limit were re-diluted. Reference, controls and unknown sera, were mixed with the coupled Luminex microsphere beads in a 96-well 1.2 micron filter plate (3500 microspheres/region/well) and incubated for 1 hour at room temperature with shaking. Microspheres were collected by vacuum filtration and washed with PBS-T. *R*-phycoerythrin-conjugated anti-human IgG was added across all wells and incubated for 30 min, in a shaker, at room temperature. Following incubation, the plates were washed and the beads were re-suspended in PBS-T buffer and transferred into a 96-well round-bottom plate, and read with a Luminex platform Bio-Plex 200 instrument (Bio-Rad Laboratories, Hercules, CA, USA) for quantification of IgG antibodies. Antibody concentrations in arbitrary units were determined

relative to the standard curve, with a five parameter logistic log model with the BioPlex software (Bio-Rad Laboratories, Hercules, CA, USA) (Pickering et al., 2002a).

4.7 Validation of the Luminex method

4.7.1 Competitive inhibition studies

To determine the specificity of both ELISA and Luminex assays, inhibition studies were performed as described by Pickering *et al* with slight modifications (Pickering et al., 2002a). A serum with known high concentrations of IgG antibodies directed against PspA, PspC, PsaA and PdB was diluted 1:500 times. Four aliquots of each serum dilution were prepared. Each aliquot was incubated with an equal volume of one of the four purified antigens in a concentration of 15µg/ml for 1 hour at room temperature. A 1:1000 dilution of the serum was used as a control. The serum aliquots were analysed as per fluorescent covalent microsphere immunoassay (FCMIA). Inhibition studies were initially performed for PspA, PspC, PsaA and PdB proteins, for comparison and validation of the Luminex assay and then optimized for the remaining 11 proteins that were included in the second phase of this study.

4.7.2 Assay sensitivity

For the determination of the sensitivity of the assay, the reference serum was diluted in eight-steps of 4-fold dilutions (1:50 to 1: 204800) respectively. The average mean fluorescent intensity

(MFI) and the standard deviation of the blank were calculated for each protein. The lower limit of quantification (LLOQ) was interpolated from the standard curve of the reference serum and was calculated by adding 3 SD to the MFIs of twenty blanks and reported in arbitrary units per millilitre (U/ml). Estimation of the upper limit of quantification (ULOQ) was based on the cut-off value of 2.5 optical densities for the ELISA method and for the Luminex method; it was the highest fluorescent value where there is still sufficient linearity to accurately quantify a sample relative to the standard curve of the reference serum. The ULOQ was reported in U/ml for the ELISA and Luminex methods (Pickering et al., 2002a).

4.7.3 Assay reproducibility

Reproducibility of the assay was assessed by determining the variation between intra- and inter-assays of replicate ELISA results with that of the Luminex assay. Intra-assay variation was determined from testing sera assayed on different wells within one plate. Inter-assay variation was assessed by testing samples in different assays and time- points, and then calculating the percentage of variation between results from each assay (Lal et al., 2005).

4.8 Data analysis

Data were analyzed using STATA software (version 11.0, StataCorp, Tx, USA). Antibody titers were reported as geometric mean titers (GMTs) in U/ml. The correlation between the ELISA and Luminex assay was measured with Spearman's correlation coefficients. Data were log transformed (log base 10) to obtain normal distributions. Student t-test was used for comparison

between two groups. $P \leq 0.05$ was considered to be statistically significant. Analysis of variance (ANOVA) test was used for comparison of more than two groups. Multivariate linear regression analysis was used to determine whether antibody titers were associated with PCV vaccination, HIV status and/or nasopharyngeal pneumococcal exposure.

Chapter 5: Results

5.1 Validation of Luminex assay

5.1.1 Comparison of Luminex with ELISA

The correlation of antibody titers measured by a multiplex Luminex assay was compared to a single-plex ELISA for IgG antibodies against PspA, PspC, PsaA and PdB. The same reference sera, controls and sera (n=100) were used for comparison of antibodies against all four proteins. Twenty serum samples from each of five different age groups of HIV-uninfected subjects; i.e. children at ages 10 months, 18 months, 24 months, 5 years and mothers, were tested with both methods. The mean age for mothers was 27 years (standard deviation [SD] ± 6.2 years). Samples that fell outside of the upper detection limit for the assays were repeated at a higher dilution. Serum antibody titers against the proteins were determined by both methods and were subjected to linear regression analysis and the correlation coefficients were determined as shown in Figure 3. The linear regression model was adjusted for age. The correlation coefficients for PspA, PspC, PdB and PsaA were 0.85, 0.90, 0.86 and 0.96 respectively. The correlation coefficients between age groups were greater than 0.90 for PspA, PspC, PsaA and PdB. Overall, there was good agreement between the two methods for all the antigens tested.

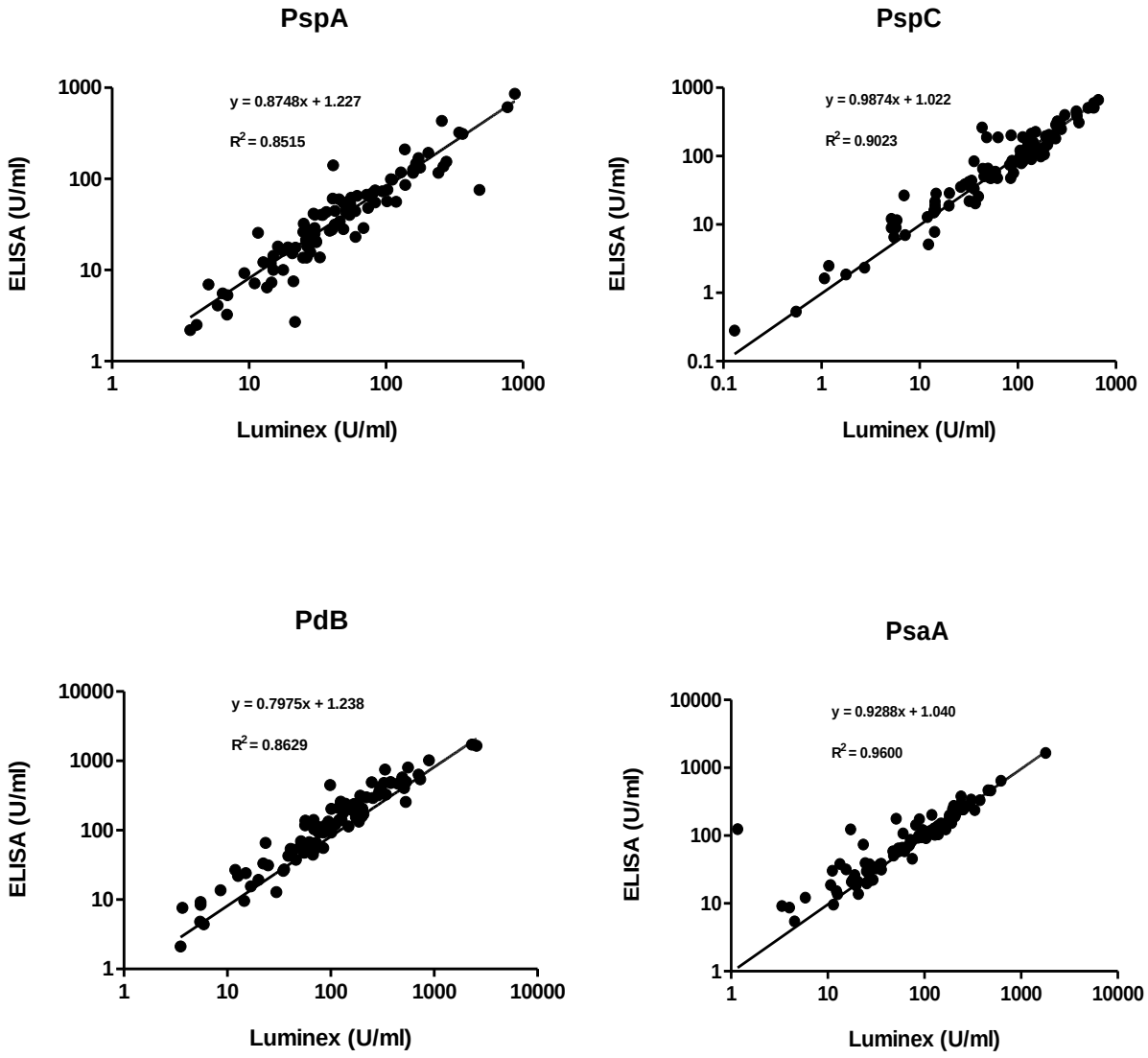
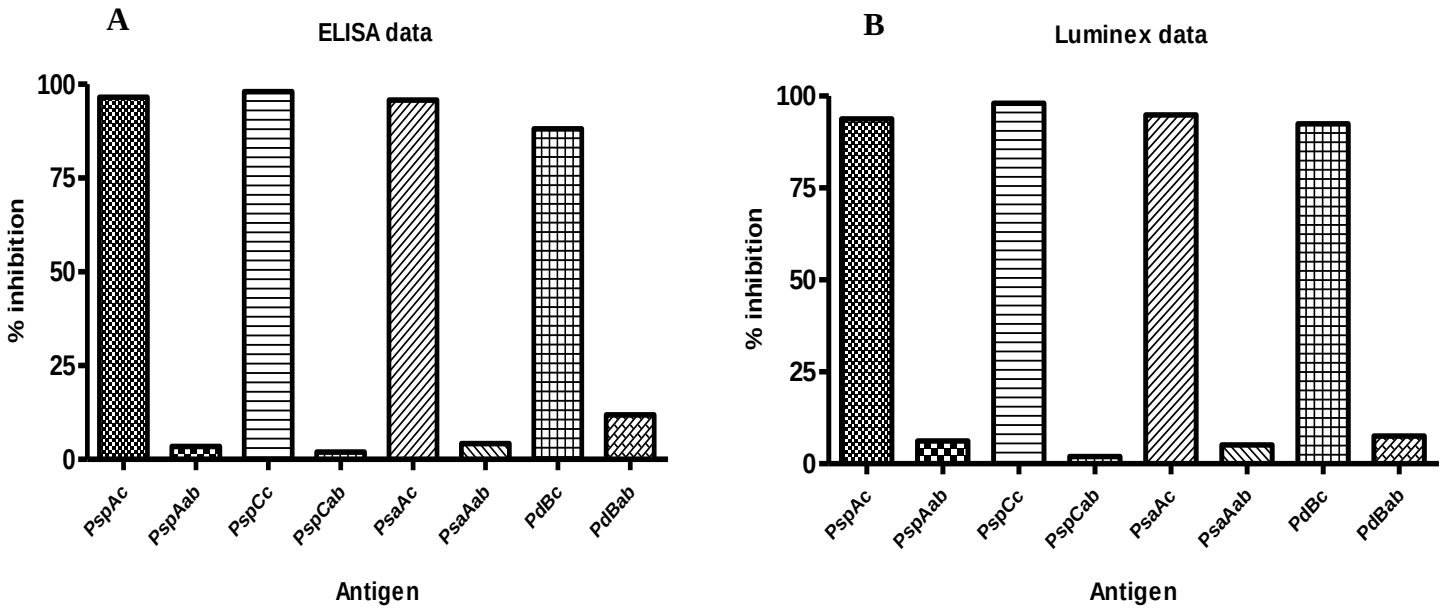


Figure 3: Comparison of the IgG concentrations against PspA, PspC, PdB and PsaA proteins determined by multiplex Luminex assay and single-plex ELISA. Sera from 100 patients were quantitated by both ELISA and Luminex methods. Antibody titers against all 4 proteins were reported in arbitrary units per ml (U/ml).

5.1.2 Specificity of Luminex and ELISA assays

Competitive inhibition experiments were performed in order to confirm the specificity of both assays, and were reported as percent change in titer in response to the addition of individual antigens to the serum. The addition of the homologous protein to the serum at saturated concentrations (15µg/ml), resulted in inhibition of over 90% of signal compared to the control sample in both ELISA and Luminex assays for PspA (93% and 97%, respectively), PspC (97% and 98%, respectively) and PsaA (94% and 96%, respectively). Inhibition of signal by the addition of PdB was slightly lower with 89% of the signal being suppressed in the ELISA, and 92 % of the signal being suppressed in the Luminex assay. The addition of non-homologous antigens to the assays had minimal effect on the assay specific titers (Figure 4 A and B). Similar results were reported for the additional 11 proteins included in the 15-plex Luminex assay (Figure 4 C). These data suggest that both assays were highly specific for the antigens tested.



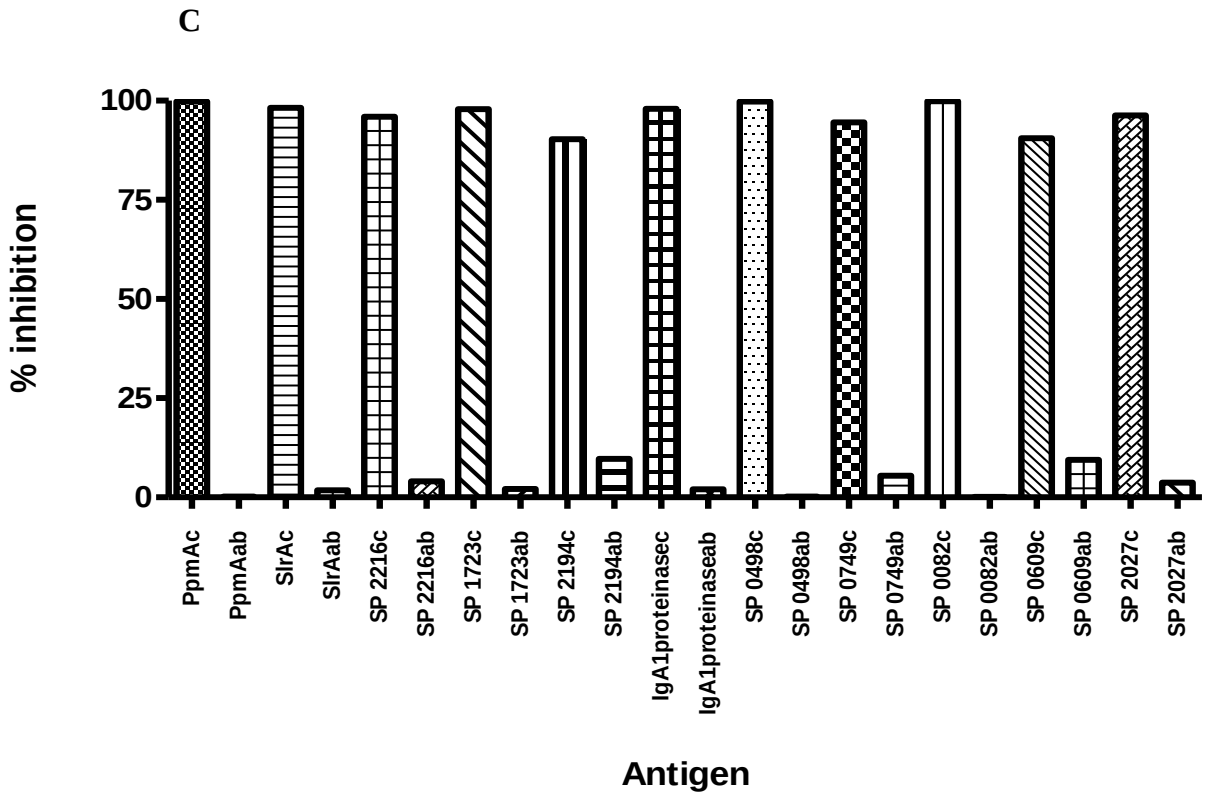


Figure 4: Inhibition of antigen specific titers by the addition of homologous and non-homologous antigen for PspA, PspC, PsaA and PdB with ELISA (A) and Luminex (B). Additional antigen targets included in the multiplex Luminex assay are shown in (C). The subscript (c) in each antigen represents the % change for serum absorbed with non-homologous antigen and (ab) represents the serum absorbed with homologous antigen.

5.1.3 Sensitivity of Luminex and ELISA assays and reproducibility

The lower limit of quantitation (LLOQ) for the Luminex assay was determined from the recorded blank values from independent runs (n=20) for each protein. This value was given as the equivalent antibody concentration of the reference at the point where the fluorescence intensity (FI) was equal to the blank plus three standard deviations. A similar method was employed to calculate the LLOQ of ELISA for PspA, PspC, PsaA and PdB proteins. The calculated LLOQ and the fold difference between assays are shown in Table 2A.

The Luminex assays were 67-, 75-, 100-, and 89-fold more sensitive than ELISA for PdB, PsaA, PspA and PspC respectively. The LLOQs for the additional 11 proteins were also calculated as part of optimization for the Luminex method as shown in Table 2B. There was no upper limit of detection since all the samples which were above the measurable range were re-diluted to fall within the measurable range of the standard curve of the reference serum. The Luminex method was reproducible; the percentage for the coefficient of variation (CV) of the high and low control samples was less than 30% in both intra- and inter-assay comparisons.

Table 2A: Lower limit of quantitation of specific IgG antibodies (U/ml) against PspA, PspC, PsaA and PdB proteins determined by ELISA and Luminex methods, and the fold difference in sensitivity of Luminex over ELISA

Antigen	ELISA *LLOQ(U/ml)	Luminex *LLOQ(U/ml)	**Fold difference between assays
PdB	2	0.03	67
PsaA	6	0.08	75
PspA	2	0.02	100
PspC	8	0.09	89

LLOQ: lower limit of quantification (arbitrary units/ml). **Fold difference between assays was calculated as ELISA LLOQ/ Luminex LLOQ

Table 2B: Lower limit of quantitation of specific IgG antibodies (U/ml) against the 11 additional proteins under study, calculated by the Luminex method

Antigen	SP 0498	SP 0749	SP 0082	SP 0609	SP 2027	SP 2194	SP 1723	SP 2216	IgA1-proteinase	SlrA	PpmA
***LLOQ**	0.02	0.11	0.03	0.04	0.03	0.08	0.03	0.01	0.03	0.11	0.01

*LLOQ: lower limit of quantification (arbitrary units/ml).

5.2 Antibody responses against pneumococcal proteins in PCV-7 unvaccinated children aged between 4 and 24 months

Serum samples from PCV-7 unvaccinated children at 4, 10, 18 and 24 months of age were analyzed. The number of samples analyzed varied between time-points depending on sample availability. The demographic features of study participants are indicated in Table 3.1. Of the 250 PCV-7 unvaccinated childhood cohort, samples were available for 90% (451/500) of the four time- points in M-/I- children and 83% (414/500) of the four time-points in M+/I- children (as shown in Figure 5 below).

Table 3.1: Demographic features of the PCV-7 unvaccinated children less than 2 years of age, children were stratified according to vaccination, HIV and pneumococcal colonization status at the time of blood sampling.

Study group	Sampling time-points	Total number of samples (N)	Median age (months)	M-/I- (N) NT = 125	Colonized (M-/I-) infants	M+/I- (N) NT = 125	Colonized (M+/I-) infants
PCV unvaccinated children	1st time-point	243	5 (3-8)	124 [99 %]	(65/124) 52 %	119 (96 %)	(77/119) 64 %
	2nd time-point	225	10 (5-12)	114 [91 %]	(76/114) 65 %	111 (89 %)	(78/111) 70 %
	3rd time-point	206	17 (10-20)	107 [86 %]	(73/107) 68 %	99 (79 %)	(76/99) 75 %
	4th time-point	191	26 (19-30)	106 [85 %]	(77/106) 72 %	85 (70 %)	(68/85) 79 %
Total number of samples evaluated (N)	-	**865**	-	-	-	-	-

Nasopharyngeal swabs from children were collected at each time- point (4, 10, 18 and 24 months). NT = total number of participants recruited for the study. N = total number of samples that were present at that time- (visit).M = mother's HIV status and I = infant's HIV status.

M+/I-: HIV exposed, uninfected children and M-/I-: HIV unexposed, uninfected, children.

** The values in square brackets indicate the percentage of samples that were available at each time-point for each group of children.

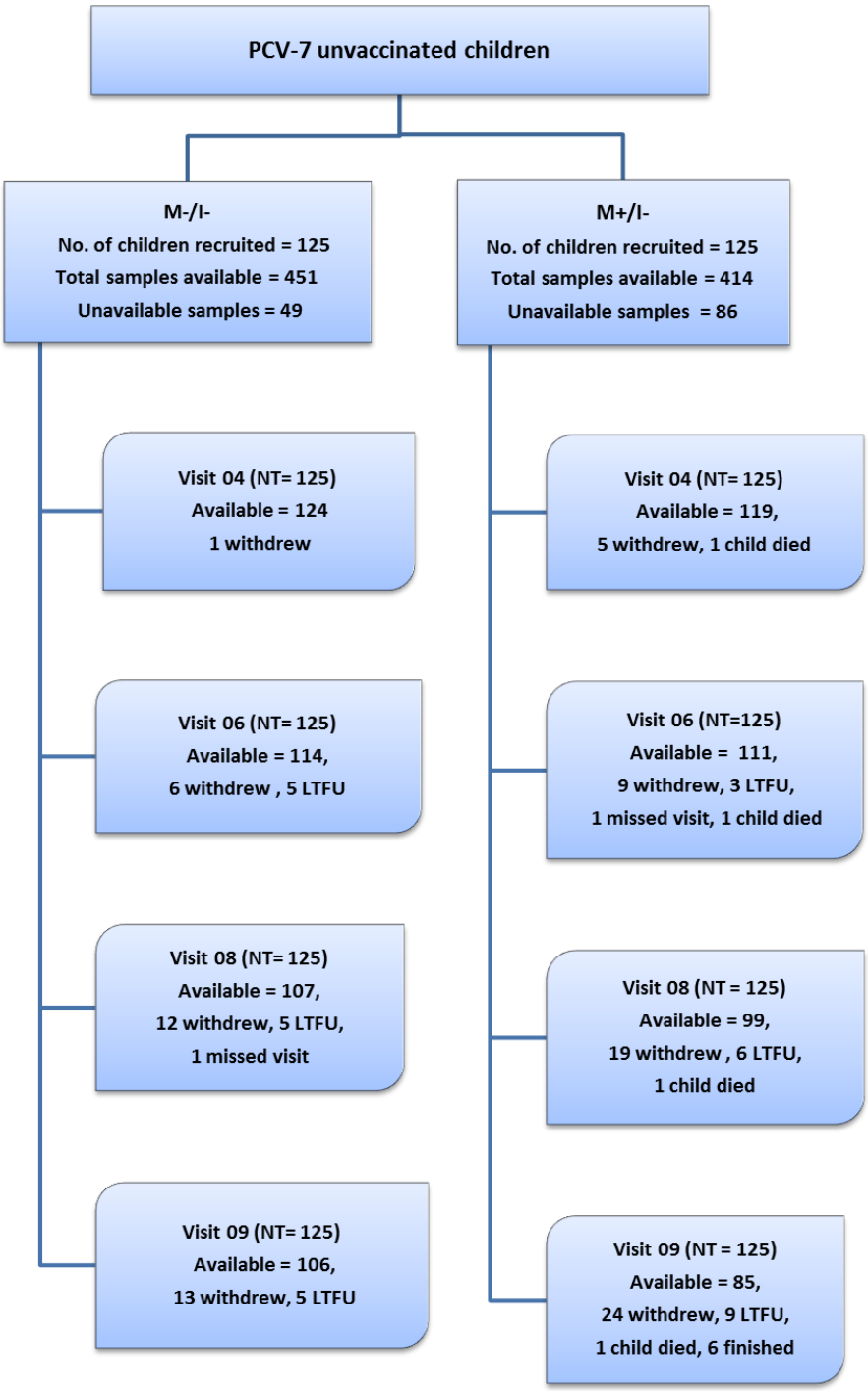


Figure 5: Flow diagram indicating sample availability at different time-points for PCV-7 unvaccinated children. Children were stratified by HIV exposure and pneumococcal colonization status.
LTFU = loss to follow up, *finished = sample used up.

5.2.1 Anti-pneumococcal protein antibodies in M-/I- and M+/I- children

Comparison of antibody titers at each of the four time-points was adjusted for prevailing pneumococcal nasopharyngeal colonization status. Significantly higher antibody titers were observed in M-/I- infants compared to M+/I- infants against 10 of the 15 proteins, namely: PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, SlrA, PcsB and StkP, at 4 months of age. Differences in GMTs (in U/ml) between M-/I- and M+/I- infants at this time-point are shown in Table 3.2. However, lower antibody titers against PsaA were observed in M-/I- children compared to M+/I- children at this age, as shown in Table 3.2.

At the 10 month time-point, M-/I- children had higher antibody titers against PpmA (47 vs 30 U/ml; p = 0.037) and SrlA (185 vs 138 U/ml; p = 0.012) and lower antibody titers against SP 0609 (88 vs 136 U/ml; p = 0.002) compared to M+/I- children. However, no differences were observed at the subsequent time-point of 18 months between M-/I- and M+/I- children to any of the analyzed proteins.

At 24 months, significantly higher antibody titers were also observed in M-/I- children compared to M+/I- children against PspA (158 vs. 119 U/ml; p = 0.040), PspC (169 vs. 139 U/ml; p = 0.009), PsaA (176 vs 137 U/ml; p = 0.036), SP 0082 (189 vs. 146 U/ml; p = 0.009), SP 0609 (113 vs 64 U/ml; p = 0.001) and IgA1-proteinase (134 vs. 84 U/ml; p = 0.015) as shown in Table 3.2).

5.2.2 Relationship between antibody titers and concurrent pneumococcal colonization in PCV unvaccinated children

The overall prevalence of nasopharyngeal pneumococcal colonization in M-/I- and M+/I- children increased with age from 59% (142/243) at 4 months, 68% (154/225) at 10 months, 72% (149/206) at 18 months and 76% (145/191) by 24 months. There were differences between the M-/I- and M+/I- groups, in that the latter had a higher prevalence of colonization at the 1st time-point (52% [65/124] in M-/I- infants vs 64% [77/119] in M+/I- infants, p = 0.015). No differences were observed in the prevalence of nasopharyngeal pneumococcal colonization between M-/I- and M+/I- infants at subsequent time-points including the 2nd time-point (65% [76/114] in M-/I- infants vs 70% [78/111] in M+/I- infants, p = 0.10), at the 3rd time-point (68% [73/107] in M-/I- infants vs 75% [76/99] in M+/I- infants, p = 0.37)] and at the 4th time-point (72% [77/106] in M-/I- infants vs 79% [68/85] in M+/I- infants, p = 0.68) of the children were colonized with pneumococci.

In the M-/I- group, at 4 months, higher antibody titers against PcsB (39 vs 28; p = 0.050) were observed in children colonized with pneumococci compared to uncolonized children. At 10 months, higher antibody titers against PspC (126 vs 44; p < 0.0001), PdB (114 vs 57; p = 0.003), SP 0082 (105 vs 37; p < 0.0001), LytB (106 vs 38; p < 0.0001), PpmA (60 vs 23; p = 0.007), PcsB (131 vs 32; p < 0.0001) and StkP (35 vs 17; p = 0.011) were also observed in children colonized with pneumococci than -uncolonized children. Differences in antibody titers between the pneumococcal-colonized and -uncolonized

children declined with an increase in age in the M-/I- group, such that by 24 months of age, children had similar antibody titers regardless of pneumococcal colonization status (refer to Tables 3.3 – 3.6).

In the M+/I- group, at 4 months, pneumococcal-colonized children had higher antibody titers compared -uncolonized children for PspC (54 vs 37; p = 0.016) and PcsB (35 vs 14; p = 0.005). At 10 months, higher antibody titers against PspC (106 vs 70; p = 0.043) and lower antibody titers against SP 0609 (120 vs 180; p = 0.022) were observed in pneumococcal-colonized compared to -uncolonized children, respectively. There were no differences in antibody titers between pneumococcal-colonized and -uncolonized children at 24 months of age (see Tables 3.3 – 3.6).

Overall, when adjusting for HIV exposure status, higher antibody titers against PspC were observed in pneumococcal-colonized children compared to -uncolonized children at all four time-points. Most of the differences in antibody titers between pneumococcal-colonized and -uncolonized children were observed early in infancy at approximately 4 and 10 months of age. These differences at the 1st and 2nd time-points were higher antibody titers against PdB, SP 0082, LytB, PpmA, PcsB and StkP in pneumococcal-colonized children compared to -uncolonized children (refer to Tables 3.3 – 3.6). Differences in antibody titers between pneumococcal-colonized children and -uncolonized children were less evident at the 3rd and 4th time-points; i.e. at approximately 18 and 24 months of age.

