

B CELL RESPONSE TO PNEUMOCOCCAL VACCINES

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ABSTRACT

Streptococcus pneumoniae is a significant cause of mortality and morbidity in both children and older adults, with infection resulting in invasive disease, pneumonia and otitis media. The inclusion of pneumococcal conjugate vaccines in routine infant immunisation programmes has had a major impact on disease rates. Vaccine-induced protection against pneumococcal infection is thought to be mediated by the generation of persistent serotype-specific functional antibodies and antigen-specific memory B cells, the latter capable of generating a rapid secondary antibody response on re-exposure to antigen. Although many studies have investigated the immunogenicity of pneumococcal vaccines in different age groups by measuring serotype-specific antibodies, there is more limited information about the B cells underlying such an immune response. Important areas to investigate include the identity of the B cell subsets involved in antibody production and the potential link between memory B cells (B_{MEM}) and persistent antibody production by long-lived plasma cells.

In this thesis I have investigated in detail the immune response to pneumococcal vaccines given to children and adults by a variety of different methods. By examining the variability of a B_{MEM} ELISpot method, it was shown that this assay is robust and reproducible and can be performed on fresh or frozen samples and in different laboratories. Using this technique, in a study of pre-school children, it was demonstrated for the first time that the level of pre-existing serotype 3-specific antibody is negatively correlated with, and may directly impair the B_{MEM} response to a booster dose of 13-valent pneumococcal conjugate vaccine (PCV-13) containing serotype 3 glycoconjugate. In the same study, it was shown that antibody persistence against most vaccine serotypes can be expected until the age of 3.5 years. A novel antigen-labelling technique was used in a detailed kinetics study of antigen-specific B cell subsets in response to either PCV-13 or 23-valent pneumococcal polysaccharide vaccine in adults. The results of this study revealed distinct B cell subset response patterns that were observed in all study participants indicating that IgM B_{MEM} seem to play a major role in the immune response to pneumococcal vaccines. In addition, in the same study, genome wide analysis of gene expression was performed and it was shown that vaccination with either a pneumococcal conjugate or polysaccharide vaccine results in a marked difference in numbers of differentially expressed genes 8 days following vaccination. A further tool likely to be of use in investigating B cell responses is the analysis of the antibody repertoire using next-generation sequencing techniques. In order to test the ability of these methods to detect vaccine responses, a large dataset of

high-throughput B cell receptor sequences was analysed and revealed convergence of antigen-specific complementary-determining region (CDR)₃ amino acid (AA) sequences following vaccination and identified antigen-specific sequences. It was further demonstrated that for sequences directed against the *H. influenzae* type b (Hib) polysaccharide, diversity of immunoglobulin gene rearrangements is much greater than previously recognised. Frequencies of Hib-specific CDR₃ AA sequences were linked with anti-Hib avidity indices highlighting the potential of this method as an alternative (functional) measure of vaccine immunogenicity.

These data suggest that studying the B cells and antibody repertoire post-vaccination can give novel insights into the biology that underlies the immune responses.

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*Anybody who has been seriously engaged in scientific work
of any kind realises that over the entrance to the gates of the temple
of science are written the words: 'Ye must have faith.'
It is a quality which the scientist cannot dispense with.*

— Max Planck 1858-1947

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ABBREVIATIONS

ASC	antibody secreting cell
BCR	B cell receptor
CAP	community-acquired pneumonia
CDR	complementary-determining region
CLSI	Clinical and Laboratory Standards Institute (USA)
CpG	cytosine phosphorothioate guanine
CSF	cerebrospinal fluid
CVID	common variable immunodeficiency
DC	dendritic cells
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunoSPOT
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FACS	fluorescence-activated cell sorter
FOB	follicular B cells
GC	germinal centre
GMT	geometric mean titre
GMC	geometric mean concentration
Hib	<i>Haemophilus influenzae</i> type b
HIV	human immunodeficiency virus
Ig	immunoglobulin
IL	interleukin
IPD	invasive pneumococcal disease
LAMP	Loop-mediated isothermal amplification
LPS	Gram-negative bacterial cell membrane component lipopolysaccharide
MHC	major histocompatibility complex

MIC	minimum inhibitory concentration
MZB	marginal zone B cells
NGS	next-generation sequencing
OPA	Opsonophagocytic assay
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PBP _s	penicillin-binding proteins
MBL	mannose-binding lectin
PCV	pneumococcal conjugate vaccine
PCV-7	7-valent pneumococcal conjugate vaccine
PCV-10	10-valent pneumococcal conjugate vaccine
PCV-13	13-valent pneumococcal conjugate vaccine
PPV-23	23-valent pneumococcal polysaccharide vaccine
PPS	pneumococcal plain polysaccharide
PWM	pokeweed mitogen
RB	rinse buffer
SAC	<i>S. aureus</i> Cowan strain
TCR	T cell receptor
TD	thymus dependent
TH	T helper cell
TI	thymus independent
TLR	Toll-like receptor
TT	tetanus toxoid
WHO	World Health Organization
VH	variable heavy chain
AIDS	acquired immunodeficiency syndrome
PRRs	pattern recognition receptors
MAMPs	microbe-associated molecular patterns
IRAK	interleukin-1 receptor-associated kinase

Part I

INTRODUCTION

MICROBIOLOGY AND DRUG RESISTANCE OF *S. PNEUMONIAE*

1.1 CLASSIFICATION AND IDENTIFICATION

S. pneumoniae is a lancet-shaped Gram-positive diplococcus whose outer surface is covered by a polysaccharide capsule (Figure 1.1). This capsule is the most important virulence factor of pneumococci as it protects the bacteria from phagocytosis. The second layer – the cell wall – consists of polysaccharides and teichoic acid and serves as an anchor for cell-wall-associated surface proteins (Figure 1.1).

Pneumococci are frequently identified as α -haemolytic, optochin sensitive colonies on blood agar and growth is enhanced in 5% CO₂ and under anaerobic conditions. Based on agglutination using pooled typing sera, they can be classified into 46 serogroups, which can be further subdivided into more than 90 serotypes, not all of which cause disease [2]. This classification is based on a type-specific immune response directed against the pneumococcal polysaccharide capsule, which results in a swelling of the capsule that in turn can be seen under a phase contrast microscope (Quellung reaction, Figure 1.2). Type-specific sera can cross-react with other serotypes in the same serogroup but there is no cross-reactivity between different serogroups.

An alternative to pneumococcal serotyping by the Quellung reaction is the detection of capsular synthesis genes using direct sequencing or amplification followed by restriction fragment length polymorphism analysis [3–5]. Genomic microarrays have been available for more than 10 years allowing the identification of most of the pneumococcal genes [6]. Using probes from all known capsular polysaccharide genes, a very robust and rapid microarray based serotyping tool is available. This method also allows identification of novel serotypes, detection of multiple serotypes e.g. from swab samples, and their relative abundance [7].

Multi Locus Sequence Typing (MLST) is an alternative method frequently used to classify bacteria, in which submitted sequences of seven 'house-keeping' genes are compared with an existing database to determine an "allelic profile" and a sequence type (ST) [8].

1.2 VIRULENCE

The major virulence factor of *S. pneumoniae* is the capsule and the invasive potential differs by serotype [9, 10], which may have to do with

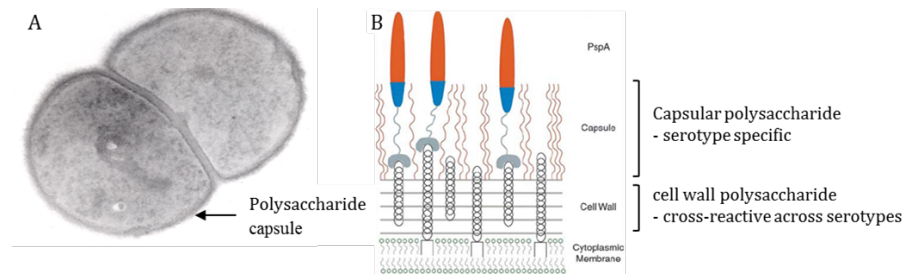


Figure 1.1: (A) Electron micrograph of *S. pneumoniae*, reproduced from the former PneumoADIP website (now: www.preventpneumonia.org); (B) Schematic diagram of the outer part of *S. pneumoniae*, which is composed of the cytoplasmic membrane, the serotype-specific capsular polysaccharide and the conserved cell wall polysaccharide, the latter being common to all pneumococcal serotypes. Reproduced from [1] with permission from the American Society of Microbiology.

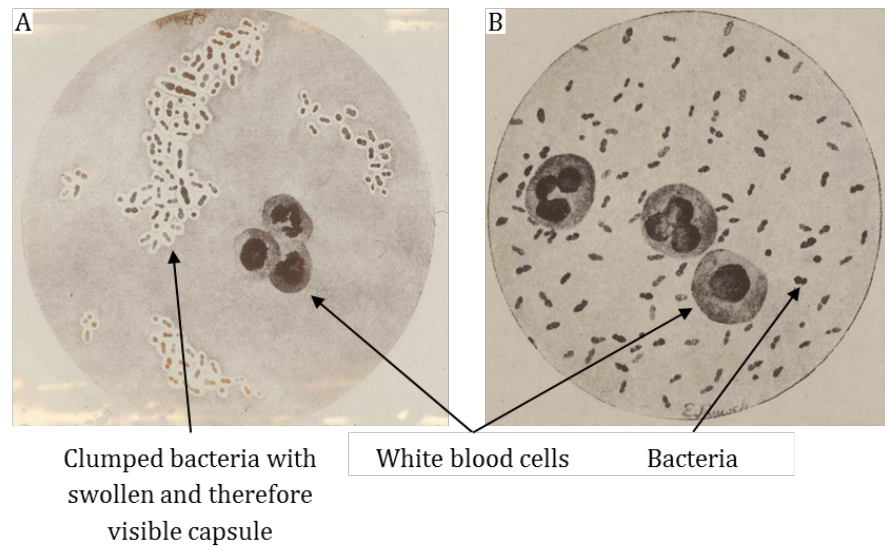


Figure 1.2: (A) Positive and (B) negative Quellung reaction using antibodies directed against pneumococcal capsular polysaccharide. Reproduced from the former PneumoADIP website (now: www.preventpneumonia.org)

the chemical structure of the capsular polysaccharide and the thickness of the capsule [11]. Further virulence factors such as the cell wall, choline-binding protein, pneumococcal surface proteins A and C are mainly situated on the bacterial surface in contrast to the intracellular toxin pneumolysin [11, 12]. Pneumococci are able to reversibly switch off capsule production, to down-regulate the amount of capsule or lose the ability to produce capsule. The pneumococcus is also very efficient in taking up nucleic acids from co-colonising pneumococci and integrate their capsular genes due to e.g. vaccine-induced selection pressure [13] resulting in a different “serotype”.

1.3 THE ANTIMICROBIAL RESISTANCE PROFILE OF *S. pneumoniae*

Antimicrobial resistance is identified by measuring the minimum concentration of an antimicrobial that will inhibit pneumococcal growth, which is referred to as minimum inhibitory concentration (MIC). Break-points are MICs that define infections as susceptible (treatable), intermediate (possibly treatable with higher doses), and resistant (not treatable) to certain antimicrobials. Antimicrobial susceptibility break-points are established based on a) the pharmacokinetic and pharmacodynamic properties of an antimicrobial agent and b) data correlating individual MIC results with patient outcomes [14].

Over the past 3 decades, antimicrobial resistance among *S. pneumoniae* has escalated dramatically worldwide. Strains of pneumococci with resistance to penicillin were found first in South Africa and Spain in the 1970s, followed by a spread across Europe and globally. Resistance to macrolides and other antibiotic classes emerged parallel to penicillin resistance. Of more concern, 40% of pneumococci display multi-drug resistant phenotypes, with highly variable prevalence among countries [15]. Serotypes 6A, 6B, 9V, 14, 19F, and 23F are responsible for most of these resistant isolates. Multiple factors may have added to the development and survival of various pneumococcal serotype strains with the selection pressure resulting from human misuse of antibiotics probably being the most important one [16]. This view is supported by the fact that most of the serotypes showing high rates of resistance frequently colonise young children. However, some of the serotypes, e.g. 3, 15, 21, 23, and 35, often carried in the nasopharynx, show low rates of resistance. Thus, there must be other factors influencing the acquisition and selection of resistant strains, e.g. the duration of the colonising event or an inherent type-specific genetic susceptibility.

In 2006, the prevalence of penicillin non-susceptible *S. pneumoniae* in Europe varied widely: 25-50% in Spain, France, Greece and Israel; 10-25% in Portugal, Ireland, Finland and Turkey; 5-10% in Italy; and 1-5% in the UK, Germany, Austria, Norway and Sweden [17]. Beta-

lactam antibiotics act via binding to penicillin-binding proteins (PBPs) whose genes are fixed on the bacterial chromosome. PBPs are located in the cytoplasmic membrane of many bacteria and have some essential functions in the cell wall synthesis process. Penicillin resistance in pneumococci is due to the modification in the structure of PBPs resulting in reduced affinity for the antibiotic. Inhibition of PBPs produces an imbalance in cell wall metabolism, resulting in growth inhibition or lysis. In *S. pneumoniae*, 6 PBPs have been described: 1a, 1b, 2x, 2a, 2b and 3. Penicillin-resistant strains show mosaicism in genes encoding 1a, 2b, 2x and sometimes 2a PBPs [18]. Mosaic sequence blocks are the result of genetic exchange between related streptococcal species, especially from *S. mitis* and *S. oralis* [19]. The prevalence of macrolide-resistant pneumococcal strains also varies extensively in Europe: 25-50% in France, Italy and Greece; 10-25% in Spain, Portugal, the UK, Germany, Poland, Norway and Finland; and 1-5% in Latvia and Sweden [17]. Resistance to macrolides is mediated by different mechanisms. Macrolide-resistance determinants are found on mobile genetic elements (transposons). The *erm*(B) gene encodes a methylase and results in macrolides-lincosamide-streptogramin B resistance. The *erm*(A) gene is found only infrequently in pneumococci. Alternatively, resistance may be mediated by the *mef*(A) gene, which encodes an antibiotic efflux pump. Other mechanisms include mutations in ribosomal RNA (23S rRNA) and ribosomal proteins L4 and L22 [20, 21]. In addition to the *mef*(A) efflux pump gene, another variant, *mef*(E), is expressed. These genes are 90% identical in nucleotide sequence, and were assigned to the same class of macrolid-resistance determinants.

The clinical MIC breakpoints of some of the most used antimicrobial drugs for upper and lower respiratory tract infections are shown in Table 1.1. These differ to a certain extent between the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the American Clinical and Laboratory Standards Institute Clinical and Laboratory Standards Institute (USA) (CLSI).

Table 1.1: Comparison of penicillin and erythromycin breakpoints (MIC) for *S. pneumoniae* defined by EUCAST and CLSI. Penicillin and erythromycin can be used to determine susceptibility to all penicillins and macrolides, respectively.

PENICILLIN			
	Susceptibility category MIC (µg/ml)		
	Susceptible	Intermediate	Resistant
EUCAST (by clinical syndrome and dose)			
Overall (incl. meningitis)	≤ 0.064	0.12 – 1	≥ 2
Pneumonia, 4 times 1.3 g	≤ 0.5		
Pneumonia, 4 times 2.4 g or 6 times 1.3 g	≤ 1.0		
Pneumonia, 6 times 2.4 g	≤ 2.0		
CLSI (by clinical syndrome and administration route)			
Meningitis, intravenous	≤ 0.064	-	≥ 0.12
Non-meningitis, intravenous	≤ 2	4	≥ 8
Non-meningitis, oral	≤ 0.064	0.12 – 1	≥ 2
ERYTHROMYCIN			
	Susceptibility category MIC (µg/ml)		
	Susceptible	Intermediate	Resistant
EUCAST	≤ 0.25	-	≥ 0.5
CLSI	≤ 0.25	0.5	≥ 1.0

PNEUMOCOCCAL CARRIAGE AND PATHOGENESIS OF DISEASE

2.1 NASOPHARYNGEAL CARRIAGE OF *s. pneumoniae*

S. pneumoniae is part of the commensal flora of the upper respiratory tract. Pneumococci are frequently and asymptotically carried in the nasopharynx of healthy individuals with the highest colonisation rates being among young children, who are thought to be the main transmitters in the population [10, 22]. Crowding, which occurs in hospitals, day-care centres, and prisons, increases the horizontal spread of pneumococci. Pneumococcal acquisition starts in the first few weeks of life [10] but acquisition is also dependent on the socioeconomic setting (Figure 2.1). Interestingly, in infants there seems to be an inverse relationship between colonisation prevalence of *S. pneumoniae* and *S. aureus* [24, 25]. However, the reason for this negative association is unknown and differences in serum antibodies against anti-pneumococcal or anti-staphylococcal antibodies don't seem to play a role [26]. In the UK, pneumococcal carriage rates were around 50%, 25% and 8-10% in the under 5 years, 5-20 years and over 20 years age groups, respectively [27]. Following introduction of the 7-valent pneumococcal conjugate vaccine into routine immunisation schedules, colonisation prevalence of vaccine-serotypes declined dramatically although overall carriage rates remained similar compared to the pre-vaccine era [27, 28].

Although mostly symptomless, colonisation can progress to respiratory or even systemic disease. Pneumococcal disease will not occur without preceding nasopharyngeal colonisation with the homologous strain [29]. In addition, several studies have shown a correlation between the duration of carriage and the occurrence of invasive disease with recently acquired serotypes more likely causing disease [9, 30]. There is also a serotype-dependent relationship between carriage and disease, which is usually referred to as serotype-specific case-carrier ratios or serotype-specific attack rates [9, 27]. For example, before routine infant immunisation in the UK, serotype 4 was rarely found in carriage studies but represented a relatively high proportion of invasive disease cases whereas the opposite was true for serotype 6B [9]. This picture, however, changed completely in studies that were conducted following the implementation of the infant pneumococcal vaccination program with an almost complete disappearance of vaccine serotypes from carriage and disease isolates [27, 31].

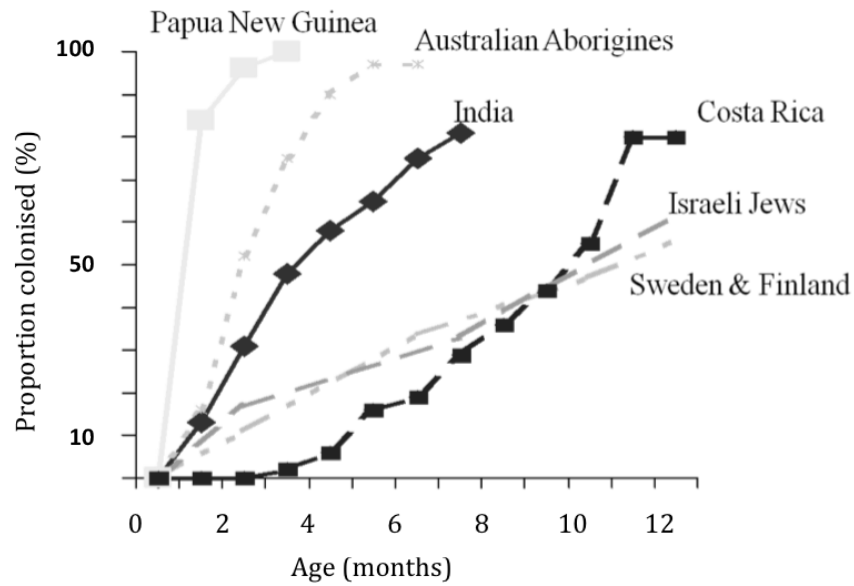


Figure 2.1: Cumulative prevalence of pneumococcal nasopharyngeal colonisation by age in various populations. Reproduced from O'Brien et al. [23], with permission from Wolters Kluwer Health.

2.2 PATHOGENESIS OF PNEUMOCOCCAL DISEASE

Colonisation of the nasopharynx is the first step in the pathogenesis of disease caused by *S. pneumoniae*. Colonisation is a frequent event, especially in pre-school children but only very few individuals further develop disease, which follows the acquisition of recently acquired serotypes rather than after prolonged carriage [11, 30]. This implies that the balance between bacterial virulence factors and the immune status of the host determines whether invasion and disease occurs. It has been shown that down-regulation of capsule production appears to be necessary for attachment and invasion of host cells through a mechanism called phase variation [12, 32–34]. Several virulence factors seem to be important in promoting bacterial adhesion to epithelium including phosphorylcholine, a constituent of both cell-wall-associated acids and lipoteichoic acids, which interacts with the receptor for platelet-activating factor [12]. Other factors facilitating attachment or invasion of bacteria are prior viral infections (especially with influenza strains) as well as inflammation and damage of the epithelial cell barrier by the exotoxin pneumolysin. This acute localised inflammation, however, does not lead to bacterial clearance and may therefore result in a prolonged carrier status. The observation of this phenomenon has even led to the suggestion that the pneumococcus might have evolved to cause this response as the resulting secretions may help transmission [12]. Other primary host defence mechanisms are either specific such as secretory IgA or are non-specific (e.g. cough reflex or mucociliar clearance). Most of the

Table 2.1: Site-specific disease potential of different pneumococcal serogroups.

SEROGROUP	COMMON SITE OF INFECTION
1, 14	Blood
6, 10, 23	Cerebrospinal fluid
3, 19, 23	Middle ear fluid

IgA in the human airway is of the type IgA₁ and the pneumococcus has developed a very efficient way to evade this mechanism by cleavage of bound IgA₁ through the secretion of a zinc metalloprotease [35], which may explain the unchanged incidence of infection in IgA-deficient individuals. Important factors for invasion are pneumococcal choline binding protein A binding to a host polymeric immunoglobulin receptor [36], which is used by the pneumococcus [37]. Once in the bloodstream, the pneumococcal capsule provides a physical barrier against the host defence mechanisms limiting the interaction of phagocyte receptors with opsonised complement and antibodies [38]. From the blood, pneumococci can spread to distant sites in the body. At the blood brain barrier similar mechanisms to those used to penetrate the mucosa help breaching the endothelium and allow entry into the subarachnoid space where rapid bacterial replication occurs. Binding of pathogen structures to innate pathogen associated receptors such as Toll-like receptor (TLR)-2 and TLR-4 produce a severe inflammatory response [39], which is responsible for the clinical features of meningitis and the neuronal damage associated with disease [40]. Similarly in the alveoli of the lung, pneumococcal replication triggers an inflammatory response and the blocking of the air space with bacteria and exudates results in the pathological hallmark of pneumonia [41]. Pneumolysin seems to play an important role in the pneumococcal virulence in pneumonia [12].

Although carriage or acquisition rates of pneumococci are often used as an indirect determinant for invasive pneumococcal disease (IPD) mainly in vaccines studies, there is clear evidence that serotype prevalence differs between colonisation and invasive disease. Before widespread use of pneumococcal vaccines, serotypes 1, 5, 7F, 9A, 12F, and 14 contributed disproportionately to invasive pneumococcal disease whereas serotypes 6B, 15A, 33F, and 35F were often carried but less invasive [9, 27, 42] (Figure 2.2). The prevalence of serotypes also differs by the site of clinical infection (Table 2.1) and the age of the host [43]. Non-typeable pneumococci, which are uncapsulated and do not react with typing sera are found in approximately 2% of pneumococci causing invasive disease [44].

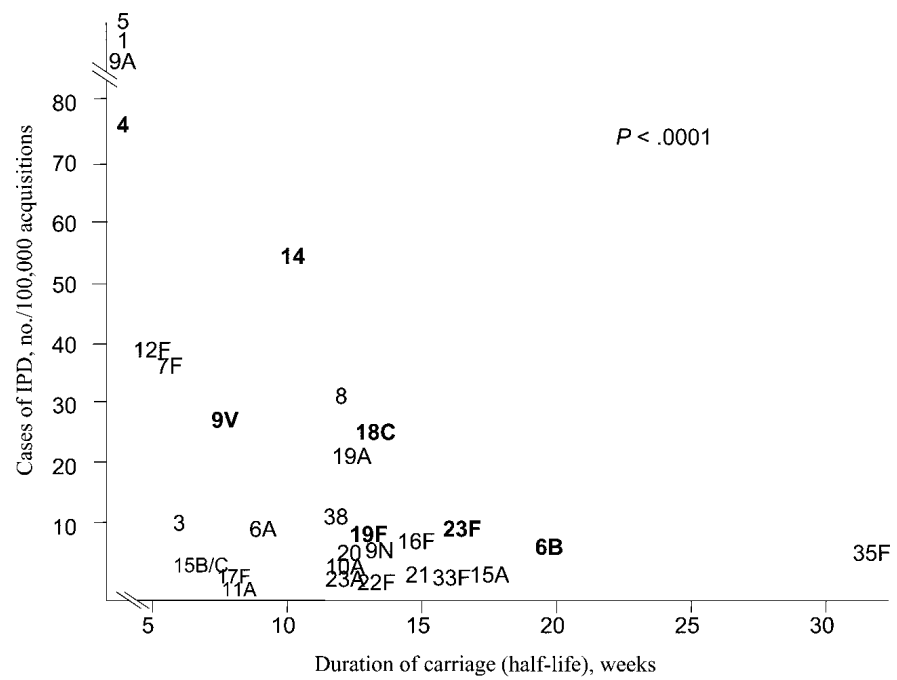


Figure 2.2: Correlation between type-specific invasive disease potential and duration of carriage. Reproduced from Sleeman et al. [9] with permission from Oxford University Press.

PNEUMOCOCCAL DISEASE AND EPIDEMIOLOGY

3.1 PNEUMOCOCCAL DISEASE

S. pneumoniae is a major public health problem worldwide causing high morbidity and mortality in young children who suffer pneumococcal pneumonia, meningitis and septicaemia. More common but less severe manifestations of pneumococcal infection are otitis media and sinusitis [45, 46], which collectively place a huge burden on health services. Peritonitis, arthritis and osteomyelitis are very rare manifestations of pneumococcal disease. Invasive pneumococcal disease (IPD) is defined as an infection in which pneumococci are isolated from an individual from a normally sterile site, such as blood, cerebrospinal fluid (CSF) and less frequently from surgical aspirate or pleural, pericardial, peritoneal, bone or joint fluid. IPD can range from mild, usually transient, occult bacteraemia without a focus of infection through severe disease with a focus of infection such as pneumonia or meningitis to a sepsis with cardiovascular compromise. Meningitis and bacteraemia account for the majority of IPD cases whereas complicated pneumonia, in which the organism is isolated from pleural fluid or blood, is rare. The majority of pneumonia cases are considered to be a localised disease and the definition of pneumonia is based upon clinical signs and symptoms and radiographic characteristics. It is the most common serious manifestation of pneumococcal disease and is far more frequent than can be confirmed by positive blood cultures [47]. Less than 10% of patients with a clinical diagnosis of pneumonia have a positive blood culture [48]. Pleural tap to obtain fluid or infected lung tissue for culture increases the identification of a causative bacterium to above 50% in children with pneumonia [47] but this procedure is rarely performed in children.

Pneumococci are spread person to person by contact with respiratory droplets containing the bacteria, such as through coughing or sneezing. Any person carrying the pneumococcus in their nasopharynx and some persons with disease can spread the bacteria. However, not everyone exposed to contaminated respiratory droplets will develop disease. Some exposed persons will become carriers of the pneumococcus, and some will progress to develop pneumococcal disease (Figure 3.1).

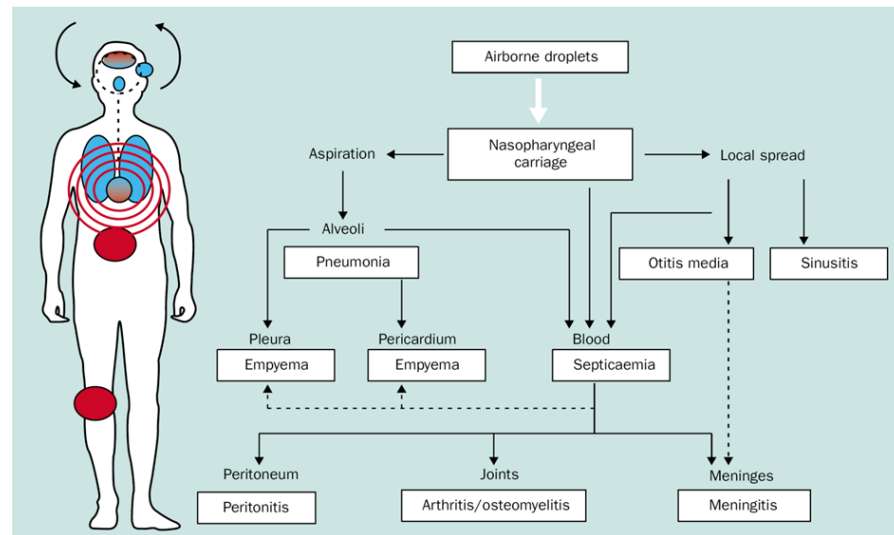


Figure 3.1: Pathogenesis of *S. pneumoniae* infection following nasopharyngeal colonisation. Reproduced from Bogaert et al. [29] with permission from Elsevier.

Meningitis

Meningitis describes inflammation of the meninges and is difficult to diagnose in young children, especially infants, as the classic clinical symptoms and signs with headache and stiff neck (which are commonly found in older children and adults) cannot be reported or are absent. Apart from high fever, presentation in children may include various subtle or non-specific symptoms such as nausea, vomiting, photophobia, sleepiness, confusion or seizures. Infants may only present with lethargy, sleepiness, irritability or have a tense, bulging fontanelle or poor feeding [49]. In developed countries, about half of acute bacterial meningitis cases in children are due to *S. pneumoniae* and also contributes to meningitis epidemics in Africa. Community-acquired pneumococcal meningitis has a very high fatality rate (20% and 50% in more and less developed countries, respectively). Depending on age, 30-60% of survivors develop long-term sequelae including hearing loss, neurological deficits, and neuropsychological impairment [50], which is much higher than is found in other causes of bacterial meningitis.

Bacteraemia and sepsis

In bacteraemia, the pneumococcus is isolated from the bloodstream. Sepsis is defined as bacteraemia combined with a severe inflammatory reaction in the host in response to bacterial infection and involving several organ systems. The signs of sepsis may include fever or abnormally low body temperature, rigors, tachycardia, low blood pressure, lethargy, confusion or irritability, skin rash, inability to eat

or drink and/or decreased urine output. There may or may not be signs of localised infection, such as pneumonia or meningitis, associated with these clinical signs. Sepsis can progress rapidly to septic shock and multi-organ failure due to lack of oxygen delivery to various organs in the body.

Pneumonia

It is thought that aspiration of *S. pneumoniae* in individuals with nasopharyngeal colonisation results in direct spread to the lungs, leading to community-acquired pneumonia (CAP), with pleural empyema as a complication occurring in less than 5% of CAP cases [29]. Pneumonia is the leading cause of death in children accounting for 17% of childhood deaths worldwide [51, 52]. Risk factors for pneumonia include a compromised immune system (such as e.g. human immunodeficiency virus (HIV) infection) or malnutrition and also environmental factors such as overcrowding in homes and exposure to tobacco smoke or indoor air pollution [45, 53, 54]. In addition, climate change might contribute to an increase in lower respiratory tract infections due to predicted increases in rainfall in tropical setting where disease incidence is generally highest in the rainy season [55]. Vaccine probe studies using pneumococcal conjugate vaccine (PCV) indicate that up to 37-39% of pneumonia cases in children are caused by the pneumococcus [56, 57]. Of the deaths due to pneumonia, 25-45% are attributed to the pneumococcus, making the organism the most common bacterial cause of pneumonia deaths (www.preventpneumonia.org). The World Health Organization (WHO) estimates that more than 1.6 million people die of pneumococcal pneumonia each year, and over half of these deaths – up to 1 million deaths – are in children under the age of five years [45].

Otitis media and sinusitis

Otitis media and sinusitis are considered non-invasive pneumococcal diseases, resulting from contiguous spread from the nasopharyngeal mucosa. Thirty to 50% of all cases of otitis media are attributed to the pneumococcus [58] with acute otitis media being more frequent with newly acquired nasopharyngeal carriage compared to established carriage [59].

Diagnosis of pneumococcal disease

Pneumococcal infections can be diagnosed by Gram stain and isolation of the organism from microbiological culture of normally sterile sites such as blood, CSF, pleural fluid or joint fluid. The highest yield of positive cultures can be expected if specimens are taken prior to

initiation of antimicrobial therapy. Culture is still the gold standard diagnostic method but its yield in most patients with pneumococcal infection is low and may be further reduced by prior antibiotic therapy. As discussed in section 1.1, there are several nucleic acid amplification tests available, with the potential to detect small amounts of bacterial DNA although sensitivity seems to be variable [60]. A number of different pneumococcal genes including autolysin, pneumolysin, and pneumococcal surface adhesin have been used to identify pneumococci by polymerase chain reaction (PCR). Loop-mediated isothermal amplification (LAMP) may be another – potentially more sensitive tool – to diagnose IPD, especially pneumococcal meningitis [61].

A further method, which may be helpful in some patients, is the rapid detection of pneumococcal antigens in urine, CSF, or pleural fluid. A urinary immunochromatographic test (BinaxNOW®), which detects the C-polysaccharide wall antigen of *S. pneumoniae* is commercially available and takes only 15 minutes to get a result. However, in children, this test lacks specificity as it cannot distinguish colonisation from infection whereas a negative test may help ruling out a pneumococcal infection [62]. In addition, this test doesn't allow antimicrobial susceptibility testing making culture still the mainstay of diagnosis.

Treatment of pneumococcal disease

Penicillin is the traditional antibiotic used for pneumococcal infections. It acts through binding to cytoplasmic membrane-bound proteins, called PBPs, which are a crucial part of the biosynthesis of the bacterial cell wall. Inhibition of PBPs produces an imbalance in cell wall metabolism, resulting in growth inhibition or lysis. Despite the escalation in the rate of antimicrobial resistance among pneumococci worldwide, the clinical impact of antimicrobial resistance is difficult to define. Although treatment failures due to antibiotic-resistant strains have been reported, the relation between drug resistance and treatment failure has not been convincingly established [63]. Other factors independent of antimicrobial susceptibility strongly influence prognosis, such as host factors (e.g. extremes of age, immunodeficiency, and comorbidities) or factors that affect intrinsic virulence of the organisms (e.g. capsular subtype).

3.2 EPIDEMIOLOGY AND RISK FACTORS OF PNEUMOCOCCAL DISEASE

More than 14 million episodes of serious pneumococcal disease and about 800 000 - 1 million deaths in children under the age of five occur annually [45, 46, 64]. Worldwide children under the age of five years and the elderly suffer the highest burden of disease. The ma-

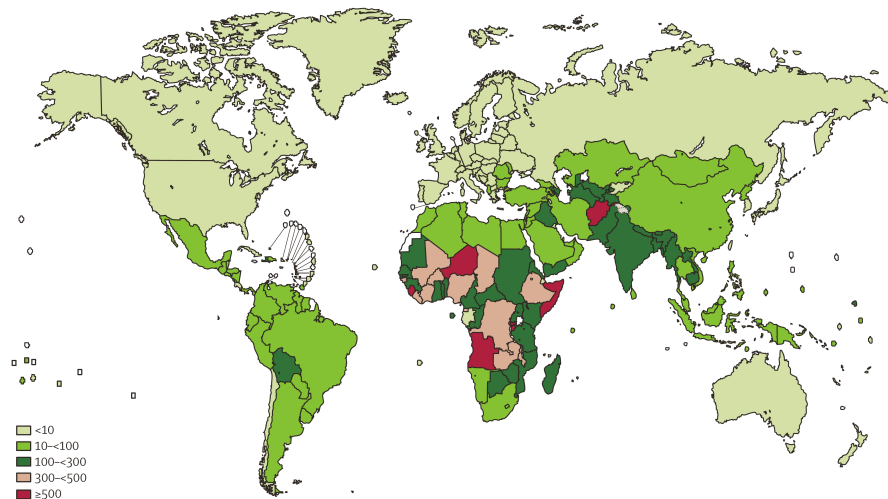


Figure 3.2: Total mortality rates from *S. pneumoniae* in children under 5 for the year 2000. Reproduced from O'Brien et al. [46] with permission from Elsevier.

majority of the morbidity and mortality due to *S. pneumoniae* occurs in developing countries (Figure 3.2).

Serotypes are not contributing equally to IPD cases in different parts of the world. The pneumococcal global serotype project aims to systematically estimate the global and regional distributions of serotypes causing IPD in children younger than 5 years of age [65]. In this project it was found that overall only a small number of serotypes cause most IPD worldwide with 10 serotypes being responsible for more than 60% of disease isolates (Figure 3.3).

Individuals with immune deficiency, such as HIV infection or congenital deficiencies in immunoglobulin or complement are also at risk of serious pneumococcal infection [66]. Further, functional or anatomical asplenia and cirrhosis of the liver predispose to severe pneumococcal infection and for the former IPD risk is highest in the first 1-2 years after splenectomy. Other conditions apart from defects in the immune system that render individuals susceptible to high rates of IPD are primarily due to respiratory disease. In adults, smoking is a strong risk factor for IPD. In children, the same is true for those who are exposed to cigarette smoke. In developing countries, malnutrition and HIV infection are the most significant risk factors.

The incidence of pneumococcal disease varies widely depending on the geographic area, the ethnic background and the stage of development [66]. Risk factors for pneumococcal colonisation and disease are summarised in Table 3.1. Individuals with inborn or acquired errors of the immune system are particularly prone to develop pneumococcal diseases. A recent study from the UK calculated odds ratios of several risk factors on IPD and found that IPD is significantly increased

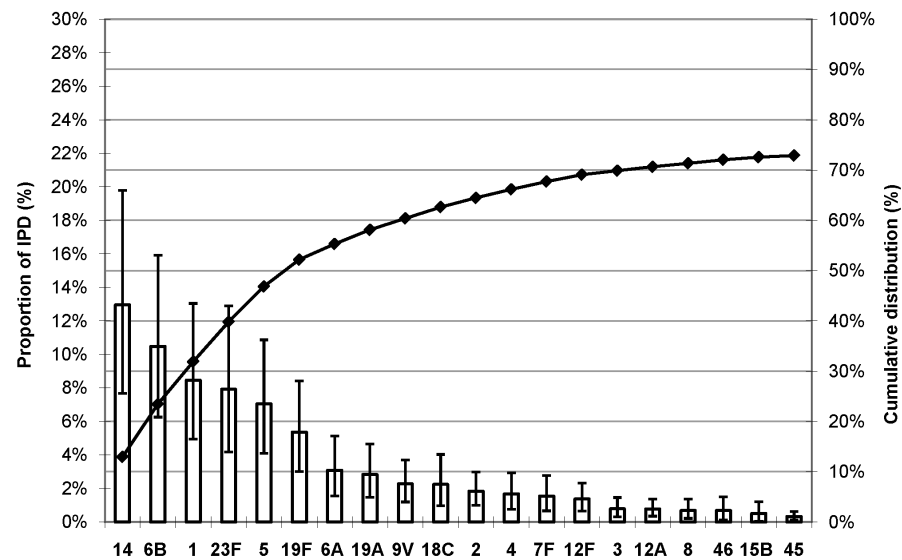


Figure 3.3: Proportion of IPD in children younger than 5 years of age due to the most common serotypes worldwide. Reproduced from Johnson et al. [65].

Age group (years)	2-15	16-64	65+
Asplenia/splenic dysfunction	5	2	1
Chronic respiratory disease	13	17	5
Chronic heart disease	4	7	3
Chronic kidney disease	12	7	1
Chronic liver disease	30	33	7
Diabetes	4	5	2
Immunosuppression	41	17	12
HIV infection	101	61	5
One or more risk factors	12	8	3

Figure 3.4: Odds ratios for IPD cases in at risk groups compared to the non-risk group. Reproduced from van Hoek et al. [67] with permission from Elsevier.

for all of the risk groups assessed but in particular for children with immunosuppression or chronic liver disease (Figure 3.4).

Table 3.1: Risk factors for pneumococcal carriage, carriage with antibiotic-resistant strains, IPD, outbreaks of IPD and mortality of IPD in children, adapted from [66].

Carriage	Young age (<5 years) Having young siblings Attendance in day care centres Inhaled corticosteroids in children with asthma [68]
Carriage with antibiotic-resistant strains	Young age (<2 years) Attendance in day care centres Recent (<3 months) antibiotic administration
IPD	Young age (<2 years), especially in winter months Exposure to cigarette smoke Multiple children in the same household Chronic illness in immunocompetent hosts (severe asthma, chronic cardiac or pulmonary disease, chronic renal disease [particularly nephrotic syndrome], diabetes, cochlear implant, cerebrospinal fluid leaks) Defects in the immune defence (HIV infection, antibody deficiency, solid and haematological malignancy, bone marrow and organ transplant recipients, functional or anatomical asplenia) Ethnic background (indigenous people of Alaska and the Canadian arctic, Inuits in Greenland, American Indians, blacks in the USA, Australian aborigines, Maoris of New Zealand and Bedouins of Israel) – socioeconomic factors might be more important than the ethnic background in these populations
Outbreaks of IPD	Crowded settings such as schools, day care centres, households with multiple children, long-term care facilities, military camps, jails, hospitals and closed communities

NATURAL AND VACCINE-INDUCED IMMUNITY TO *S. PNEUMONIAE*

4.1 NATURAL IMMUNITY TO *s. pneumoniae*

Natural immunity to *S. pneumoniae* is provided by a combination of physical barriers, innate immune mechanisms and parts of the adaptive immune system including CD4⁺ T cells and antibodies. It can be divided into preventing acquisition, clearing colonisation, preventing invasion and clearance of bacteria that have invaded the bloodstream. The nasopharynx represents the first barrier consisting of viscous mucous secretions and cilia movement [69] followed by mucosal defence mechanisms.