Table 3.2: Comparison of the geometric mean titers against the 15 pneumococcal proteins between unvaccinated M-/I- and M+/I- children at approximately 4, 10, 18 and 24 months of age.

Protein	4 months age		**p (adj.)	10 months age		**p (adj.)	18 months age		**p (adj.)	24 months age		**p (adj.)
	M-/I- *GMT (95 % CI)	M+/I- *GMT (95 % CI)		M-/I- *GMT (95 % CI)	M+/I- *GMT (95 % CI)		M-/I- *GMT (95 % CI)	M+/I- *GMT (95 % CI)		M-/I- *GMT (95 % CI)	M+/I- *GMT (95 % CI)	
PspA	47 (40-55)	33 (29-37)	**0.001**	116 (98-138)	101 (84-120)	0.25	131 (105-164)	111 (91-135)	0.26	158 (129-192)	119 (101-140)	**0.040**
PspC	60 (54-67)	47 (42-55)	**0.002**	100 (83-120)	93 (78-110)	0.97	131 (110-155)	113 (95-134)	0.31	169 (152-188)	139 (126-154)	**0.009**
PsaA	182 (158-211)	249 (223-278)	***0.001***	174 (153-199)	193 (167-222)	0.27	158 (134-186)	180 (155-209)	0.24	176 (150-207)	137 (116-162)	**0.036**
PdB	45 (39-52)	35 (30-41)	**0.008**	98 (80-119)	73 (58-93)	0.09	130 (108-156)	108 (86-135)	0.20	147 (125-173)	131 (111-156)	0.33
SP 2027	571 (491-663)	556 (444-696)	0.82	561 (445-706)	483 (366-638)	0.40	285 (222-365)	191 (125-290)	0.07	476 (403-562)	449 (310-648)	0.75
SP 0609	79 (67-94)	67 (56-81)	0.23	88 (73-107)	136 (115- 162)	***0.002***	121 (90-164)	171 (140-209)	0.07	113 (91-139)	64 (50-81)	**0.001**
SP 0082	39 (35-45)	30 (24-36)	**0.010**	83 (67-104)	72 (58-89)	0.55	130 (106-159)	101 (87-118)	0.05	189 (116-215)	146 (125-170)	**0.009**
SP 0749	124 (102-151)	107 (91-126)	0.26	57 (44-73)	56 (42-75)	0.99	84 (58-121)	87 (62-122)	0.89	106 (84-134)	99 (80-121)	0.63
(SP 0498) LytB	38 (34-43)	26 (21-31)	**< 0.0001**	85 (67-106)	81 (67-99)	0.98	164 (135-200)	155 (132-181)	0.67	204 (176-236)	187 (159-218)	0.41
IgA1-proteinase	61 (53-69)	31 (26-38)	**< 0.0001**	77 (60-10)	100 (80-123)	0.14	90 (65-125)	121 (97-151)	0.17	134 (106-169)	84 (61-115)	**0.015**
PpmA	41 (35-48)	24 (20-30)	**< 0.0001**	47 (36-62)	30 (24-39)	**0.037**	63 (46-85)	42 (32-56)	0.07	98 (75-128)	87 (65-118)	0.57
SlrA	185 (158-216)	119 (104-136)	**< 0.0001**	185 (157-217)	138 (117-163)	**0.012**	124 (97-158)	96 (79-116)	0.96	124 (98-158)	123 (101-149)	0.94
SP 2216 (PcsB)	33 (28-39)	26 (20-34)	**0.027**	96 (74-124)	88 (68-113)	0.99	157 (120-205)	142 (121-166)	0.56	208 (185-234)	197 (169-230)	0.55
SP 1723 (StkP)	29 (26-33)	21 (18-23)	**< 0.0001**	30 (24-37)	22 (17-29)	0.14	39 (29-52)	34 (26-45)	0.56	61 (49-75)	46 (37-57)	0.07
SP 2194	141 (119-168)	168 (145-193	0.10	159 (131-191)	153 (126-185)	0.75	144 (113-185)	150 (124-181)	0.96	125 (101-153)	128 (100-162)	0.88

*GMTs (with 95% CI) were reported in arbitrary units per milliliter (U/ml). M-/I- : HIV unexposed, uninfected children and M+/I-: HIV exposed, uninfected children. GMTs in M-/I- significantly higher than in M+/I- are highlighted in red; GMTs in M-/I- significantly lower than in M+/I- are highlighted in blue. **For comparison between M-/I- and M+/I- children, p values were adjusted for pneumococcal exposure.

Table 3.3: GMTs (with 95% CI) against the 15 pneumococcal proteins in children at approximately 4 months of age

Protein	Overall *GMT (95 % CI)	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**p (adj.)	M+/I- *GMT (95 % CI)			M-/I-, *GMT (95 % CI)		
					Colonized	Uncolonized	P	Colonized	Uncolonized	p
PspA	39 (36-44)	38 (34-42)	42 (35-50)	0.74	34 (30-38)	31 (25-40)	0.61	44 (36-55)	50 (40-64)	0.45
PspC	54 (49-59)	59 (52-66)	47 (41-55)	**0.006**	54 (46-63)	37 (29-48)	**0.016**	65 (56-76)	55 (47-65)	0.14
PsaA	213 (194-233)	214 (190-241)	210 (180-245)	0.73	258 (227-293)	232 (186-289)	0.40	170 (139-208)	197 (159-244)	0.32
PdB	40 (35-44)	42 (37-49)	36 (30-43)	0.08	39 (32-47)	28 (21-36)	0.052	47 (38-58)	43 (34-53)	0.50
SP 2027	563 (493-644)	567 (477-673)	558 (449-692)	0.89	570 (437-743)	527 (339-819)	0.76	563 (458-694)	578 (462-725)	0.86
SP 0609	73 (65-83)	70 (60-81)	79 (64-97)	0.40	65 (53-81)	72 (50-103)	0.65	75 (60-94)	84 (65-108)	0.53
SP 0082	34 (30-38)	36 (31-42)	32 (26-38)	0.17	32 (26-41)	25 (17-35)	0.21	41 (35-48)	38 (31-46)	0.51
SP 0749	115 (102-131)	114 (97-134)	118 (95-145)	0.97	118 (96-144)	87 (67-113)	0.07	110 (84-143)	143 (106-192)	0.18
(SP 0498) LytB	32 (28-35)	33 (29-38)	29 (24-34)	0.06	28 (22-34)	22 (15-31)	0.25	42 (36-49)	35 (29-41)	0.09
IgA1-proteinase	44 (39-49)	45 (39-52)	43 (35-52)	0.21	35 (29-44)	25 (18-34)	0.06	60 (49-73)	61 (51-75)	0.86
PpmA	32 (28-36)	34 (29-40	28 (23-36)	**0.043**	27 (22-33)	20 (13-30)	0.17	46 (37-57)	36 (28-46)	0.16
SlrA	149 (134-166)	139 (122-158)	166 (139-199)	0.30	122 (105-141)	115 (87-152)	0.73	165 (132-206)	211 (169-263)	0.11
SP 2216 (PcsB)	30 (25-34)	36 (31-43)	22 (17-28)	**0.001**	35 (29-45)	14 (8-25)	**0.005**	39 (31-47)	28 (22-36)	**0.050**
SP 1723 (StkP)	25 (22-27)	26 (23-29)	23 (20-27)	0.06	22 (19-25)	18 (14-23)	0.18	31 (27-37)	27 (22-32)	0.19
SP 2194	154 (137-172)	150 (129-174)	159 (134-189)	0.45	174 (148-204)	155 (116-207)	0.48	125 (96-162)	162 (131-202)	0.13

**GMTs (with 95% CI) were reported in arbitrary units per milliliter (U/ml). M-/I- : HIV unexposed, uninfected children and M+/I-: HIV exposed, uninfected children. GMTs in M-/I- significantly higher than in M+/I- are highlighted in red; GMTs in M-/I- significantly lower than in M+/I- are highlighted in blue. **For comparison between M-/I- and M+/I- children, p values were adjusted for pneumococcal exposure.

Table 3.4: GMTs (with 95% CI) to the 15 pneumococcal proteins in children at approximately 10 months of age

Protein	Overall *GMT (95 % CI)	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**p (adj.)	M+/I- *GMT (95 % CI)			M-/I-, *GMT (95 % CI)		
					Colonized	Uncolonized	p	Colonized	Uncolonized	p
PspA	108 (96-123)	108 (93-125)	110 (87-137)	0.82	100 (82-123)	102 (71-146)	0.95	115 (93-142)	122 (97-152)	0.70
PspC	96 (85-109)	116 (103-131)	58 (43-77)	**< 0.0001**	106 (89-127)	70 (49-101)	**0.043**	126 (107-148)	44 (27-73)	**< 0.0001**
PsaA	183 (167-201)	187 (167-209)	174 (146-208)	0.45	199 (168-236)	179 (138-233)	0.50	177 (151-206)	167 (133-211)	0.69
PdB	85 (73-99)	93 (78-111)	65 (48-89)	0.06	74 (56-98)	71 (45-113)	0.89	114 (92-142)	57 (38-86)	**0.003**
SP 2027	521 (435-622)	516 (415-640)	535 (388-739)	0.78	494 (351-697)	459 (279-755)	0.80	534 (404-707)	664 (464-951)	0.34
SP 0609	109 (96-126)	102 (87-119)	133 (105-169)	0.14	120 (97-148)	180 (136-239)	***0.022***	88 (71-111)	87 (60-127)	0.94
SP 0082	77 (66-91)	91 (77-109)	50 (36-67)	**0.001**	78 (60-101)	61 (40-91)	0.31	105 (83-133)	37 (23-60)	**< 0.0001**
SP 0749	57 (47-68)	58 (46-73)	52 (38-74)	0.64	60 (42-86)	49 (30-78)	0.47	57 (42-76)	59 (35-97)	0.90
(SP 0498) LytB	83 (71-96)	92 (77-110)	62 (47-83)	**0.027**	78 (60-100)	89 (65-121)	0.52	106 (83-135)	38 (23-63)	**< 0.0001**
IgA1-proteinase	88 (74-104)	88 (72-107)	87 (63-121)	0.83	89 (69-116)	126 (86-183)	0.14	87 (64-117)	52 (30-90)	0.10
PpmA	38 (32-45)	45 (37-56)	23 (16-33)	**0.003**	34 (25-45)	24 (16-37)	0.19	60 (43-78)	23 (12-41)	**0.007**
SlrA	160 (142-180)	159 (138-183)	162 (132-199)	0.67	135 (110-166)	144 (108-193)	0.70	183 (150-222)	191 (143-256)	0.80
SP 2216 (PcsB)	92 (77-110)	116 (96-139)	49 (33-73)	**< 0.0001**	100 (76-134)	66 (40-111)	0.16	131 (103-167)	32 (17-60)	**< 0.0001**
SP 1723 (StkP)	26 (22-31)	29 (24-35)	19 (13-27)	**0.048**	23 (17-32)	21 (12-35)	0.72	35 (28-45)	17 (10-28)	**0.011**
SP 2194	156 (136-178)	153 (130-180)	164 (130-206)	0.64	157 (124-198)	145 (103-204)	0.70	150 (119-187)	194 (143-264)	0.17

**GMTs (with 95% CI) were reported in arbitrary units per milliliter (U/ml). M-/I- : HIV unexposed, uninfected children and M+/I-: HIV exposed, uninfected children. GMTs in M-/I- significantly higher than in M+/I- are highlighted in red; GMTs in M-/I- significantly lower than in M+/I- are highlighted in blue. **For comparison between M-/I- and M+/I- children, p values were adjusted for pneumococcal exposure.

Table 3.5: GMTs (with 95% CI) to the 15 pneumococcal proteins in children at approximately 18 months of age

Protein	Overall *GMT (95 % CI)	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**p (adj.)	M+/I- *GMT (95 % CI)			M-/I-, *GMT (95 % CI)		
					Colonized	Uncolonized	p	Colonized	Uncolonized	p
PspA	121 (104-140)	118 (99-141)	131 (101-169)	0.51	108 (85-135)	121 (81-181)	0.60	128 (97-168)	143 (101-201)	0.61
PspC	122 (108-138)	135 (119-153)	90 (67-122)	**0.005**	122 (101-148)	93 (64-135)	0.19	147 (125-173)	87 (52-147)	0.06
PsaA	168 (150-188)	170 (149-194)	162 (131-201)	0.67	189 (159-225)	159 (117-215)	0.31	155 (128-188)	167 (120-232)	0.69
PdB	118 (102-137)	118 (100-140)	118 (90-158)	0.91	109 (84-143)	103 (65-162)	0.81	126 (101-157)	141 (101-198)	0.58
SP 2027	234 (185-298)	203 (153-270)	357 (233-547)	***0.032***	153 (92-254)	332 (159-690)	0.08	260 (193-349)	389 (253-597)	0.12
SP 0609	144 (120-172)	134 (108-168)	174 (129-236)	0.26	160 (125-204)	203 (141-292)	0.27	115 (81-165)	146 (87-247)	0.45
SP 0082	115 (101-131)	116 (99-135)	113 (90-141)	0.95	94 (77-114)	122 (98-152)	0.07	139 (111-176)	102 (67-157)	0.20
SP 0749	85 (67-109)	86 (64-117)	82 (55-122)	0.85	91 (59-141)	76 (45-128)	0.59	82 (53-127)	89 (46-174)	0.83
(SP 0498) LytB	159 (141-181)	164 (142-190)	147 (114-188)	0.46	144 (119-176)	184 (142-238)	0.14	183 (148-227)	113 (72-177)	0.05
IgA1-proteinase	104 (86-127)	97 (76-123)	130 (93-181)	0.24	114 (88-148)	141 (90-221)	0.41	84 (56-124)	118 (69-201)	0.30
PpmA	52 (42-64)	55 (43-70)	44 (29-65)	0.38	43 (31-60)	40 (23-71)	0.81	68 (48-97)	48 (26-89)	0.32
SlrA	109 (93-128)	105 (87-126)	124 (93-165)	0.30	90 (72-111)	114 (76-171)	0.29	120 (89-161)	136 (88-211)	0.62
SP 2216 (PcsB)	149 (128-175)	154 (128-186)	136 (103-181)	0.53	146 (123-173)	132 (92-190)	0.62	161 (117-223)	142 (89-226)	0.64
SP 1723 (StkP)	37 (30-45)	38 (30-48)	33 (23-48)	0.54	33 (24-46)	37 (22-62)	0.74	43 (30-60)	29 (16-50)	0.22
SP 2194	147 (126-172)	127 (105-154)	226 (181-282)	***0.001***	131 (104-164)	214 (156-293)	***0.012***	124 (92-167)	240 (173-334)	***0.004***

**GMTs (with 95% CI) were reported in arbitrary units per milliliter (U/ml). M-/I- : HIV unexposed, uninfected children and M+/I-: HIV exposed, uninfected children. GMTs in M-/I- significantly higher than in M+/I- are highlighted in red; GMTs in M-/I- significantly lower than in M+/I- are highlighted in blue. **For comparison between M-/I- and M+/I- children, p values were adjusted for pneumococcal exposure.

Table 3.6: GMTs (with 95% CI) to the 15 pneumococcal proteins in children at approximately 24 months of age

Protein	Overall *GMT (95 % CI)	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**p (adj.)	M+/I- *GMT (95 % CI)			M-/I-, *GMT (95 % CI)		
					Colonized	Uncolonized	p	Colonized	Uncolonized	p
PspA	140 (122-160)	145 (125-168)	125 (92-169)	0.32	118 (99-141)	122 (79-189)	0.89	169 (135-212)	127 (82-197)	0.24
PspC	156 (144-168)	163 (153-174)	133 (104-169)	**0.016**	147 (136-159)	115 (80-166)	0.18	177 (161-195)	146 (105-205)	0.27
PsaA	159 (141-178)	165 (143-189)	141 (112-177)	0.23	141 (117-172)	124 (88-177)	0.51	185 (156-224)	152 (111-209)	0.29
PdB	140 (124-157)	148 (129-168)	118 (90-153)	0.10	133 (110-161)	124 (82-189)	0.75	160 (133-191)	113 (79-162)	0.09
SP 2027	460 (369-574)	414 (320-536)	647 (425-986)	0.09	446 (365-544)	600 (455-790)	0.08	391 (253-605)	680 (338-1368)	0.18
SP 0609	88 (75-104)	88 (73-107)	88 (64-121)	0.89	61 (46-81)	72 (43-123)	0.56	117 (92-150)	100 (65-154)	0.51
SP 0082	169 (153-187)	175 (159-194)	151 (115-199)	0.18	155 (137-177)	117 (67-205)	0.32	192 (166-223)	179 (135-236)	0.64
SP 0749	103 (88-120)	104 (87-125)	98 (71-137)	0.74	106 (84-134)	76 (47-122)	0.20	103 (78-135)	117 (74-185)	0.63
(SP 0498) LytB	196 (176-218)	201 (178-226)	182 (143-232)	0.79	189 (158-225)	179 (123-261)	0.79	210 (179-248)	184 (131-259)	0.48
IgA1-proteinase	109 (90-133)	117 (94-146)	88 (60-131)	0.19	84 (58-123)	81 (43-152)	0.91	150 (116-194)	94 (55-160)	0.11
PpmA	93 (76-113)	95 (75-119)	88 (58-133)	0.73	83 (59-117)	104 (52-206)	0.55	105 (77-143)	78 (45-137)	0.35
SlrA	124 (105-145)	126 (105-151)	117 (81-168)	0.70	126 (102-156)	112 (67-186)	0.65	126 (102-156)	112 (67-186)	0.65
SP 2216 (PcsB)	203 (185-223)	209 (190-230)	185 (143-239)	0.26	208 (180-240)	163 (98-271)	0.34	210 (185-239)	201 (151-269)	0.78
SP 1723 (StkP)	54 (47-63)	58 (49-69)	43 (31-60)	0.08	49 (38-63)	37 (24-58)	0.26	66 (52-83)	47 (29-78)	0.22
SP 2194	126 (108-147)	124 (104-148)	131 (93-186)	0.76	133 (103-170)	111 (56-220)	0.61	118 (92-151)	147 (99-218)	0.34

**GMTs (with 95% CI) were reported in arbitrary units per milliliter (U/ml). M-/I- : HIV unexposed, uninfected children and M+/I-: HIV exposed, uninfected children. GMTs in M-/I- significantly higher than in M+/I- are highlighted in red; GMTs in M-/I- significantly lower than in M+/I- are highlighted in blue. **For comparison between M-/I- and M+/I- children, p values were adjusted for pneumococcal exposure.

5.3 Antibody titers against pneumococcal proteins in PCV-vaccinated children aged between 4 and 24 months.

Serum samples from PCV-7 vaccinated children at approximately 4, 10, 18 and 24 months of age were analyzed. The number of samples analyzed varied between time-points based on availability. The demographic features of study participants are indicated in Table 4.1. Of the 565 PCV-7 vaccinated children enrolled, samples were available for 87% (433/496) of the four time-points in M-/I- children, 82% (412/500) of the four time-points in M+/I- children and 75% (940/1260) of the 4 time-points in M+/I+ children as shown in figure 6.

Table 4.1: Demographic features of the PCV-7 vaccinated children less than 2 years of age, stratified by vaccination, HIV and pneumococcal colonization status at the time of blood sampling.

Study group	Sampling time points	Total number of samples (N_T)	Median age (months)	**M+/I+ (N)	Colonized (M+/I+) infants	M+/I- (N)	Colonized (M+/I-) infants	M-/I- (N)	Colonized (M-/I-) infants
				NT=315		NT=125		NT=125	
PCV-7 vaccinated children	1st time point	508	5 (4-7)	276 [88 %]	(114/276) 41 %	117 [94 %]	(59/117) 50 %	115 [92 %]	(61/115) 53 %
	2nd time point	462	10 (5-18)	243 [77 %]	(135/243) 55 %	106 [85 %]	(63/106) 59 %	113 [90 %]	(66/113) 58 %
	3rd time point	438	17 (9-25)	235 [75 %]	(138/235) 58 %	96 [77 %]	(60/96) 62 %	107 [86 %]	(64/107) 60 %
	4th time point	377	27 (17-33)	186 [60 %]	(55/186) 64 %	93 [75 %]	(62/93) 66 %	98 [78 %]	(70/98) 71 %

Nasopharyngeal swabs from children were collected at each time-point (i.e. approximately 4, 10, 18 and 24 months of age) to determine pneumococcal colonization status. N: total number of samples that was present at that time-point (visit). M+/I+: HIV exposed, infected children, M+/I-: HIV exposed, uninfected children and M-/I-: HIV unexposed, uninfected children. NT: total number of participants that were recruited for the study.

* M+/I+ children who were on ART were combined with M+/I+ children who were not on ART since no statistically significant differences in antibody titers against all proteins were observed between these groups.

** The values in square brackets indicate the percentage of samples that were available at each time-point in each group of children.

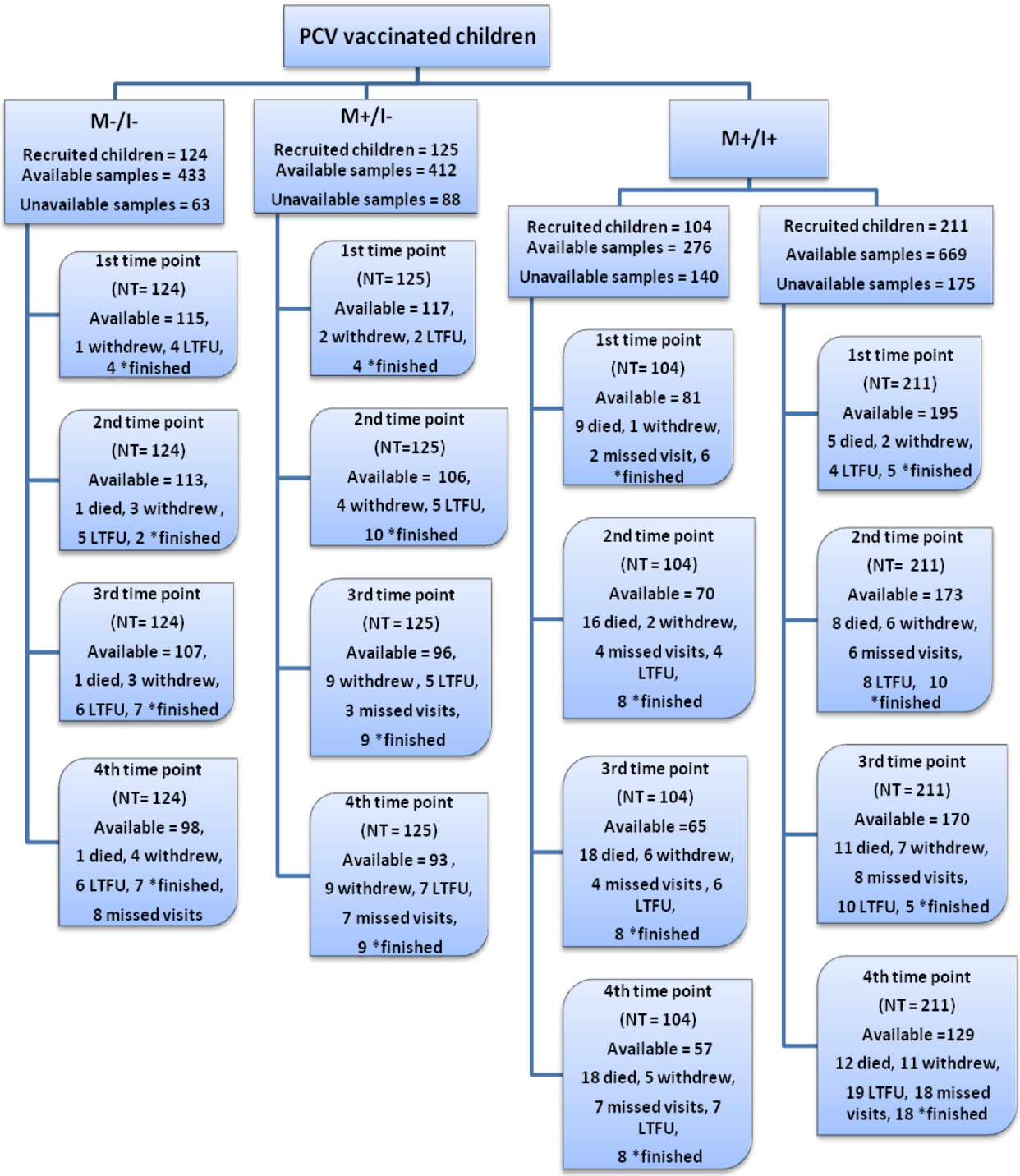


Figure 6: Flow diagram indicating sample availability at different time-points for PCV-7 vaccinated children. Children were stratified according to HIV exposure and pneumococcal colonization status.

LTFU = loss to follow up, *finished = sample used up. M/I- children were stratified according to those who were on ART (NT = 211) and children who were not on ART (NT = 104).

5.3.1 Antibody titers against pneumococcal proteins in PCV-vaccinated infants in relation to HIV status

M-/I- children had significantly higher antibody titers than M+/I+ children against PspA, PspC, SP 0082, IgA1-proteinase, PpmA, and StkP at all four time-points. In addition, antibody titers against SP 2027, SlrA, PcsB, were consistently higher in M-/I- children compared to M+/I+ children, reaching statistical significance in at least two time-points (see Table 4.2 and 4.3).

Compared to M+/I- children at 4 months, M-/I- children had higher antibody titers (in U/ml) against PspA (11 vs 4; $p < 0.0001$), PspC (21 vs 9; < 0.0001), PdB (8 vs 5; $p = 0.011$) SP 0082 (39 vs 22; $p = 0.006$), LytB (29 vs 17; $p < 0.0001$), IgA1-proteinase (25 vs 13; $p < 0.0001$) PpmA (16 vs 9; $p = 0.002$), PcsB (39 vs 21; $p = 0.005$) and StkP (16 vs 12; $p = 0.012$). At 10 months higher antibody titers against SP 0082 (162 vs 88; $p = 0.028$), PpmA (78 vs 37; $p = 0.006$), SlrA (109 vs 62; $p = 0.004$) and StkP (17 vs 9; $p = 0.009$) were also evident in M-/I- children compared to M+/I- children. Differences in antibody titers between these groups was less evident with increasing age, such that by 24 months M+/I- children had similar antibody titers as M-/I- children against all proteins (see table 4.2 and 4.3).

Table 4.2: Comparison of the geometric mean titers against the 15 pneumococcal proteins between M-/I- , M+/I- and M+/I+ children at approximately 4 and 10 months of age

Protein	4 months age					10 months age				
	**M-/I- GMT (95 % CI)	M+/I+ GMT (95 % CI)	M+/I- GMT (95 % CI)	*p1 adjusted	*p2 Adjusted	**M-/I- GMT (95 % CI)	M+/I+ GMT (95 % CI)	M+/I- GMT (95 % CI)	*p1 adjusted	*p2 adjusted
PspA	11 (9-14)	2 (2-3)	4 (3-6)	**< 0.0001**	**< 0.0001**	46 (37-57)	26 (22-30)	34 (28-43)	**< 0.0001**	0.08
PspC	21 (17-(25)	6 (5-8)	9 (7-12)	**< 0.0001**	**< 0.0001**	75 (55-104)	40 (30-54)	66 (47-93)	**0.020**	0.96
PsaA	13 (10-17)	11 (9-14)	11 (8-14)	0.74	0.39	93 (83-104)	88 (78-98)	81 (72-92)	0.62	0.21
PdB	8 (6-9)	6 (5-7)	5 (5-6)	0.13	**0.011**	50 (37-67)	30 (24-38)	31 (23-43)	**0.027**	0.06
SP 2027	258 (219-303)	220 (195-248)	227 (195-268)	0.08	0.28	611 (512-729)	357 (288-443)	624 (517-752)	**0.001**	0.89
SP 0609	131 (108-159)	117 (101-135)	107 (88-130)	0.31	0.17	134 (107-168)	125 (100-156)	103 (86-124)	0.71	0.17
SP 0082	39 (32-47)	14 (11-18)	22 (17-28)	**< 0.0001**	**0.006**	162 (121-218)	72 (54-97)	88 (64-121)	**0.001**	**0.028**
SP 0749	30 (25-35)	41 (35-47)	33 (27-40)	***0.015***	0.45	79 (56-112)	98 (74-130)	66 (47-93)	0.36	0.52
SP 0498 (LytB)	29 (24-35)	15 (13-17)	17 (15-21)	**< 0.0001**	**< 0.0001**	54 (40-73)	38 (30-49)	53 (39-72)	0.16	0.91
IgA1-proteinase	25 (22-30)	13 (12-15)	13 (11-16)	**< 0.0001**	**< 0.0001**	49 (36-66)	28 (23-34)	32 (24-44)	**0.003**	0.06
PpmA	16 (12-20)	7 (6-9)	9 (7-11)	**< 0.0001**	**0.002**	78 (58-105)	32 (24-42)	37 (26-52)	**< 0.0001**	**0.006**
SlrA	26 (21-31)	25 (21-29)	29 (23-35)	0.88	0.49	109 (85-138)	62 (51-76)	62 (48-79)	**0.002**	**0.004**
SP 2216 (PcsB)	39 (30-51)	14 (11-17)	21 (15-28)	**< 0.0001**	**0.005**	72 (51-102)	50 (35-71)	70 (52-94)	0.30	0.82
SP 1723 (StkP)	16 (14-20)	8 (7-9)	12 (10-14)	**< 0.0001**	**0.012**	17 (13-23)	8 (6-10)	9 (7-13)	**0.001**	**0.009**
SP 2194	127 (105-154)	117 (102-134)	110 (90-134)	0.41	0.29	134 (109-166)	124 (101-151)	105 (86-128)	0.56	0.16

GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml). *P-values: statistical differences between different groups of children and they were adjusted for pneumococcal exposure. **M-/I- children were used as a reference group for calculating the adjusted p-values. M+/I+: HIV exposed, infected; M+/I-: HIV exposed, uninfected and M-/I-: HIV unexposed, uninfected.

*p1= M-/I- vs M+/I+, p2 = M-/I- vs M+/I-. 'GMTs in M-/I- significantly higher than in M+/I- (p2) or in M+/I+ (p1) are highlighted in red; GMTs in M-/I- significantly lower than in M+/I- (p2) or in M+/I+ (p1) are highlighted in blue.

Table 4.3: Comparison of the geometric mean titers against the 15 pneumococcal proteins between M-/I- , M+/I- and M+/I+ children at approximately 18 and 24 months of age

Protein	18 months age					24 months age				
	M-/I- GMT (95 % CI)	**M+/I+ GMT (95 % CI)**	**M+/I- GMT (95 % CI)**	***p1 adjusted**	***p2 adjusted**	**M-/I- GMT (95 % CI)**	**M+/I+ GMT (95 % CI)**	**M+/I- GMT (95 % CI)**	***p1 adjusted**	***p2 adjusted**
PspA	53 (43-66)	35 (31-40)	40 (33-49)	**< 0.0001**	**0.054**	70 (59-85)	50 (44-58)	67 (54-82)	**0.016**	0.68
PspC	144 (110-189)	64 (50-80)	78 (58-105)	**< 0.0001**	**0.003**	152 (126-185)	86 (65-114)	107 (74-155)	**0.013**	0.11
PsaA	156 (142-171)	129 (111-150)	122 (111-135)	0.09	**0.045**	290 (268-315)	322 (291-357)	260 (228-297)	0.30	0.15
PdB	87 (72-106)	75 (63-90)	81 (64-103)	0.38	0.58	111 (91-135)	85 (68-105)	98 (78-125)	0.15	0.50
SP 2027	693 (585-822)	444 (372-530)	512 (415-633)	**0.001**	0.08	457 (389-537)	365 (296-452)	383 (308-476)	0.14	0.28
SP 0609	102 (83-125)	102 (86-120)	53 (40-70)	0.94	***< 0.0001***	106 (86-130)	106 (87-130)	77 (61-99)	0.58	0.07
SP 0082	264 (209-332)	185 (149-231)	224 (160-314)	0.054	0.40	434 (357-529)	178 (135-234)	310 (199-484)	**< 0.0001**	0.15
SP 0749	218 (171-278)	276 (228-335)	173 (123-242)	0.15	0.33	162 (122-215)	130 (95-178)	149 (105-212)	0.41	0.72
SP 0498 (LytB)	75 (57-97)	63 (52-76)	72 (58-88)	0.29	0.72	92 (68-123)	48 (36-65)	94 (66-134)	**0.007**	0.99
IgA1-proteinase	96 (74-123)	62 (52-72)	48 (33-69)	**0.006**	**< 0.0001**	73 (53-99)	36 (28-46)	76 (50-116)	**0.001**	0.81
PpmA	126 (96-165)	64 (50-81)	99 (78-127)	**< 0.0001**	0.30	146 (104-205)	77 (56-104)	120 (83-174)	**0.003**	0.41
SlrA	135 (115-159)	94 (79-110)	80 (67-97)	**0.005**	**0.001**	93 (68-126)	61 (47-79)	101 (81-125)	**0.034**	0.74
SP 2216 (PcsB)	151 (115-196)	108 (83-140)	143 (98-209)	0.13	0.71	275 (224-338)	179 (138-233)	216 (140-335)	**0.051**	0.32
SP 1723 (StkP)	30 (22-41)	15 (12-19)	25 (17-35)	**0.001**	0.39	59 (41-84)	30 (23-40)	55 (37-81)	**0.002**	0.72
SP 2194	150 (119-190)	167 (141-199)	90 (70-116)	0.47	***0.007***	74 (59-93)	72 (55-94)	70 (57-86)	0.99	0.80

GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml). *P-values: statistical differences between different groups of children and they were adjusted for pneumococcal exposure. **M-/I- children were used as a reference group for calculating the adjusted p-values. M+/I+: HIV exposed, infected; M+/I-: HIV exposed, uninfected and M-/I-: HIV unexposed, uninfected.

*p1= M-/I- vs M+/I+, p2 = M-/I- vs M+/I-. 'GMTs in M-/I- significantly higher than in M+/I- (p2) or in M+/I+ (p1) are highlighted in red; GMTs in M-/I- significantly lower than in M+/I- (p2) or in M+/I+ (p1) are highlighted in blue.

5.3.2 Relationship between antibody titers and concurrent pneumococcal colonization in PCV vaccinated children

Significance testing between groups was adjusted for concurrent pneumococcal colonization status. The overall prevalence of nasopharyngeal pneumococcal colonization increased with age in all groups (M-/I-, M+/I- and M+/I+). The prevalence of pneumococcal colonization in M-/I- children was 53 % (61/115) at 4 months of age, 58 % (66/113) at 10 months, 60 % (64/107) at 18 months and 71 % (70/98) by 24 months of age. The prevalence of pneumococcal colonization in M+/I- children was 50% (59/117) at 4 months of age, 59 % (63/106) at 10 months, 62 % (60/96) at 18 months and 66 % (62/93) by 24 months of age. The prevalence of nasopharyngeal pneumococcal colonization in M+/I+ children was lower compared to M-/I- children at all time-points, but only significantly so at 4 months of age, [41% (114/276) vs 53 % (61/115); p = 0.024)]. Other time-point comparisons included 55% (135/243 vs 58 % (66/113); p =0.13) at 10 months, 58% (138/235 vs 60 % (64/107); p = 0.51) at 18 months and 64% (55/186) vs 71 % (70/98); p = 0.33) by 24 months age.

Differences were observed among M-/I- PCV-vaccinated children, between pneumococcal-colonized compared to -uncolonized at the initial three sampling time-points. Significantly higher antibody titers in pneumococcal-colonized children compared to –uncolonized children were observed at the 4 month age time-point for PsaA, SP 0082

and PcsB; at the 10 month sampling point to PspC, PdB, LytB and PcsB and at the 18-month sampling point to PspC, PsaA, and SP 0082 (refer to tables 5.1 to 5.3). By 24 months of age, with the exception of PspA, there were no differences in antibody titers in PCV-7 vaccinated M-'I- children in relation to prevailing pneumococcal-colonization status.

Differences in antibody titers among M+/I- children between pneumococcal-colonized and -uncolonized children followed similar patterns to M-/I- children in the first year of life; i.e. significantly higher titers were observed at both the 4 and 10 month sampling time-points for PspC, PdB, and PcsB (tables 5.1 and 5.2). In addition, higher titers were also observed in the pneumococcal-colonized children compared to -uncolonized children for PspA, PspC, PdB, and StkP at 4 months of age; and for SlrA and StkP at 10 months of age. Antibody titers against PdB, and PcsB were significantly higher in pneumococcal-colonized compared to -uncolonized children at 18 months of age.

M+/I+ infants colonized by pneumococci were similar to colonized M-/I- infants and M+/I- infants in terms of the antigens for which antibody titers showed an increase at 4 and 10 months of age. In addition M+/I+ children who were colonized with pneumococci had higher antibody titers against PpmA at the 4 and 10 month age time-points compared to -uncolonized children. After one year of age, higher antibody titers associated with pneumococcal colonization were less evident in the M+/I+ group. Exceptions to this were

higher titers in pneumococcal-colonized compared to -uncolonized children against PspC and PcsB at 18 months and titers against PspC, PsaA and LytB at 24 months.

Adjusting for HIV infection status, overall pneumococcal-colonized children had higher antibody titers against PspC compared to -uncolonized children at all four sampling time-points. In addition higher antibody titers to PsaA, PdB, SP 0082, LytB, PpmA, SlrA, PcsB and StkP in pneumococcal-colonized compared to -uncolonized children were observed at the 4 and 10 month age time-points. These differences were however less evident in older children at the 18 to 24 months age-group sampling points; tables 5.1 to 5.4.

Table 5.1: Comparison of the GMT's against the 15 pneumococcal proteins between PCV vaccinated M-/I-, M+/I- and M+/I+ children at approximately 4 months of age, in relation to pneumococcal colonization status.

Protein	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**p (adj.)	M-/I-, *GMT (95 % CI)			M+/I- *GMT (95 % CI)			M+/I+-, *GMT (95 % CI)		
				Col.	Un-col.	P	Col.	Un-col.	P	Col.	Un-col.	P
PspA	6 (5-7)	3 (3-4)	< 0.0001	13 (10-18)	9 (7-14)	0.11	6 (4-8)	3 (2-5)	0.008	3 (2-5)	2 (2-3)	0.004
PspC	16 (13-19)	6 (5-7)	< 0.0001	24 (18-30)	18 (14-25)	0.18	16 (12-23)	5 (4-7)	< 0.0001	12 (9-17)	4 (3-5)	< 0.0001
PsaA	17 (14-20)	9 (7-10)	< 0.0001	19 (15-25)	8 (5-13)	0.002	14 (10-20)	8 (5-13)	0.041	16 (12-22)	9 (7-11)	0.002
PdB	9 (7-10)	4 (4-5)	< 0.0001	9 (7-11)	7 (5-9)	0.11	7 (5-10)	4 (3-4)	< 0.0001	10 (7-13)	4 (3-5)	< 0.0001
SP 2027	216 (191-243)	242 (215-271)	0.13	268 (211-342)	246 (197-308)	0.60	194 (159-237)	264 (205-340)	0.06	200 (166-241)	231 (197-270)	0.25
SP 0609	113 (96-132)	120 (106-137)	0.50	129 (99-168)	133 (99-177)	0.90	97 (72-131)	117 (90-153	0.34	114 (88-148)	117 (98-140)	0.84
SP 0082	27 (22-34)	16 (13-19)	0.001	47 (36-61)	31 (23-42)	0.040	28 (19-40)	18 (13-25)	0.07	19 (13-29)	11 (9-15)	0.022
SP 0749	34 (30-40)	37 (32-42)	0.68	31 (25-39)	28 (21-36)	0.47	33 (24-44)	33 (25-44)	0.95	37 (29-47)	43 (35-52)	0.37
(SP 0498) LytB	21 (18-25)	16 (14-18)	0.015	32 (26-41)	26 (19-34)	0.21	19 (15-25)	16 (13-20)	0.36	18 (13-23)	13 (11-16)	0.06
IgA1-proteinase	16 (14-19)	15 (13-16)	0.55	24 (20-30)	27 (21-34)	0.46	15 (11-20)	12 (10-14)	0.16	13 (11-17)	13 (11-15)	0.80
PpmA	12 (10-15)	7 (6-9)	< 0.0001	18 (13-24)	13 (9-19)	0.22	10 (7-14)	8 (6-10)	0.20	10 (7-15)	6 (5-7)	0.002
SlrA	30 (25-35)	23 (20-27)	0.033	30 (22-39)	22 (17-29)	0.15	31 (23-41)	27 (20-35)	0.48	29 (22-38)	22 (18-27)	0.13
SP 2216 (PcsB)	34 (27-43)	12 (10-15)	< 0.0001	51 (35-74)	29 (20-42)	0.030	37 (25-56)	12 (8-17)	< 0.0001	25 (17-38)	9 (7-12)	< 0.0001
SP 1723 (StkP)	12 (11-14)	9 (8-10)	0.003	18 (14-23)	15 (11-19)	0.25	14 (11-17)	10 (8-13)	0.051	9 (7-12)	7 (6-8)	0.034
SP 2194	110 (95-126)	124 (109-142)	0.19	138 (106-179)	116 (87-156)	0.38	97 (73-130)	124 (93-164)	0.24	102 (83-125)	128 (107-153)	0.11

*GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **For differences between overall colonized and uncolonized children, p-values were adjusted for HIV exposure. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMTs in pneumococcal-colonized children significantly higher than in –uncolonized children are highlighted in red; GMTs in pneumococcal-colonized children significantly lower than in uncolonized children are highlighted in blue.

Table 5.2: Comparison of the GMT's against the 15 pneumococcal proteins between PCV vaccinated M-/I-, M+/I- and M+/I+ children at approximately 10 months of age, in relation to pneumococcal colonization status.

Protein	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**p (adj.)	M-/I-, *GMT (95 % CI)			M+/I- *GMT (95 % CI)			M+/I+-, *GMT (95 % CI)		
				Col.	Un-col.	P	Col.	Un-col.	P	Col.	Un-col.	P
PspA	34 (29-39)	30 (25-36)	0.45	48 (37-64)	41 (29-58)	0.17	39 (30-50)	29 (19-43)	0.17	25 (20-31)	27 (21-34)	0.66
PspC	108 (90-129)	19 (14-26)	**< 0.0001**	118 (88-160)	31 (16-61)	**< 0.0001**	119 (86-166)	28 (15-51)	**< 0.0001**	96 (70-131)	12 (8-19)	**< 0.0001**
PsaA	94 (86-103)	78 (69-87)	**0.009**	98 (84-114)	83 (69-99)	0.18	85 (73-99)	76 (61-95)	0.40	97 (84-112)	76 (64-90)	**0.032**
PdB	50 (42-61)	20 (15-25)	**< 0.0001**	66 (47-93)	29 (17-49)	**0.007**	43 (30-62)	19 (11-33)	**0.010**	47 (35-63)	17 (12-24)	**< 0.0001**
SP 2027	314 (392-342)	448 (360-558)	0.64	671 (542-830)	508 (368-703)	0.14	666 (534-829)	566 (402-798)	0.40	340 (259-447)	376 (260-543)	0.66
SP 0609	126 (107-148)	114 (92-142)	0.51	149 (112-198)	109 (75-158)	0.19	112 (88-141)	92 (68-126)	0.31	121 (91-160)	131 (91-189)	0.72
SP 0082	147 (119-182)	47 (34-64)	**< 0.0001**	195 (138-277)	113 (65-195)	0.07	170 (121-238)	34 (21-55)	**< 0.0001**	113 (79-163)	38 (24-62)	**< 0.0001**
SP 0749	88 (69-113)	76 (58-101)	0.43	91 (57-146)	61 (39-95)	0.27	65 (41-103)	69 (41-114)	0.87	103 (70-151)	89 (57-138)	0.63
(SP 0498) LytB	64 (53-78)	30 (21-35)	**< 0.0001**	72 (52-100)	31 (18-55)	**0.007**	87 (61-123)	26 (15-42)	**< 0.0001**	50 (37-69)	26 (18-39)	**0.009**
IgA1-proteinase	38 (32-46)	27 (22-34)	**0.041**	51 (35-74)	45 (27-76)	0.70	40 (26-60)	24 (16-37)	0.10	31 (24-41)	23 (17-32)	0.15
PpmA	62 (50-77)	23 (17-31)	**< 0.0001**	91 (65-127)	58 (32-107)	0.16	48 (32-74)	25 (13-46)	0.06	56 (39-80)	15 (10-23)	**< 0.0001**
SlrA	91 (76-108)	50 (42-61)	**< 0.0001**	111 (82-149)	105 (69-159)	0.84	80 (58-110)	42 (29-62)	**0.012**	86 (64-116)	40 (31-53)	**< 0.0001**
SP 2216 (PcsB)	111 (90-137)	24 (16-34)	**< 0.0001**	106 (77-146)	34 (16-74)	**0.002**	92 (64-133)	46 (28-76)	**0.023**	126 (87-183)	14 (8-25)	**< 0.0001**
SP 1723 (StkP)	15 (12-18)	6 (4-7)	**< 0.0001**	20 (14-28)	13 (8-23)	0.20	13 (9-19)	5 (3-9)	**0.006**	14 (10-19)	4 (3-6)	**< 0.0001**
SP 2194	123 (105-143)	117 (96-143)	0.76	153 (116-200)	105 (75-146)	0.10	107 (83-139)	101 (71-142)	0.75	115 (88-151)	133 (97-183)	0.49

*GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **For differences between overall colonized and uncolonized children, p-values were adjusted for HIV exposure. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMT's in pneumococcal-colonized children significantly higher than in –uncolonized children are highlighted in red; GMTs in pneumococcal-colonized children significantly lower than in uncolonized children are highlighted in blue.

Table 5.3: Comparison of the GMT's against the 15 pneumococcal proteins between PCV vaccinated M-/I-, M+/I- and M+/I+ children at approximately 18 months of age, in relation to pneumococcal colonization status.

Protein	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**p (adj.)	M-/I-, *GMT (95 % CI)			M+/I- *GMT (95 % CI)			M+/I+-, *GMT (95 % CI)		
				Col.	Un-col.	P	Col.	Un-col.	P	Col.	Un-col.	P
PspA	40 (35-46)	42 (36-48)	0.60	56 (42-73)	50 (36-70)	0.63	40 (31-51)	41 (29-58)	0.89	33 (28-40)	38 (32-46)	0.28
PspC	107 (90-128)	60 (45-79)	< **0.0001**	198 (154-256)	93 (56-157)	**0.005**	92 (67-127)	55 (29-107)	0.10	82 (62-107)	49 (33-72)	**0.03**
PsaA	142 (127-160)	126 (111-143)	0.14	173 (153-196)	134 (116-155)	**0.008**	124 (109-140)	118 (101-138)	0.68	136 (109-169)	124 (100-153)	0.53
PdB	90 (77-104)	69 (57-83)	**0.027**	102 (80-129)	70 (50-97)	0.06	98 (79-122)	55 (31-97)	**0.021**	80 (62-103)	72 (56-92)	0.56
SP 2027	500 (428-584)	540 (457-639)	0.43	676 (546-835)	717 (537-957)	0.73	518 (392-685)	500 (362-690)	0.87	415 (320-538)	482 (377-615)	0.41
SP 0609	83 (70-98)	99 (84-117)	0.28	96 (75-1250	111 (78-156)	0.52	51 (36-74)	57 (37-87)	0.73	95 (73-124)	109 (89-134)	0.41
SP 0082	236 (195-287)	184 (146-231)	0.11	320 (248-414)	202 (133-308)	**0.050**	252 (176-361)	175 (82-375)	0.32	193 (139-268)	178 (133-238)	0.71
SP 0749	203 (168-245)	293 (239-358)	***0.021***	202 (146-279)	240 (164-352)	0.49	131 (88-196)	306 (169-552)	***0.018***	251 (190-331)	318 (243-415)	0.23
(SP 0498) LytB	77 (66-90)	57 (46-70)	**0.027**	80 (60-107)	68 (41-111)	0.53	81 (65-100)	56 (35-91)	0.11	73 (56-96)	52 (41-68)	0.08
IgA1-proteinase	67 (57-80)	65 (52-79)	0.65	97 (70-134)	93 (61-142)	0.87	51 (33-78)	42 (21-86)	0.63	61 (50-78)	61 (48-77)	0.85
PpmA	88 (72-109)	79 (61-102)	0.65	145 (104-203)	103 (64-165)	0.22	91 (67-123)	120 (77-187)	0.29	66 (47-93)	62 (43-90)	0.83
SlrA	102 (88-118)	100 (86-116)	0.73	141 (114-174)	128 (99-165)	0.56	83 (65-104)	76 (57-103)	0.69	94 (73-121)	95 (76-118)	0.97
SP 2216 (PcsB)	154 (127-188)	94 (70-128)	**0.008**	156 (113-215)	143 (90-228)	0.74	188 (133-265)	82 (33-206)	**0.039**	140 (100-194)	80 (53-122)	**0.038**
SP 1723 (StkP)	23 (18-29)	17 (13-22)	0.11	35 (24-52)	24 (14-41)	0.26	27 (17-40)	21 (9-44)	0.52	17 (12-25)	13 (9-19)	0.33
SP 2194	134 (114-159)	159 (132-191)	0.35	140 (102-191)	167 (116-241)	0.45	96 (69-135)	79 (56-113)	0.48	154 (120-197)	187 (145-240)	0.28

*GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **For differences between overall colonized and uncolonized children, p-values were adjusted for HIV exposure. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMT's in pneumococcal-colonized children significantly higher than in –uncolonized children are highlighted in red; GMTs in pneumococcal-colonized children significantly lower than in uncolonized children are highlighted in blue.

Table 5.4: Comparison of the GMT's against the 15 pneumococcal proteins between PCV vaccinated M-/I-, M+/I- and M+/I+ children at approximately 24 months of age, in relation to pneumococcal colonization status.

Protein	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**p (adj.)	M-/I-, *GMT (95 % CI)			M+/I- *GMT (95 % CI)			M+/I+-, *GMT (95 % CI)		
				Col.	Un-col.	P	Col.	Un-col.	P	Col.	Un-col.	P
PspA	65 (57-74)	53 (45-62)	0.08	85 (67-108)	48 (37-63)	**0.004**	60 (46-79)	86 (64-115)	0.13	56 (46-68)	46 (36-58)	0.20
PspC	129 (105-158)	82 (62-109)	**0.015**	160 (126-203)	133 (95-188)	0.37	98 (58-163)	134 (100-181)	0.44	131 (95-180)	52 (32-84)	**0.001**
PsaA	301 (277-327)	276 (257-296)	0.10	293 (261-328)	287 (258-319)	0.83	257 (214-308)	269 (244-297)	0.74	341 (298-391)	271 (240-307)	**0.020**
PdB	103 (89-120)	85 (66-108)	0.18	114 (92-141)	102 (67-154)	0.59	97 (72-131)	101 (69-148)	0.89	99 (77-129)	71 (48-106)	0.15
SP 2027	404 (347-470)	384 (313-471)	0.73	451 (374-545)	467 (337-647)	0.84	391 (298-515)	361 (247-527)	0.74	379 (283-507)	352 (252-491)	0.74
SP 0609	107 (91-127)	87 (73-103)	0.06	115 (89-149)	85 (60-122)	0.18	81 (58-113)	69 (56-85)	0.55	123 (93-161)	95 (72-126)	0.21
SP 0082	287 (228-362)	254 (192-336)	0.71	409 (315-531)	490 (359-667)	0.40	280 (154-508)	403 (242-670)	0.46	224 (158-317)	147 (94-229)	0.13
SP 0749	152 (120-193)	137 (100-187)	0.64	177 (123-255)	139 (85-228)	0.43	133 (89-197)	201 (94-430)	0.29	149 (96-232)	117 (73-190)	0.48
(SP 0498) LytB	80 (64-101)	54 (38-75)	0.08	97 (68-139)	81 (45-146)	0.58	90 (57-140)	105 (56-140)	0.68	65 (45-94)	33 (19-55)	**0.031**
IgA1-proteinase	58 (46-73)	44 (33-60)	0.32	71 (47-106)	71 (42-121)	0.99	75 (44-128)	80 (42-151)	0.88	42 (31-58)	27 (18-41)	0.10
PpmA	113 (88-144)	84 (59-119)	0.25	155 (101-236)	135 (72-254)	0.72	113 (71-179)	140 (75-263)	0.60	89 (60-132)	53 (32-89)	0.11
SlrA	85 (70-104)	67 (50-89)	0.24	100 (70-142)	80 (43-150)	0.50	98 (75-128)	109 (74-162)	0.64	69 (49-99)	50 (33-75)	0.23
SP 2216 (PcsB)	217 (172-273)	214 (169-271)	0.97	249 (189-328)	326 (239-444)	0.22	201 (110-367)	261 (187-363)	0.59	205 (144-292)	156 (104-233)	0.31
SP 1723 (StkP)	46 (35-59)	37 (27-50)	0.45	61 (39-96)	57 (30-108)	0.87	50 (30-84)	68 (44-106)	0.48	35 (23-51)	23 (15-35)	0.14
SP 2194	74 (62-89)	70 (54-91)	0.68	73 (54-97)	76 (53-111)	0.83	71 (54-92)	69 (51-95)	0.96	78 (55-111)	67 (42-106)	0.56

*GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **For differences between overall colonized and uncolonized children, p-values were adjusted for HIV exposure. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMT's in pneumococcal-colonized children significantly higher than in –uncolonized children are highlighted in red; GMTs in pneumococcal-colonized children significantly lower than in uncolonized children are highlighted in blue.

5.4 Natural immune responses in M-/I- and M+/I- children aged between 4 and 24 months in relation to PCV-vaccination status

Vaccination with PCV-7 was associated with significant differences in antibody titers to selected proteins during the first two years of life in M-/I- and M+/I- children, including when adjusted for overall pneumococcal colonization status. At 4 months of age, PCV vaccinated children had lower antibody titers to PspA, PspC, PsaA, PdB, SP 2027, SP 0749, LytB, IgA1-proteinase, PpmA, SlrA and StkP, among M-/I- and M+/I- groups compared to PCV-unvaccinated children. Conversely, PCV-recipients had higher antibody titers against SP 0609 compared to PCV-unvaccinated children. Antibodies against SP 0082 and PcsB did not differ by PCV vaccination status in either M-/I- or M+/I- infants at 4 months (refer to tables 6.1 and 6.5).

At 10 months of age, antibodies titers against PspA, PsaA, PdB, IgA1-proteinase, SlrA, and StkP remained higher in PCV-unvaccinated compared to -vaccinated infants in M-/I- and M+/I- groups. Receipt of PCV was, however, associated with higher antibody titers in the M-/I- group to SP 0609, SP 0082, and PpmA. In the M+/I- groups, PCV-vaccination was associated with significantly lower antibody titers to SP 0609, LytB, and SP 2194 (refer to tables 6.2 and 6.6).

At 18 months of age, PCV-recipients still had lower antibody titers against PspA, and LytB, but higher titers against SP 2027, SP 0082, SP 0749 and PpmA in both M-/I- and M+/I- groups. M-/I- children had significantly lower titers against PdB in the PCV-vaccinated group, and PCV-

vaccinated M+/I- children had significantly lower antibody titers against PspC, PsaA, SP 0609, IgA1-proteinase and SP 2194 (refer to tables 6.3 and 6.7).

At 24 months of age, differences in the effect of PCV-vaccination and HIV exposure on titers against pneumococcal antigens were still noticeable. PCV-vaccination was associated with lower antibody titers against PspA, PdB, LytB and SP 2194; but higher antibody titers against PsaA and SP 0082 in both M-/I- and M+/I- groups. Additionally, PCV-vaccinated, M+/I- children had significantly lower antibody titers against IgA1-proteinase and higher titers against PpmA, SP 0749 and PcsB. Antibody titers against SP 0749 were observed to be higher in PCV-vaccinated M+/I- children at this time-point (refer to tables 6.4 and 6.8).