Innate immunity

The innate immune response acts as the first line of host defence against microorganisms that have entered the body. It is a rapid system against invading pathogens that focuses on a limited number of microbial determinants shared by a large number of pathogens. The defence mechanisms of the innate immune system rely on a limited number of germline-encoded receptors that recognise features common to many pathogens and do not rely on the clonal expansion of antigen-specific lymphocytes. Cells of the innate immune system comprise of tissue-residing cells (macrophages and dendritic cells (DC)s) and cells circulating through the body within the blood and the lymph stream (neutrophils, eosinophils, monocytes, and natural killer cells). Pathogens such as *S. pneumoniae* that have invaded the body are first recognised by innate pattern recognition receptors (PRRs), which include a number of different receptors such as the TLR family, which are activated by conserved microbe-associated molecular patterns (MAMPs). Activation of these receptors results in up-regulation of inflammatory mediators and cytokines, which in turn promote a local immune response, activate the acute-phase response, recruit neutrophils and macrophages, and control the adaptive immunity [70]. By evaluation of primary immunodeficiency patients at increased risk for pneumococcal disease, the NF- κ B pathway was found to be fundamental to control pneumococci, both by ensuring the production of polysaccharide-specific antibodies and by controlling interleukin-1 receptor-associated kinase (IRAK)-4-dependent signalling downstream of Toll-like and interleukin (IL) receptors [71].

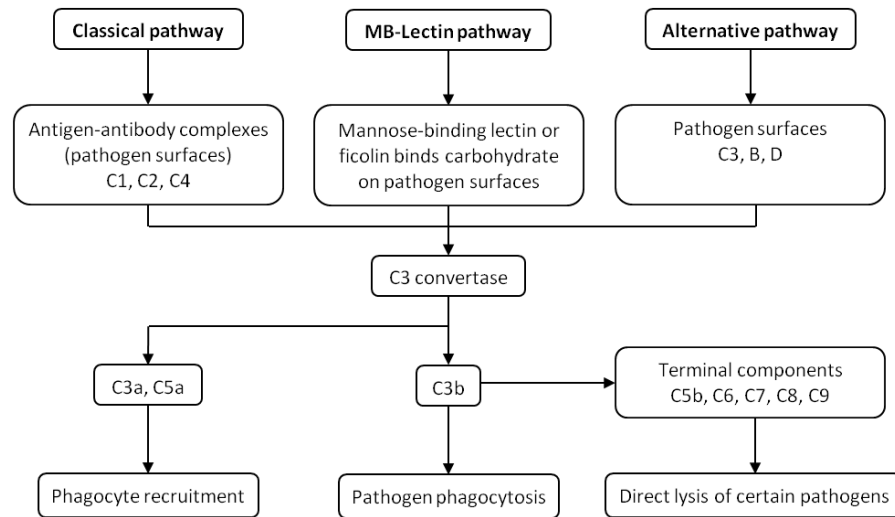


Figure 4.1: Activation of the complement pathway by pneumococci, adapted from [69].

Complement system

The complement system consists of a large number of plasma proteins that are involved in a complex biochemical cascade. There are three pathways of complement activation, the classical, the mannose-binding lectin (MBL) and the alternative pathway (Figure 4.1). Following invasion, components of the complement system are bound and activated on the pneumococcal surface resulting in opsono-phagocytosis and the initiation of an inflammatory response, which links both the innate and the adaptive arm of the immune system.

The classic complement pathway is activated by antibody-dependent opsonisation or binding of acute-phase proteins to the bacterial surface, e.g. binding of natural IgM, possibly to C-polysaccharide, or C-reactive protein to phosphorylcholine and subsequent interaction of complement component C1q. The mechanism of clearance depends on the interaction of type-specific antibodies, complement, and neutrophils or phagocytic cells from lung, liver and spleen. MBL is a plasma protein produced by the liver in response to acute infection and the MBL pathway is triggered by MBL recognising and binding carbohydrate on the microbial surface. Complement component C3 is naturally abundant in the plasma and is converted into C3b in the alternative pathway spontaneously at a low rate. However, further complement activation only occurs if C3b is bound to the surface of a pathogen. All three pathways join at the point when C3 is transformed into C3b (Figure 4.1) with the latter being able to bind to pathogen surfaces eventually resulting in phagocytosis of the pathogen, which is thought to be the main mechanism by which pneumococci are killed in humans.

Recent studies in mice have dissected the relative contribution of each pathway in the innate response to *S. pneumoniae* and found that the classical pathway is predominant whereas the alternative and MBL pathways having some and very little contribution to protective innate responses, respectively [72]. The importance of the classical pathway again with some contribution of the alternative pathway has also been suggested in vitro in humans using serum from patients with selective complement component deficiencies [71, 73]. In contrast to mice, however, a small but significant increased susceptibility to IPD was found in individuals with mutations in the MBL gene [74].

The role of antibodies in natural immunity

Similar to complement, surface bound antibodies facilitate opsonophagocytosis of the pneumococcus by recognition of the Fc portion of the antibody through phagocytes, which then initiates bacterial uptake. Although anti-polysaccharide antibodies alone may result in opsonophagocytosis, adding complement to the system greatly increases the uptake of bacteria by phagocytes [75, 76]. Early studies of serum therapy in the 1930s showed that specific anti-polysaccharide antibodies are protective against pneumococcal infection in humans [77, 78]. Similarly, *S. pneumoniae* is one of the most prevalent pathogens causing invasive disease in children with X-linked agammaglobulinaemia and disease incidences fall following immunoglobulin (Ig) substitution therapy. Only 5% of these patients, however, develop IPD [71]. Together these findings suggest that both Ig-mediated as well as non-Ig-mediated mechanisms contribute to protective immunity to the pneumococcus, although their relative contribution remains unknown. Work in mice implies that IgG mediated protection is mainly through activation of the complement cascade [79]. In humans there seems to be a difference between IgG subclasses and their relative contribution to complement activation with greater protection offered by IgG₁ than IgG₂ [80]. Equally, IgG₁ is up to 1000-fold more efficient in activation of the complement cascade [81–83]. In unvaccinated individuals, however, IgG₂ is the predominant serotype-specific IgG subclass [84].

Natural antibodies

B cells produce antibodies also in the absence of microorganisms, which has been shown in experiments with germ-free mice [85–87]. Furthermore, B cells may proliferate and produce antibody independently of their specific B cell receptor, e.g. in response to Gram-negative bacterial cell membrane component lipopolysaccharide (LPS) (thymus independent (TI) type 1 response) through recognition by TLR-4. The expression of TLR-4 and other PRRs like TLR-3, TLR-7, TLR-8 and TLR-

9 links B cells to the innate immune system [88]. These non-specific antibodies are produced early in the immune response, are predominantly IgM antibodies and may be considered part of the innate response. Most natural IgM is produced by so-called B1 cells [88, 89]. In mice it has been shown that the antibody repertoire of B1 cells is mainly derived from germline-encoded sequences and shows minimal non-templated nucleotide (N-region) insertions and only a small amount of somatic hypermutation [90]. These antibodies are highly cross-reactive and can recognise antigens displayed on the surface of several pathogens. It has been shown in mice that depleting natural IgM producing B cells makes the animals more susceptible to pneumococcal infection [91]. Antibodies that bind to the polysaccharide capsule may also play an important role in natural immunity against the pneumococcus although in humans there are only limited data available. Carsetti et al. investigated frequency of IgM memory B cells (B_{MEM}) and levels of anti-polysaccharide IgM antibodies in a group of patients with common variable immunodeficiency (CVID) and found an inverse correlation between both measurements and the susceptibility to bacterial infection of the lower respiratory tract [92]. Specific antibodies against pneumococcal surface proteins are produced naturally in adults [93] but less so in children [94] and their role in protection against pneumococcal disease remains elusive. Musher et al. took anti-pneumolysin IgG antibodies from patients with pneumonia and transferred into mice, which protected them against IPD [95].

Mucosal immunity

Mucosal immunity seems to mature earlier than systemic immunity, and might be present from the age of 6 months [29]. Traditionally it was thought that the local immune response is mediated by secretory IgA antibodies and IgG antibodies “leaking” from serum directed against capsular polysaccharides and surface-associated proteins as these have been found in saliva of children in response to colonisation with *S. pneumoniae* [96, 97]. There is, however, some evidence that T cells, in particular $CD4^+$ TH_{17} cells, and not humoral immunity plays a major role in the mucosal immunity to pneumococci [98]. For example, the rates of IPD are higher in patients with acquired immunodeficiency syndrome (AIDS) and there is a correlation between low $CD4^+$ T cell counts and a persistent pneumococcal carriage in HIV positive patients [99, 100]. Further evidence to support a mechanism for protection against pneumococcal acquisition and subsequent invasion that is not serotype-specific is the fact that IPD incidences for the seven most important serogroups seem to decline simultaneously with age [101]. Using a mouse model of pneumococcal colonisation, the importance of $CD4^+$ T cells in the clearance of

colonisation and the protection of disease through acquisition of T cell immunity has clearly been demonstrated. In this study, antibody-deficient mice that were colonised with live pneumococci were protected from re-colonisation with the same and a different serotype through an anti-capsular antibody-independent mechanism [102]. A similar phenomenon was observed when a killed, non-encapsulated whole-cell vaccine was used for intranasal immunisation indicating protection that is independent of antibodies to any pneumococcal antigen. T cell deficient mice, however, were not protected by intranasal vaccination with the whole-cell vaccine suggesting a T cell dependent mechanism for protection [102]. The relationship between colonisation and the development of immunity is not fully understood and only few studies have looked at the generation of CD4⁺ T cell immunity in response to pneumococcal colonisation in humans [103].

4.2 THE HUMORAL IMMUNE RESPONSE

Most infectious agents activate innate immune cells, which then release cytokines and chemokines resulting in an inflammatory response. This helps antigen-bearing cells migrate to the lymphoid tissues, where they will also activate lymphocytes so that an early adaptive immune response is initiated. Hallmarks of adaptive immunity are (i) specificity, i.e. the response is restricted to a particular antigen or epitope and (ii) memory, which means that after recovering from an infection, the immune system is primed to respond more rapidly upon subsequent encounter with the same antigen.

Lymphoid organs play an important role in the adaptive immune system and can be divided into primary (central) lymphoid organs, the place where B and T lymphocytes are generated, and secondary (peripheral) organs where adaptive immune responses are initiated (Figure 4.2). Although both B and T cells are being produced in the bone marrow, only the B cells mature there whereas the precursor T cells migrate to the thymus where they mature [105]. Both B and T cells have receptors (B and T cell receptors, respectively) that specifically recognise an antigen by binding to a 3-dimensional epitope and a major histocompatibility complex (MHC)-peptide complex in the case of B and T cells, respectively. The concept of thymus dependent (TD) and TI responses is of particular importance in the context of vaccine responses. The main aim of most currently used vaccines is to induce a humoral response, i.e. generation of an antigen-specific antibody response. Antibodies are produced by B cells that have been triggered and activated by antigen and have undergone differentiation into antibody-secreting plasma cells. Extracellular spaces, which have been invaded by pathogens and which are usually the place where they multiply, are protected by antibodies, which allow destruction of extracellular microorganisms and prevention of spread-

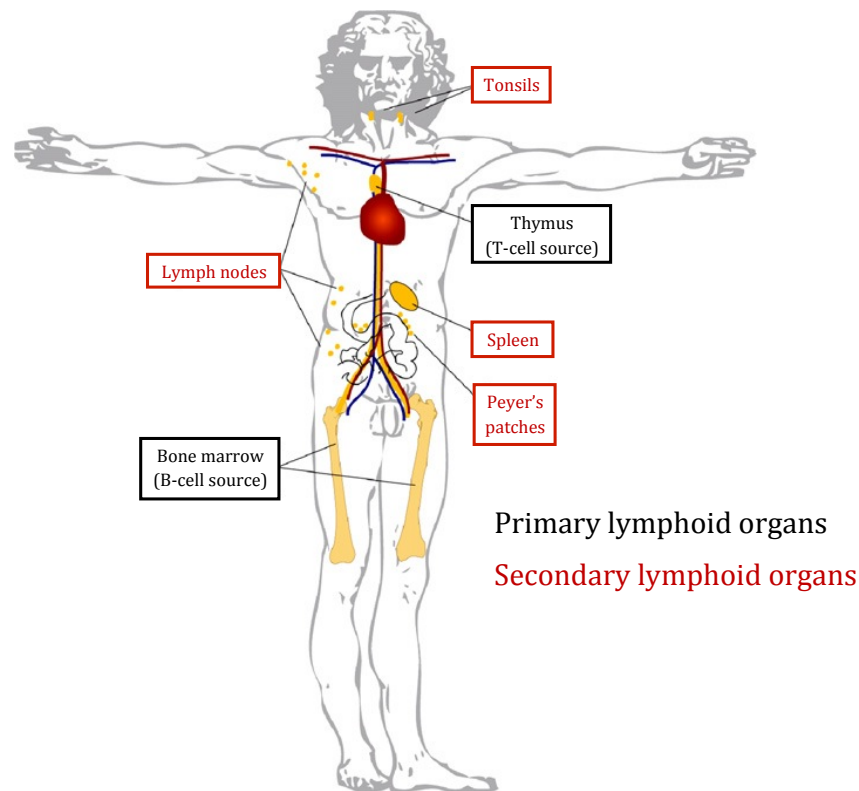


Figure 4.2: Lymphoid organs in humans, reproduced and adapted from [104] with permission from Elsevier.

ing intracellular infections. Antibody-mediated immunity is achieved by binding of antibodies to the surface of pathogens resulting in (i) neutralisation, i.e. prevention of bacterial adherence, (ii) opsonisation, i.e. promoting phagocytosis, and (iii) complement activation, which enhances opsonisation [105]. Some B cells differentiate into memory B cells rather than plasma cells as is discussed below.

Thymus dependent response

In order to proliferate and differentiate, naïve B cells need further signals in addition to activation through their B cell receptor by antigen. These signals are provided by T cells in an antigen-specific manner and/or by TLR agonists or by cytokines produced by activated DCs [106]. In most cases, responses to whole pathogens or proteins involve T cell help, which is why they are called thymus dependent (TD) responses. T cell help is achieved by the B cell presenting antigen to a T cell via MHC-II. In vaccinology, this concept is used in glycoconjugate vaccines to generate a TD response to polysaccharides, which are hydrophilic and can therefore not bind to the MHC-II cavity. In the traditional model for antigen processing and presentation of a glycoconjugate vaccine, the polysaccharide part of the covalently linked antigens binds to polysaccharide-specific B cells via their B cell

receptor (BCR), followed by internalisation, processing and presentation of the peptide epitope to T cells, which bind via their T cell receptor (TCR) resulting in release of cytokines. In this case the B and the T cell clones that are involved in the immune response recognise different antigens (Figure 4.3a). However, this theory has never been backed up by experimental data and the mystery that the covalent link between polysaccharide and protein seems fundamental for the generation of a TD response has never been resolved as the co-delivery of both antigens separately does not elicit an immune response to the polysaccharide. The traditional view has been recently challenged by Lai and co-workers suggesting that the polysaccharide of a pneumococcal glycoconjugate may be localised to the surface of an antigen presenting cell and co-localised with MHC-II [107]. More detailed work exploring the mechanism of the processing and presentation of conjugate vaccines was performed by Avci and colleagues [108] who showed that T cells may well be able to recognise carbohydrates presented in the context of MHC-II. In this model, internalisation is achieved by binding of the polysaccharide to the BCR but the covalent link between protein and carbohydrate is not broken up within an endosome. A fragment of the vaccine consisting of both a carbohydrate and a peptide is then presented to T cells through binding of the peptide in the MHC-II groove therefore serving as an anchor for the carbohydrate part to be displayed to T cells (Figure 4.3b). In this model, B cells and T cells recognise the same antigen, i.e. carbohydrates in the case of a glycoconjugate vaccine. During an immune response to a TD antigen, these interactions between B and T cells occur in a specialised anatomical structure, called germinal centre (GC), to which antigen has been brought by macrophages or DCs and in which the direct outcome of a TD reaction is produced. There is, however, some early B cell differentiation outside of GC, which is then called an extrafollicular response and represents an early defence delivered by unmutated and short-lived plasma cells [109, 110].

Thymus independent response

Some bacterial components such as capsular polysaccharides, polymeric proteins, and lipopolysaccharides are able to induce an antibody production in the absence of helper T cells, achieved through strong BCR cross-linking along with danger signals provided by TLR agonists or inflammatory cytokines, e.g. [109]. There are two classes of TI antigens. At high concentrations, TI-1 antigens such as LPS are able to induce a B cell response, i.e. proliferation and differentiation of naïve B cells, regardless of their antigen specificity, which is called polyclonal activation. This response is predominantly mediated by binding to receptors other than the BCR, e.g. LPS-binding protein and CD14. At low concentrations, only B cells that are able to bind the TI-1

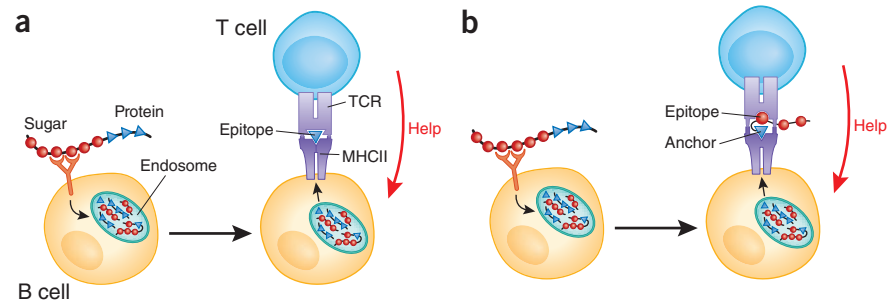


Figure 4.3: Two models for antigen processing and presentation of a conjugate vaccine. (a) Traditional view of how glycoconjugate vaccines are processed with only the fragmented protein being presented to T cells. (b) An alternative model that has been proposed by Avci et al. [108], which is based on mice work demonstrating that in contrast to previous assumptions T cells are in fact able to recognise (processed) polysaccharide with the carrier protein serving as an anchor to allow binding to the MHC-II groove. Reproduced from Rappuoli et al. [111] with permission from Nature publishing group.

antigen with their BCR will respond. It is believed that *in vivo* during an infection the concentration of TI-1 antigens are low so that only antigen-specific B cells respond resulting in early targeted antibody production although without affinity maturation, class switching and also probably less production of B_{MEM}. TI-2 antigens such as bacterial polysaccharides, show highly repetitive structures and are not able to intrinsically stimulate naïve B cells in the absence of other stimuli and are also not capable of activating immature B cells in contrast to TI-1 antigens, which can activate both immature and mature B cells [105]. Plain polysaccharide vaccines such as the 23-valent pneumococcal polysaccharide vaccine (PPV-23, Pneumovax II, Merck Inc.) are thought to behave like TI-2 antigens in humans and do not produce B_{MEM} or generate class switched antibodies that contain somatically mutated genes. There is, however, increasing evidence (at least in mice) suggesting the existence of TI memory consisting of (i) B_{MEM} that are phenotypically and functionally different from TD B_{MEM} and (ii) memory plasma cells (PC_{MEM}), which play a fundamental role in protection [109, 112].

Children under the age of 2 years are not able to make an efficient immune response to TI-2 antigens such as plain polysaccharide vaccines, an observation which led to the development of glycoconjugate vaccines. This phenomenon is not well understood but present models suggest the following:

- A. Immaturity of most B cells, e.g. lack of CD21/complement receptor 2 on neonatal B cells [113–116].

- B. Differences in activation requirements between primary and secondary B cells with young children having primary B cells that may need BCR signalling and T cell help to get activated [117].
- C. Low frequency of marginal zone B cells (MZB), which are thought to be the B cell subset mainly responding to TI-2 antigens [118, 119].
- D. Low levels of complement C₃ in neonates reaching adult levels at around 1 year of age.
- E. Generalised immaturity of marginal zone DCs – immaturity of the marginal zone in young children [120].

In contrast to mouse data, assessment of the immune response to TI-2 antigens in humans does not show a similar clear picture, at least when using plain polysaccharide vaccines, which may contain additional antigens providing low-level T cell help or serving as adjuvants. Although infants respond less well than adults to these vaccines, recent data suggest that a large proportion still mount an “adequate” immune response by generation of functional antibodies to some of the antigens included in the vaccine [121].

For both TD and TI antigens, the strength of the B cell signal seems to influence the type of immune response. Strong BCR signalling favours an extrafollicular response over a germinal centre response whereas in the case of a weak signal it is the other way round [109]. The epitope density of antigen determines the B cell fate, too. TI-2 antigens show a high epitope density resulting in strong BCR cross-linking and stimulation of mainly B₁ cells and MZB. TD antigens have lower density epitopes, which leads to reduced BCR cross-linking and activation of follicular B cells (FOB) and MZB [109].