Table 6.1: Comparison of the GMT's against the 15 pneumococcal proteins between PCV-vaccinated and unvaccinated M-/I- children at approximately 4 months of age

Protein	M-/I- PCV-vaccinated *GMT (95 % CI)	M-/I- PCV-unvaccinated *GMT (95 % CI)	**P adj.	Uncolonized PCV-vaccinated GMT (95 % CI)	Uncolonized PCV-unvaccinated GMT (95 % CI)	p	Colonized PCV-vaccinated *GMT (95 % CI)	Colonized PCV-unvaccinated *GMT (95 % CI)	p
PspA	11 (9-14)	47 (40-55)	**< 0.0001**	9 (7-14)	50 (40-64)	**< 0.0001**	13 (10-18)	44 (36-55)	**< 0.0001**
PspC	21 (17-(25)	60 (54-67)	**< 0.0001**	18 (14-25)	55 (47-65)	**< 0.0001**	24 (18-30)	65 (56-76)	**< 0.0001**
PsaA	13 (10-17)	182 (158-211)	**< 0.0001**	8 (5-13)	197 (159-244)	**< 0.0001**	19 (15-25)	170 (139-208)	**< 0.0001**
PdB	8 (6-9)	45 (39-52)	**< 0.0001**	7 (5-9)	43 (34-53)	**< 0.0001**	9 (7-11)	47 (38-58)	**< 0.0001**
SP 2027	258 (219-303)	571 (491-663)	**< 0.0001**	246 (197-308)	578 (462-725)	**< 0.0001**	268 (211-342)	563 (458-694)	**< 0.0001**
SP 0609	131 (108-159)	79 (67-94)	***< 0.0001***	133 (99-177)	84 (65-108)	***0.019***	129 (99-168)	75 (60-94)	***0.002***
SP 0082	39 (32-47)	39 (35-45)	0.88	31 (23-42)	38 (31-46)	0.30	47 (36-61)	41 (35-48)	0.40
SP 0749	30 (25-35)	124 (102-151)	**< 0.0001**	28 (21-36)	143 (106-192)	**< 0.0001**	31 (25-39)	110 (84-143)	**< 0.0001**
SP 0498 (LytB)	29 (24-35)	38 (34-43)	**0.008**	26 (19-34)	35 (29-41)	0.07	32 (26-41)	42 (36-49)	**0.051**
IgA1-proteinase	25 (22-30)	61 (53-69)	**< 0.0001**	27 (21-34)	61 (51-75)	**< 0.0001**	24 (20-30)	60 (49-73)	**< 0.0001**
PpmA	16 (12-20)	41 (35-48)	**< 0.0001**	13 (9-19)	36 (28-46)	**< 0.0001**	18 (13-24)	46 (37-57)	**< 0.0001**
SlrA	26 (21-31)	185 (158-216)	**< 0.0001**	22 (17-29)	211 (169-263)	**< 0.0001**	30 (22-39)	165 (132-206)	**< 0.0001**
SP 2216 (PcsB)	39 (30-51)	33 (28-39)	0.28	29 (20-42)	28 (22-36)	0.89	51 (35-74)	39 (31-47)	0.17
SP 1723 (StkP)	16 (14-20)	29 (26-33)	**< 0.0001**	15 (11-19)	27 (22-32)	**< 0.0001**	18 (14-23)	31 (27-37)	**< 0.0001**
SP 2194	127 (105-154)	141 (119-168)	0.41	116 (87-156)	162 (131-202)	0.07	138 (106-179)	125 (96-162)	0.60

*****GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **for differences between overall vaccinated and un-vaccinated children, p-values were adjusted for nasopharyngeal pneumococcal colonization status. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMTs in PCV-7 unvaccinated children significantly higher than in –vaccinated children are highlighted in red whilst GMTs in PCV-7 unvaccinated children significantly lower than in –vaccinated children are highlighted in blue.

Table 6.2: Comparison of the GMT's against the 15 pneumococcal proteins between PCV-vaccinated and unvaccinated M-/I- children at approximately 10 months of age

Protein	M-/I- vaccinated *GMT (95 % CI)	M-/I- unvaccinated *GMT (95 % CI)	**P adj.	Uncolonized vaccinated *GMT (95 % CI)	Uncolonized unvaccinated *GMT (95 % CI)	p	Colonized vaccinated *GMT (95 % CI)	Colonized unvaccinated *GMT (95 % CI)	p
PspA	46 (37-57)	116 (98-138)	**< 0.0001**	41 (29-58)	122 (97-152)	**< 0.0001**	48 (37-64)	115 (93-142)	**< 0.0001**
PspC	75 (55-104)	100 (83-120)	0.34	31 (16-61)	44 (27-73)	0.35	118 (88-160)	126 (107-148)	0.71
PsaA	93 (83-104)	174 (153-199)	**< 0.0001**	83 (69-99)	167 (133-211)	**< 0.0001**	98 (84-114)	177 (151-206)	**< 0.0001**
PdB	50 (37-67)	98 (80-119)	**0.001**	29 (17-49)	57 (38-86)	**0.045**	66 (47-93)	114 (92-142)	**0.006**
SP 2027	611 (512-729)	561 (445-706)	0.51	508 (368-703)	664 (464-951)	0.35	671 (542-830)	534 (404-707)	0.22
SP 0609	134 (107-168)	88 (73-107)	***0.004***	109 (75-158)	87 (60-127)	0.45	149 (112-198)	88 (71-111)	***0.004***
SP 0082	162 (121-218)	83 (67-104)	***< 0.0001***	113 (65-195)	37 (23-60)	***0.006***	195 (138-277)	105 (83-133)	***0.003***
SP 0749	79 (56-112)	57 (44-73)	0.10	61 (39-95)	59 (35-97)	0.95	91 (57-146)	57 (42-76)	0.07
SP 0498 (LytB)	54 (40-73)	85 (67-106)	0.06	31 (18-55)	38 (23-63)	0.58	72 (52-100)	106 (83-135)	0.06
IgA1-proteinase	49 (36-66)	77 (60-10)	**0.040**	45 (27-76)	52 (30-90)	0.76	51 (35-74)	87 (64-117)	**0.027**
PpmA	78 (58-105)	47 (36-62)	***0.004***	58 (32-107)	23 (12-41)	***0.036***	91 (65-127)	60 (43-78)	***0.045***
SlrA	109 (85-138)	185 (157-217)	**< 0.0001**	105 (69-159)	191 (143-256)	**0.022**	111 (82-149)	183 (150-222)	**0.004**
SP 2216 (PcsB)	72 (51-102)	96 (74-124)	0.47	34 (16-74)	32 (17-60)	0.96	106 (77-146)	131 (103-167)	0.29
SP 1723 (StkP)	17 (13-23)	30 (24-37)	**0.007**	13 (8-23)	17 (10-28)	0.50	20 (14-28)	35 (28-45)	**0.005**
SP 2194	134 (109-166)	159 (131-191)	0.25	105 (75-146)	194 (143-264)	**0.006**	153 (116-200)	150 (119-187)	0.91

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Table 6.3: Comparison of the GMT's against the 15 pneumococcal proteins between PCV-vaccinated and unvaccinated M-/I- children at approximately 18 months of age

Protein	M-/I- vaccinated *GMT (95 % CI)	M-/I- unvaccinated *GMT (95 % CI)	**P adj.	Uncolonized vaccinated *GMT (95 % CI)	Uncolonized unvaccinated *GMT (95 % CI)	p	Colonized vaccinated *GMT (95 % CI)	Colonized unvaccinated *GMT (95 % CI)	p
PspA	53 (43-66)	131 (105-164)	**< 0.0001**	50 (36-70)	143 (101-201)	**0.001**	56 (42-73)	128 (97-168)	**< 0.0001**
PspC	144 (110-189)	131 (110-155)	0.15	93 (56-157)	87 (52-147)	0.84	198 (154-256)	147 (125-173)	***0.042***
PsaA	156 (142-171)	158 (134-186)	0.64	134 (116-155)	141 (104-189)	0.74	173 (153-196)	155 (128-188)	0.49
PdB	87 (72-106)	130 (108-156)	**0.020**	70 (50-97)	114 (101-198)	**0.051**	102 (80-129)	126 (101-157)	0.14
SP 2027	693 (585-822)	285 (222-365)	***< 0.0001***	717 (537-957)	389 (253-597)	***0.023***	676 (546-835)	260 (193-349)	***< 0.0001***
SP 0609	102 (83-125)	121 (90-164)	0.39	111 (78-156)	116 (87-247)	0.81	96 (75-1250	115 (81-165)	0.39
SP 0082	264 (209-332)	130 (106-159)	***< 0.0001***	202 (133-308)	102 (67-157)	***0.031***	320 (248-414)	139 (111-176)	***< 0.0001***
SP 0749	218 (171-278)	84 (58-121)	***< 0.0001***	240 (164-352)	89 (46-174)	***0.020***	202 (146-279)	82 (53-127)	***0.002***
SP 0498 (LytB)	75 (57-97)	164 (135-200)	**< 0.0001**	68 (41-111)	113 (72-177)	0.16	80 (60-107)	183 (148-227)	**< 0.0001**
IgA1-proteinase	96 (74-123)	90 (65-125)	0.82	93 (61-142)	108 (69-201)	0.67	97 (70-134)	84 (56-124)	0.61
PpmA	126 (96-165)	63 (46-85)	***< 0.0001***	103 (64-165)	48 (26-89)	***0.014***	145 (104-203)	68 (48-97)	***0.007***
SlrA	135 (115-159)	124 (97-158)	0.53	128 (99-165)	136 (88-211)	0.70	141 (114-174)	120 (89-161)	0.61
SP 2216 (PcsB)	151 (115-196)	157 (120-205)	0.96	143 (90-228)	142 (89-226)	0.95	156 (113-215)	161 (117-223)	0.92
SP 1723 (StkP)	30 (22-41)	39 (29-52)	0.46	24 (14-41)	29 (16-50)	0.93	35 (24-52)	43 (30-60)	0.40
SP 2194	150 (119-190)	144 (113-185)	0.88	167 (116-241)	240 (173-334)	0.41	140 (102-191)	124 (92-167)	0.75

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Table 6.4: Comparison of the GMT's against the 15 pneumococcal proteins between PCV-vaccinated and unvaccinated M-/I- children at approximately 24 months of age

Protein	M-/I- vaccinated *GMT (95 % CI)	M-/I- unvaccinated *GMT (95 % CI)	**P adj.	Uncolonized vaccinated *GMT (95 % CI)	Uncolonized unvaccinated *GMT (95 % CI)	p	Colonized vaccinated *GMT (95 % CI)	Colonized unvaccinated *GMT (95 % CI)	p
PspA	70 (59-85)	158 (129-192)	**< 0.0001**	48 (37-63)	127 (82-197)	**< 0.0001**	85 (67-108)	169 (135-212)	**< 0.0001**
PspC	152 (126-185)	169 (152-188)	0.35	133 (95-188)	146 (105-205)	0.69	160 (126-203)	177 (161-195)	0.38
PsaA	290 (268-315)	176 (150-207)	***< 0.0001***	287 (258-319)	152 (111-209)	***< 0.0001***	293 (261-328)	185 (156-224)	***< 0.0001***
PdB	111 (91-135)	147 (125-173)	**0.036**	102 (67-154)	113 (79-162)	0.70	114 (92-141)	160 (133-191)	**0.018**
SP 2027	457 (389-537)	476 (403-562)	0.96	467 (337-647)	680 (338-1368)	0.31	451 (374-545)	391 (253-605)	0.60
SP 0609	106 (86-130)	113 (91-139)	0.69	85 (60-122)	100 (65-154)	0.57	115 (89-149)	117 (92-150)	0.91
SP 0082	434 (357-529)	189 (116-215)	***< 0.0001***	490 (359-667)	179 (135-236)	***< 0.0001***	409 (315-531)	192 (166-223)	***< 0.0001***
SP 0749	162 (122-215)	106 (84-134)	***0.020***	139 (85-228)	117 (74-185)	0.60	177 (123-255)	103 (78-135)	***0.016***
SP 0498 (LytB)	92 (68-123)	204 (176-236)	**< 0.0001**	81 (45-146)	184 (131-259)	**0.019**	97 (68-139)	210 (179-248)	**< 0.0001**
IgA1-proteinase	73 (53-99)	134 (106-169)	**0.002**	71 (42-121)	94 (55-160)	0.46	71 (47-106)	150 (116-194)	**0.001**
PpmA	146 (104-205)	98 (75-128)	***0.048***	135 (72-254)	78 (45-137)	0.19	155 (101-236)	105 (77-143)	0.13
SlrA	93 (68-126)	124 (98-158)	0.15	80 (43-150)	112 (67-186)	0.32	100 (70-142)	126 (102-156)	0.31
SP 2216 (PcsB)	275 (224-338)	208 (185-234)	***0.024***	326 (239-444)	201 (151-269)	***0.024***	249 (189-328)	210 (185-239)	0.22
SP 1723 (StkP)	59 (41-84)	61 (49-75)	0.99	57 (30-108)	47 (29-78)	0.64	61 (39-96)	66 (52-83)	0.73
SP 2194	74 (59-93)	125 (101-153)	**0.001**	76 (53-111)	147 (99-218)	**0.017**	73 (54-97)	118 (92-151)	**0.012**

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Table 6.5: Comparison of the GMT's against the 15 pneumococcal proteins between PCV-vaccinated and unvaccinated M+/I- children at approximately 4 months of age

Protein	M+/I- vaccinated *GMT (95 % CI)	M+/I- unvaccinated *GMT (95 % CI)	**P adj.	Uncolonized vaccinated GMT (95 % CI)	Uncolonized unvaccinated GMT (95 % CI)	p	Colonized vaccinated *GMT (95 % CI)	Colonized unvaccinated *GMT (95 % CI)	p
PspA	4 (3-6)	33 (29-37)	**< 0.0001**	3 (2-5)	31 (25-40)	**< 0.0001**	6 (4-8)	34 (30-38)	**< 0.0001**
PspC	9 (7-12)	47 (42-55)	**< 0.0001**	5 (4-7)	37 (29-48)	**< 0.0001**	16 (12-23)	54 (46-63)	**< 0.0001**
PsaA	11 (8-14)	249 (223-278)	**< 0.0001**	8 (5-13)	232 (186-289)	**< 0.0001**	14 (10-20)	258 (227-293)	**< 0.0001**
PdB	5 (5-6)	35 (30-41)	**< 0.0001**	7 (5-10)	28 (21-36)	**< 0.0001**	4 (3-4)	39 (32-47)	**< 0.0001**
SP 2027	227 (195-268)	556 (444-696)	**< 0.0001**	264 (205-340)	527 (339-819)	**0.004**	194 (159-237)	570 (437-743)	**< 0.0001**
SP 0609	107 (88-130)	67 (56-81)	***0.002***	117 (90-153	72 (50-103)	***0.028***	97 (72-131)	65 (53-81)	***0.024***
SP 0082	22 (17-28)	30 (24-36)	0.16	18 (13-25)	25 (17-35)	0.20	28 (19-40)	32 (26-41)	0.46
SP 0749	33 (27-40)	107 (91-126)	**< 0.0001**	33 (25-44)	87 (67-113)	**< 0.0001**	33 (24-44)	118 (96-144)	**< 0.0001**
SP 0498 (LytB)	17 (15-21)	26 (21-31)	**0.009**	16 (13-20)	22 (15-31)	0.14	19 (15-25)	28 (22-34)	**0.032**
IgA1-proteinase	13 (11-16)	31 (26-38)	**< 0.0001**	12 (10-14)	25 (18-34)	**< 0.0001**	15 (11-20)	35 (29-44)	**< 0.0001**
PpmA	9 (7-11)	24 (20-30)	**< 0.0001**	8 (6-10)	20 (13-30)	**< 0.0001**	10 (7-14)	27 (22-33)	**< 0.0001**
SlrA	29 (23-35)	119 (104-136)	**< 0.0001**	27 (20-35)	115 (87-152)	**< 0.0001**	31 (23-41)	122 (105-141)	**< 0.0001**
SP 2216 (PcsB)	21 (15-28)	26 (20-34)	0.18	12 (8-17)	14 (8-25)	0.51	37 (25-56)	35 (29-45)	0.71
SP 1723 (StkP)	12 (10-14)	21 (18-23)	**< 0.0001**	10 (8-13)	18 (14-23)	**0.001**	14 (11-17)	22 (19-25)	**< 0.0001**
SP 2194	110 (90-134)	168 (145-193	**0.001**	124 (93-164)	155 (116-207)	0.30	97 (73-130)	174 (148-204)	**< 0.0001**

*GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **for differences between overall vaccinated and un-vaccinated children, p-values were adjusted for nasopharyngeal pneumococcal colonization status. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMTs in PCV-7 unvaccinated children significantly higher than in –vaccinated children are highlighted in red whilst GMTs in PCV-7 unvaccinated children significantly lower than in –vaccinated children are highlighted in blue.

Table 6.6: Comparison of the GMT's against the 15 pneumococcal proteins between PCV-vaccinated and unvaccinated M+/I- children at approximately 10 months of age

Protein	M+/I- vaccinated *GMT (95 % CI)	M+/I- unvaccinated *GMT (95 % CI)	**P adj.	Uncolonized vaccinated *GMT (95 % CI)	Uncolonized unvaccinated *GMT (95 % CI)	p	Colonized vaccinated *GMT (95 % CI)	Colonized unvaccinated *GMT (95 % CI)	p
PspA	34 (28-43)	101 (84-120)	**< 0.0001**	29 (19-43)	102 (71-146)	**< 0.0001**	39 (30-50)	100 (82-123)	**< 0.0001**
PspC	66 (47-93)	93 (78-110)	0.17	28 (15-51)	70 (49-101)	**0.016**	119 (86-166)	106 (89-127)	0.49
PsaA	81 (72-92)	193 (167-222)	**< 0.0001**	76 (61-95)	179 (138-233)	**< 0.0001**	85 (73-99)	199 (168-236)	**< 0.0001**
PdB	31 (23-43)	73 (58-93)	**< 0.0001**	19 (11-33)	71 (45-113)	**< 0.0001**	43 (30-62)	74 (56-98)	**0.014**
SP 2027	624 (517-752)	483 (366-638)	0.18	566 (402-798)	459 (279-755)	0.49	666 (534-829)	494 (351-697)	0.25
SP 0609	103 (86-124)	136 (115- 162)	**0.026**	92 (68-126)	180 (136-239)	**0.002**	112 (88-141)	120 (97-148)	0.71
SP 0082	88 (64-121)	72 (58-89)	0.11	34 (21-55)	61 (40-91)	0.06	170 (121-238)	78 (60-101)	***< 0.0001***
SP 0749	66 (47-93)	56 (42-75)	0.52	69 (41-114)	49 (30-78)	0.41	65 (41-103)	60 (42-86)	0.82
SP 0498 (LytB)	53 (39-72)	81 (67-99)	**0.043**	26 (15-42)	89 (65-121)	**< 0.0001**	87 (61-123)	78 (60-100)	0.51
IgA1-proteinase	32 (24-44)	100 (80-123)	**< 0.0001**	24 (16-37)	126 (86-183)	**< 0.0001**	40 (26-60)	89 (69-116)	**0.001**
PpmA	37 (26-52)	30 (24-39)	0.30	25 (13-46)	24 (16-37)	0.89	48 (32-74)	34 (25-45)	0.14
SlrA	62 (48-79)	138 (117-163)	**< 0.0001**	42 (29-62)	144 (108-193)	**< 0.0001**	80 (58-110)	135 (110-166)	**0.005**
SP 2216 (PcsB)	70 (52-94)	88 (68-113)	0.35	46 (28-76)	66 (40-111)	0.36	92 (64-133)	100 (76-134)	0.67
SP 1723 (StkP)	9 (7-13)	22 (17-29)	**< 0.0001**	5 (3-9)	21 (12-35)	**< 0.0001**	13 (9-19)	23 (17-32)	**0.017**
SP 2194	105 (86-128)	153 (126-185)	**0.007**	101 (71-142)	145 (103-204)	0.11	107 (83-139)	157 (124-198)	**0.030**

*****GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **for differences between overall vaccinated and un-vaccinated children, p-values were adjusted for nasopharyngeal pneumococcal colonization status. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMTs in PCV-7 unvaccinated children significantly higher than in –vaccinated children are highlighted in red whilst GMTs in PCV-7 unvaccinated children significantly lower than in –vaccinated children are highlighted in blue.

Table 6.7: Comparison of the GMT's against the 15 pneumococcal proteins between PCV-vaccinated and unvaccinated M+/I- children at approximately 18 months of age

Protein	M+/I- vaccinated *GMT (95 % CI)	M+/I- unvaccinated *GMT (95 % CI)	**P adj.	Uncolonized vaccinated GMT (95 % CI)	Uncolonized unvaccinated GMT (95 % CI)	p	Colonized vaccinated *GMT (95 % CI)	Colonized unvaccinated *GMT (95 % CI)	p
PspA	40 (33-49)	111 (91-135)	**< 0.0001**	41 (29-58)	121 (81-181)	**< 0.0001**	40 (31-51)	108 (85-135)	**< 0.0001**
PspC	78 (58-105)	113 (95-134)	**0.029**	55 (29-107)	93 (64-135)	0.15	92 (67-127)	122 (101-148)	0.11
PsaA	122 (111-135)	180 (155-209)	**< 0.0001**	118 (101-138)	159 (117-215)	0.10	124 (109-140)	189 (159-225)	**< 0.0001**
PdB	81 (64-103)	108 (86-135)	0.11	55 (31-97)	103 (65-162)	0.08	98 (79-122)	109 (84-143)	0.56
SP 2027	512 (415-633)	191 (125-290)	***< 0.0001***	500 (362-690)	332 (159-690)	0.32	518 (392-685)	153 (92-254)	***< 0.0001***
SP 0609	53 (40-70)	171 (140-209)	**< 0.0001**	57 (37-87)	203 (141-292)	**< 0.0001**	51 (36-74)	160 (125-204)	**< 0.0001**
SP 0082	224 (160-314)	101 (87-118)	***< 0.0001***	175 (82-375)	122 (98-152)	0.32	252 (176-361)	94 (77-114)	***< 0.0001***
SP 0749	173 (123-242)	87 (62-122)	***0.008***	306 (169-552)	76 (45-128)	***0.001***	131 (88-196)	91 (59-141)	0.28
SP 0498 (LytB)	72 (58-88)	155 (132-181)	**< 0.0001**	56 (35-91)	184 (142-238)	**< 0.0001**	81 (65-100)	144 (119-176)	**< 0.0001**
IgA1-proteinase	48 (33-69)	121 (97-151)	**< 0.0001**	42 (21-86)	141 (90-221)	**0.004**	51 (33-78)	114 (88-148)	**0.001**
PpmA	99 (78-127)	42 (32-56)	***< 0.0001***	120 (77-187)	40 (23-71)	***0.004***	91 (67-123)	43 (31-60)	***0.002***
SlrA	80 (67-97)	96 (79-116)	0.21	76 (57-103)	114 (76-171)	0.12	83 (65-104)	90 (72-111)	0.68
SP 2216 (PcsB)	143 (98-209)	142 (121-166)	0.92	82 (33-206)	132 (92-190)	0.29	188 (133-265)	146 (123-173)	0.18
SP 1723 (StkP)	25 (17-35)	34 (26-45)	0.14	21 (9-44)	37 (22-62)	0.20	27 (17-40)	33 (24-46)	0.37
SP 2194	90 (70-116)	150 (124-181)	**0.001**	79 (56-113)	214 (156-293)	**< 0.0001**	96 (69-135)	131 (104-164)	0.11

*****GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **for differences between overall vaccinated and un-vaccinated children, p-values were adjusted for nasopharyngeal pneumococcal colonization status. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMTs in PCV-7 unvaccinated children significantly higher than in –vaccinated children are highlighted in red whilst GMTs in PCV-7 unvaccinated children significantly lower than in –vaccinated children are highlighted in blue.

Table 6.8: Comparison of the GMT's against the 15 pneumococcal proteins between PCV-vaccinated and unvaccinated M+/I- children at approximately 24 months of age

Protein	M+/I- vaccinated *GMT (95 % CI)	M+/I- unvaccinated *GMT (95 % CI)	**P adj.	Uncolonized vaccinated GMT (95 % CI)	Uncolonized unvaccinated GMT (95 % CI)	p	Colonized vaccinated *GMT (95 % CI)	Colonized unvaccinated *GMT (95 % CI)	p
PspA	67 (54-82)	119 (101-140)	**< 0.0001**	86 (64-115)	123 (79-189)	0.14	60 (46-79)	118 (99-141)	**< 0.0001**
PspC	107 (74-155)	139 (126-154)	0.14	134 (100-181)	115 (80-166)	0.58	98 (58-163)	147 (136-159)	0.08
PsaA	260 (228-297)	137 (116-162)	***< 0.0001***	269 (244-297)	124 (88-177)	***< 0.0001***	257 (214-308)	141 (117-172)	***< 0.0001***
PdB	98 (78-125)	131 (111-156)	**0.037**	101 (69-148)	124 (82-189)	0.33	97 (72-131)	133 (110-161)	0.07
SP 2027	383 (308-476)	449 (310-648)	0.10	361 (247-527)	600 (455-790)	**0.034**	391 (298-515)	446 (365-544)	0.44
SP 0609	77 (61-99)	64 (50-81)	0.26	69 (56-85)	72 (43-123)	0.85	81 (58-113)	61 (46-81)	0.19
SP 0082	310 (199-484)	146 (125-170)	***0.001***	403 (242-670)	117 (67-205)	***0.002***	280 (154-508)	155 (137-177)	***0.034***
SP 0749	149 (105-212)	99 (80-121)	***0.050***	201 (94-430)	83 (50-135)	***0.051***	133 (89-197)	106 (84-134)	0.31
SP 0498 (LytB)	94 (66-134)	187 (159-218)	**< 0.0001**	105 (56-140)	179 (123-261)	0.13	90 (57-140)	189 (158-225)	**0.001**
IgA1-proteinase	76 (50-116)	84 (61-115)	0.76	80 (42-151)	81 (43-152)	0.92	75 (44-128)	84 (58-123)	0.71
PpmA	120 (83-174)	87 (65-118)	0.22	140 (75-263)	104 (52-206)	0.58	113 (71-179)	83 (59-117)	0.27
SlrA	101 (81-125)	123 (101-149)	0.17	109 (74-162)	112 (67-186)	0.85	98 (75-128)	126 (102-156)	0.14
SP 2216 (PcsB)	216 (140-335)	197 (169-230)	0.70	261 (187-363)	163 (98-271)	0.13	201 (110-367)	208 (180-240)	0.90
SP 1723 (StkP)	55 (37-81)	46 (37-57)	0.46	68 (44-106)	37 (24-58)	0.06	50 (30-84)	49 (38-63)	0.96
SP 2194	70 (57-86)	128 (100-162)	**< 0.0001**	69 (51-95)	111 (56-220)	0.12	71 (54-92)	133 (103-170)	**0.001**

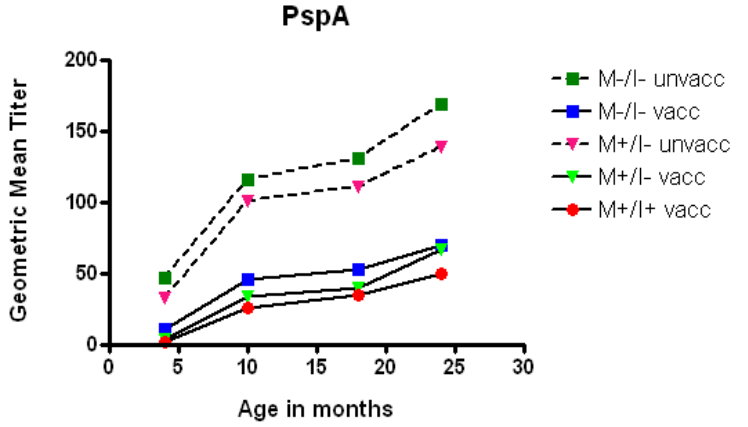
*GMT's with 95% confidence interval were reported in arbitrary units per ml (U/ml), **for differences between overall vaccinated and un-vaccinated children, p-values were adjusted for nasopharyngeal pneumococcal colonization status. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected children. GMTs in PCV-7 unvaccinated children significantly higher than in –vaccinated children are highlighted in red whilst GMTs in PCV-7 unvaccinated children significantly lower than in –vaccinated children are highlighted in blue.

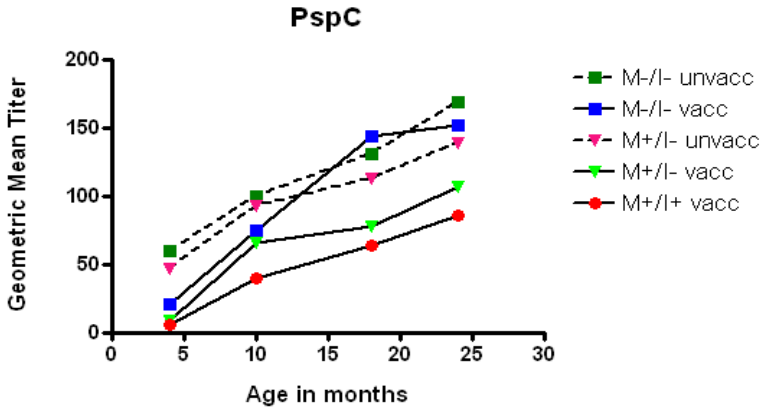
5. 5 Kinetics of antibodies against pneumococcal proteins in M-/I-, M+/I- and M+/I+ children in relation to age and vaccination status

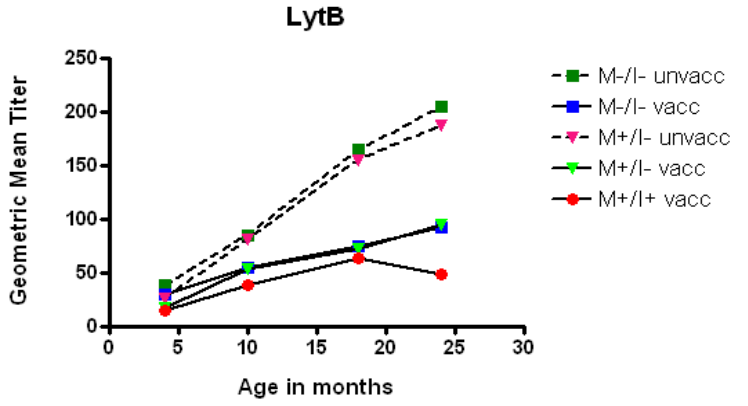
Antibody titers against select pneumococcal proteins differed by age, even when stratified by PCV-vaccination and HIV-infection status (figure 7). Antibody production against antigens which can be described as "pneumococcus specific", i.e. homologous proteins that have not been identified in related bacteria; such as Ply, and choline-binding proteins PspA, PspC, and LytB showed linear increases in titers with age as shown in figure 7a. The GMTs to these antigens at 24 months (refer to tables 6.4 and 6.8) were significantly higher than at 4 month (refer to tables 6.1 and 6.5) and 10 month (refer to tables 6.2 and 6.6) age-group time-points.

In addition, the kinetics of antibody increases differed with respect to PCV-vaccination status for these antigens. Between 4 to 24 months, PCV vaccination was associated with lower antibody titers against the choline-binding proteins: PspA, PspC and LytB (figure 7a) and the secreted protein PdB (figure 7b) as well as the integral membrane protein, StkP (figure 7c). Conversely, PCV-vaccination was associated with higher antibody titers against SP 0082 (figure 7b) and PpmA (figure 7c2) at all four time-points. In addition, HIV-infected children consistently had lower antibody titers to PspA, PspC, LytB (figure 7a) and PdB (figure 7d) and StkP (figure 7c3) compared to HIV-uninfected children. These trends were, to some extent, followed for the antigens IgA1-proteinase (figure 7b), and PcsB (figure 7d).

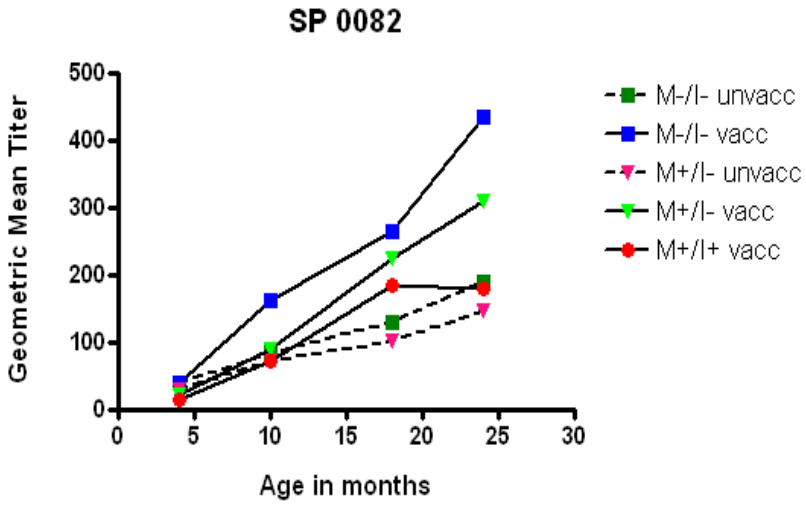
a) Choline-binding proteins:

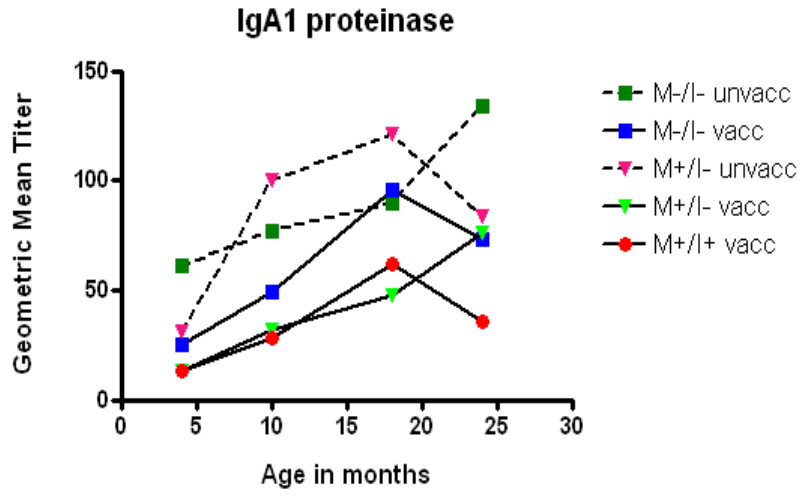






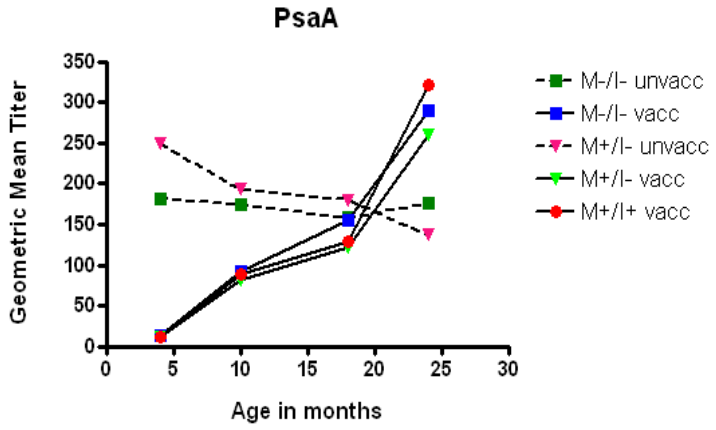
b) Sortase-dependent proteins:

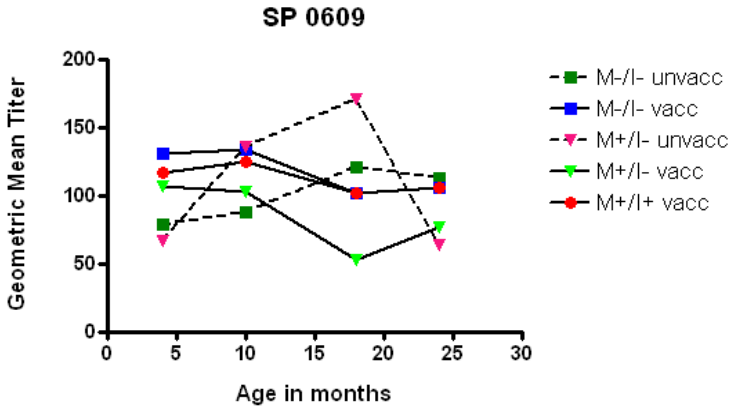


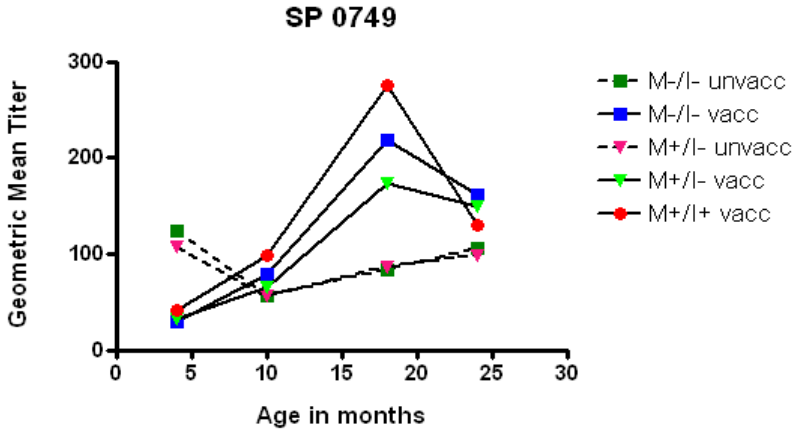


c) Lipoproteins

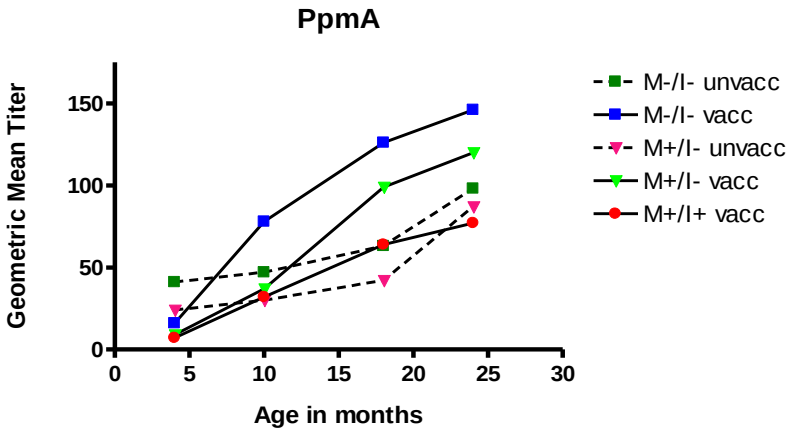
1) ABC transporters:

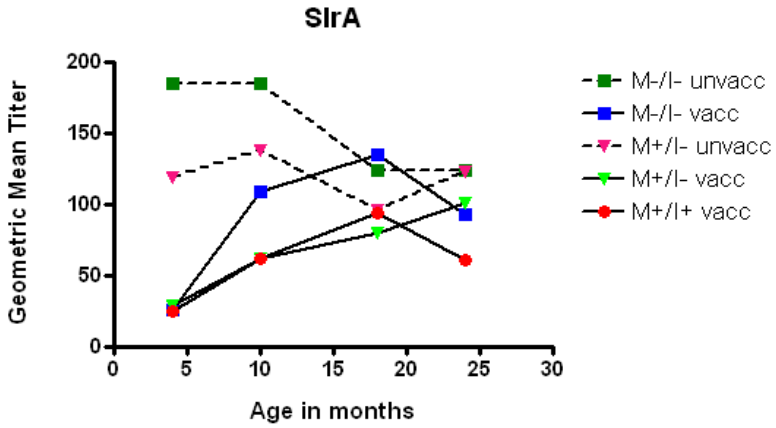




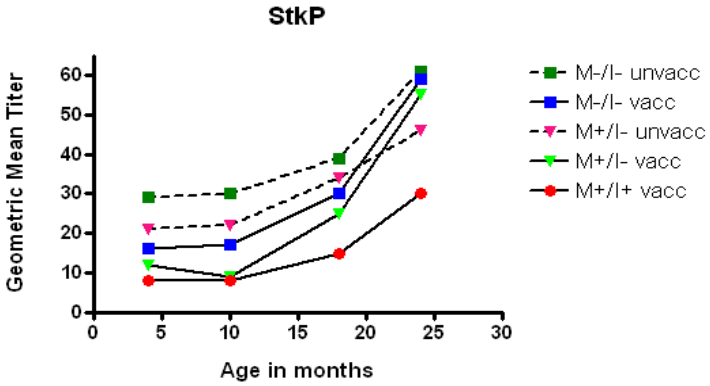


2) Cell wall associated lipoproteins (Non ABC transporters):

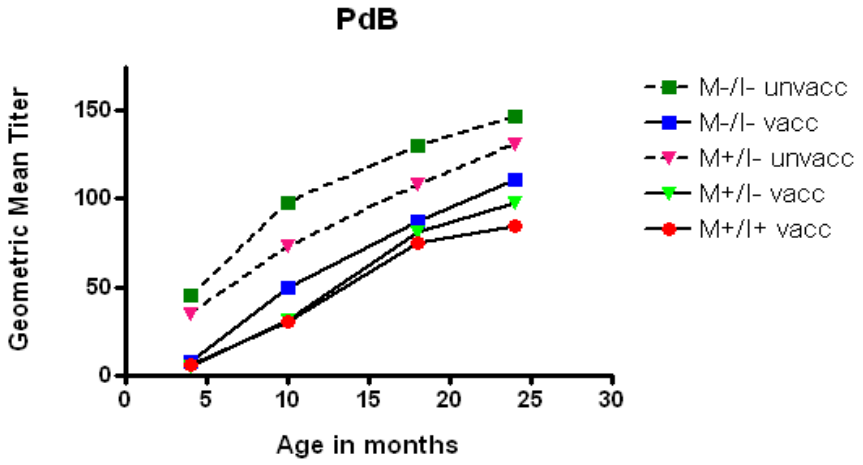


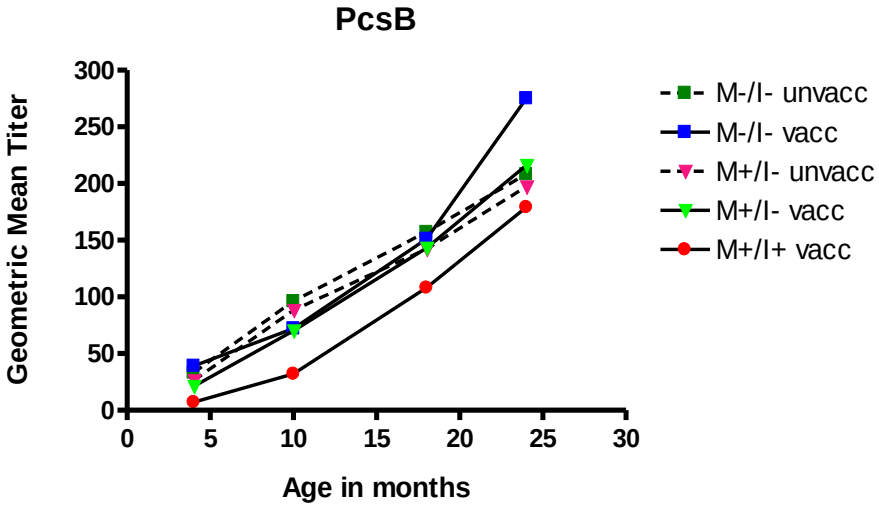


3) Integral membrane lipoproteins:



d) Secreted proteins:





e) Other pneumococcal proteins:

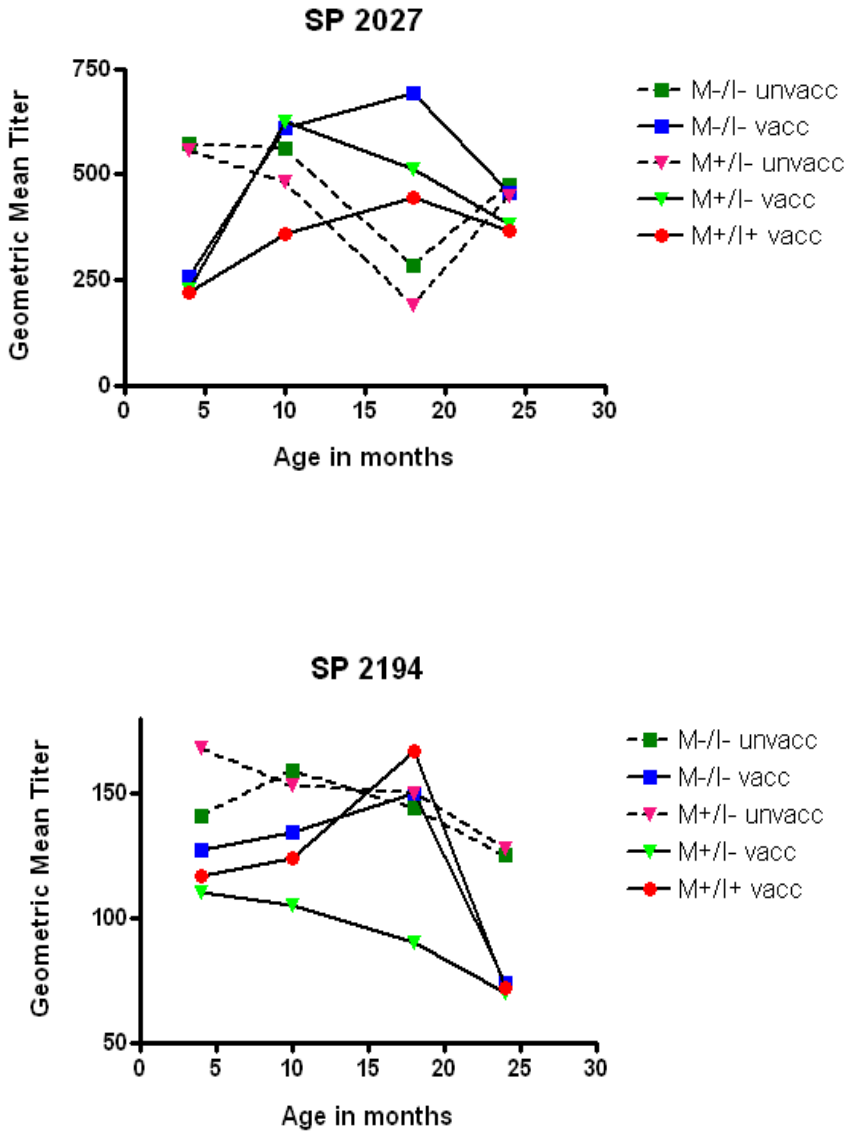


Figure 7: Kinetics of antibodies against pneumococcal proteins in PCV-vaccinated and unvaccinated children at ages 4, 10, 18 and 24 months. M-/I-: HIV unexposed, uninfected, M+/I-: HIV exposed, uninfected and M+/I+: HIV exposed, infected infants. GMT's were reported in arbitrary units per ml (U/ml

5.6 Prevalence of antibodies against pneumococcal proteins in PCV-9 vaccinated and unvaccinated children aged between 4 and 7 years

The median age of children studied in this group was 5.61 years (range: 3.89 to 7.09 years) and they were stratified according to HIV infection status, PCV-9 vaccination history during infancy and nasopharyngeal pneumococcal-colonization status. Samples from a total of 286 children; including 212 HIV uninfected (97 PCV vaccinated and 115 PCV unvaccinated) and 74 HIV infected (28 PCV-vaccinated and 46 PCV-unvaccinated) were analyzed. PCV-9 vaccinated children were immunized at 6, 10 and 14 weeks of age. The prevalence of pneumococcal colonization in HIV-infected children was higher compared to HIV-uninfected counterparts (73 % [54/74] in HIV infected children and 56 % [107/212] in HIV uninfected children, p = 0.001).

Table 7: Demographic features of the PCV9-vaccinated and -unvaccinated children aged between 4 to 7 years. Children were stratified according to vaccination, HIV and pneumococcal colonization status at the time of blood sampling.

Study group	*Total number of subjects N_T)*	*Median age (years)*	*Overall colonization*	*PCV-vaccinated (N_T)*	*PCV-unvaccinated (N_T)*
HIV-uninfected children	212	6 (4-7)	(107/212) 56 %	97	115
HIV infected children	74	6 (4-7)	(54/74) 73 %	28	46
Total number of subjects tested	286			125	161

*Nasopharyngeal swabs from children were collected at the same time-point when blood was drawn. N_T: total number of children whom samples were available for at the time-point (visit).

5.6.1 Comparison between HIV-uninfected children and HIV-infected children

Comparison of antibody titers between HIV-infected and -uninfected children was adjusted for pneumococcal nasopharyngeal colonization and PCV vaccination status. Differences in antibody titers between HIV-infected and -uninfected children were antigen specific. HIV-uninfected children had significantly higher antibody titers against antigens not associated with the cell membrane such as PdB, which are released through cell lysis, and antigens bound to the cell wall through choline and LPXTG peptidoglycan binding domains (as shown in Table 6, highlighted in bold/red). These included PspA (180 vs 63; $p < 0.0001$), PspC (176 vs 120, $p < 0.0001$), PdB (223 vs 178; $p = 0.040$), SP 0082 (519 vs 334, $p < 0.0001$), LytB (193 vs 65; $p < 0.0001$), IgA1-proteinase (101 vs 38; $p < 0.0001$) and PscB (687 vs 372; $p < 0.0001$). In contrast, membrane associated proteins such as PsaA (357 vs 198; $p < 0.0001$), SP 2027 (513 vs 361; $p = 0.007$) PpmA (214 vs 136; $p = 0.002$) and SlrA (202 vs 133; $p = 0.050$) were associated with lower antibody titres in HIV-uninfected children compared to -infected children (Table 7.2, highlighted in bold italics/blue).

The reverse cumulative distribution plots in figure 8 demonstrate the distribution of antibody titers against the pneumococcal proteins with respect to HIV and nasopharyngeal pneumococcal-colonization status. For HIV-infected children, the antibody distribution curves calculated for all cell-wall associated and “secreted” proteins (PspA, PspC, SP 0082, PdB, PcsB, LytB, IgA1-proteinase, StkP) were consistently below

the antibody distribution curves calculated for HIV-uninfected children; conversely, the antibody distribution curves calculated for all membrane associated lipoproteins and ABC transporters (PsaA, SlrA, PpmA, SP 0749, SP 0609) for HIV-infected children were consistently above the antibody distribution curves calculated for HIV-uninfected children.

5.6.2 Comparison between pneumococcal-colonized and –uncolonized children

Comparisons of GMTs between pneumococcal-colonized and –uncolonized children were adjusted for PCV-vaccination and HIV infection status. Concurrent colonization status was poorly associated with antibody titers in this age group. Higher antibody titers against PdB (235 vs 192; p = 0.048) and PcsB (688 vs 516; p = 0.013) were observed in children not colonized with pneumococci compared to pneumococcal-colonized children as shown in Table 7.2 (highlighted in bold italics/blue). No differences in antibody titers against other proteins were observed between children who were colonized with pneumococci and children who were not colonized.

For PcsB and PdB, apparent differences were observed between the distribution curves calculated for HIV-infected children, with pneumococcal-uncolonized children consistently having higher antibody titers compared to -colonized children (Figure 8d). For other proteins, the distribution curves calculated for children who were colonized with pneumococci were similar and almost parallel and those not colonized, regardless of HIV infection status.

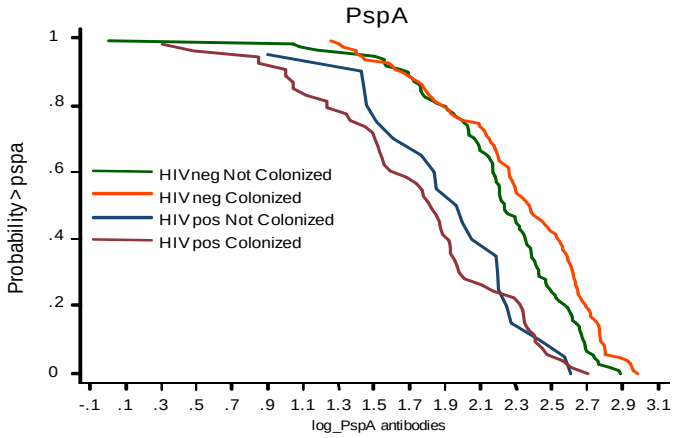
No differences in antibody titers against all proteins were observed between PCV-vaccinated and –unvaccinated children (adjusting for HIV infection status and pneumococcal nasopharyngeal colonization status).

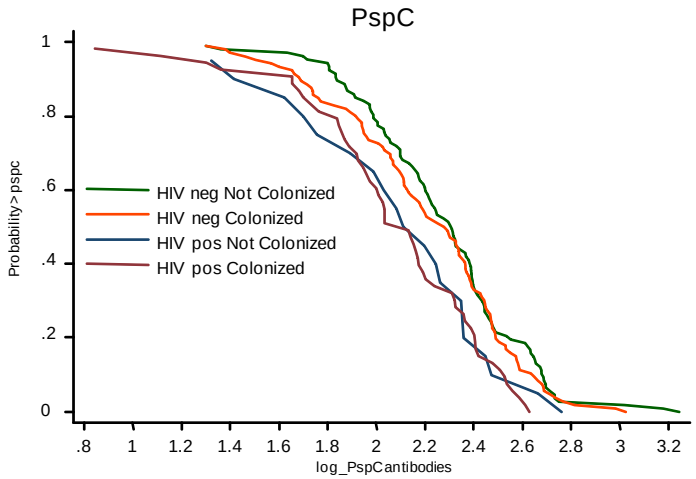
Table 7.2: Geometric mean titers to the 15 pneumococcal proteins in children aged between 4 to 7 years. Children were stratified according to HIV exposure, pneumococcal colonization status and vaccination history.

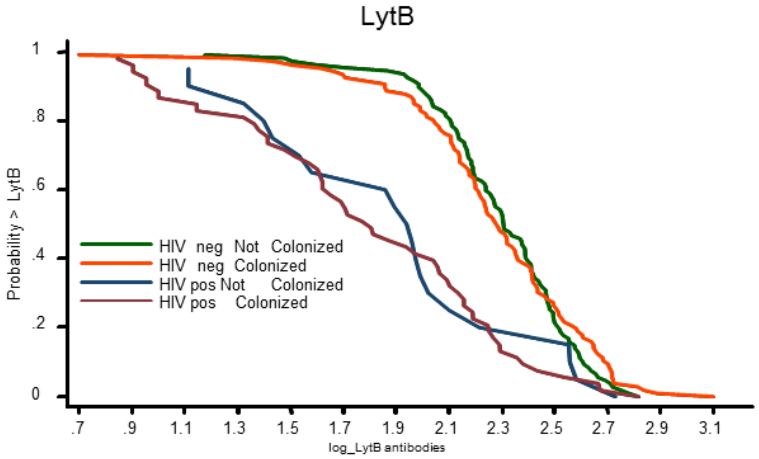
Protein	Overall *GMT (95 % CI)	HIV uninfected *GMT (95 % CI)	HIV infected *GMT (95 % CI)	**P (adjusted)	Vaccinated *GMT (95 % CI)	Unvaccinated *GMT (95 % CI)	**P (adjusted)	Colonized *GMT (95 % CI)	Uncolonized *GMT (95 % CI)	**P (adjusted)
PspA	137 (120-157)	180 (156-206)	63 (48-84)	**< 0.0001**	129 (103-162)	144 (122-170)	0.19	133 (109-162)	143 (119-172)	0.42
PspC	159 (145-176)	176 (158-196)	120 (98-147)	**< 0.0001**	158 (138-181)	160 (140-184)	0.66	146 (128-167)	178 (154-204)	0.18
PsaA	230 (209-254)	198 (179-219)	357 (292-436)	***< 0.0001***	241 (208-279)	223 (196-253)	0.20	246 (217-279)	210 (181-244)	0.52
PdB	210 (193-229)	223 (203-245)	178 (148-214)	**0.040**	194 (170-221)	224 (200-251)	0.06	192 (171-215)	235 (206-267)	***0.048***
SP 2027	396 (354-443)	361 (320-408)	513 (397-665)	***0.007***	368 (311-435)	419 (360-488)	0.32	406 (344-478))	386 (331-448)	0.88
SP 0609	101 (88-115)	96 (83-111)	116 (87-154)	0.19	99 (81-121)	102 (85-122)	0.85	101 (84-120)	101 (83-123)	0.76
SP 0082	463 (428-502)	519 (479-562)	334 (276-404)	**< 0.0001**	480 (424-542)	451 (406-501)	0.45	458 (409-512)	469 (419-525)	0.81
SP 0749	150 (133-169)	141 (122-163)	179 (145-221)	0.07	150 (124-181)	150 (128-175)	0.94	153 (131-180)	147 (122-177)	0.99
SP 0498 (LytB)	146 (130-164)	193 (175-213)	65 (49-85)	**< 0.0001**	132 (110-159)	157 (136-182)	0.15	130 (110-154)	172 (149-198)	0.31
IgA1-proteinase	78 (67-92)	101 (85-119)	38 (28-53)	**< 0.0001**	72 (57-91)	84 (68-104)	0.13	71 (57-89)	90 (72-112)	0.62
PpmA	153 (136-172)	136 (119-157)	214 (172-266)	***0.002***	159 (133-191)	148 (126-174)	0.37	162 (139-188)	142 (117-172)	0.63
SlrA	148 (127-173)	133 (110-159)	202 (153-268)	***0.050***	146 (117-183)	149 (121-185)	0.91	167 (139-202)	126 (97-163)	0.17
SP 2216 (PcsB)	586 (541-636)	687 (640-738)	372 (302-457)	**< 0.0001**	559 (484-644)	609 (556-667)	0.08	516 (455-548)	688 (630-750)	***0.013***
SP 1723 (StkP)	192 (167-220)	188 (160-254)	202 (160-254)	0.51	184 (149-226)	198 (166-237)	0.58	170 (142-204)	217 (179-270)	0.06
SP 2194	82 (73-92)	77 (67-89)	95 (77-118)	0.21	77 (65-91)	86 (73-101)	0.47	85 (72-99)	78 (65-94)	0.75

Children in this group were randomized to receive either PCV-9 or placebo at 6, 10 and 14 weeks of age. Blood was obtained from children at the time of their first visit. *GMT's were reported in arbitrary units per ml (U/ml), with 95% confidence interval. **P-values for **HIV** were adjusted for colonization and vaccination statuses, for **colonization**, p values were adjusted for vaccination status and HIV exposure and for **vaccination**, p-values were adjusted for colonization status and HIV exposure.