Germinal centres and immune memory

For TD antigens, immune memory is provided by B_{MEM} and long-lived plasma cells (PC_{MEM}) with the germinal centre being the site of production of both of these B cell subsets [110, 122–124]. Following encounter with antigen and initiation of the T helper cell (TH) adaptive immune response through DC as well as activation of naïve antigen-specific B cells, B and T lymphocytes meet and interact in the T cell zone of a secondary lymphoid organ [125]. Activated THs bind antigen on cognate B cells via TCR and MHC-II, respectively, along with co-receptors such as e.g. CD4 and CD40:CD40L resulting in release of B cell-stimulating cytokines, which induce B cell proliferation [105]. Some activated B cells differentiate into short-lived plasma cells and secrete low-affinity antigen-specific antibody of mainly IgM. Other activated B cells go to the B cell follicle and form a GC [125],

where they undergo multiple cycles of proliferation and somatic hypermutation followed by selection based on the affinity of the BCR for antigen (affinity maturation). Again through interaction with GC THs some of these B cells undergo class switching, i.e. switching their immunoglobulin constant region from IgM to e.g. IgG followed by differentiation into PC_{MEM} and B_{MEM}. However, recent data in mice indicate that generation of B_{MEM} is not restricted to the GC and that two distinct pathways involving different transcriptional regulators exist for production of GC and non-GC memory B cells [126]. There is still debate on the exact mechanisms of survival and maintenance of PC_{MEM} with the theory of “imprinting lifespan”, i.e. the plasma cell life depends on strength of combined B cell signals during the encounter with antigen, fitting best the observed antibody kinetics in humans [127]. Mechanisms of long-term maintenance of B_{MEM} are also still unclear but are most likely relying on antigen-independent processes [128].

4.3 B CELL SUBSETS IN IMMUNE RESPONSES

Human B cells can be divided into two main groups, namely B1 and B2 cells. B2 cells form the biggest population, and consist of FOB and MZB. FOB can be divided into naïve and memory B cells. Although different B cell subsets have been defined their exact contribution to the secondary immune response remains unclear [88, 110, 118, 129]. B1 cells represent the predominant source of natural IgM and IgA secretion and are rapidly activated by antigen encounter through pathogen-associated molecular patterns (PAMPs), therefore acting as innate-like B cells. B1 cells spontaneously secrete natural antibodies in the absence of specific immunisation, which account for most of the resting IgM, and a large proportion of the resting IgA, found in normal serum. Natural antibodies are broadly reactive, autoreactive, and repertoire-selected and are more germline-like compared to B2 cell derived antibody and are thought to be important in the early defence against bacterial and viral infections. The human counterpart of mouse B1 cells has only recently been identified [89], although it is less clear if the equivalent of the mouse B1a and B1b subsets exists in humans. Soon after the publication about the discovery of human B1 cells [89], controversy has risen around the nature of this circulating cell population [130, 131]. In particular, there is difficulty distinguishing these putative B1 cells from T cells and (pre-) plasma cells [131–134]. An overview of the human B cell subsets and their characteristics are shown in Table 4.1.

The relative distribution and the surface markers of known lymphocyte subpopulations in human peripheral blood are shown in Table 4.2. In both humans and mice, several functionally distinct subpopulations of mature B cells can be distinguished. Most B cells ultimately

Table 4.1: Human B cell subsets and their characteristics.

	T cell independent, extrafollicular (GC-independent)		T cell dependent, germinal centres	
	B1		B2	
	B1a/b?	MZB	FO	
			Naive	Memory
<i>Activation mechanisms</i>	PAMPs	Antigen specific activation; BCR crosslinking+C3	Antigen specific activation (BCR); requires T cell help (naïve only)	
<i>Antibody production</i>	Low avidity Ab (IgM, IgA) polyreactive	Low avidity Ab (IgM, IgA, IgG ₂); specific	High avidity Ab, (IgG ₁₋₄ , IgA); highly specific (memory only)	
<i>Ig gene repertoire</i>	Germline-like	Minimal SHM	CSR, SHM	
<i>Age-related pattern</i>	Decline with age	Decline with age, immature in <2 yrs olds	Memory established with age and antigen experience more clonal in older adults	
<i>Response to antigen</i>	Rapid, no previous antigen encounter required	Rapid, antigen-specific, some improvement in Ab quality	Antigen specific, long lasting previous antigen encounter required improved Ab specificity with each antigen encounter	
<i>Immune response</i>	Innate	Adaptive	Adaptive	

Ab, antibody; BCR, B cell receptor; CSR, class switch recombination; FO, follicular B cells; Ig, immunoglobulin; SHM, somatic hypermutation

serve to yield antibody secreting cell (ASC)s, but the subsets differ in location, ability to migrate, activation by antigen in a TI or TD manner, in the rate of ASC differentiation and the stimulation requirements for ASC differentiation [135]. Classification of B cell subsets continues to evolve. Historically, B_{MEM} were defined as being class-switched, which means that these cells lack IgM and IgD surface expression through a germinal centre reaction compared to naïve B cells being IgM⁺IgD^{hi}. Subsequent studies revealed that the expression of CD27, a TNF-receptor that binds to ligand CD70, is a universal B_{MEM} marker in humans. In the CD27⁺ population, further B cell subsets have been defined (Table 4.2). More recently, the dogma of CD27 as a marker of memory B cells has been questioned by discovery of switched and hypermutated CD27⁻ B cells and CD27⁺ B cells with a more naïve and innate phenotype, respectively, such as human B1 cells.

Table 4.2: Characteristics of major human B cell subsets in healthy 20-40 years old adults, adapted from [89, 110, 129, 134, 136, 137].

Naïve				Switched		CD27- FCRL4+		CD27- ABC B1-		B1		Switched memory		MZB		IgM memory		Plasma blasts / cells			
Frequency in PB (% of total B cells)										40-50%										<5%**	
Frequency of CD27 B cell subset (% of total B cells)		80-90% (50%)		?10-15% (5-10%)		-*		?		40% (15%)		40% (15%)		?10% (5%)		?10% (5%)					
Surface markers										CD27 ⁺										CD27 ⁺⁺	
IgM ⁺ IgD ⁺		IgM ⁻ IgD ⁻ (IgG/A/E ⁺)		IgG/A ⁺		IgG ⁺		IgM [±] IgD [±]		IgM ⁻ IgD ⁻ (IgG/A/E ⁺)		IgM ⁺ (+) IgD ⁽⁺⁾		IgM ⁺ IgD ⁻		All isotypes ^{lo/-}					
CD19 ⁺ CD20 ⁺ CD43 ⁻ CD5 ⁻		CD19 ⁺ CD20 ⁺ CD20 ^{hi}		CD19 ⁺ CD20 ⁺		CD19 ⁺ CD20 ⁺ CD24 ⁺ CD38 ⁺		CD5 [±] CD19 ⁺ CD20 ⁺ CD21 ⁺ CD43 ⁺ CD44 ⁺ CD45RB ⁺ HLA-DR ⁺		CD19 ⁺ CD20 ⁺ CD43 ⁻ CD5 ⁻		CD19 ⁺ CD20 ⁺ CD43 ⁻ CD5 ⁻		CD19 ^{lo} CD20 ^{lo/-} CD21 ^{lo/-} CD27 ⁺⁺ CD43 ⁺⁺ CD138 [±]							
IgG subclass secretion		-		IgG _{1/3}		-		-		-		IgG ₁₋₄		IgG ₂		-					

ABCB1, ATP-binding cassette B1 transporter; FCRL4 (FCRH4), Fc receptor-like protein 4; MZB, marginal zone B cells; PB, peripheral blood

*In normal individuals, this memory B cell subset has only been found in mucosal associated lymphoid tissue (MALT; tonsils) and not in the PB, but it appears in the PB of HIV infected patients [138]

** may increase to up to 30% or even higher following antigen challenge such as vaccination

4.4 PNEUMOCOCCAL VACCINES

As already discussed, there are two main types of vaccines to protect against pneumococcal disease, plain polysaccharide and glycoconjugate vaccines. The latter consists of purified capsular polysaccharides, which have been individually covalently conjugated to a carrier protein.

The efficacy of the currently available 23-valent pneumococcal polysaccharide vaccine (PPV-23) is not well established in adults and, as with many plain polysaccharide vaccines which act as TI antigens, it is far less immunogenic in infants compared with responses in adults. Some infants are able to mount a functional response to certain serotypes, such as 1, 5, 6B, 14, 19F, and 23F after PPV-23 immunisation [121]. In addition to low immunogenicity, there are concerns that PPV-23 may induce hyporesponsiveness to subsequent doses of pneumococcal vaccines [139, 140]. The vaccine is still recommended for the immunisation of children and adults at high risk of pneumococcal disease in the UK. The PCVs contain one or several carrier proteins covalently linked to the polysaccharides, converting them into TD antigens capable of inducing immunological memory and an immune response in infants.

Pneumococcal conjugate vaccines

An overview of licensed and unlicensed pneumococcal conjugate vaccines that have been used in published studies is shown in Table 4.3.

The first licensed 7-valent pneumococcal conjugate vaccine (PCV-7) included serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. Currently used vaccines are the 10-valent pneumococcal conjugate vaccine (PCV-10) (PCV-7 serotypes plus serotypes 1, 5 and 7F) and the 13-valent pneumococcal conjugate vaccine (PCV-13) (PCV-10 serotypes plus serotypes 3, 6A and 19A).

Immunisation schedules of pneumococcal conjugate vaccines in Europe

Pneumococcal immunisation schedules vary widely in different European countries (Figure 4.4). In the UK, PCV-7 was introduced in September 2006 with a 2+1 schedule (2-4-13 months) for infants younger than 2 months of age. Additionally, there was a catch-up campaign for children 2 months to 2 years of age. From April 2010, the UK was the first European country to change to the routine use of a higher-valency pneumococcal conjugate vaccine, namely PCV-13.

Table 4.3: Serotypes and carrier proteins of higher-valent pneumococcal conjugate vaccines.

Vaccine (Serotypes) [product name, manufacturer]	Antigen dose	Adjuvans	Carrier protein
<i>7-valent</i> (4, 6B, 9V, 14, 18C, 19F, 23F)			
[Prevenar, Wyeth]	2 µg; 4 µg (6B)	0.125 mg Alum phosphate	20 µg CRM ₁₉₇
[-, Merck]	Several dosages studied	?	123 µg OMPa
<i>9-valent</i> (PCV-7 + 1, 5)			
[-, Wyeth]	2 µg; 4 µg (6B)	0.125 mg Alum phosphate	CRM ₁₉₇
<i>10-valent</i> (PCV-7 + 1, 5, 7F)			
[Synflorix, GSK]	1 µg; 3 µg (4, 18C, 19F)	0,5 mg Al ₃ +	Protein Db (13 µg), Tetanus toxoid (8 µg: 18C); Diphtheria toxoid (5 µg: 19F)
<i>11-valent</i> (PCV-7 + 1, 3, 5, 7F)			
[-, Aventis Pasteur]	1 µg; 3 µg (3, 14, 18C); 10 µg (6B)	+/- Aluminium hydroxide	Tetanus toxoid; Diphtheria toxoid (3,14,18C,6B,19F)
[-, GSK]	1 µg; 3 µg (4, 18C, 19F)	0,5 mg Al ₃ +	Protein Db (13 µg), Tetanus toxoid (8 µg: 18C); Diphtheria toxoid (5 µg: 19F)
<i>13-valent</i> (PCV-7 + 1, 3, 5, 6A, 7F, 19A)			
[Prevenar 13, Wyeth/Pfizer]	2.2 µg; 44 µg (6B)	0.125 mg Alum phosphate	34 µg CRM ₁₉₇

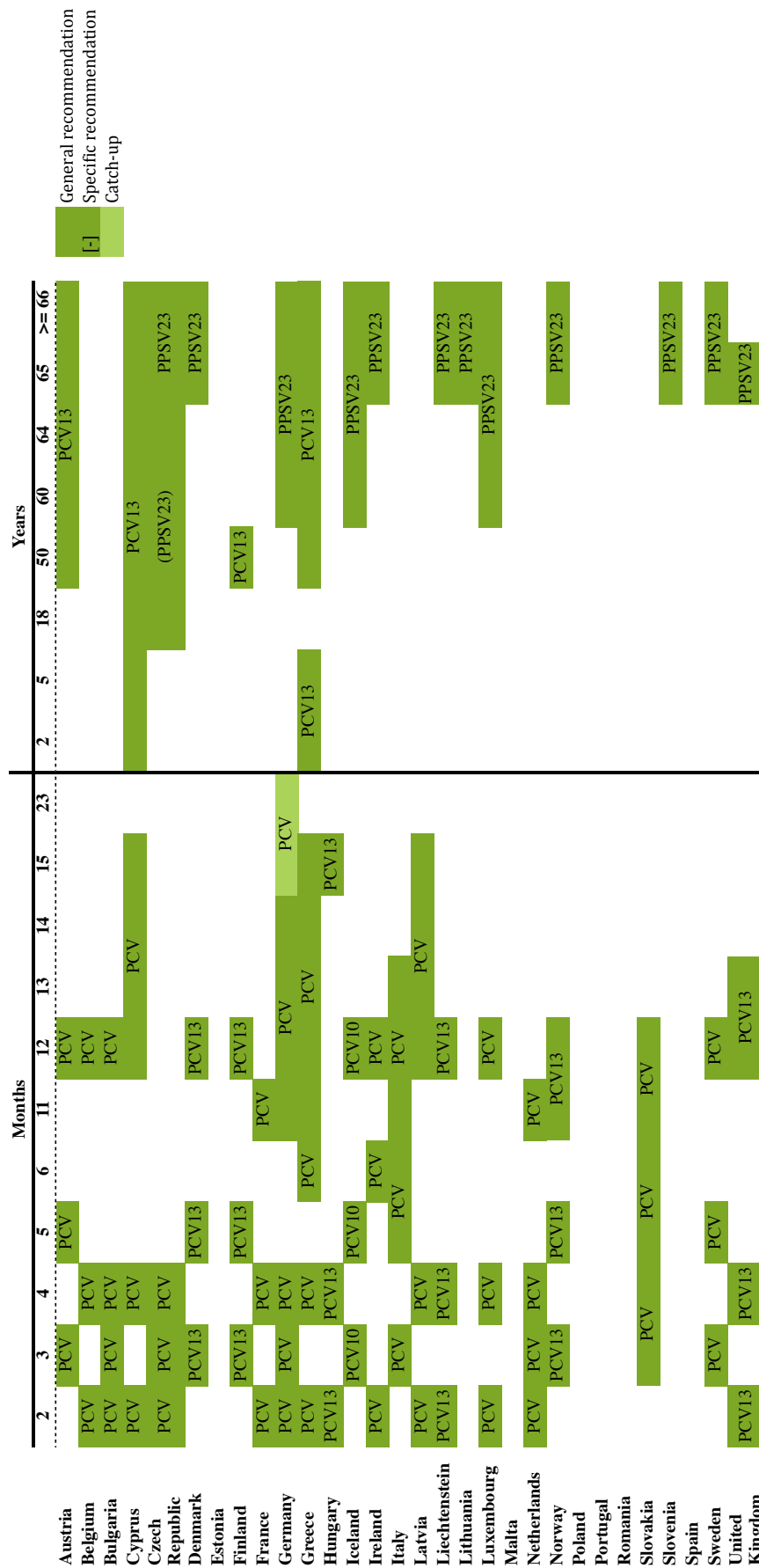


Figure 4.4: Overview of timing of pneumococcal conjugate vaccine programs across European countries. Reproduced from the European Centre for Disease Prevention and Control (ECDC) website [141].

Vaccine efficacy of pneumococcal conjugate vaccines

PCVs have been shown to be highly efficacious in infant schedules against both invasive disease and pneumonia (Table 4.4). The efficacy of PCV-7, PCV-13 and PCV-10 in age groups outside infancy has not been established. However PCV-7 and PCV-13 are immunogenic in children, adolescents and adults including the elderly although their usage is debatable because of the great impact of herd immunity in countries, which have implemented an infant vaccine programme [142, 143]. PCV-7 was introduced in the US in 2000 and successfully reduced the burden of IPD in that country [144]. The nasopharyngeal carriage rates of pneumococcal serotypes covered by the vaccine were also reduced, with the resultant herd immunity reducing IPD rates in adults [145, 146], making the vaccination programme highly cost effective [147]. A similar reduction in IPD due to vaccine serotypes has also been seen in children in the UK since the introduction of routine infant immunisation with the PCV-7 in 2006 [147].

Despite the success of the PCV-7 immunisation campaigns there remains a significant burden of disease due to serotypes that are not covered by the current vaccine [147]. These serotypes accounted for 20-30% of childhood IPD in the UK and the rest of Europe prior to the introduction of PCV-7. Less than 50% of IPD is due to PCV-7 serotypes in much of Asia and Africa, where serotypes 1 and 5 are responsible for a substantial proportion of disease [2, 148]. There is also concern regarding the emergence of disease-associated pneumococcal serotypes not contained in PCV-7 such as serotype 19A, a substantial proportion of which is penicillin resistant in the US [149]. An increase in 19A disease has also been seen in some countries not routinely using PCV-7 [150, 151], indicating that changes in epidemiology are not driven by vaccine pressure alone.

In 2010, a pneumococcal conjugate vaccine directed against 13 serotypes, PCV-13, was licensed in the UK and the US on the basis of serological non-inferiority to PCV-7. Although there are currently limited data available, marked reductions in the incidence of IPD caused by the additional serotypes in PCV-13 were noted both in the UK and the US with indirect effects of PCV-13 use in children being evident in all adult age groups [152, 153]. Of the additional serotypes included in PCV-13, the protective effect only for serotype 3 remains uncertain as indicated by early post-surveillance data from the UK [154].

Magnitude and kinetics of B cell responses

Both pneumococcal plain polysaccharide (PPS) and PCV confer protection from disease by the induction of humoral immune responses, which are conventionally described by the serum antibody concentration. However, a full understanding of the key determinants of indi-

Table 4.4: Intention-to-treat efficacy of pneumococcal conjugate vaccines against vaccine-type invasive disease, WHO X-ray defined pneumonia, otitis media in controlled double blind trials involving healthy HIV negative children <24 months of age, reviewed in [155–157].

	Number of participants		Disease cases (vaccine types)		Vaccination schedule [Vaccine valency, carrier protein]		Efficacy (95% CI)		Reference
	Control	PCV	Control	PCV					
Invasive disease	18'941	18'927	49	3	2, 4, 6, 12-15 months [PCV-7, CRM ₁₉₇]		94% (80, 98)		[158] 2000, USA
	831	831	1	0	2, 4, 6, 12 months [PCV-7, CRM ₁₉₇]		75% (-644,99)		[159] 2001, Finland
	831	835	1	0	2, 4, 6, 12 months [PCV-7, OMP]		75% (-640, 99)		[160] 2003, Finland ^a
	2'818	2'974	11	2	2, 4, 6, 12-15 months [PCV-7, CRM ₁₉₇]		83% (22, 96)		[161] 2003, USA
	18'626	18'633	17	3	6, 10, 14 weeks [PCV-9, CRM ₁₉₇]		83% (39, 97)		[162] 2003, S. Africa
	8'719	8'718	45	13	3 doses <1 year [PCV-9, CRM ₁₉₇]		71% (46,86)		[56] The Gambia ^b
X-ray pneumonia	6'094	6'097	1	3	6, 10, 14 weeks [PCV-11, TT/Dip]		-199% (-2772, 69)		[163] 2009, The Philippines
	10'273	4'941	12	0	3 doses (4 wk gap) + booster (4 mo gap, > 11mo) [PCV-10, Protein D/TT/Dip]		100% (83-100)		[164] 2012, Finland
	2'818	2'974	139	162	2, 4, 6, 12-15 months [PCV-7, CRM ₁₉₇]		-11% (-39,11)		[161] 2003, USA
	18'626	18'633	212	169	6, 10, 14 weeks [PCV-9, CRM ₁₉₇]		20% (2, 35)		[162] 2003, S. Africa
Otitis media ^e	8'719	8'718	590	388	3 doses <1 year [PCV-9, CRM ₁₉₇]		35% (26,43)		[56] 2005, The Gambia ^b
	18'941	18'927	51	38	2, 4, 6, 12-15 months [PCV-7, CRM ₁₉₇]		25.5% (6.5,40.7)		[165] 2006, USA
	6'094	6'097	141	119	6, 10, 14 weeks [PCV-11, TT/Dip]		16% (-7, 34)		[163] 2009, The Philippines
	18'941	18'927	17	6	2, 4, 6, 12-15 months [PCV-7, CRM ₁₉₇]		65%, P = 0.035		[158] 2000, USA
	831	831	250	107	2, 4, 6, 12 months [PCV-7, CRM ₁₉₇]		54% (41, 64)		[159] 2001, Finland
	831	835	250	110	2, 4, 6, 12 months [PCV-7, OMP]		56% (44,66)		[160] 2003, Finland ^{a,e}
	2'479	2'489	132	64	3, 4, 5, 12-15 months [PCV-11, Protein Dd]		58% (41,69)		[166] 2006, CZ & SK ^e
	432	424	8	3	2, 4, 6, 12-15 months [PCV-7, CRM ₁₉₇]		64% (-34, 90)		[167] 2008, USA ^e

^a22% of the children in the study group received PPV-23 as a fourth dose ^bIncludes children <29 months old ^cAny episode d Derived from non-typeable H. influenzae

^eOnly per protocol analysis available through the article OMP, group B *N. meningitidis* outer membrane protein; TT, tetanus toxoid; Dip, diphtheria toxoid

vidual and population immunity in the short and longer term (antibody persistence, and immunological memory), and the phenomenon of hyporesponsiveness will only be possible by understanding the cellular basis of humoral immunity. Antibody persistence is thought to be determined by a combination of long-lived plasma cells together with B_{MEM} that can renew the population of plasma cells by differentiating either spontaneously or through a mixture of antigen specific stimulation or non-antigen specific mechanisms of stimulation.

A number of studies have been conducted at the Oxford Vaccine Group (OVG) using cultured and ex-vivo enzyme-linked immunospot (ELISpot) methods to investigate the plasma and B_{MEM} responses to pneumococcal vaccines in children, adults and the elderly and meningococcal vaccines in children, adolescents and adults [168–173]. Circulating B cells are a dynamic population and these studies have defined the kinetics of the B cell response and permitted B cell frequencies before and after immunisation to be related to immediate and medium-term antibody responses. There is some evidence from these studies that plasma cell frequencies after immunisation correlate with immediate increases in antibody but not longer term antibody production. By contrast B_{MEM} responses following primary immunisation may be related to longer-term antibody production, at least in infants. Infants produce relatively low frequencies of B_{MEM} compared to older age groups and this may be one reason behind their relatively poor antibody persistence. However, a variety of other B cell subsets have been described of particular relevance for polysaccharide encapsulated pathogens, such as the pneumococcus. There is a lack of information about the nature of cells involved in the B cell response to pneumococcal vaccines in humans, despite the likely importance of subsets in determining the magnitude and persistence of the immune response following immunisation, most notably in infants.

B CELL RECEPTOR HEAVY CHAIN REPERTOIRE IN RESPONSE TO VACCINATION

The immunoglobulin (Ig) molecule consists of paired heavy (H) and light (L) polypeptide chains, both containing variable (V) and constant (C) regions. Genes encoding heavy chain V regions are split into variable heavy chain (VH), diversity (DH) and joining (JH) segments, which together with light chain VL and JL genes form the antigen-binding part of the antibody. During B cell development, initial diversity is generated by assembly of one V, D (heavy chain only), J gene segments into a functional VH gene by an event called “VDJ recombination”, which forms the basis of the antibody function to bind foreign antigen. In addition to randomly assembling different V to D and to J segments, further diversity is made possible by random removal and addition of nucleotides at the recombination sites (junctional diversity). During response to an antigen, further diversification occurs through rounds of somatic hypermutation, followed by selection of B cells for improved antigen binding in the GC (Figure 5.1). Variability is concentrated in three hypervariable domains, found in both heavy and light chain V regions. An individual’s antibody repertoire may be determined by a variety of factors including immunoglobulin locus genotype and surrounding chromatin structure [174], antigen exposure history, age and sex. Because of the extensive diversity, the antibody repertoire is difficult to study, but improvements in next-generation sequencing (NGS) over the last decade have made in-depth studies of the antibody repertoire possible.

An alternative approach to exploring the nature of the populations of B cells involved in the immune response to vaccination is to examine the repertoire of antibody forming gene segments used in the response. In contrast to more traditional methods such as studying the surface markers and stimulation properties of newly generated circulating B cells, studying the antibody repertoire permits a fundamental insight into vaccine biology, may serve as an alternative assay of immunogenicity, identify important Ig properties, such as broadly cross-reactive Ig and autoimmune Ig, and may also identify antigen-specific Ig for monoclonal antibody synthesis.

Several studies have investigated the antibody repertoire following vaccination (influenza [175–180], tetanus toxoid (TT) [176, 181, 182], *Haemophilus influenzae* type b (Hib) [183, 184], and *S. pneumoniae* [179, 185]), and natural infection (rotavirus [186], HIV [187, 188], and dengue [189]), with the majority of these studies being based on low-resolution methods (Table 5.1) but there are four recent studies that use NGS for in-depth characterisation (Table 5.2).

Table 5.1: Summary of publications where the antibody repertoire has been investigated after vaccination using a low-resolution approach (courtesy of Jacob Galson).

Vaccine	Ref.	Cells Used	Methodology	Key Findings
Influenza TIV	[180]	Day 7 IgG plasmablasts	Single-cell VH and VL PCR	50 mAbs from 14 individuals, against 3 different influenza strains. Showed that influenza-specific antibody response is pauci-clonal, with extensive intradonal diversification of the influenza-specific lineages due to mutations.
Tetanus TT	[181]	Day 6 plasmablasts following 3 vaccinations ≥ 1.5 yrs apart	Single-cell VH:VL linkage PCR, cloning and sequencing of TT-positive clones.	Similar number of mutations between individuals, no increase through the study, indicating the limit had already been reached through previous routine vaccinations.
TT	[182]	Day 6 and day 9 TT-specific plasmablasts and B _{MEM} , respectively	Single-cell isotype-specific VH and VL PCR	VDJ segment usage, CDR3 length and distribution of mutations were similar among TT-specific plasmablasts and memory cells.
<i>S. pneumoniae</i> PPV-23	[190]	Day 7 IgG plasmablasts	Single-cell VH and VL PCR	Cloned 137 mAbs against 19/23 vaccine serotypes from 4 individuals. Most antibodies were serotype-specific, but 12% cross-reacted with 2 or more serotypes.
PPV-23	[191]	PPS4 or PPS14 specific B cells 6 weeks after vaccination	Single-cell culture followed by VH PCR and sequencing of pooled, cultured cells	>1300 sequences from 40 individuals. Antibody repertoire different in young and elderly participants, the latter having a more clonal repertoire with less mutations.
Hib PS or PS-DT or OC-CRM	[183]	Day 7 lymphocytes	Fusion to mouse myeloma cells followed by culture, VH and VL PCR	15 cell lines from 10 individuals, secreting antibody against Hib. Demonstrated clonality of the antibody repertoire after vaccination. Showed that these mAbs had undergone mutations.
PS or PS-DT	[192]	Day 7 lymphocytes	Fusion of lymphocytes to mouse myeloma cells followed by culture, VH PCR	5 cell lines from 4 individuals, secreting antibody against Hib. Demonstrated that they all used the VH3 segment, but different D and J segments.

mAbs, monoclonal antibodies; TT, tetanus toxoid; PS, polysaccharide; OC, oligosaccharide; DT, diphtheria toxoid; TIV, trivalent influenza vaccine

Table 5.2: Summary of publications where the antibody repertoire has been investigated after vaccination using a high-throughput approach and next-generation sequencing (courtesy of Jacob Galson).

Vaccine	Ref.	Cells Used	Methodology	Key Findings
Influenza TIV	[176]	B _{MEM} 14 days after vaccination	High-throughput single-cell VH:VL linkage PCR, 2 x 250 Illumina sequencing	Validated accuracy of VH:VL pairings identified using their high-throughput method. Identified 240 putatively influenza-specific CDR-H3:CDR-L3 pairings.
TIV or LAIV	[175]	PBMCs on the day of vaccination, and on day 7 and 28 after two vaccinations given a year apart	VH-specific reverse transcription and 2nd strand synthesis, followed by PCR. Custom Illumina 100 x 120 sequencing	Different repertoire dynamics after TIV and LAIV vaccination. TIV with stronger responses & more abundant IgG lineages. Found shared sequences on day 7 after two TIV vaccinations. Hypothesised that these lineages are due to memory B cell recall. Suggested that this method could be used to identify cross-specific antibodies.
TIV or LAIV	[177]	Naïve B cells, and plasmablasts, on the day of vaccination and on days 7 or 8 and day 28 after vaccination	VH-specific multiplex PCR, and 454 sequencing	Influenza-specific antibody repertoire in older individuals was more clonal and with more mutations. In twins, no. of mutations in the IgM repertoire were similar, but diverged for the IgG repertoire, indicating that the naïve repertoire is more influenced by individual genetics, but the memory repertoire is more influenced by environmental stimuli.
Tetanus TT	[176]	Plasmablasts 7 days after vaccination	High-throughput single-cell VH:VL linkage PCR, 2 x 250 Illumina sequencing	Identified 86 putatively TT-specific CDR-H3:CDR-L3 pairings. Cloning ten of these showed them to be TT-specific.
Co-administered TIV & PPV-23	[179]	PBMCs on the day of vaccination and on day 7 and 28 after vaccination	Semi-nested isotype and VH-specific multiplex PCR, and 454 sequencing	The repertoire changed at day 7 post-vaccination, but returns to a baseline-like state after 28 days. Clonal expansion following vaccination was delayed in older individuals, mainly involving IgA and IgM response, with little difference in IgG.
TIV, trivalent inactivated influenza vaccine; LAIV, live attenuated influenza vaccine				

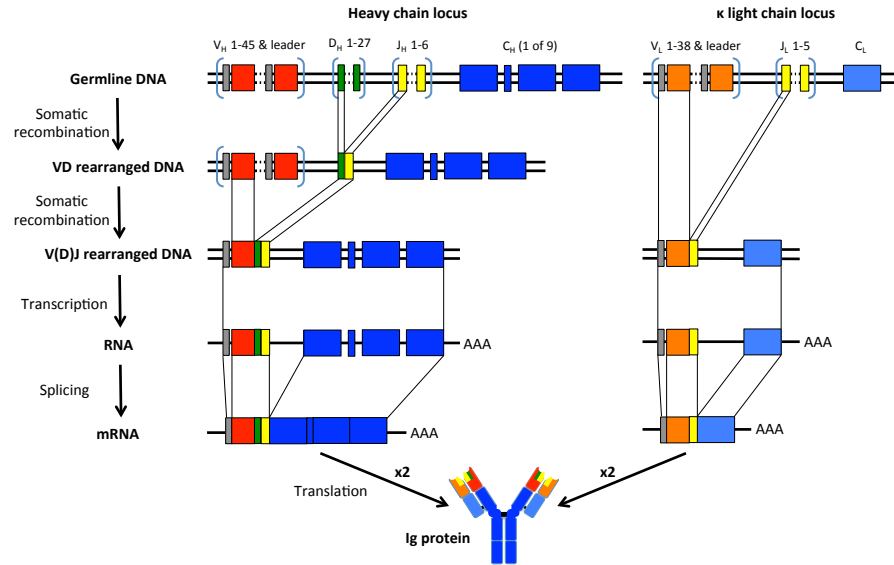


Figure 5.1: Overview of the structure and formation of antibodies.

For low-resolution methods, sorting B cells using fluorescence-activated cell sorter (FACS) for precise definition of the B cells studied in combination with single-cell Sanger sequencing and functional characterisation of cloned antibody sequences has helped enormously in investigating monoclonal antibodies generated against a variety of antigens. For example, we now know a number of antibody “signatures” and antigen-specific VH gene segments in response to vaccination with influenza, TT, Hib polysaccharide, and some serotypes of *S. pneumoniae* (Table 5.1). The antibody repertoire generated in response to Hib vaccination, containing the Hib polysaccharide - a relatively simple antigen of repeated “sugar chains”, appears markedly oligoclonal and dominated by a small number of VH (IGHV3-23) and JH (IGHJ4/6) segments in combination with a kappa A2 light chain V segment and either J κ 1 or J κ 3. The complementary-determining region (CDR)₃ often contains the sequence motif Gly–Tyr–Gly–Phe–Met–Asp (GYGMD or GYGFD) and the CDR₃ of the light chain is 10 amino acids long. Most of the Hib repertoire studies were performed in adults [183, 192–195] but one study looked into the B cell repertoire following Hib conjugate vaccination in infancy [196], which showed similar results. In the latter study, 2 different types of conjugate vaccines were used, one in which Hib is coupled to CRM₁₉₇ and the other to an outer membrane protein of *N. meningitidis*. Specificity of the cloned antibodies was confirmed by binding of Fab fragments to radio-labelled Hib polysaccharide. In these studies, Hib-specific antibodies contained sequences that are unmutated, therefore the structurally conserved antibody combining site containing IGHV3-23 is referred to as “canonical” although germline sequences were usually not determined in these studies [196]. Interestingly, by construction of Fab fragments using the canonical combination of IGHV3-23, IGHJ6,

IGKV A2, and IGKJ1 including two different alleles with respect to the IGHV3-23 sequence (in the CDR2 region), marked differences of these two antibody fragments were found in the complement dependent bactericidal activity against Hib [184].

For protein antigens (e.g. TT), repertoire diversity appears to be greater. One study described a method to isolate human antibody repertoires with preservation of the heavy and light chain pairing using tetanus-toxoid specific B cells [197]. The immunoglobulin genes taken from plasma cells of only 2 individuals were cloned before being screened for antigen specificity. The repertoires found were highly diverse and not oligoclonal. However, numbers of productive isolates were low corresponding to only 8% of the total number of single-sorted plasma cells. In a follow-on study, the same authors analysed TT-specific single-sorted B cells derived from two individuals following TT vaccination and found extensive diversity in their V gene family and J segment usage for the heavy and kappa light genes without any preferential usage of certain gene segments [181]. Antigen-specific VDJ sequences had, however, longer CDR3 regions than random repertoires.

In a second study using a flow cytometry based labelling technique, antigen-binding B cells (PC_{MEM} and B_{MEM}) were single-cell sorted and IgG, IgA and IgM mRNA transcripts were sequenced [182]. In this study, recovery rate as defined by the numbers of sequences per isolated B cells was very low especially for transcripts derived from B_{MEM}. In keeping with the already described study, the B cell repertoire was polyclonal with VH4 and VH3 families being most frequently expressed in both plasma and B_{MEM}, respectively, which is similar to the naïve B cell repertoire [198]. It has to be noted that in this study, antigen-specific B cells were sorted based on their binding to a protein that only contains around 50% of the tetanus toxoid epitopes [182].

The advent of NGS enabled the sequencing of antibody genes from millions of cells in a single run, giving insight into a much larger proportion of the antibody repertoire. To date, however, the focus of published NGS work has been on developing methods and visualisation tools. In contrast to studies using low-resolution methods, the NGS approach has allowed identification of rare antigen-specific sequences within the context of bulk-sequenced B cells or peripheral blood mononuclear cells (PBMC)s [175–177]. In addition, these NGS studies have shown how different vaccines have different effects on the repertoire, and that these effects are dependent on the age of the vaccinated individual.

AIMS AND OBJECTIVES OF THIS THESIS

This thesis aims to detail the investigation of the immune response to pneumococcal vaccines given to children and adults with a focus on B cells. The data obtained provide a better understanding of the way in which these vaccines work and which cells are generated through the immune response. In addition, this work can give novel insights into the biology that underlies the immune responses and the practical application of these data may have a profound effect on the development of new vaccines.

The more specific objectives of this thesis included the following:

- A. To standardise and refine a novel method for detection of antigen-specific B cells by [FACS](#).
- B. To investigate the B cell [ELISpot](#) assay variability between laboratories/frozen or fresh [PBMC](#).
- C. To investigate and compare pneumococcal polysaccharide-specific memory B cell frequencies by [ELISpot](#) following immunisation with pneumococcal polysaccharide and conjugate vaccines.
- D. To investigate and compare the phenotype of pneumococcal polysaccharide-specific B cells by [FACS](#) and the detailed kinetics of antigen-binding B cell subsets following immunisation with pneumococcal polysaccharide and conjugate vaccines.
- E. To investigate and compare the kinetics and long-term persistence of pneumococcal serotype-specific antibody concentrations following immunisation with pneumococcal polysaccharide and conjugate vaccines.
- F. To investigate and compare the whole blood gene expression profile and its kinetic following immunisation with pneumococcal polysaccharide and conjugate vaccines.
- G. To describe the B cell repertoire of immunoglobulin heavy chain gene segment usage following immunisation (using a model vaccine containing [Hib](#) polysaccharide).

Part II

METHODS

PBMC ISOLATION, ELISPOT AND ANTI-PNEUMOCOCCAL ANTIBODY

7.1 BUFFERS

Phosphate buffered saline (PBS)

Five phosphate buffered saline (PBS) tablets (Sigma-Aldrich, Dorset, UK: P4417) were dissolved in 1000 ml sterile, pyrogen free water. The pH was adjusted to 7.2 with 10 M sodium hydroxide (NaOH: Sigma-Aldrich; S5881) or 50% hydrochloric acid (HCl; Sigma-Aldrich; S814-8). The solution was autoclaved and kept for up to 6 months at room temperature.

PBS-Tween (0.25%)

PBS-Tween was used as ELISpot wash solution and was prepared by adding 2.5 ml of Tween detergent (Sigma-Aldrich; P1754) to 1000 ml of PBS. The solution was kept for up to 6 months at room temperature.

PBS-EDTA (Rinse buffer, RB)

EDTA (0.744 mg; ICH Biochemicals, Aurora, USA; 195173) was added to 1000 ml of PBS and the solution autoclaved to be kept for up to 6 months at room temperature.

PBS-EDTA-NBBS (0.5%RB, Running buffer)

Five ml of newborn bovine serum (NBBS, Sigma-Aldrich; N4637) was added to 1000ml of PBS-EDTA and stored at 4°C for up to 6 months.

RPMI + L-glutamine + penicillin + streptomycin (Ro)

Five ml of L-glutamine at 2 mM (Sigma-Aldrich; G7513) and 5 ml of penicillin-streptomycin solution containing 50 U/ml and 0.05 g/ml (Sigma-Aldrich; P4458) was added to 500 ml of RPMI-160 with phenol red indicator and 25 mM hepes modification (Sigma-Aldrich; R5886). The medium was stored at 4°C.

Ro + 10%NBBS (R10)

Fifty ml of heat inactivated NBBS was added to 450 ml Ro. The medium was stored at 4°C.

R10 complete

Five ml of MEM non-essential Amino Acids (Invitrogen; 11140-035) and MEM Sodium Pyruvate 100mM (Invitrogen; 11360-039) each and 0.5 ml of 2-Mercaptoethanol 1000x (Invitrogen; 31350-010) was added to 500 ml of R10 to make up the R10 complete medium.

RB + bovine serum albumin (0.5%BSA)

One gram of bovine serum albumin (Sigma-Aldrich; A7030) was added to 200 ml RB and final solution was stored at 4°C.

7.2 ISOLATION OF PBMCs FROM WHOLE BLOOD

Fresh heparinised blood (taken ≤ 6 hours previously) was diluted 1:2 in Ro for the ELISpot or in rinse buffer (RB) for B cell phenotyping and then layered on top of lymphoprep, which was prepared in a fresh tube with the volume equivalent to half the volume of the diluted blood. The PBMCs were then separated by density gradient centrifugation according to the manufacturer's instructions by spinning the tubes at 1000 g for 30' (break off). The buffy coat layer of PBMC was transferred into a fresh tube using sterile Pasteur pipettes, the tube filled with Ro or RB and then centrifuged at 800 g (ELISpot) or 300 g (phenotyping) for 20' to wash cells. PBMCs that were prepared for CD19 bead separation (phenotyping) were washed further 2 times at 200 g for 10' to remove platelets. After the final wash, cell pellets were re-suspended in 1-4 ml of R10 (ELISpot) or cold 0.5%RB (phenotyping) for quantification by adding 50 μ l of cell solution to 50 μ l trypan blue (0.4%) and 50 μ l phosphate buffered saline (PBS). The cells in a 10 μ l volume of this mix were counted using a haemocytometer (Neubauer improved) with a central 5x5 row by column grid and counting 5 squares within this grid (volume of 20 nl). The number of cells obtained was multiplied by 5 to give the number of cells in 25 squares (volume of 0.1 μ l), then by 3 allowing for the cell dilution, and then by 10^4 to give the number of cells per ml of PBMC suspension. For the culture step (ELISpot), an adequate volume of R10 was added to achieve the required cell concentration (2×10^6 cells/ml). For phenotyping, PBMC were centrifuged at 300 g for 10' after an adequate volume of PBMC (usually 100-200 μ l) from each sample was put on ice in a 1.5 ml Eppendorff tube until further processing.

7.3 FREEZING AND THAWING OF ISOLATED PBMCs

The freezing and thawing procedure of PBMC was performed using a standard protocol. Following PBMC separation as described in section 7.2, the cells were re-suspended at a concentration of 5×10^6 cells/ml in

Recovery™ cell freezing mix (Invitrogen; 12648010) and aliquoted in 1 ml volumes in cryovials. These aliquots were placed into a pre-chilled freezing container “Mr Frosty” (Nalgene®, VWR International, UK), which was then put overnight in a -80 °C freezer before transferred into liquid nitrogen for long-term storage. For thawing, the vials were immersed into a water bath (at 37 °C) and the obtained PBMC solution of each vial was transferred into 10 ml of pre-warmed complete R10. Following a wash step, the cells were then ready to be processed further.

7.4 ACTIVATION OF MEMORY B CELLS IN CULTURE WITH POLYCLONAL STIMULI

The cultured ELISpot assay to detect low frequencies of antigen-specific memory B cells was performed as previously described [171, 172]. First, a culture mix containing 1:5000 *S. aureus* Cowan strain (SAC) (Pansorbin cells; Calbiochem; 507858) + 1:6000 pokeweed mitogen (PWM) (Sigma-Aldrich; L-9379) + 1:40 cytosine phosphorothioate guanine (CpG) (Biotin oligodeoxynucleotide 2006 (biotin labelled CpG type B – human TLR9 ligand: reconstituted in 1 ml endotoxin free water); Bioscience for Invivogen; tlrl-2006b) was made up in R10. 100 µl of the PBMC solution (prepared at a concentration of 2×10^6 cells/ml) was added onto round-bottom 96-well culture plates and mixed with 100 µl of the culture mix per well. The cells were incubated at 37 °C in 5% CO₂ for 5 to 6 days. Once incubation was complete the cells were re-suspended by pipetting up and down with the electronic multichannel pipette and transferred into a fresh tube, which was centrifuged once at 1000 g for 20 minutes with 30 ml of 0.5%RB, then twice more at 800 g for 15 minutes with 10 ml of 0.5%RB. Cells were then seeded at 2×10^5 (viable) cells per well onto a 96-well plate with a PVDF-membrane, pre-coated with different antigens (see section 7.5) and incubated at 37 °C in 5% CO₂ for 18 to 26 hours before development.