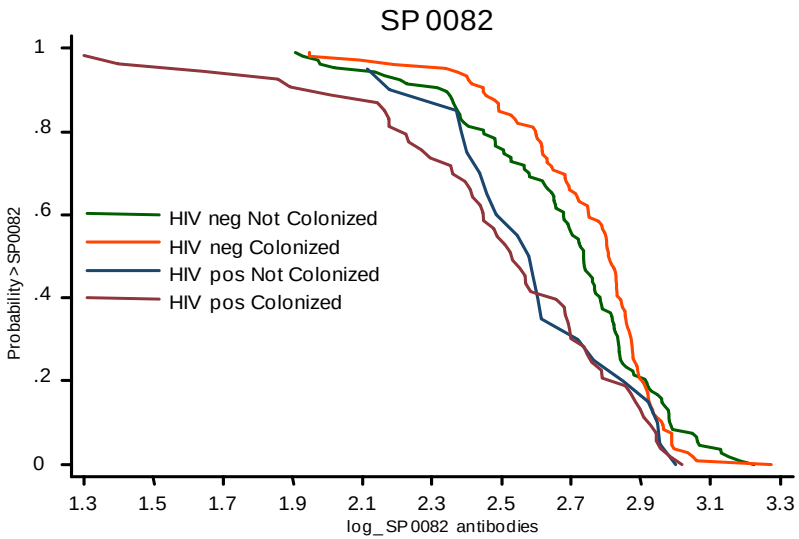
a) Choline-binding proteins: Children aged between 4 to 7 years colonization data

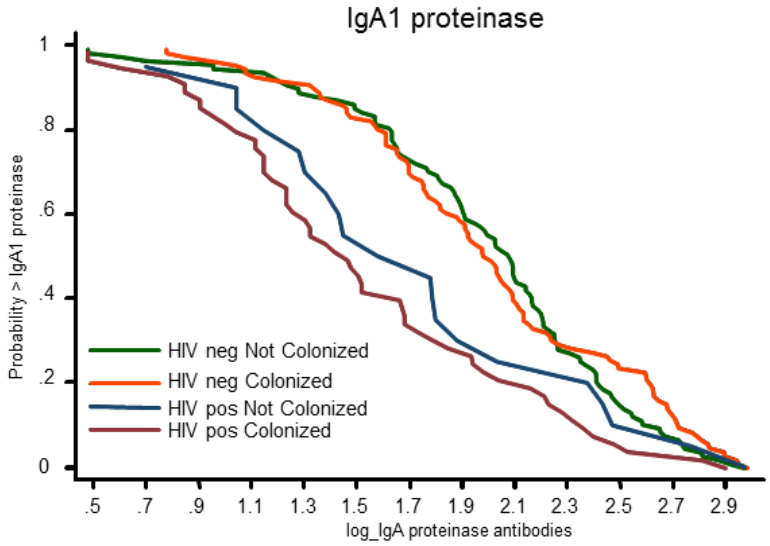






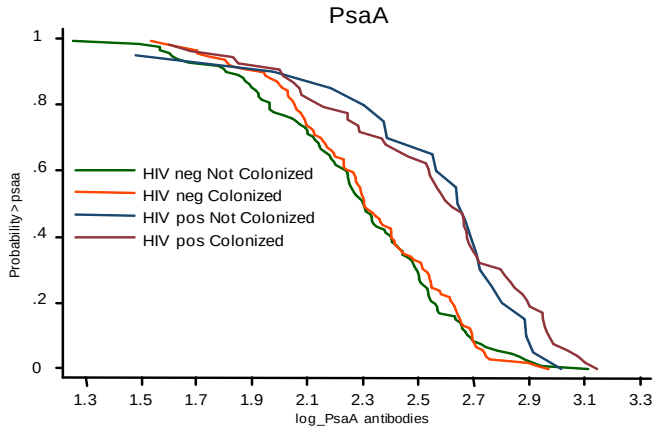
b) Sortase-dependent proteins:

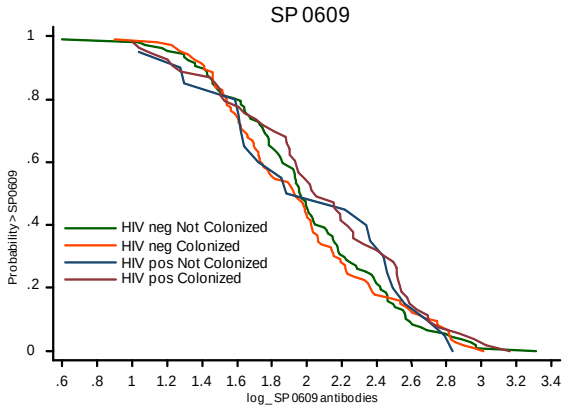


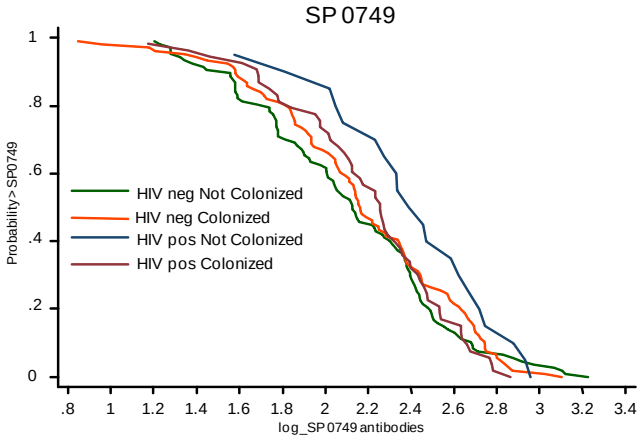


c) Lipoproteins:

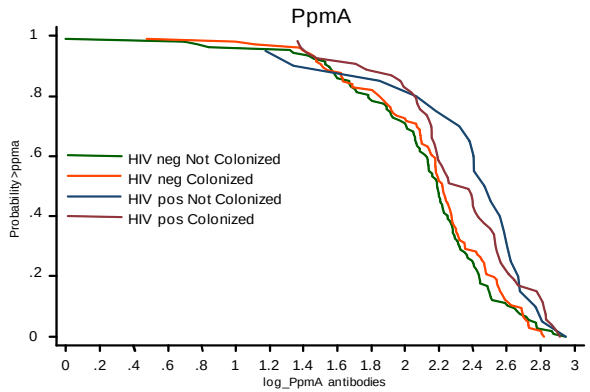
1.) *ABC* transporters:

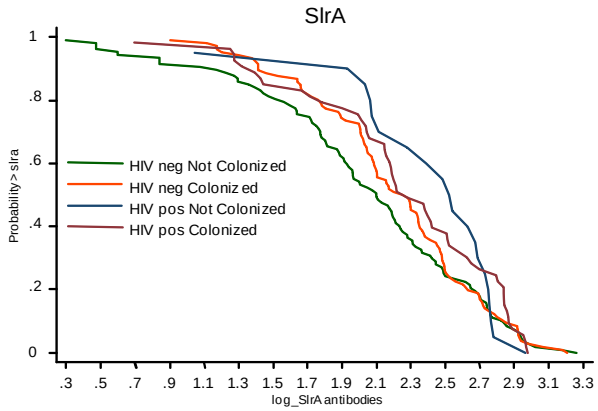




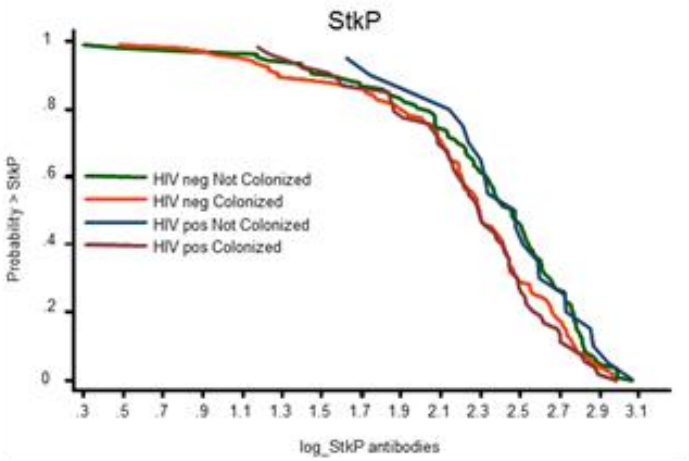


2.) Non *ABC* transporters (cell-wall associated lipoproteins):

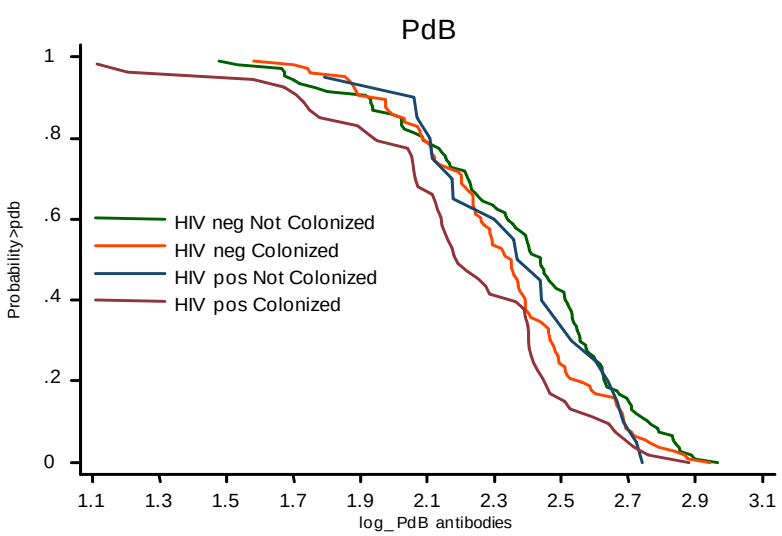


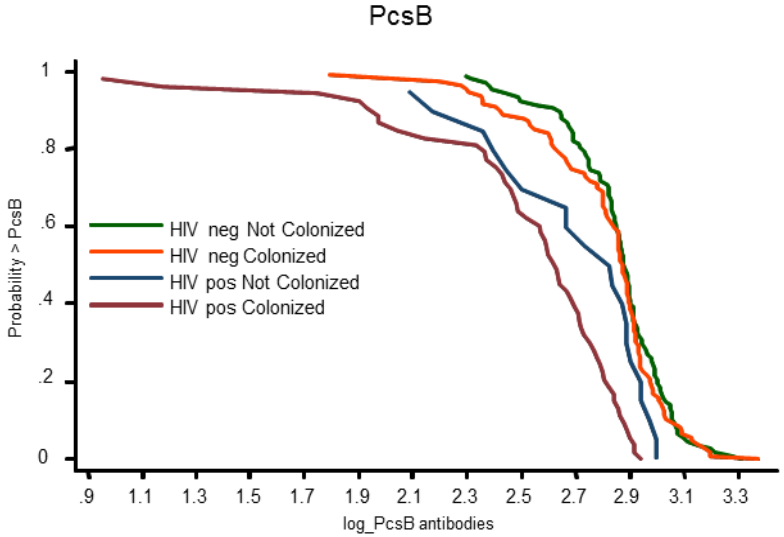


3.) Integral membrane lipoprotein(s):



d) Secreted proteins:





e) Other pneumococcocal proteins:

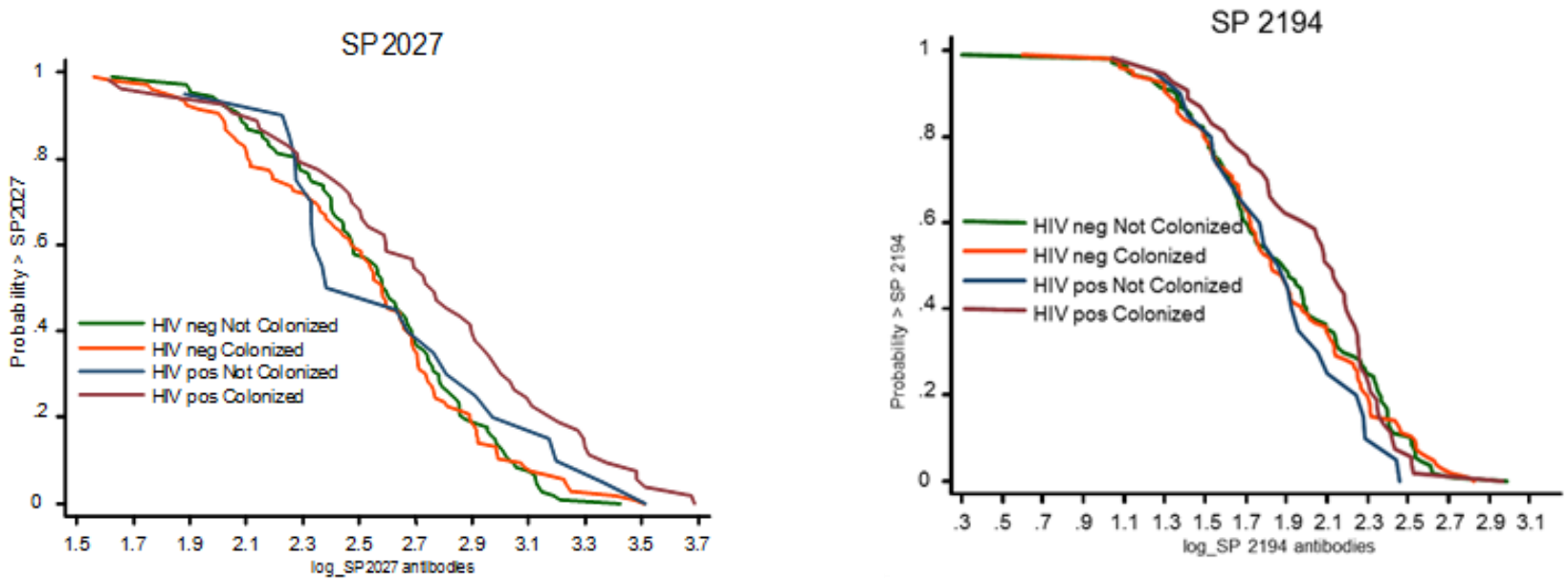


Figure 8: Reverse cumulative distribution curves of IgG antibodies against the pneumococcal proteins measured in HIV uninfected and HIV infected children aged between 4 to 7 years for comparison of the pneumococcal-colonized and uncolonized groups.

5.7 Comparison of antibody titers against pneumococcal proteins in PCV-vaccinated and HIV uninfected children between two years of age and older children

Antibodies against PspA, PspC, PdB, SP 0082, LytB, PpmA, PcsB, and StkP showed increases in titer with respect to increasing age, and either continued peaking in the 4 to 7 year old (PspA, PdB, SP 0082, LytB, PcsB and StkP) or declined by 24 months and remained constant until 4 to 7 years of age (PspC and PpmA). Antibodies against IgA1-proteinase declined by 24 months of age and had already reached levels observed in older children (4 to 7 years) by 18 months of age (Figure 9).

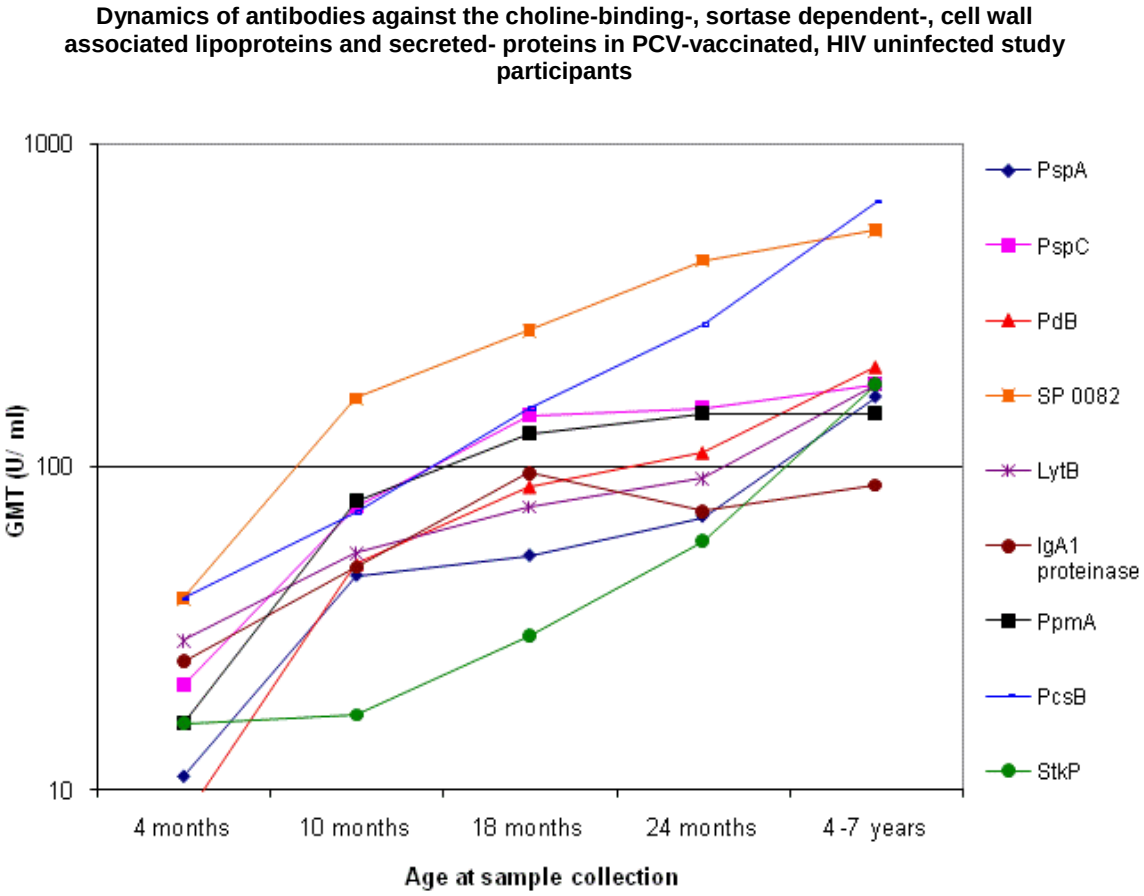


Figure 9: Natural development of antibodies against the choline-binding, sortase-dependent, secreted and cell-wall associated pneumococcal proteins in PCV-vaccinated, HIV uninfected children until two years of age and children between 4-7 years of age. Children aged between 4 to 24 months were vaccinated with PCV-7 and children between 4 to 7 years were vaccinated with PCV-9. GMT = Geometric Mean Titer.

Antibody titers against SP 2027, SP 0609, and SP 2194 did not show any relationship to age as shown in figure 10. Antibody titers against PsaA, SP 0749 and SlrA showed an increase with respect to increasing age and either peaked at 18 months (SP 0749 and SlrA) or at 24 months (PsaA) then declined until 4 to 7 years of age.

Dynamics of antibodies against the ABC transporter lipoproteins, cell-wall associated lipoproteins and other pneumococcal protein antigens in PCV-vaccinated HIV uninfected study participants

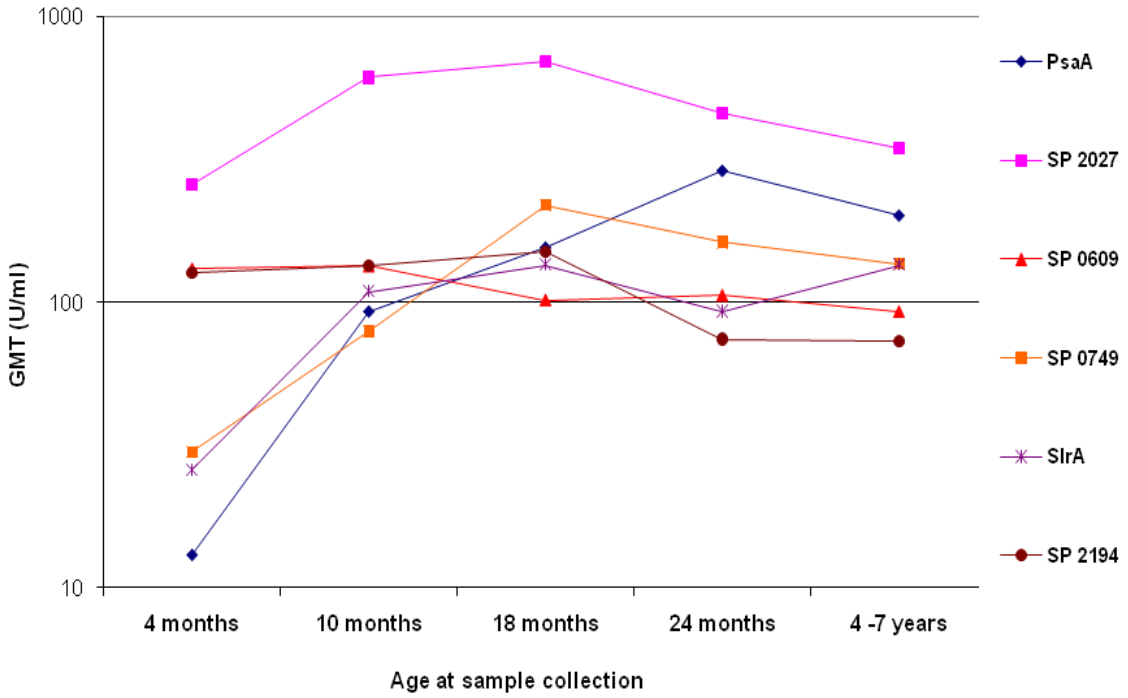


Figure 10: Natural development of antibodies against the ABC transporter lipoproteins, cell-wall associated lipoproteins and other pneumococcal proteins in PCV-vaccinated HIV children until two years of age and children between 4-7 years of age. Children aged between 4 to 24 months were vaccinated with PCV-7 and children between 4 to 7 years were vaccinated with PCV-9. GMT = Geometric Mean Titer.

5.8 Prevalence of antibodies against pneumococcal proteins in HIV uninfected and HIV infected women

The median age of the women was 27 years (range: 15 to 48) and they were stratified according to pneumococcal colonization status and HIV infection status. These women were the mothers of the PCV-7 vaccinated and -unvaccinated children less than two years of age. Thirty five percent (197/565) of the women were HIV-uninfected and 65% (368/565) were HIV-infected. Nasopharyngeal and oropharyngeal swabs were collected and analyzed for pneumococcal colonization from women at the blood sampling time-point. The prevalence of pneumococcal colonization was similar between HIV-uninfected women [12 % (24/197)] and HIV-infected women [15 % (55/368), p = 0.43] at the blood sampling time-point as shown in Table 8.

Table 8: Demographic features of women at the blood withdrawal time-point stratified by HIV exposure and pneumococcal colonization status

Study group	*Total number of samples (N_T)*	*Median age (years)*	*Overall colonization*	*HIV infected (N_T)*	*Colonized HIV infected*	*HIV uninfected (N_T)*	*Colonized HIV uninfected*
Mothers of the PCV-vaccinated and PCV unvaccinated children	**565**	**27 (15-48)**	**(79/565) 14 %**	**368**	**(55/368) 15 %**	**197**	**(24/197) 12 %**

*Nasopharyngeal and oropharyngeal sampling of women for pneumococcal colonization was done at the blood withdrawal time. N_T: total number of women who were present at each time-point

5.8.1 Comparison of antibody titers in HIV-uninfected and HIV-infected women in relation to pneumococcal colonization

Higher antibody titers against PspC (211 vs 153; p = 0.029)], SP 2027 [(141 vs 75; p = 0.009), SP 0082 (357 vs 204; p = 0.008), LytB (191 vs 127; p = 0.048) and PcsB (248 vs 163; p = 0.020) were evident in HIV-uninfected women not colonized with pneumococci compared to pneumococcal-colonized women (see table 9). In contrast, lower antibody titers against PsaA 126 vs 200; p = 0.010) were observed in HIV-uninfected pneumococcal-uncolonized women compared to –colonized women (table 9). No differences in antibody titers against all proteins were evident in HIV infected women when comparing pneumococcal-colonized women to women who were not colonized with pneumococci.

The reverse distribution plots in figure 11 indicate the distribution of antibody titers produced by women in relation to HIV infection and pneumococcal colonization status. In HIV uninfected women, distribution curves calculated for pneumococcal-uncolonized women against PspC, SP 2027 and SP 0082 were parallel and above the distribution curves calculated for women who were colonized with pneumococci. In HIV infected women, distribution curves calculated for pneumococcal-colonized women were similar to the distribution curves calculated for women who were not colonized with pneumococci.

Adjusting for HIV infection status, differences in antibody titers were only evident for PdB and SP 2027. These included higher antibody titers against PdB (223 vs 157; p = 0.010) and SP 2027 (195 vs 135; p = 0.039) were observed in women who were not colonized compared to those colonized with pneumococci (table 9). For PdB (figure 11d) and SP 2027 (figure 11c), the distribution curves were almost parallel but a deviation was observed around 100 U/ml, where the distribution curve calculated for women who were not colonized indicated a higher proportion above this threshold compared to women who were colonized with pneumococci.

5.8.2 Comparison of antibody titers in HIV infected and HIV uninfected women

Comparisons of the GMTs between HIV-uninfected and -infected women were adjusted for pneumococcal colonization status. HIV-uninfected women had significantly higher antibody titers than HIV-infected women against antigens that were associated with the cell wall, either through choline-binding domains or LPXTG motifs, and 'secreted' proteins. These included higher antibody titers against PspA (162 vs 103, $p < 0.0001$), PspC (202 vs 138; $p < 0.0001$), PdB (249 vs 195; $p = 0.015$), SP 0082 (332 vs 257, $p = 0.015$), LytB (181 vs 116, $p < 0.0001$), IgA1-proteinase (307 vs 212, $p < 0.0001$), and PcsB (235 vs 132, $p < 0.0001$). Exceptions to this were the membrane anchored proteins PpmA (278 vs 187, $p < 0.0001$) StkP (142 vs 80, $p < 0.0001$), antibodies against which were also elevated in HIV-uninfected women. Lower antibody titers were observed in HIV- uninfected women compared to -infected women against the membrane associated proteins: SP 2027 (130 vs 222; $p < 0.0001$) and SP 0749 (140 vs 204; $p = 0.004$ as indicated in Table 10, (highlighted in bold italic/blue).

The distribution curves calculated for PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, PcsB, and StkP in HIV uninfected women were consistently higher than the distribution curves calculated in HIV-infected women, whilst the distribution curves calculated for SP 2027 and SP 0749 in HIV-uninfected women were lower than those observed in HIV-infected women (Figure 11).