7.5 PREPARATION OF ELISPOT PLATES

ELISpot plates were coated with 100 µl/well of either purified pneumococcal serotypes (LGC Standards) conjugated to methylated human serum albumin (mHSA, NIBSC UK), purified diphtheria toxoid, tetanus toxoid (both Statens Seruminstitut, Denmark) or goat-antihuman Ig in sterile filtered PBS (Table 7.1). PBS was added to the blank wells. The ELISpot plates were then stored at 4 °C until use (no longer than 1 month). Before adding the cell suspension that was harvested after activation with polyclonal stimuli as described in 7.4, pre-coated ELISpot plates were washed three times with PBS and blocked with R10 at 37 °C in 5% CO₂ for a minimum of 30 minutes. Antigen concentrations used to coat plates are summarised in Table 7.1.

Table 7.1: Antigen dilutions used for coating ELISpot plates. Sp, serotype.

	Sp 1 [†]	Sp 3 [†]	Sp 4 [†]	Sp 9V [†]	Sp 14 [†]	Sp 19A [†]
Stock ($\mu\text{g/ml}$)	1000	1000	1000	1000	1000	1000
Dilution on plate ($\mu\text{g/ml}$)	10	10	10	10	10	10

	Sp 23F [†]	mHSA [†]	Dip tox	Tet tox	Ig [±]
Stock ($\mu\text{g/ml}$)	1000	1000	4777.5	2052.5	2500
Dilution on plate ($\mu\text{g/ml}$)	20	10/20 [‡]	10	5	10

[†] Final solution made by putting polysaccharide solution drop wise into individual mHSA solution while mixing the latter simultaneously to achieve a final concentration of 10-20 $\mu\text{g/ml}$ each. [±] Goat anti-human polyvalent Ig [‡] only Sp 23F

7.6 DEVELOPING AND READING ELSPOT PLATES

Following overnight incubation, plates were washed before wells were incubated with a goat anti-human IgG alkaline phosphatase conjugate to detect bound antibody. The antibody was visualised using the alkaline phosphatase substrate kit, the reaction stopped with distilled water and the plate dried over-night in a drying-oven before read using an automated [ELISpot](#) reader (AID ELR03, serial no. ELR030408215). AID [ELISpot](#) software (Autoimmune Diagnostika, version 3.3) was used to automatically count cells. Basic cell count settings were adjusted to have an emphasis on 'tiny' and the thresholds were used to optimise automated spot recognition. Identical settings were used for all plates but different settings were used for the different antigens (Table 7.2). The operator was blinded to which sample was being counted. All wells were manually verified and any artefacts were deleted. Saturated, fungal contaminated, and damaged wells were not used for the analysis. For each sample and each antigen, there were usually 3-4 replicate wells and the mean number of spots was calculated for each antigen from the replicates. Results were usually expressed as the number of spots, i.e. antibody-secreting cells (ASCs) per million cultured [PBMCs](#). Mean spots detected in the [PBS](#) wells were subtracted from the mean spot count for each antigen.

Table 7.2: Setting for counting spots on ELISpot plates using the AID ELISpot reader.

Antigen	Intensity (min-max)	Size (min-max)	Gradient (min-max)	Emphasis (target spot size)
Pneumococcal polysaccharides	15 - 255	30 - 5000	1 - 90	tiny
Diphtheria / tetanus toxoid	15 - 255	30 - 5000	1 - 90	big
Total IgG	5 - 255	30 - 5000	1 - 90	small

7.7 PNEUMOCOCCAL ANTIBODY CONCENTRATIONS MEASURED BY MULTIPLEX BEAD ASSAY

Sera from samples described in chapter 12 were sent to the Health Protection Agency laboratories in Manchester for quantification of total IgG to 12 pneumococcal serotypes: 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F. A validated bead based multiplex assay was used as described previously [199]. Briefly, pneumococcal polysaccharides were conjugated to carboxylated microspheres. Serum samples were then incubated with the conjugate microspheres in 96 well plates, with cell wall polysaccharide and 22F polysaccharide adsorption. After 20 minutes anti-human IgG was added to each well and left for a further 20 minutes. Beads were finally washed and results read on a Bio-plex, using real time Bio-plex software. Serotype-specific antibody concentrations of $\geq 0.35 \mu\text{g/ml}$ were used as putative correlates of immunity against serotype-specific IPD [200].

7.8 PNEUMOCOCCAL ANTIBODY CONCENTRATIONS TESTED BY ELISA

Sera from samples described in chapter 10 were tested by enzyme-linked immunosorbent assay (ELISA) for pneumococcal serotype-specific antipolysaccharide IgG concentrations [201, 202] against an international reference serum 89-SF [203] at Pfizer Vaccine Research, Pearl River, NY. The reference and test sera were preabsorbed with a crude pneumococcal cell wall extract, containing cell wall polysaccharide, and purified serotype 22F polysaccharide (test and control sera) to enhance the specificity of the assay by removal of nonspecific antibodies [200].

7.9 PNEUMOCOCCAL OPA TITRES

Opsonophagocytic assay (OPA) titres were tested for samples described in chapter 10 at Pfizer Vaccine Research, Pearl River, USA and were calculated as the interpolated reciprocal dilution of test serum that causes a 50% reduction of bacteria surviving the opsonophagocytic reaction incubation. A titer of 4 was allocated to sera with undetectable OPAs, the titer of 8 being the lowest positive result. Serotype-specific OPA titers ≥ 8 were used as putative correlates of immunity against serotype-specific IPD [204].

LABELLING AND ISOLATION OF ANTIGEN-SPECIFIC B CELLS

8.1 DETECTION OF B CELL SUBSETS AND ANTIGEN-SPECIFIC B CELLS BY FLOW CYTOMETRY

PBMCs were separated as described in section 7.2 as soon as possible following blood collection. B cells were then be enriched by positive magnetic selection using CD19 MicroBeads or the Plasma cell isolation Kit II (negative selection by depleting non-B cells by using a cocktail of biotin-conjugated antibodies against CD2, CD14, CD16, CD36, CD43, CD235a [glycophorin A]) and the AutoMacs™ (all Miltenyi Biotec) according to the manufacturer's instructions and following Fc receptor blockage. Subsequently, enriched B cells were immunofluorescently stained with various surface B cell markers to phenotypically characterise different B cell subpopulations (see chapter 12) using the following commercially available antibodies: CD19-FITC (eBioscience), CD43-PE (eBioscience), Propidium Iodide (eBioscience), IgM-PerCPCy5.5 (Biolegend), CD27-PECy7 (eBioscience), CD3-V500 (BD Biosciences), CD14-V500 (BD Biosciences), CD16-V500 (BD Biosciences), IgD-APC (Miltenyi), CD20-APCH7 (BD Biosciences).

FACS based antigen labelling method

A method, based on the technique described by Diaz Romero and Outschoorn [205] and Ferry et al. [206], to biotinylate and fluorescently label polysaccharide and protein antigens was used (Figure 8.1).

Purified diphtheria and tetanus toxoid (both Statens Seruminstitut Denmark) were dialysed overnight by injecting a 1 mg/ml dilution of the antigen (in sterile PBS) into a dialysis cassette (Slida-A-Lyzer dialysis cassette, 0.5-3ml, Perbio Science), placed into a float holder and immersed into sodium acetate buffer (0.1 M, pH 5.5, Sigma Aldrich). The antigen was biotinylated by agitation in the dark for 2 h at room

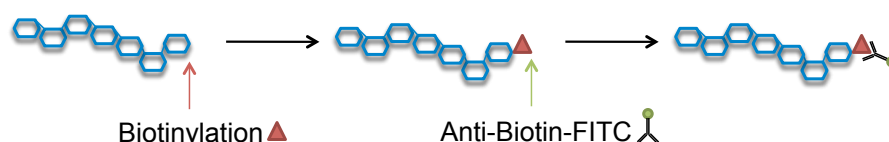


Figure 8.1: Principle of biotinylation and fluorescent labelling of polysaccharides.

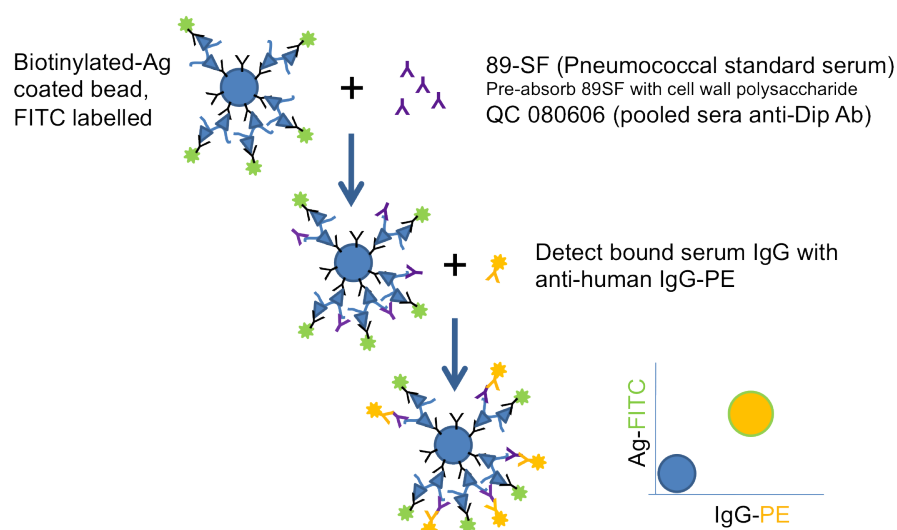


Figure 8.2: Testing the retention of epitopes for specific antibody binding.

temperature of 1 part of a biotin hydrazide solution (50 mM, Sigma Aldrich) with 9 parts of the antigen solution. This biotinylated antigen was dialysed a second time overnight as before. Purified pneumococcal polysaccharides (LCG Standards) were processed by dialysing overnight in sodium acetate buffer, then mixed with a 20 mM sodium periodate solution (Sigma Aldrich) and incubated at 0 °C for 5 min before being dialysed a second time and finally biotinylated by mixing with biotin hydrazide. After overnight dialysis, the biotinylated polysaccharides and the biotinylated proteins were stored at 4 °C until use. To allow visualisation of antigen-binding B cells, the biotinylated antigens were incubated with 1 µl of either anti-biotin FITC antibody (Miltenyi) or streptavidin Pacific Blue conjugate (Invitrogen) and the following volumes of biotinylated antigen, which gave the best signal-to-noise ratio when tested with anti-biotin MicroBeads (Miltenyi): 0.25 µl (serotypes 3 and 23F), 0.5 µl (serotypes 1 and 9V); 1 µl (serotype 18C); and 2 µl (diphtheria and tetanus toxoid).

Epitopes remain after antigen biotinylation

The presence of remaining epitopes on the antigens after the antigen has been biotinylated was tested by incubation of antigen labelled beads with increasing dilutions of standard sera (Figure 8.2 and 8.3). Incubation with a less concentrated serum resulted in a lower percentage of IgG-PE positive beads suggesting that there are still epitopes on the antigens available for binding of antibody specific for pneumococcal antigens despite the antigen had already bound biotin.

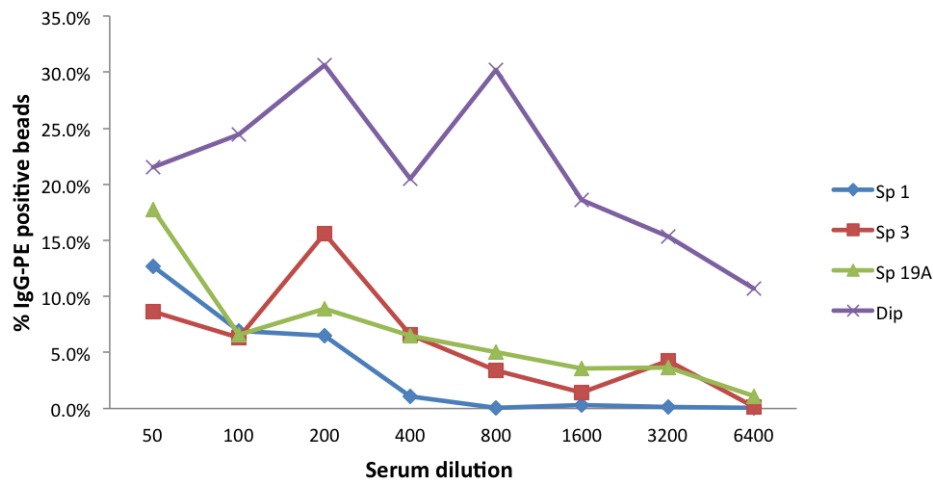


Figure 8.3: Incubation with increasing dilutions of standard serum results in decreasing IgG-PE positive antigen labelled beads (shown for pneumococcal serotypes 1, 3 and 19A and diphtheria toxoid).

Testing the specificity of the FACS based antigen labelling method

To test the specificity of the above described labelling assay, the following experiments were performed:

- A. Antigen labelled beads were incubated with a standard serum and with increasing concentrations of homologous antigen to remove diphtheria toxoid or polysaccharide-specific antibodies (Figure 8.4 and 8.5) [E.A. Clutterbuck, unpublished].
- B. After enrichment by negative isolation using the B cell isolation kit (see above), B cells were incubated with FITC labelled and biotinylated diphtheria toxoid. Subsequently, diphtheria toxoid-binding B cells were enriched by magnetic bead separation using anti-FITC beads (Miltenyi Biotec). Finally, 4 different cell populations (PBMC, B cells, Dip tox-positive B cells and Dip tox-negative B cells) were cultured in the presence of SAC, PWM and CpG DNA as well as IL-2 (50 U/ml) for 5 days (Figure 8.6). The frequencies of diphtheria toxoid and tetanus toxoid IgG ASCs were detected by ELISpot (Figure 8.7).

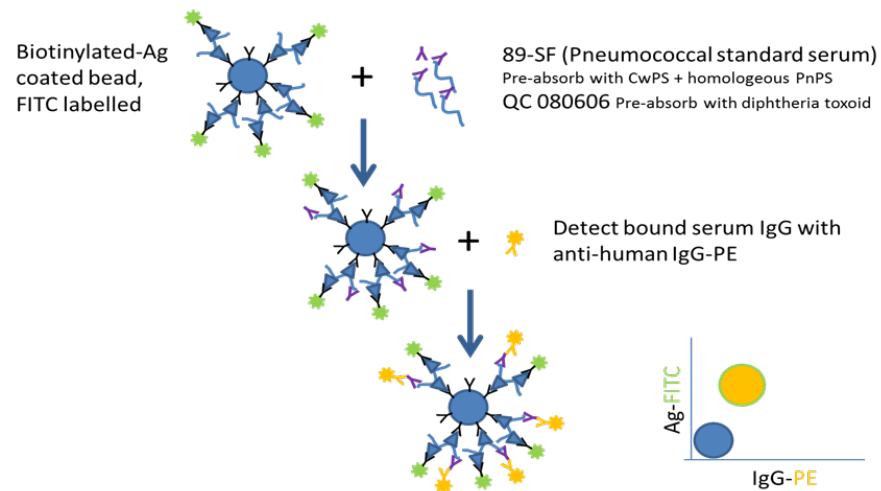


Figure 8.4: Testing the specificity of the antigen labelling method.

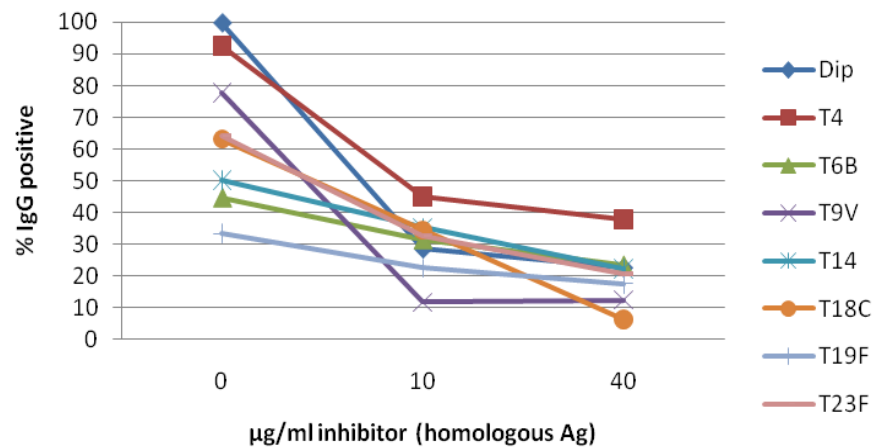


Figure 8.5: Incubation with increasing concentrations of the homologous antigen results in reducing numbers of IgG-PE positive antigen labelled beads (shown for pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F and diphtheria toxoid).

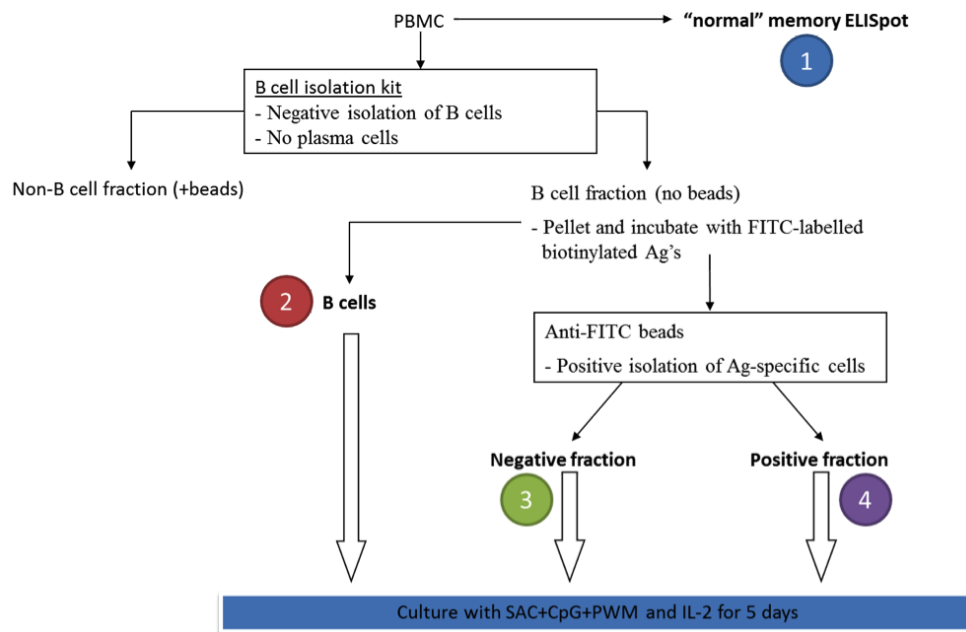


Figure 8.6: Experimental setup for isolation of 4 different B cell subsets (see text for details).

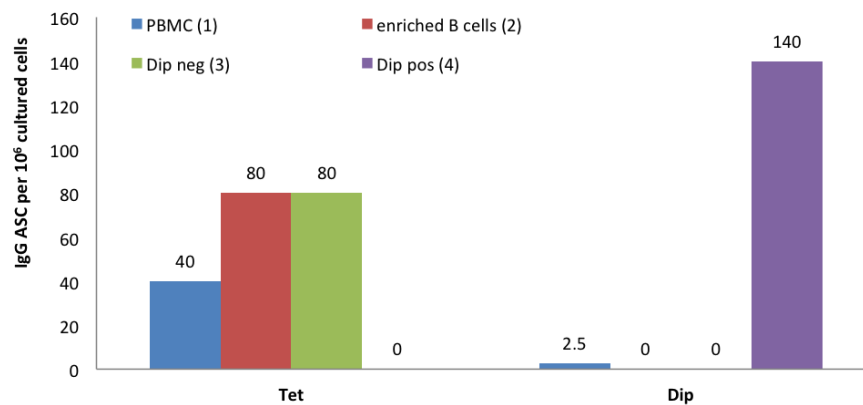


Figure 8.7: The B cell population enriched for diphtheria toxoid binding B cells (Dip pos) resulted in higher numbers of diphtheria toxoid specific ASC compared to the other 3 populations. In contrast, no tetanus-specific ASC could be detected in the Dip tox enriched population compared to the other 3 populations.

Part III

RESULTS

VARIABILITY OF THE MEMORY B CELL ELISPOT ASSAY

9.1 BACKGROUND

The measurement of antigen-specific B cells through [ELISpot](#) assays has provided new insight into the biology of the immune response following immunisation and is now more frequently used in clinical vaccine trials [173, 207–209]. This method relies on the isolation of [PBMCs](#) from blood specimens collected at study visits before and after a stimulus to the immune system, i.e. an immunisation. Although used in a large number of studies, the variability of the B cell [ELISpot](#) assay between different laboratories or operators or between fresh and frozen [PBMC](#) samples has never been formally studied. These parameters are, however, important in order to combine data produced in different laboratories or at different time points e.g. in multi-centre studies.