Table 9: Geometric mean titers (with 95 % confidence interval) of antibodies against pneumococcal proteins in women, stratified by HIV and pneumococcal colonization status

Protein	**Overall** GMT (95 % CI)	**Colonized** GMT (95 % CI)	**Uncolonized** GMT (95 % CI)	****p adjusted**	**HIV uninfected, GMT (95 % CI**			**HIV, infected, GMT (95 % CI)**		
					Colonized	**Uncolonized**	**p**	**Colonized**	**Uncolonized**	**p**
PspA	120 (109-133)	117 (93-148)	120 (108-135)	0.96	165 (115-236)	162 (137-192)	0.93	101 (75-135)	103 (90-118)	0.91
PspC	158 (146-170)	140 (117-168)	161 (148-175)	0.26	153 (121-194)	211 (181-245)	***0.029***	134 (106-171)	139 (126-152)	0.81
PsaA	127 (114-142)	142 (113-178)	125 (110-141)	0.41	200 (146-272)	126 (103-152)	**0.010**	122 (91-163)	125 (107-145)	0.89
PdB	212 (194-233)	157 (119-207)	223 (203-246)	***0.010***	174 (99-308)	262 (222-310)	0.17	150 (109-206)	204 (182-230)	0.07
SP 2027	185 (162-211)	135 (96-189)	195 (169-225)	***0.039***	75 (49-115)	141 (112-178)	***0.009***	175 (112-272)	232 (193-278)	0.24
SP 0609	108 (97-120)	94 (70-126)	110 (98-124)	0.32	118 (68-204)	119 (95-148)	0.99	85 (59-121)	106 (92-122)	0.24
SP 0082	281 (254-309)	233 (184-295)	289 (260-322)	0.15	204 (139-299)	357 (293-434)	***0.008***	248 (184-335)	258 (228-292)	0.81
SP 0749	179 (159-202)	189 (135-264)	178 (156-202)	0.80	114 (71-181)	145 (122-172)	0.33	237 (154-366)	198 (166-237)	0.45
SP 0498 (LytB)	135 (124-147)	113 (91-141)	139 (127-153)	0.13	127 (88-185)	191 (161-226)	***0.048***	108 (81-142)	117 (105-131)	0.56
IgA1-proteinase	241 (221-263)	241 (194-300)	241 (219-265)	0.91	274 (206-363)	312 (269-361)	0.41	227 (170-305)	210 (186-237)	0.61
PpmA	215 (194-238)	218 (164-288)	214 (192-240)	0.82	247 (167-365)	283 236-340)	0.52	206 (142-299)	184 (161-211)	0.58
SlrA	192 (174-213)	163 (124-214)	198 (177-220)	0.21	194 (135-278)	211 (177-253)	0.67	151 (105-218)	190 (166-219)	0.24
SP 2216 (PcsB)	161 (145-179)	151 (125-181)	163 (144-184)	0.74	163 (123-217)	248 (199-309)	***0.020***	146 (115-185)	129 (113-149)	0.40
SP 1723 (StkP)	97 (86-111)	94 (68-129)	98 (85-113)	0.90	138 (77-249)	142 (111-182)	0.93	79 (53-116)	80 (68-95)	0.93
SP 2194	93 (84-102)	97 (74-128)	92 (83-102)	0.67	104 (62-173)	100 (84-119)	0.88	94 (67-132)	88 (78-100)	0.70

GMT's were reported in arbitrary units per ml (U/ml), with 95% confidence interval. *Nasopharyngeal swabs from children were collected at the time-point when blood was withdrawn. Swabs were stored in STGG medium at -80°C. **for comparison between overall pneumococcal-colonized and uncolonized women, p-values were adjusted for pneumococcal HIV exposure.

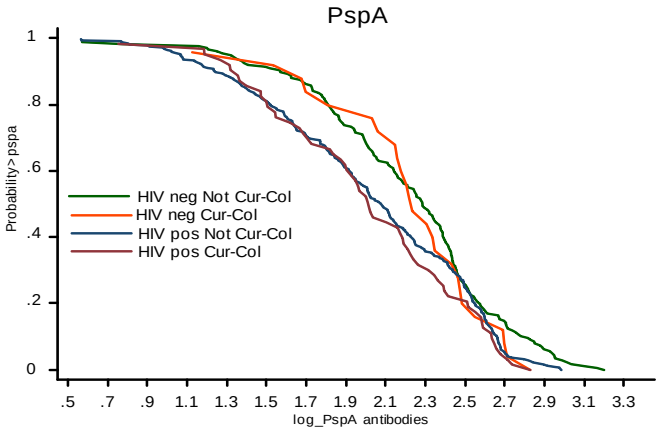
Table 10: Geometric mean titers of antibody titers against pneumococcal proteins in women, stratified by HIV and pneumococcal-colonization status at the time of sampling

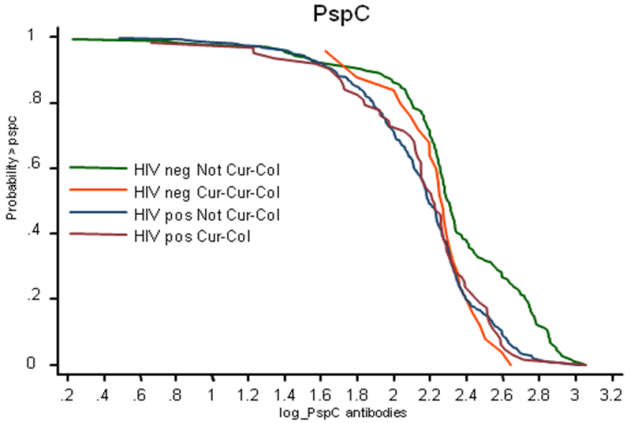
Protein	**HIV uninfected** *GMT (95 % CI)	**HIV infected** *GMT (95 % CI**)**	****p adjusted**
PspA	162 (139-189)	103 (91-116)	**< 0.0001**
PspC	202 (177-232)	138 (126-151)	**< 0.0001**
PsaA	133 (112-159)	124 (108-142)	0.51
PdB	249 (212-293)	195 (174-218)	**0.015**
SP 2027	130 (106-161)	222 (188-263)	***< 0.0001***
SP 0609	119 (97-145)	103 (90-117)	0.23
SP 0082	332 (277-397)	257 (229-288)	**0.015**
SP 0749	140 (120-165)	204 (173-241)	***0.004***
SP 0498 (LytB)	181 (155-211)	116 (105-128)	**< 0.0001**
IgA1-proteinase	307 (269-350)	212 (189-237)	**< 0.0001**
PpmA	278 (235-328	187 (165-213)	**< 0.0001**
SlrA	209 (178-246)	184 (161-209)	0.25
SP 2216 (PcsB)	235 (193-286)	132 (116-149)	**< 0.0001**
SP 1723 (StkP)	142 (113-178)	80 (69-93)	**< 0.0001**
SP 2194	100 (85-118)	89 (79-100)	0.24

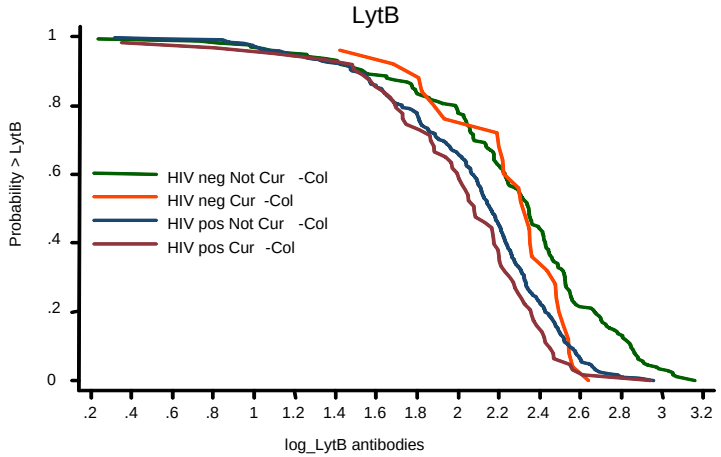
*GMT's (with 95% confidence interval) were reported in arbitrary units per ml (U/ml).
**for comparison between HIV uninfected and HIV infected women, p-values were adjusted for pneumococcal colonization status.

Women's current colonization data

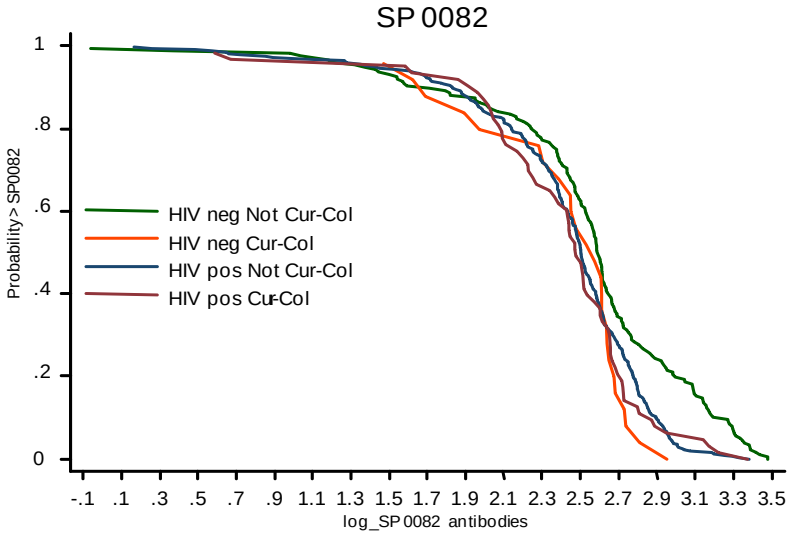
a) Choline-binding proteins:

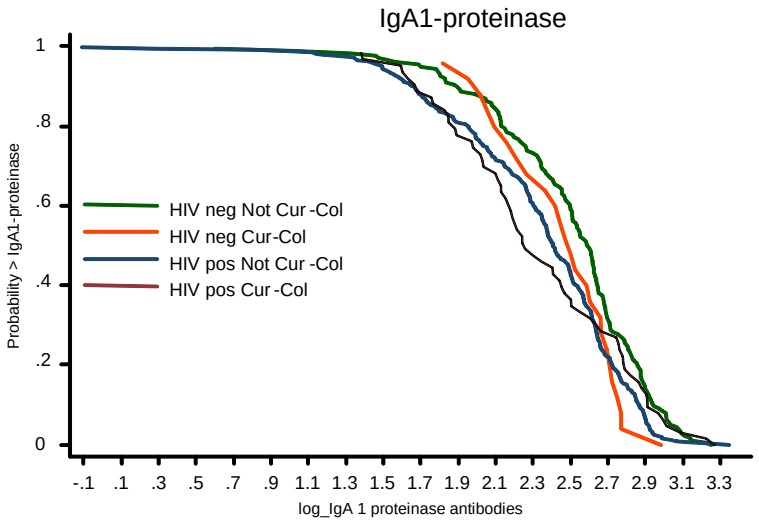






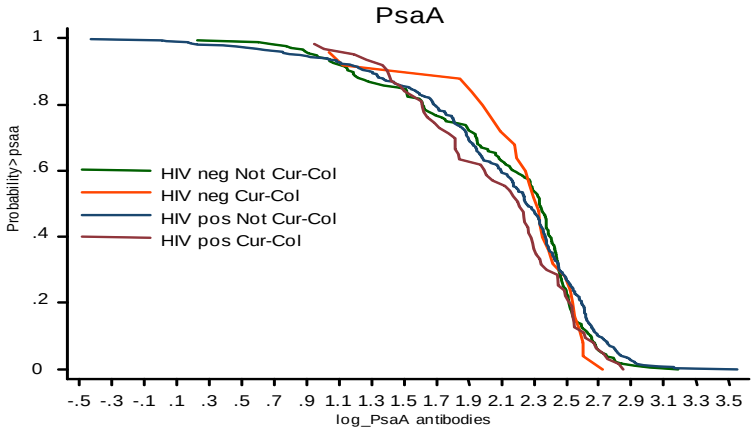
b) Sortase-dependent proteins:

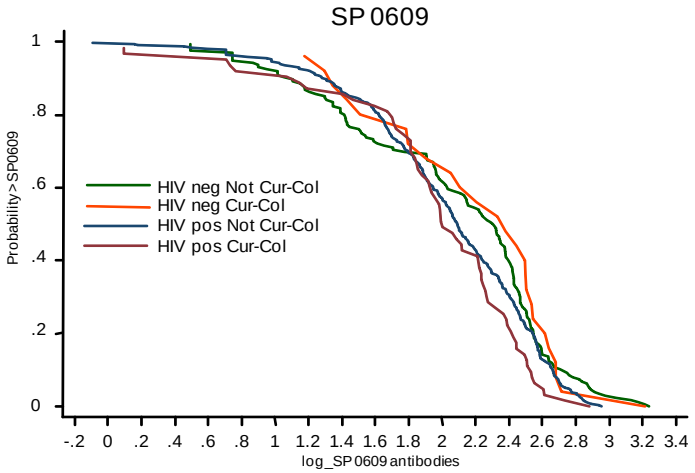


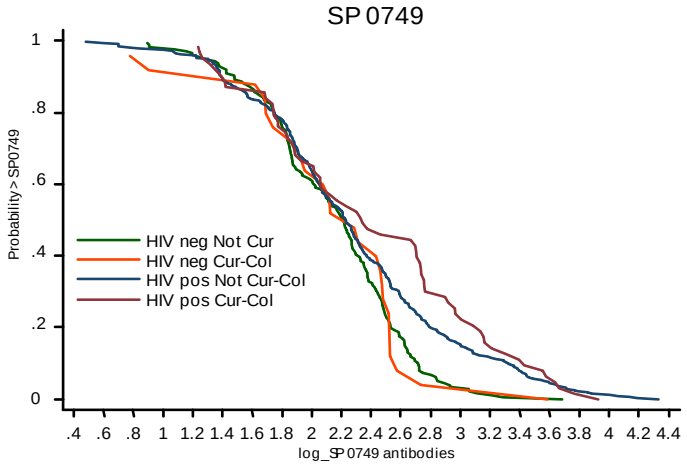


c) Lipoproteins

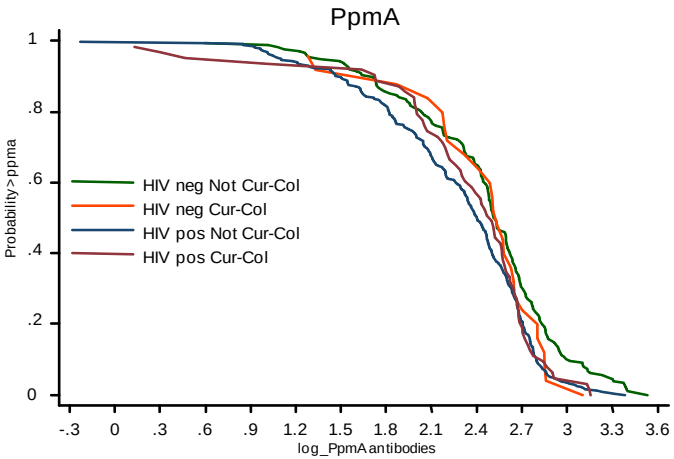
1) ABC transporters:

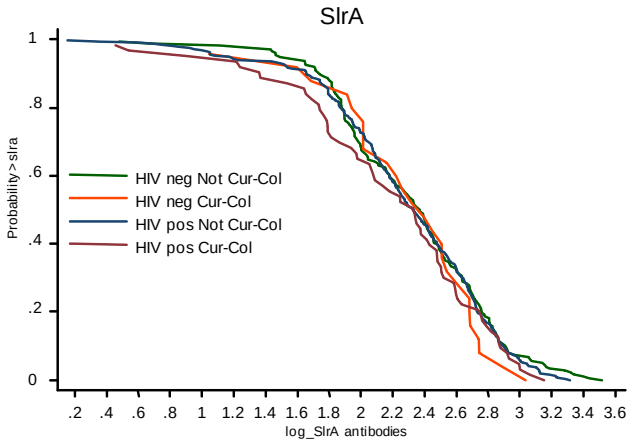




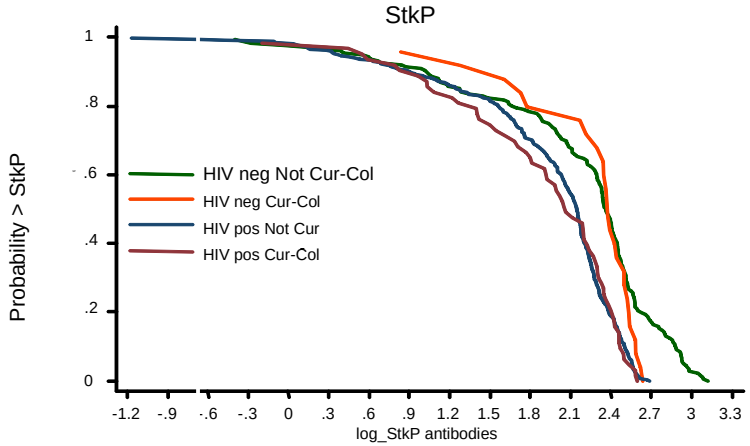


2) <u>Non ABC transporters (cell-wall associated lipoproteins):</u>

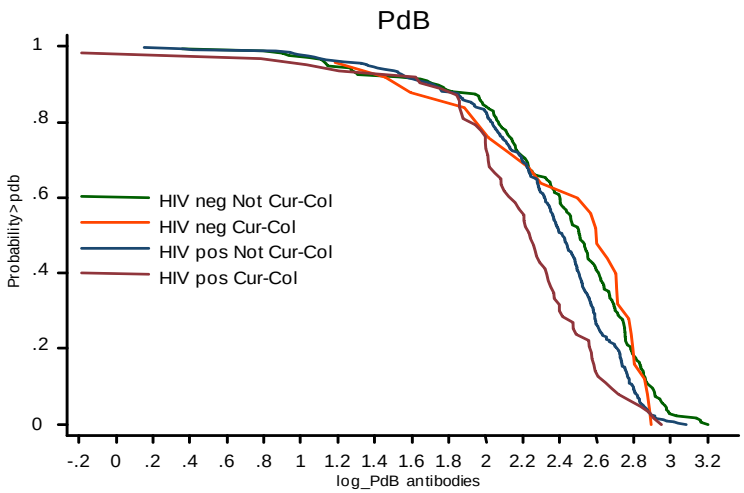


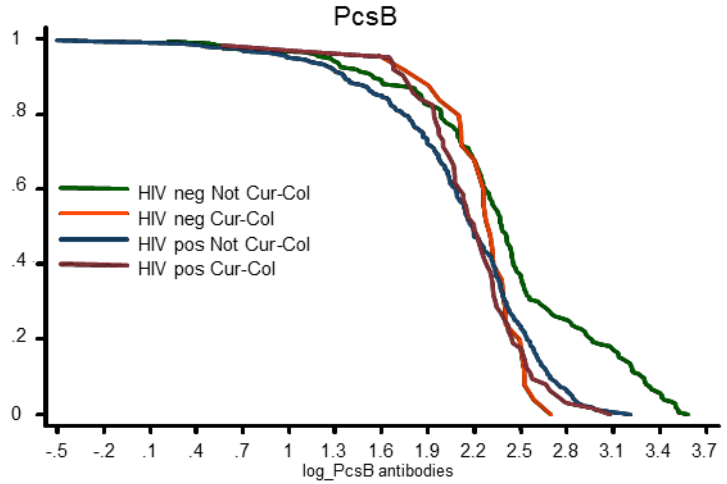


3) Integral membrane lipoprotein(s):



d) Secreted proteins:





e) Other pneumococcal proteins:

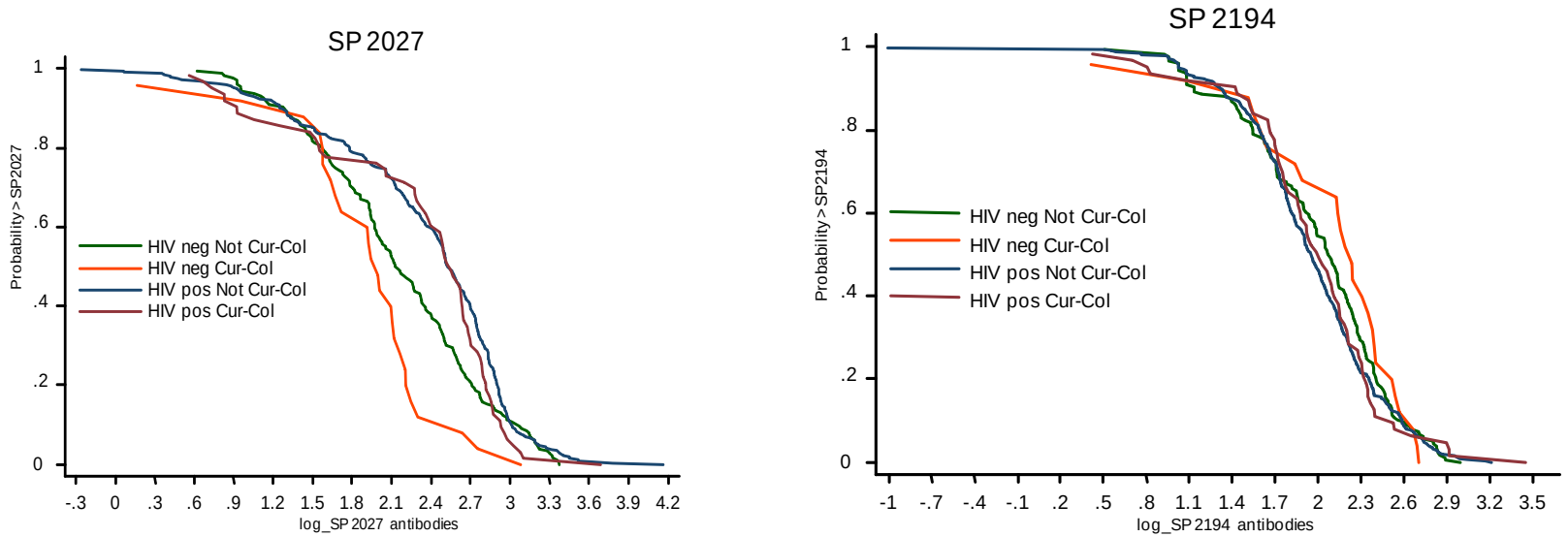


Figure 11: Reverse cumulative distribution curves of IgG antibodies against the pneumococcal proteins measured in the sera from mothers of the PCV-vaccinated and PCV-unvaccinated children for the comparison of women who were colonized with pneumococcus at the time of blood withdrawal (cur-col) and women who were not colonized at the time of blood withdrawal (not cur-col). Nasopharyngeal and oropharyngeal swabs were collected and stored in STGG medium at -80°C **.**

5.9 Kinetics of antibodies against pneumococcal proteins in pneumococcal-unvaccinated HIV uninfected children between 2 years of age, older children and adult females

Antibodies against PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, PcsB, and StkP showed increases in titer with respect to increasing age, and peaked in either the 4 to 7 year old and remained constant until adulthood (PspC, PdB and LytB) or declined by adulthood (PcsB, SP 0082 and StkP) as shown in figure 12.

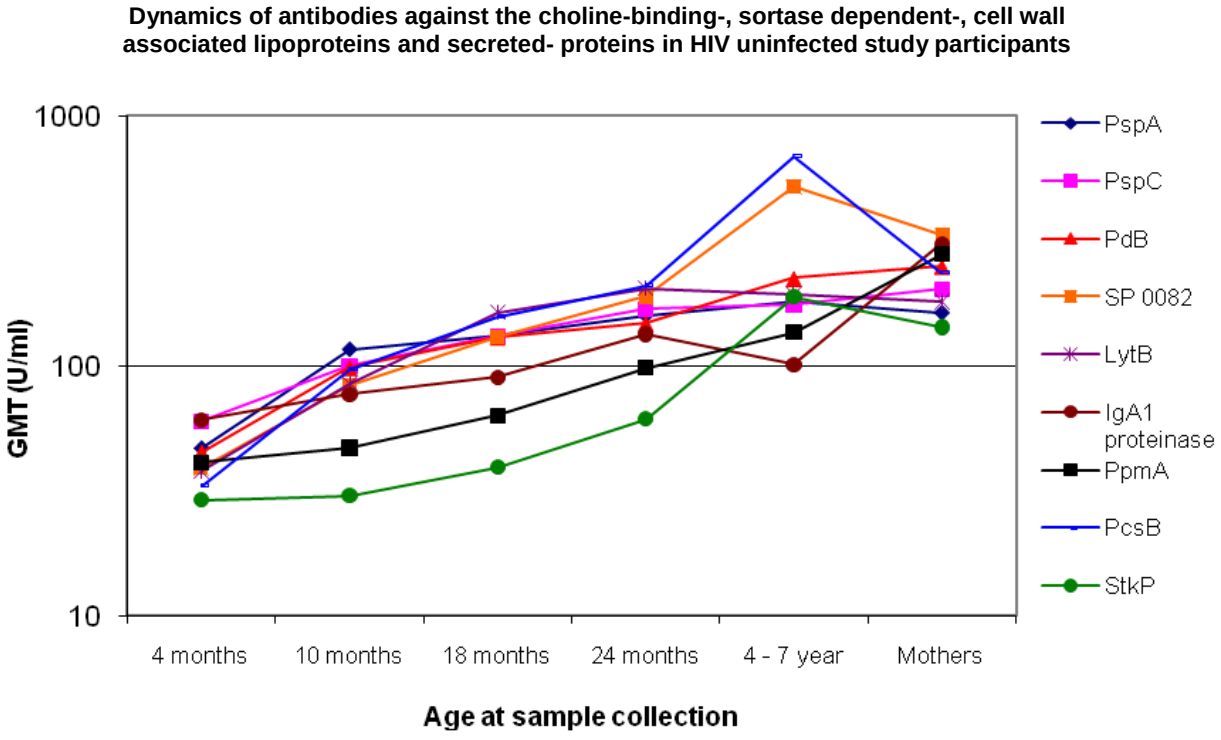


Figure 12: Natural development of antibodies against the choline-binding, sortase-dependent, secreted and cell-wall associated pneumococcal proteins in PCV unvaccinated HIV uninfected infants, older children and adults. GMT = Geometric Mean Titer.

In contrast, antibody titers against PsaA, SP 2027, SP 0609, SP 0749, SlrA, and SP 2194 did not show any relationship to age as shown in figure 13.

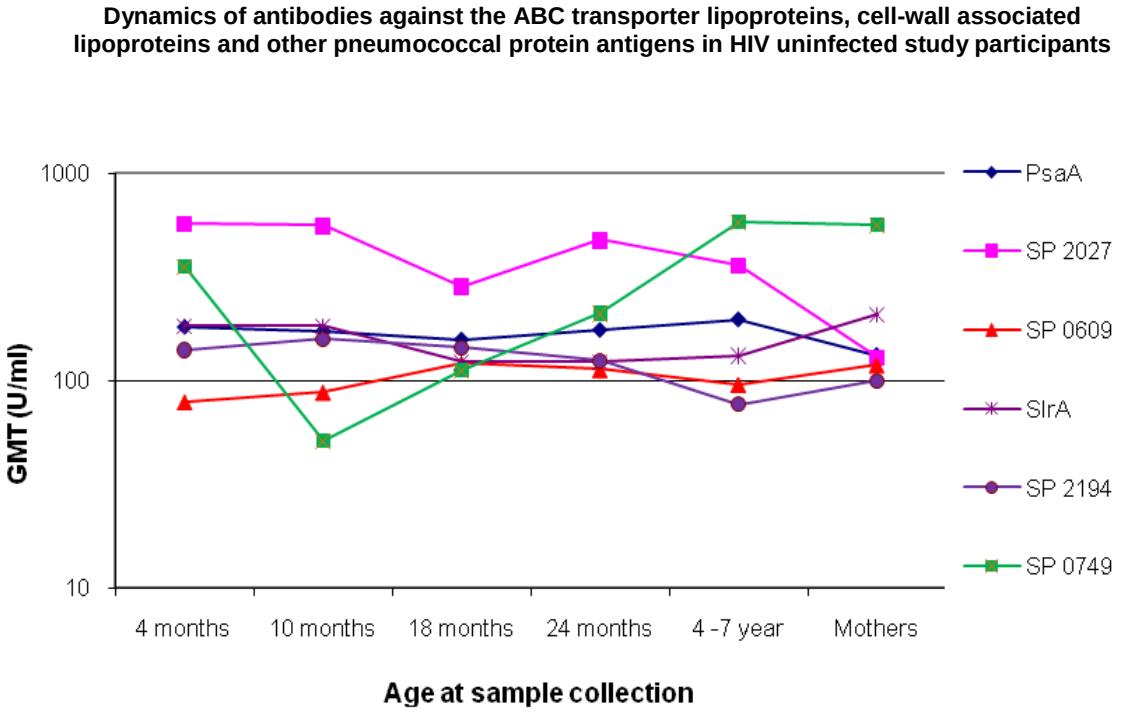


Figure 13: Natural development of antibodies against the ABC transporter lipoproteins, cell wall associated lipoproteins and other pneumococcal proteins in PCV unvaccinated HIV uninfected infants, older children and adults. GMT = Geometric Mean Titer.

Chapter 6: Discussion

i) Validation of the Luminex assay

Many pneumococcal proteins have been reported to possess immune protective potential (Adrian et al., 2004, Bogaert et al., 2006, Cao et al., 2007, Giefing et al., 2008, Gosink et al., 2000, Hermans et al., 2006, Holmlund et al., 2006, McCool et al., 2002, Ogunniyi et al., 2007b, Rapola et al., 2000). Unfortunately most of these studies have come from different laboratories which have employed incomparable methods to evaluate different antigens and as a result, there are very few studies that compare numerous antigens side by side using similar methods to evaluate their potential as possible vaccine candidates. One of the priorities of this study was to establish and validate a common platform for the simultaneous quantification of IgG antibodies against a variety of pneumococcal proteins in order to compare them side by side.

The multiplex Luminex assay was compared and validated against the standard ELISA method for four pneumococcal proteins: PspA, PspC, PsaA and PdB. The multiplex Luminex method showed good correlation with singleplex ELISAs for all four antigens, with similar specificity and was more sensitive than the ELISA method. The broader dynamic range of the Luminex method and the capacity to analyse 15 antigens simultaneously made it an ideal high throughput platform with which to compare data for these antigens (Pickering et al., 2002b).

ii) Effects of age on antibody response

In this study, we have shown that the antibody responses to different proteins can differ markedly. The immune response to pneumococcal proteins PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, PcsB, and StkP increased linearly with age in the first two years of life (refer to Figure 12). Our findings are consistent with the Finnish and Filipino studies, where serum antibodies against PspA and PdB were measured at 6, 12, 18 and 24 months of age. In these studies, the GMC of serum antibodies against PspA, PspC and PdB increased with age (Holmlund et al., 2006, Rapola et al., 2000, Simell et al., 2009). Consistent with our findings, studies by Adrian et al and Bogaert et al also showed that antibody responses to IgA1-proteinase and PpmA developed early in infancy and increased with age (Adrian et al., 2004, Bogaert et al., 2006).

Also consistent with findings from Rapola *et al* (Rapola et al., 2000) and Holmund *et al* (Holmlund et al., 2006) antibodies against PspA never reached levels observed in adults within the first 5 years of life, antibodies against PdB reached adult levels by 5 years of age and by 10 months of age, antibody titers against PsaA in PCV-vaccinated infants reached levels observed in adults and exceeded it afterwards (Rapola et al., 2000). Giefing *et al* (Giefing et al., 2008) studied the natural immune responses against PcsB and StkP in children aged between 2 months to 18 years and adults and demonstrated the development of antibodies against PcsB and StkP increased linearly with age, peaked in the 4 to 7 year old group and declined until adulthood. These observations were consistent with our findings. In study, we have also shown that antibodies against SP 0082, which is a new uncharacterized

protein, increase linearly with age, peaked in the 4 to year old group and declined until adulthood (refer to Figure 12).

iii) Effects of HIV on antibody response

Our data revealed important differences according to HIV exposure and infection status among the study subjects. In PCV-vaccinated children, M+/I+ children had lower antibody titers to PspA, PspC, SP 0082, LytB, IgA1-proteinase, PpmA, SlrA, PcsB and StkP proteins from 4 to 24 months of age compared to M-/I- children (see Tables 4.2 and 4.3). Previous studies have demonstrated that M+/I+ infants with low CD4+ T-cells have an impaired immune response upon T-cell dependent antigens like the influenza vaccine, tetanus toxoid, diphtheria toxoid and the conjugated *Haemophilus infuenzea* type b (Kroon et al., 1994, Kroon et al., 1997). Differences in antibody titers from 4 to 18 months of age were also observed between M-/I- and M+/I- children, whereby M-/I- children had higher antibody titers against PspA, PspC, IgA1-proteinase and SlrA compared to M+/I- children.

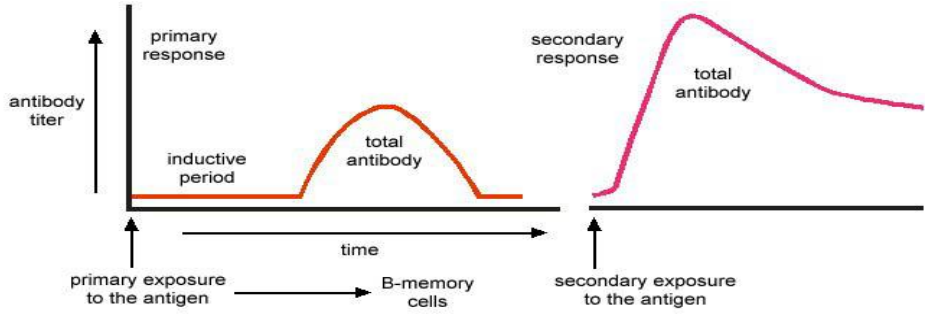
Risk factors contributing to morbidity of M+/I+ and M+/I- children include the severity of maternal HIV disease (Kuhn et al., 2005), poor placental transfer of protective maternal antibodies (de Moraes-Pinto et al., 1996, de Moraes-Pinto et al., 1998), replacement feeding rather than breast feeding (WHO 2000), perinatal exposure to antiretroviral drugs (Feiterna-Sperling et al., 2007) and increased exposure to pathogens from immune-deficient individuals in the household (Mermin et al., 2005). Previous studies have described the clinical profile and morbidity of infants born to HIV infected mothers and they reported that 44% of M+/I- children had clinical signs suggestive of HIV infection. These included hepatomegaly, splenogamy, lymphadenopathy, oral candida and pneumoniae (Adhikari et al., 2006). A

Zambian study showed a relationship between advanced maternal HIV disease and increased infant morbidity and mortality in the HIV uninfected (M+/I- equivalent) offspring (Kuhn et al., 2005). A Zimbabwean study also showed that M+/I- infants had twice the risk of mortality compared to M-/I- infants (Marinda et al., 2008).

M+/I- infants have demonstrable HIV-specific cellular immune responses for both $CD4^+$ and $CD8^+$ T-lymphocytes in peripheral and cord blood, suggesting exposure to HIV antigens (Cheynier et al., 1992, Kuhn et al., 2002, Rowland-Jones et al., 1993). Both M+/I+ and M+/I- infants have reduced IL-12 production in the cord blood, which can also be demonstrable until 6 months of age (Chougner et al., 2000). The early age at onset of a wide spectrum of infections observed in M+/I- infants suggests an immunodeficiency perhaps of innate immune function. The antigen presentation or co-stimulation signals of M+/I- infants immune systems may possibly be transiently impaired by HIV exposure (Slogrove et al., 2010). The difference observed in antibody titers between M+/I+, M+/I- and M-/I- could be due to all these factors.

In PCV-unvaccinated children, higher antibody titers against PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, SlrA, PcsB and StkP were observed in M-/I- children compared to M+/I- children at 4 months of age. Higher antibody titers against PspA, PspC, PsaA, SP 0082 and IgA1-proteinase were observed in M-/I- children compared to M+/I- children at 24 months of age. As observed in PCV-vaccinated children, differences in antibody titers at 4 months of age were most likely due the risk factors observed in M+/I- infants compared to M-/I- infants. Also, the prevalence of colonization in M+/I- children was higher compared to M-/I- children, especially at earlier time-points, suggesting that immune aberrations may exist in M+/I- children compared to M-/I- children (Madhi et al., 2010).

The higher antibody titers against PspA, PspC, PsaA, SP 0082 and IgA1-proteinase in M-/I- children compared to M+/I- children observed at 24 months of age could possibly be due to enhanced memory responses in the former group induced by preceding nasopharyngeal colonization as demonstrated in the figure below.



M+/I+ children are at an increased risk for pneumococcal infections, especially bacteremia (Malaspina, 2003). The poor antibody responses to the proteins may in part be responsible for the higher incidence of systemic pneumococcal disease observed among M+/I+ compared to M-/I- children. Our findings agree with observations that M+/I+ children have reduced antibody responses to systemically administered antigens such as routine childhood vaccines compared to M-/I- children (Arpadi et al., 1994, Kale et al., 1995).

In the 4 to 7 year old group, HIV uninfected children had significantly higher antibody titers against antigens which are theoretically surface located, such as choline-binding proteins, secreted proteins and antigens which posess peptidoglycan binding motifs such as LPXTG (PspA, PspC, SP 0082, LytB, IgA1-proteinase, PdB and PcsB) compared to HIV infected children. In contrast, antibody titers against antigens which are anchored to the cell

membrane e.g. cell-wall associated lipoproteins and ABC transporters (PsaA, SP 2027, PpmA and SlrA) were higher in HIV-infected compared to HIV-uninfected children.

These differences suggest that depleted T-cell function in HIV-infected individuals are associated with differences in natural immune responses to common pneumococcal proteins based on where they are found in the bacterium.

HIV-uninfected women had higher antibody titers against PspA, PspC, PdB, SP 0082, IgA1-proteinase, PpmA, PcsB, and StkP compared to HIV-infected women. Lower antibody titers against membrane associated proteins: SP 2027 and SP 0749 were observed in HIV-uninfected women compared to HIV-infected women. Our data are in agreement with Amdahl's and Sullivan's studies where they reported lower antibody titers against PdB in HIV-infected adults compared to HIV-uninfected adults (Amdahl et al., 1995).

HIV is a major risk factor to developing invasive pneumococcal disease. The mechanisms behind this are poorly understood, especially in the context that the risk of developing pneumococcal disease appears to be more closely related to T-cell function than to antibody titer. Antibody responses to proteins depend on $CD4^+$ T-cell function (King et al., 1996, Feikin et al., 2001). This T-cell dependent response should provide long term protection against pneumococcal disease by inducing memory cell response. High HIV loads contribute to the loss of memory cell resulting in impaired interactions between B cells and CD4+ T cells (Moir and Fauci, 2009).

iv) Effects of colonization on antibody response

In children less than 2 years of age, differences in antibody titers between children who were colonized with pneumococci and those not colonized were evident for the choline-binding proteins, secreted proteins, membrane associated proteins and sortase-depend (LPXTG motif) proteins, namely; PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, PcsB and StkP proteins at ages 4 and 10 months. These data suggested that antibody titers against these proteins are stimulated by antigen exposure through pneumococcal colonization. At 18 and 24 months, the differences between colonized children and uncolonized children became less apparent. This is expected, since by this age, the majority of children have had prior pneumococcal exposure through colonization and thus stimulation of natural antibody responses.

Our results are in agreement with Adrian *et al* (Adrian et al., 2004) whereby antibodies against IgA1-proteinase and PpmA increased in response to pneumococcal carriage in the first two years of life. Our data are also consistent with findings from Rapola *et al* (Rapola et al., 2000) and Holmlund *et al* (Holmlund et al., 2006) whereby the increase in antibody titers against PspA and PdB was associated with pneumococcal exposure as children who were colonized with pneumococci produced more antibody against these proteins compared to children who were not colonized with pneumococci.

In children between 4 to 7 years of age, higher antibody titers against PdB and PcsB were observed in children who were not colonized with pneumococci compared to pneumococcal-colonized children (refer to Table 7.2). Similarly, HIV uninfected women who were not colonized with pneumococci at the time of blood sampling had significantly higher antibody titers than pneumococcal-colonized women against PspC, SP 2027, SP 0082, LytB and PcsB

(Table 9). This inverse association between colonization and antibody titers suggests that antibodies against these proteins might potentially protect against the risk of future acquisition.

In our study, HIVuninfected women who were colonized with pneumococci produced similar antibody titers against PspA and PdB, and lower antibody titers against PspC compared to women who were not colonized with pneumococci. These findings contrast with those of McCool *et al* (McCool et al., 2002) in which adults colonized with pneumococci were found to produce higher titers against PspA, PspC and PdB compared to subject who were not colonized with pneumococci.

No differences in antibody titers against the studied pneumococcal proteins were observed in HIV-infected women, regardless of their pneumococcal colonization status (Table 9). Similarly, no differences in antibody titers against Ply have also been reported in HIV-infected adults with and without invasive pneumococcal diseases (Amdahl et al., 1995, Etuwewe et al., 2009, Sullivan et al., 2001).

v) Effects of vaccination on antibody response

Our data demonstrates that PCV-vaccination dampened the immune response against some of the proteins. This was observed in children at 4, 10 and 18 months of age, whereby PCV-vaccinated children had lower antibody titers against PspA, PspC, LytB, PdB, IgA1-proteinase, PcsB and StkP compared to PCV-unvaccinated children (Tables 6.1 to 6.3). Lower antibody titers observed in PCV-vaccinated children compared to PCV-unvaccinated children, could most likely be due to PCV vaccination affecting pneumococcal colonization

and modulating the natural immune responses to colonization events resulting in reduced antibody induction against some of the proteins.

vi) Evaluation of vaccine candidates in the context of natural immunity

There is no evidence based method for predicting the potential effectiveness of a pneumococcal protein as a vaccine candidate against nasopharyngeal colonization. However, analysis of the natural immune response in the context of variables such as age, PCV vaccination status, HIV status, and colonization status provide data which highlight significant differences between antigens. Combining these differences with our knowledge of pneumococcal biology, immunology, and dynamics of colonization can help to provide a rationale as to which of these antigens are most likely to be effective candidate vaccine molecules.

There are numerous limitations to this study in extrapolating natural acquired antibody titers to clinical vaccine efficacy. For example there is no guarantee that antibodies that are present and antigen-specific *in vitro* are able to protect against colonization and invasive disease, either as a result of the antigen being inaccessible to antibody binding through being 'hidden' by the cell wall and capsule, or from antibody exposure by the cell wall and capsule, or that the most reactive epitopes are in a conformation which is able to prevent antibody recognition and binding. In general, there is a rule which suggests that the more the antibody is present, the more likely there is to be some protective benefit. However, through the use of a pooled reference, where titers for each antigen are described as arbitrary units relative to this reference, this study design did not allow us to measure the empirical amount of antibody, and compare it between antigens. Despite the limitations of the methods used to quantify the

antibody responses, we were able to obtain data on responses relative to age, HIV, PCV vaccination and colonization status which can assist in identifying antigens which may be useful vaccine candidates. A summary of the candidates and their relative responses are shown in Table 11.

Age: The risk of getting invasive pneumococcal disease is associated with age, and is at its highest in the 4 - 24 month age group, and gradually decreases with age until adulthood is reached (Aniansson et al., 1992, Granat et al., 2007, Hammitt et al., 2006). By comparing the age-related risk of developing invasive pneumococcal disease to the titers of natural acquired antibodies, it is likely that antigens against which there is an inverse correlation between titer and risk of developing pneumococcal disease are likely to be effective candidates. Antibodies against PspA, PspC, PdB, SP 0082, LytB, IgA1-proteinase, PpmA, PcsB, and StkP showed increases in titer with respect to increasing age, and peaked in either the 4 to 7 year old age group or in the mothers. Antibodies against PspC, SP 2027, SP 0082, LytB and PcsB proteins were inversely associated with pneumococcal colonization in adults. In contrast antibody titers against all the ABC transporters; PsaA, SP 0609 and SP 0749, cell wall associated lipoprotein; SlrA and uncharacterized pneumococcal proteins; SP 2027 and SP 2194, did not show any relationship with respect to age.

The kinetics of pneumococcal antigens which can be described as being “non-pneumococcus specific” i.e. conserved antigens with housekeeping functions, and having homologues in related species, such as ABC transporters (PsaA, SP 0609, SP 0749), cell-wall associated lipoproteins (PpmA and SlrA) and conserved uncharacterized proteins (SP 2027 and SP 2194) were different from the previously described “pneumococcus specific” antigens, and did not show the comparable kinetics with respect to increasing with age or lower titers in

PCV immunized children. For these antigens, the absence of predictable kinetics may be influenced by exposure to homologous antigens present elsewhere in the commensal flora.

PCV vaccination and colonization status: Natural antibody responses induced by contact with pneumococci can provide guidance as to selection of effective vaccine candidates. Since many of the proposed antigens have homologues in related bacteria which are associated with our normal flora, candidates that stand out are those that are unique to the pneumococcus and are able to be induced specifically through pneumococcal contact. Since PCV vaccination results in prevention of acquisition of vaccine serotypes, one would expect that vaccination should act as a probe to measure the effects of pneumococcal exposure on the acquisition of natural antibodies against pneumococcal specific antigens (Obaro et al., 1996).

There are limitations to relating antibody titers to concurrent colonization, because the duration of colonization prior to antibody measurement is thought to play a major role in determining serum antibody concentrations (Cauchemez et al., 2006). Without detailed colonization data which includes both the time of acquisition and a quantitative measure of colonizing organisms, the usefulness of antibody titers in relation to colonization is frequently compromised (Kauppi-Korkeila et al., 1996, Lloyd-Evans et al., 1996). In addition, many antigens are heterogeneous to the point where there is limited cross-reactivity between the different clades and families of an antigen (Brooks-Walter et al., 1999, Crain et al., 1990). This has potential to mute the comparisons of natural responses to pneumococcal exposure through colonization, as there is no information matching the antigen family used in the immunoassay with that of the colonizing types. Despite this, our data showed good agreement between the effects of vaccination and concurrent colonization in that significantly higher antibody titers to PspC, LytB, PdB, PcsB, PpmA and StkP were directly associated

with concurrent colonization in the first year of life (Table 5.1), and PCV vaccination had the greatest effect on titers to PspA, PspC, LytB, PdB, IgA1-proteinase, PcsB, and StkP in the first 18 months of life (Tables 5.1 to 5.3).

In the older age groups one would expect that all individuals have had at least some contact with pneumococci, and thus acquired some level of antibody against pneumococcal antigens by the age of 2 years. With this in mind, ideal vaccine candidates can potentially be identified as antigens against which elevated titers protect against colonization. High antibody titers against PcsB and PdB were associated with lower prevalence of colonization, suggesting a protective effect. In addition, high titers of antibodies against PspC, SP 2027, SP 0082, LytB and PcsB were associated with lower prevalence of colonization in mothers (refer to Table 9).

HIV status: HIV is a major risk factor to developing pneumococcal disease, and hence by inference, one would expect that antigens against which titers were suppressed in the HIV infected groups may well be involved in protection from disease. In the 4 to 7 year old group, HIV-uninfected children had significantly higher titers to antigens which are theoretically surface located, such as choline-binding proteins, and antigens which posess peptidoglycan-binding motifs such as LPXTG. In contrast, antibody titers against antigens which are anchored to the cell membrane e.g. lipoproteins and ABC transporters were higher in HIV-infected children. These differences are possibly related to depleted cellular immunity in HIV- infected individuals, whereby antibody titers are driven by direct contact between immune cells and the outer surface of pneumococci. Interestingly, in 2 year olds and mothers, the lipoprotein PpmA and membrane anchored StkP were exceptions to this rule, and this might be because young children and mothers (adults) may have been on antiretroviral therapy, unlike the 4 to 7 year old group.

PspC, PdB, LytB, SP 0082, PcsB and StkP are exceptionally conserved among clinical isolates. Previous studies have shown that antibodies against PcsB and StkP are cross-protective against different serotypes in lethal sepsis and pneumonia in human models, and they play a crucial role in bacterial growth (Giefing et al., 2008). PspC is a highly variable antigen and previous studies have shown that PspC is cross-reactive and cross-protective among heterologous strains, however the suitability of this antigen as a vaccine candidate might be limited by the fact that it is present in only 75% of *S. pneumoniae* strains (Brooks-Walter et al., 1999), therefore a vaccine containing this antigen may need to represent more than 1 family of this heterogeneous antigen.

Ply is produced by all strains of pneumococci. Previous studies (Bogaert et al., 2004a, Rapola et al., 1997) and our study have shown that PdB is potentially a good vaccine antigen; however, PdB might not provide enough protection to be an effective stand-alone vaccine antigen as it is not located on the surface of the pneumococcus. Antibodies against Ply are predicted to provide protection by neutralizing the biological properties of the toxin, rather than by stimulating opsonophagocytic clearance of the invading bacteria (Rubins and Janoff, 1998). Therefore a vaccine combining surface proteins and PdB would be more effective than a vaccine consisting of PdB alone.

SP 0082 is a highly conserved surface anchor protein. It contains an LPXTG binding motif and is therefore predicted to play a role in pneumococcal adherence and colonization. Like other proteins that are located on the surface of the pneumococcus (choline-binding proteins), it is presumed to elicit opsonophagocytic antibodies.

LytB is a highly conserved choline-binding hydrolase that plays a crucial role in cell division and pneumococcal colonization (Gosink et al., 2000). A vaccine containing LytB and SP 0082 will potentially prevent against nasopharyngeal colonization, which is the crucial step in the pathogenesis of *S. pneumoniae.*

Based on the results presented in this study, a subunit vaccine comprising a combination of the antigens highlighted in table 11 (PspC, PdB, LytB, SP 0082, PcsB and StkP) consistently match our hypothesised criteria in terms of titers increasing with an increase in age and the association of antibody titers against these proteins with HIV infection status, PCV vaccination history and pneumococcal colonization in young children.

In conclusion, pneumococcal proteins offer the potential advantage of being immunogenic even when administered early in life, providing serotype independent protection and being less expensive compared to polysaccharide-based vaccines. This study contributes significantly to the identification of potential candidates in the development of a protein-based pneumococcal vaccine. Therefore, the development of a vaccine composed of PspC, PdB, LytB, SP 0082, PcsB and StkP might protect against pneumococcal colonization and consequently prevent pneumococcal disease.

Table 11: Summary of differences in natural antibody responses in relation to age, HIV, PCV vaccination and colonization status. Antigens with potential as vaccine candidates are highlighted in red.

Proteins	PCV-vaccinated children			PCV-unvaccinated children			Response to vaccination		Older children			Adults (Women)	
	Titers ↑ with age?	Effect of HIV on titer	Response to colonization	Titers ↑ with age?	Effect of HIV on titer	Response to colonization	4 to 10 months	18 to 24 months	Effect of HIV on titer	Titer suppressed by PCV?	Titers protect from colonization ?	Effect of HIV	Titers protect from colonization?
PspA	Y	Incr.	Incr.	Y	Incr.	NC	Suppr.	Suppr.	Incr.	No	ND	Incr.	ND
PspC	**Y**	**Incr.**	**Incr.**	**Y**	**Incr.**	**Incr.**	**Suppr.**	**Suppr.**	**Incr.**	**No**	**ND**	**Incr.**	**Yes**
PsaA	Y	Decr.	Incr.	N	Decr.	NC	Suppr.	Enhanc.	Decr.	No	ND	ND	ND
PdB	**Y**	**Incr.**	**Incr.**	**Y**	**Incr.**	**Incr.**	**Suppr.**	**Suppr.**	**Incr.**	**No**	**Yes**	**ND**	**ND**
SP 2027	N	NC	NC	N	NC	NC	Suppr.	Enhanc.	Decr.	No	ND	Decr.	Yes
SP 0609	N	NC	ND	N	Decr.	Decr.	Enhanc.	NC	ND	No	ND	ND	ND
SP 0082	**Y**	**Incr.**	**Incr**	**Y**	**Incr.**	**Incr.**	**Enhanc**	**Enhanc.**	**Incr.**	**No**	**ND**	**Incr.**	**Yes**
SP 0749	N	Decr.	Decr.	N	NC		Suppr.	Enhanc.	ND	No	ND	Decr.	ND
SP 0498 (LytB)	**Y**	**Incr.**	**Incr.**	**Y**	**Incr.**	**Incr.**	**Suppr.**	**Suppr.**	**Incr.**	**No**	**ND**	**Incr.**	**Yes**
IgA1-proteinase	N	Incr.	ND	Y	NC	NC	Suppr.	Suppr.	Incr.	No	ND	Incr.	ND
PpmA	Y	Incr.	Incr.	Y	Incr.	Incr.	Suppr.	Enhanc.	Decr.	No	ND	Incr.	ND
SlrA	N	Incr.	NC	N	Decr.	Decr.	NC	NC	Decr.	No	ND	ND	ND
SP 2216 (PcsB)	**Y**	**Incr.**	**Incr.**	**Y**	**Incr.**	**Incr.**	**NC**	**Enhanc.**	**Incr.**	**No**	**Yes**	**Incr.**	**Yes**
SP 1723 (StkP)	**Y**	**Incr.**	**Incr.**	**Y**	**Incr.**	**Inc.**	**Suppr.**	**NC**	**ND**	**No**	**ND**	**Incr.**	**ND**
SP 2194	N	NC	NC	N	NC	NC	NC	NC	ND	No	ND	ND	ND

* Incr. = Increase in antibody titers. Decr = Decrease in antibody titers. Suppr. = suppressed acquisition of antibody titers. Enhanc. = enhanced acquisition of antibody titers.

ND = no differences. NC = no change.

Limitations

The findings in this study do not suggest that the measured antibody titers represent the mechanism for protection. Also, through the use of a pooled reference, where titers for each antigen are described as arbitrary units relative to this reference, this study design did not allow us to measure the empirical amount of antibody, and compare it between antigens. Another limitation of this study is that a large number (15) of proteins was investigated, due to this; some of the statistical differences observed could have been purely by chance.

Appendices

Appendix A

Enzyme-linked immuno-sorbent assay (ELISA)

1. Protein dilution buffer (1X Phosphate buffer saline, pH 7.3)

    - 8 g of sodium chloride (NaCl)
    - 0.2 g of potassium chloride (KCl)
    - 1.1g sodium phosphate (dibasic salt) Na_2HPO4
    - 0.3 g potassium phosphate (monobasic salt) KH_2PO4
    - Make up to 1 liter with distilled water

2. Assay buffer (Phosphate buffer saline containing 10% foetal bovine serum and 0.05% sodium azide)

    - Measure 100 ml of foetal bovine serum (FBS)
    - Add 0.5 g of sodium azide
    - Make up to 1 liter with 1X PBS

3. Wash buffer (Phosphate Buffer Saline containing 0.05% Tween 20)

    - Measure 500 µl of Tween 20
    - Make up to 1 liter with 1X phosphate buffer saline

4. Secondary antibody (alkaline phosphatase-conjugated goat anti-human IgG, γ-chain specific)

    - Dilute 10 µl of the secondary antibody in 10 ml to make a 1: 1000 dilution

5. Substrate [(4- nitrophenyl phosphate disodium salt hexadydrate (1.14 mg/ml)]

    - Weigh 0.098 g 4- nitrophenyl phosphate disodium salt hexadydrate
    - Make up to 70 ml with carbonate buffer, pH 9.1

6. 0.05M Carbonate buffer, pH 9.6

    - Weigh 1.59 g of sodium carbonate
    - Weigh 2.93 g of sodium hydrogen carbonate
    - Make up to 1 liter with distilled water

Appendix B

Multiplexed fluorescent covalent microsphere immunoassay (FCMIA)

1. Conjugation buffer , 1X PBS, pH 6.1

    - Weigh 8 g of NaCl
    - Weigh 0.2 g of KCl
    - Weigh 1.1 g of NaH_2PO_4
    - Weigh 0.3 g of KH_2PO_4
    - Make up to 1 liter with distilled water

2. 50 mg/ml 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl

    - 1 ampule contains 10 mg EDC
    - Dilute with 200 µl 1X PBS, pH 6.1 to make a 50mg/ml solution

3. 50 mg/ml *N*-hydroxy-sulphosuccinimide

    - Weigh 0.05 g of *N*-hydroxy-sulphosuccinimide
    - Make up to 1 ml with 1X PBS, pH 6.1

Appendix C

Other trials referred to in protocol

HREC reference number: 040703

Abbreviated title: CIPRA 2

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

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

HREC reference number: 040704

Abbreviated title: CIPRA 4

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

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

HREC reference number*:* 031013

Abbreviated title: Durability study

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

Date of initial approval: 08 December 2003

HREC reference number: M06-03-59

Abbreviated title: M2C unvax

Trial title: Dynamics of pneumococcal colonization in HIV-exposed and HIV unexposed infants and their mothers.

Date of initial approval: 03 April 2006

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