Storage and cryopreservation of human lymphocytes

The cryopreservation process allows human lymphocytes to be stored long-term before being used in immunological assays. Cryopreservation involves cooling the sample to a low temperature before transferring it into liquid nitrogen for long-term storage. In this way, samples from multiple time points may be stored until they can be analysed simultaneously, reducing variation arising from different assay conditions. Frozen samples may also be transported to a centralised laboratory for all testing to be carried out; thereby reducing variation introduced by different operators and laboratory conditions. It is important that this process of freezing, storage and thawing does not have a negative impact on cell function and response in the subsequent assay. One parameter reported to be affected following cryopreservation is cell viability. A median viable cell recovery of 50% following the thawing of frozen [PBMCs](#) was reported in one study [210], however there was no significant reduction in the percentage of viable cells recovered over the 12 year study period. A more recent study reported over 80% cell viability, attributed to having all samples processed by a single experienced technician in the same laboratory [211]. The effects of cryopreservation on B_{MEM} responses have not previously been studied, however assays of T cell response using frozen [PBMCs](#) have had mixed results. Cryopreservation of [PBMCs](#) has been shown to alter cell surface markers [212–214], cytokine production [212, 215]

and cell proliferation [216], however responses have been shown to be equivalent between fresh and frozen samples when an optimised protocol is used [213, 217–219]. Preservation of cell function has been shown to be improved if PBMCs are frozen <12 hours post blood collection [219] and temperature fluctuations are minimised during storage [208].

Variability of the ELISpot assay

The B cell ELISpot assay was initially designed for the detection of IgG-producing B cells [220], but has been further developed to enable quantification of B_{MEM} by the inclusion of a stimulation step in which B_{MEM} differentiate into detectable antibody-secreting cells [221]. Although the basic principles of the assay remain unchanged, protocols are not standardised between laboratories and are continually undergoing optimisation [222]. Protocols are similarly variable for ELISpot assays detecting T cell cytokine secretion [223], and studies of the reproducibility of T cell cytokine release ELISpots between different laboratories have shown substantial inter-laboratory variation [224–226]. Reproducible T cell ELISpot results have however been shown to be achievable if assay procedures and data analysis are standardised across centres [227]. Inter-laboratory variation is therefore more likely to result from differences in protocol than inherent unreproducibility of the ELISpot assay, suggesting that the compilation of T cell ELISpot results from different laboratories is appropriate in the context of multi-centre clinical trials. The aim of this study was to establish whether the quantification of antigen-specific B_{MEM} using ELISpot is also reproducible when performed by different operators and laboratories and a standardised protocol and using fresh and frozen samples.

9.2 METHODS

Two different methods were used to study the variability of the B cell ELISpot and the effect of storage of lymphocytes.

PBMC isolation and ELISpot

The cultured ELISpot assay to detect low frequencies of antigen-specific B_{MEM} was performed as described in methods sections 7.2 and 7.4. PBMCs were first isolated from whole blood using the lymphoprep™ (Axis-Shield Diagnostics, UK) density gradient centrifugation. After a wash step, the PBMCs were cultured at a concentration of 2×10^5 cells/well in a mixture of SAC (1:5000, Calbiochem, UK), CpG ODN 2006 (1.8 µg/ml, Source Bioscience, UK) and PWM (83 ng/ml, Sigma Aldrich, UK) for 5–6 days at 37 °C, 5% CO₂ and 95% humidity. Cells were

then harvested, washed and seeded at 2×10^5 (viable) cells per well onto a 96-well plate with a PVDF-membrane (Millipore, UK), pre-coated with different antigens. Following overnight incubation, plates were washed before incubating the wells with a Goat anti-human IgG-alkaline phosphatase conjugate (Calbiochem, UK) to detect bound antibody. The antibody was visualised using the alkaline phosphatase substrate kit (Bio-Rad, UK) and the reaction stopped with distilled water. Plates were then dried over-night in a drying-oven before being read using an automated [ELISpot](#) reader (AID ELRo3, AID Diagnostika, Strassberg, Germany).

Cryopreservation of PBMCs

The freezing and thawing procedure of [PBMCs](#) was performed using a standard protocol as describe in section 7.3. Following [PBMC](#) separation as described above, the cells were re-suspended at a concentration of 5×10^6 cells/ml in a cell freezing mix (Recovery™, Invitrogen, UK) and aliquoted in 1 ml volumes in cryovials. These aliquots were placed into a pre-chilled freezing container “Mr Frosty” (Nalgene®, VWR International, UK), which was put overnight into a -80°C freezer before being transferred into liquid nitrogen for long-term storage. For thawing, vials were immersed into a water bath (at 37°C) and the [PBMC](#) solution obtained from each vial was transferred into 10 ml of pre-warmed medium (“complete medium”), containing RPMI-1640 (hepes modification), 10% NBBS, 1% penicillin-streptomycin, 1% L-glutamine (all from Sigma-Aldrich, UK), 1% MEM non-essential amino acids, 1% MEM sodium pyruvate 100mM and 0.1% 2-mercaptoethanol 1000x (all from Invitrogen, UK). Following a wash step, the cells could be further processed as described above.

Study design

A prospective study was set up in collaboration with the Bristol Children’s Vaccine Centre to establish the effect of freezing of [PBMCs](#), inter-operator and inter-laboratory variability, and the reproducibility of the memory B cell [ELISpot](#) method. For this analysis, 12 healthy volunteers were asked to give 40 ml of peripheral blood divided into two aliquots. The blood was transferred directly into 50 ml falcon tubes containing 350 μl of heparin to prevent clotting and processed within 2 hours as described above. A summary of the study design is outlined in Figure 9.1. To cover a wide range of B_{MEM} frequencies, [ELISpot](#) was performed using pre-coated plates with 2 antigens, diphtheria and tetanus toxoid, both of which are contained in routine vaccines. Correlation between samples was assessed by calculation of the Pearson correlation coefficient. Agreement between B_{MEM} numbers obtained from the same participants’ samples was assessed by gen-

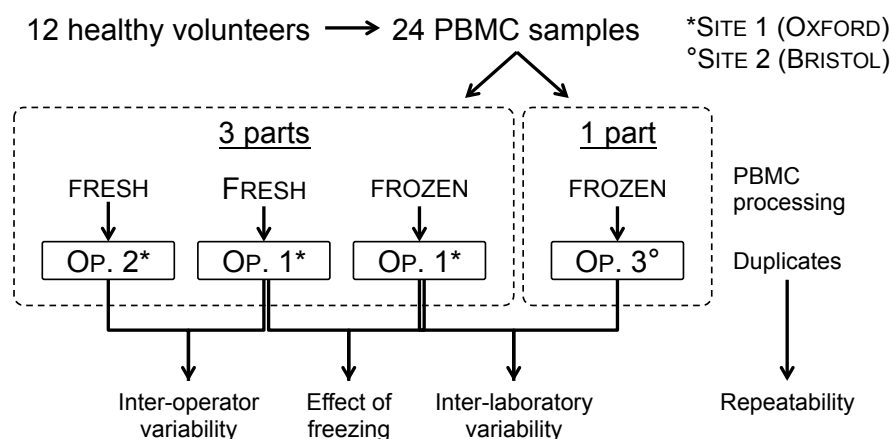


Figure 9.1: Study design used to evaluate memory B cell ELISpot variability.

erating Bland-Altman plots for each antigen and each group. ELISpot plates were read and counted automatically using pre-defined settings and spot numbers were subsequently verified independently by two operators for the presence of artefacts, in which case spot numbers were adjusted by removing counts obtained by artefacts. All operators were blinded to the samples that were processed, read and counted.

9.3 RESULTS

Effect of cryopreservation, operator, and laboratory on antigen-specific B_{MEM} frequencies

Numbers of PBMC obtained after thawing of frozen samples were significantly lower in samples, which were kept on dry ice for transport to the other laboratory (Figure 9.2a). Of note, shipment and storage was not optimal in this study due to unintentional prolonged shipment of 2 days and storage of frozen cells at -80°C for 1 week prior to processing. This resulted in lower numbers of viable cells before and after culture (Figure 9.2b). For the culture step, the numbers of viable cells put into each culture well were the same. After the 6 day culture, viable cell counts per well were not different between the 2 groups (data not shown).

A subset of plates was counted independently by two operators who were blinded to the samples. A very high correlation between the two spots counts was found when numbers of spots for each sample were compared (Figure 9.3). For subsequent analysis, only counts from operator 1 were used.

When comparing diphtheria and tetanus toxoid-specific B_{MEM} between two independent operators within the same laboratory using fresh PBMC samples (operator 1 fresh vs. operator 2 fresh), correla-

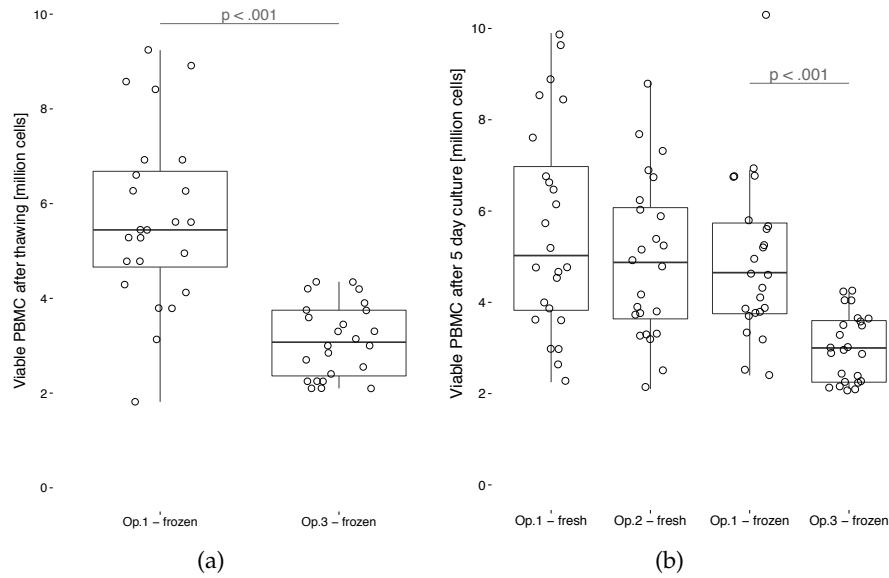


Figure 9.2: Number of viable PBMCs (a) following thawing of the samples and (b) after the 6 day culture. Significant differences in the PBMC counts were found both before and after culturing the cells. P-values were calculated by two-tailed paired t-tests. Horizontal bars and box borders indicate medians and interquartile ranges. Op. = operator.

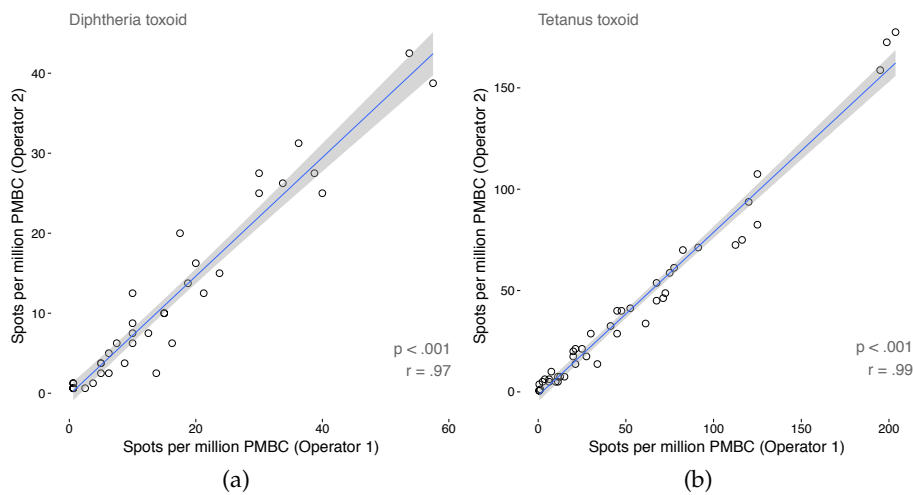


Figure 9.3: Counting of 2 groups of samples (operator 1 & 2 fresh) showed very good correlation for (a) diphtheria toxoid-specific B_{MEM} ($n=43$) and (b) tetanus toxoid-specific B_{MEM} ($n=46$).

tion was relatively high (Figures 9.4 and 9.5). Comparison of fresh vs. frozen samples showed highest correlation coefficients for both diphtheria and tetanus toxoid-specific B_{MEM} frequencies when processed by the same operator. Inter-laboratory correlation, i.e. samples being processed by two different operators in two different laboratories (operator 1 frozen vs. operator 3 frozen), was lower but still relatively high when comparing diphtheria and tetanus toxoid-specific B_{MEM} frequencies. Not unexpectedly, the lowest correlation was measured for both diphtheria and tetanus toxoid-specific B_{MEM} when samples were processed by different operators in different laboratories with one operator working with fresh and the other operator working with frozen samples (Figure 9.4 and 9.5). Figures 9.6 and 9.7 show intra-operator agreements between 2 samples taken from the same study participant for diphtheria and tetanus toxoid-specific B_{MEM} and fresh and frozen samples, respectively. Almost all values were found to be within the 95% CI with diphtheria toxoid-specific B_{MEM} numbers generally being lower than those for tetanus toxoid B_{MEM} .

9.4 DISCUSSION

In this study an ELISpot assay measuring B_{MEM} was assessed for inter- and intra-operator variability as well as inter-laboratory performance and the effect of freezing of PBMCs. Here, we show that repeatability of this method is acceptable and that B_{MEM} frequencies are well correlated when two operators within the same laboratory are processing samples. This is an encouraging finding, as there are a number of stages in the ELISpot assay where variability may be introduced, particularly when different operators are processing samples. For example, the final B_{MEM} count in the assay is dependent on the correct number of cells (2×10^5) being placed into each well, therefore errors in the initial cell count may introduce systematic bias into the rest of that particular assay [228]. Furthermore, the method used to count B_{MEM} when analysing plates may also introduce variability [229], however in this study, counting of spots in a semi-automated manner [229], resulted in good correlation when assessed by two different operators. We also found that frozen cells are sensitive to suboptimal conditions during shipment and storage, consistent with a previous study showing reduced cell viability associated with unstable storage temperatures [208]. However, in the present study, even when numbers of viable cells that could be used in the assay were significantly reduced, antigen-specific B_{MEM} frequencies could still be reliably measured. It has previously been reported that a low cell viability following cryopreservation compromises lymphocyte proliferation in response to mitogens including PWM [230]. In the present study, after allowing for lower viable cell numbers following storage and shipment, freezing of PBMC did not affect the numbers of viable PBMCs that were

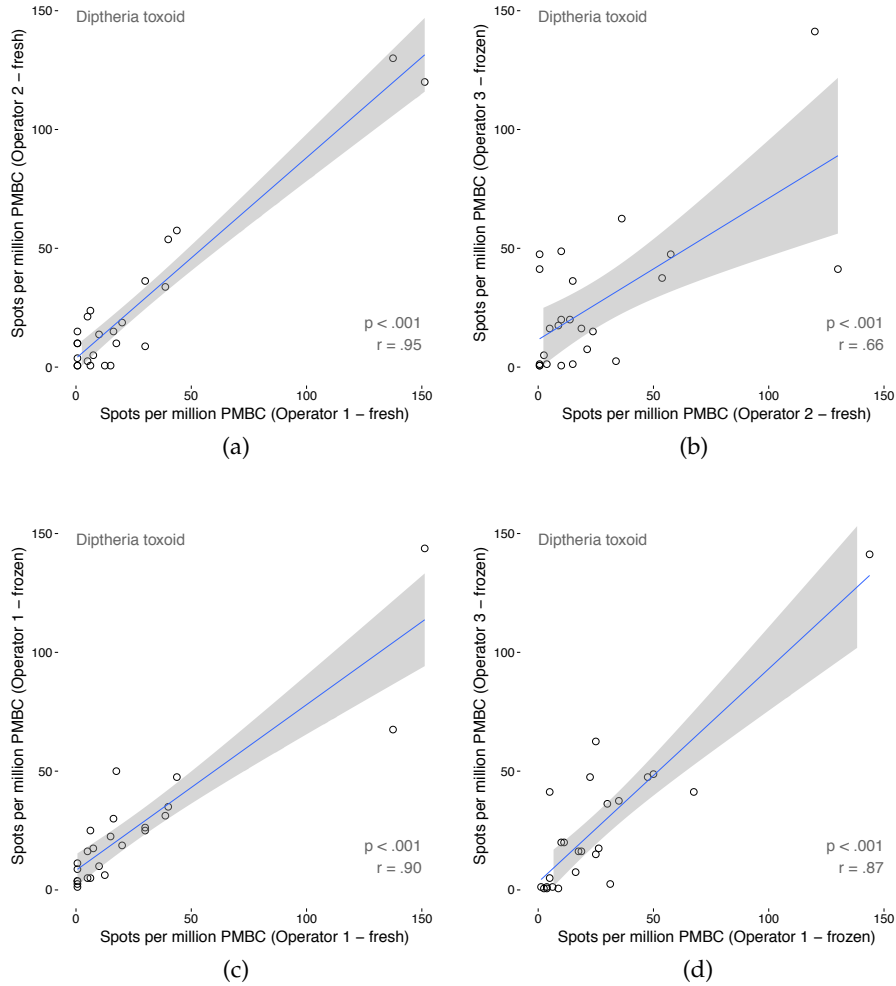


Figure 9.4: Scatterplot matrix of diphtheria toxoid-specific ASC per 10^6 cultured PBMC using (a) fresh samples operator 1 vs. operator 2, (b) fresh operator 2 [Oxford] vs. frozen operator 3 [Bristol], (c) fresh vs. frozen samples operator 1, and (d) frozen samples operator 1 [Oxford] vs. operator 3 [Bristol].

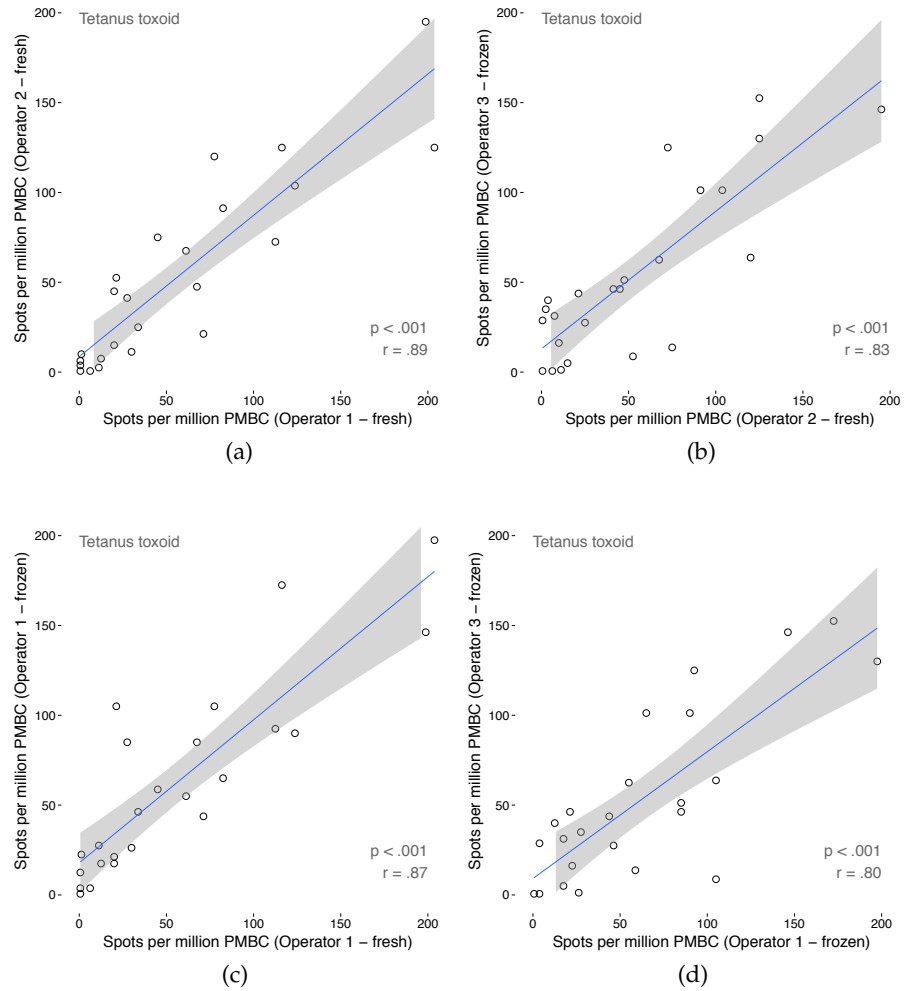


Figure 9.5: Scatterplot matrix of tetanus toxoid-specific ASC per 10^6 cultured PBMC using (a) fresh samples operator 1 vs. operator 2, (b) fresh operator 2 [Oxford] vs. frozen operator 3 [Bristol], (c) fresh vs. frozen samples operator 1, and (d) frozen samples operator 1 [Oxford] vs. operator 3 [Bristol].

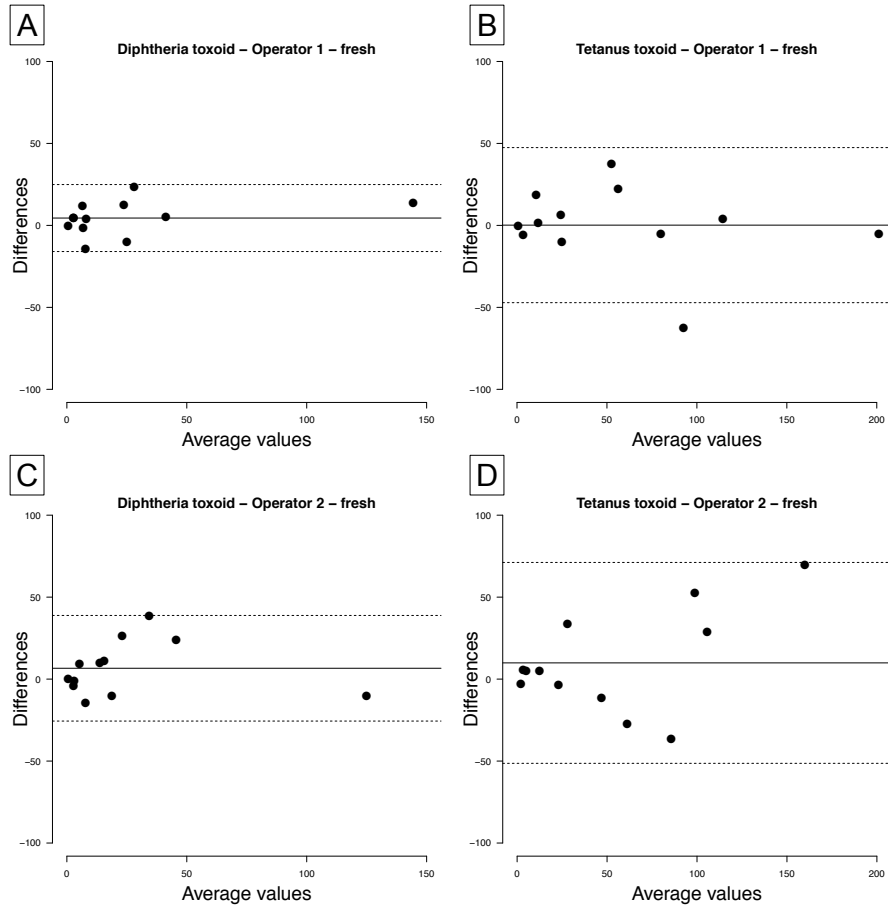


Figure 9.6: Bland-Altman plots of intra-operator variability on fresh samples showing the difference against the mean of two measurements. Bias (mean difference, dotted line) and 95%-limits of agreement (dashed lines) are shown. (A) Operator 1, diphtheria toxoid; (B) Operator 1, tetanus toxoid; (C) Operator 2, diphtheria toxoid; (D) Operator 2, tetanus toxoid.

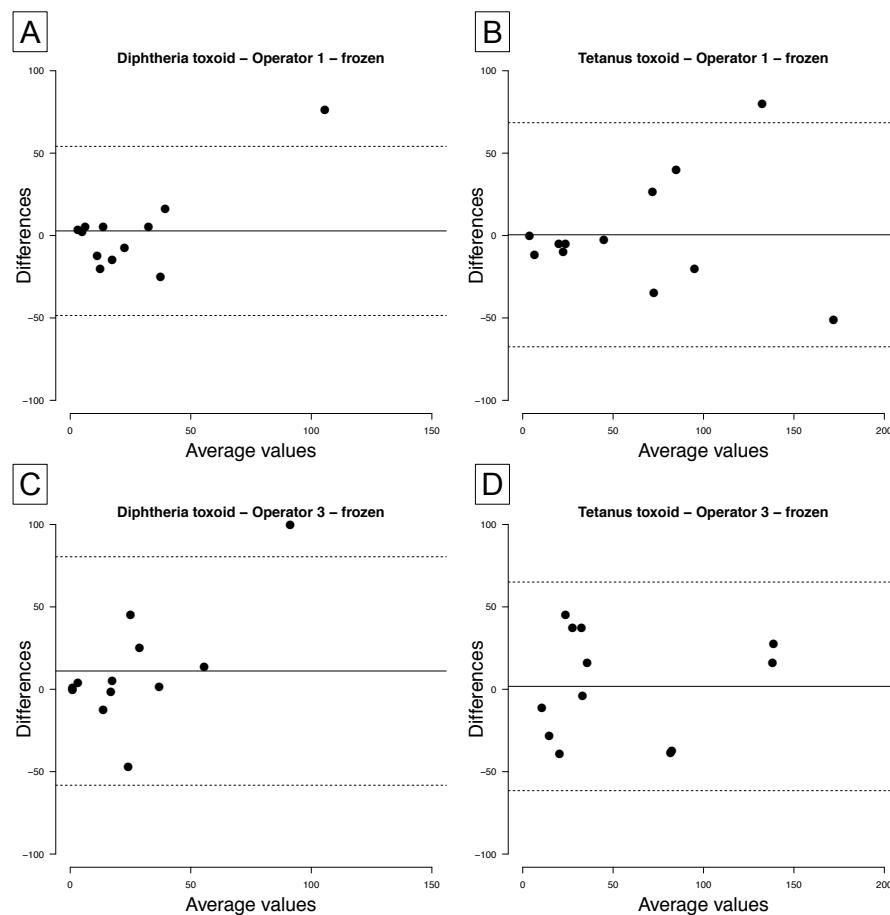


Figure 9.7: Bland-Altman plots of intra-operator variability on frozen samples showing the difference against the mean of two measurements. Bias (mean difference, dotted line) and 95%-limits of agreement (dashed lines) are shown. (A) Operator 1, diphtheria toxoid; (B) Operator 1, tetanus toxoid; (C) Operator 3, diphtheria toxoid; (D) Operator 3, tetanus toxoid.

harvested after a 6-day culture compared to fresh samples. This suggests that in this case, the remaining viable cells responded to the polyclonal stimulators (PWM, CpG and SAC) in the culture medium to the same degree as the fresh cells. Although cryopreserved PBMCs transported to the Bristol laboratory experienced suboptimal storage conditions during transit, frozen samples processed in Oxford were stored according to protocol. There was no significant difference in the number of viable PBMCs recovered from cryopreserved samples after culture compared with fresh samples, and B_{MEM} counts from the two samples were well correlated. This demonstrates that cryopreservation does not have a negative impact on the detection of B_{MEM} in the cultured ELISpot, consistent with studies demonstrating reliable T cell ELISpot results when using cryopreserved samples [217–219]. Inter-laboratory variability was relatively low and within the range of the variability that was found when two different operators within the same laboratory processed fresh samples. Intra-operator agreement of sample replicates was similar between fresh and frozen samples and overall acceptable in all groups and in all 3 operators. Reproducibility of the ELISpot assay between laboratories is important in order to combine data in the context of multi-centre studies. We, along with a study investigating the reproducibility of a T cell ELISpots [227], have demonstrated that low inter-laboratory variability is achievable when using a standardised protocol.

9.5 CONCLUSION

In this study we found that the cultured B cell ELISpot is a robust and reproducible assay that can be performed on fresh or frozen samples and in different laboratories. Specific caution is advised when shipment of frozen samples to a different laboratory is considered although antigen-specific B_{MEM} frequencies can still be reliably measured even with a lower number of viable cells resulting from suboptimal shipment and storage.

PERSISTENCE OF PNEUMOCOCCAL ANTIBODY UP TO PRE-SCHOOL AGE

10.1 BACKGROUND

The encapsulated bacterium *Streptococcus pneumoniae* (pneumococcus) is responsible for a considerable burden of disease in young children, both invasive disease such as meningitis, as well as mucosal infection such as otitis media and pneumonia. Worldwide more than 14 million episodes of serious pneumococcal disease and about 800 000 deaths in children under the age of five years occur annually [45, 46, 64].

Healthy pre-school children frequently carry pneumococci in the nasopharynx [10]. Young children are thought to be the main transmitters of pneumococci throughout the population including to other young children and the elderly [231], which may result in disease in these populations. Maintaining adequate (functional) antibody levels in pre-school children is the key to sustained direct protection of these vaccinated individuals, as well as blocking transmission of the organism to achieve herd immunity. Both are presumed to be mediated by antibody. PCVs are highly effective in preventing carriage of the serotypes contained in the vaccine and thus disease [27]. Previous immunogenicity studies showed that with the currently used infant immunisation schedules, immunity to pneumococcal serotypes wanes rapidly during the 2nd year of life [232, 233].

In a previously reported phase II parallel-group, randomised, active-controlled, double-blind, multicenter trial [234], 278 children received either PCV-7 or PCV-13 at 2, 4 and 12 months of age along with routine immunisations for the United Kingdom. In this follow-on study, we evaluated the persistence of serotype-specific antibody at 3.5 years of age in individuals who had been enrolled into the earlier trial. In addition, we investigated the immunogenicity and reactogenicity of a PCV-13 vaccine booster at this age, comparing individuals previously primed with PCV-7 or PCV-13.

10.2 METHODS

Participants, recruitment and design

This follow-on, multicenter, open label, phase IV clinical trial was conducted from May to December 2010 in four sites (Oxford, London, Bristol, Southampton) across the United Kingdom. Five sites, enrolling 66 participants in the original study, did not take part in this

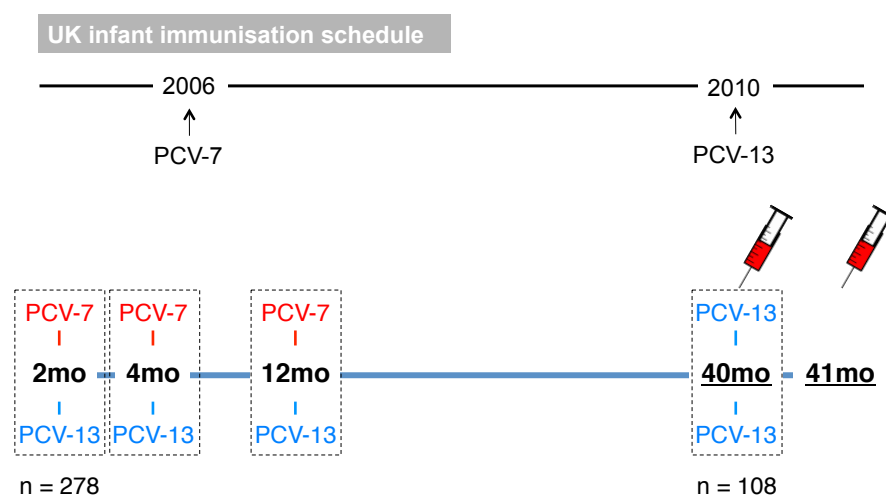


Figure 10.1: Study design of the follow-on study along with the years of the implementation of the 7- and 13-valent pneumococcal conjugate vaccines into the routine UK infant immunisation schedule.

follow-on study. Ethical approval was obtained from the Oxfordshire Research Ethics Committee C (Reference Number 10/Ho606/09). Potential study participants were those who had completed the initial infant trial (Clinicaltrials.gov registration number: [NCT00384059](#)), were healthy and 39-46 months of age. Exclusion criteria were similar to the initial study [234] and the receipt of further doses of pneumococcal vaccines since the initial study. Written informed consent was taken by members of the research team, either a doctor or nurse trained in taking informed consent. The parent/guardian and the person who presented and obtained consent had to personally date and sign the latest approved version of the consent form before any study-specific procedures were performed, which was then retained at the study site. This procedure of taking and documenting consent was approved by the Oxfordshire Research Ethics Committee (Reference Number 10/Ho606/9).

At visit 1, participants had a blood sample taken, followed by a dose of Prevenar 13® (PCV-13; Pfizer Inc., Pearl River, USA). A second blood sample was drawn at visit 2 (28 to 42 days after visit 1; Figure 10.1), together with doses of the routine pre-school immunisations, if not already received. Laboratory staff remained blinded to original trial vaccine group allocation.

Study objectives

The primary objective of this study was to determine the proportion of participants immunised with PCV-13 at 2, 4 and 12 months of age, who had serotype-specific IgG concentrations $\geq 0.35 \mu\text{g/ml}$ [235] for PCV-13 serotypes at pre-school age. The secondary objectives were to

determine the proportion of participants with pre-booster serotype-specific IgG ≥ 0.35 $\mu\text{g/ml}$ for PCV-13 serotypes in the PCV-7 group; the proportion of participants with OPA titres ≥ 8 for PCV-13 serotypes in both groups; and the immunogenicity as well as the reactogenicity of a booster dose of PCV-13 given at approximately 3.5 years of age.

Vaccines and interventions

PCV-13 is formulated in a similar manner to PCV-7 containing serotypes 4, 6B, 9V, 14, 18C, 19F, 23F and includes the additional serotypes 1, 3, 5, 6A, 7F, and 19A [236]. PCV-13 was administered to all participants intramuscularly into the deltoid muscle during visit 1, which took place at the participants' homes by trained members of the research team. Routine pre-school booster immunisations were also administered to those who required them following the UK immunisation program at visit 2. All vaccines were presented in pre-filled syringes.

Laboratory measurements

Serum was separated within 24 hours of sampling and stored at -80 °C. PCV-13 serotype-specific IgG antibody concentrations and OPA titres were tested at Pfizer Vaccine Research, Pearl River, USA as described in sections 7.8 and 7.9.

Statistical analysis

Data were log₁₀-transformed prior to analysis and results of group comparisons on log scales were presented as geometric means in each group, 95% confidence interval (CI, calculated using the binomial exact method) and associated p-value for the group comparison. All statistical tests were 2-sided and p-values less than 0.05 were considered significant. For each participant the number of serotypes above threshold was calculated and standardised to 13 serotypes (to account for a small number of participants with some missing serotypes). Participants were analysed according to the group to which they had been randomised in the initial study. Statistical analysis were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) and R [237].

10.3 RESULTS

Out of 278 participants enrolled in the original study, 191 were eligible for the follow-on study and 108 children were enrolled into the study and received the booster vaccine. The parents of one child withdrew consent before visit 2 and 1 child was lost to follow-up, result-

Table 10.1: Summary of study participants' characteristics.

	PCV-13 (N=56)	PCV-7 (N=52)
Proportion of female study participants	28 (50%)	28 (54%)
Proportion of White Caucasian study participants	54 (96%)	48 (92%)
Mean age at Visit 1 in years (range)	3.50 (3.16 - 4.09)	3.51 (3.15 - 4.04)
Mean time since 12-month booster to Visit 1 in years (range)	2.49 (2.13 - 3.07)	2.51 (2.15 - 2.97)
Mean time interval between Visit 1 - Visit 2 in days (range)	34.9 (26.5 - 56.5)	35.0 (27.6 - 42.6)

ing in 106 participants who completed visit 2 (Figure 10.2). The age, gender, time since last pneumococcal conjugate vaccination and the time interval between visit 1 and visit 2 were similar in both groups (Table 10.1).

Antibody persistence to pre-school years

Approximately 2.5 years after completion of a 2+1 immunisation schedule with either PCV-13 or PCV-7, most children had serotype-specific IgG concentrations ≥ 0.35 $\mu\text{g/ml}$, for the majority of serotypes against which they had been immunised (median number of serotypes with IgG above threshold: 10). This was also true for children immunised with PCV-7 as the primary vaccine (Figure 10.3). Before the pre-school booster $\geq 76\%$ of participants had IgG concentrations ≥ 0.35 $\mu\text{g/ml}$ for 5 of the 7 shared serotypes, the exceptions being serotypes 4 and 18C (Table 10.2 and Figure 10.4). For the 6 additional PCV-13 serotypes, IgG concentrations ≥ 0.35 $\mu\text{g/ml}$ were present in almost all participants in both groups for serotypes 6A and 19A (and at least 83% for serotype 5). Between the groups the proportions of participants with IgG concentrations ≥ 0.35 $\mu\text{g/ml}$ were significantly higher in PCV-13 than PCV-7 recipients for serotypes 1, 5 and 7F and lower for serotype 4 (Table 10.2 and Figure 10.4).

Serotype specific IgG geometric mean concentration (GMC)s were significantly higher in PCV-13 than PCV-7 recipients for serotypes 1, 7F and 19A (Figure 10.5 and Table 10.4), but did not significantly differ for the other three serotypes contained only in PCV-13. For serotypes included in both vaccines, only serotype 4 was significantly different between the groups (slightly higher in the PCV-7 group).

Serotype-specific functional antibodies were also assessed by OPA. Similar to the IgG data, a high proportion of participants had OPA titres ≥ 8 for most of the serotypes in the PCV-13 group (median number of serotypes with OPA above threshold: 9), which was significantly lower for the PCV-7 group with a median of 7.1 (Figure 10.2, $p < 0.001$). Before the PCV-13 booster, $\geq 71\%$ of participants had OPA titres ≥ 8

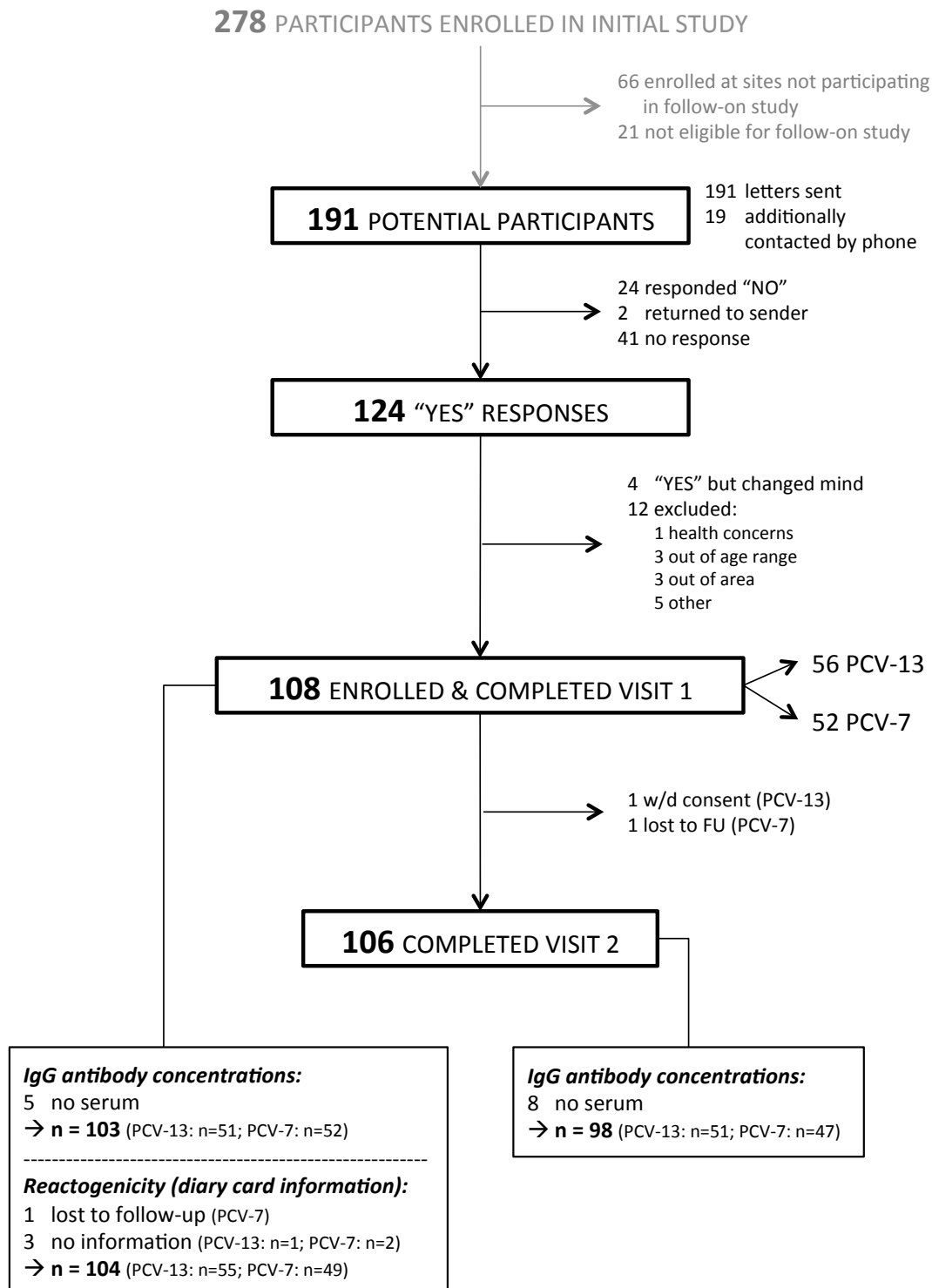


Figure 10.2: Flow chart of participants through the study.

Table 10.2: Proportion of participants with serotype-specific IgG concentrations $\geq 0.35 \mu\text{g/mL}$.

Serotype	PRE-BOOSTER (40 MONTHS)			POSTBOOSTER (41 MONTHS)	
	Proportion $\geq 0.35 \mu\text{g/mL}$ (95% CI) *		P value **	Proportion $\geq 0.35 \mu\text{g/mL}$ (95% CI) *	
	PCV-13	PCV-7		PCV-13	PCV-7
1 [†]	0.45 (0.31, 0.60)	0.14 (0.06, 0.27)	0.0006	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
3 [†]	0.52 (0.37, 0.66)	0.59 (0.43, 0.73)	0.5099	0.96 (0.87, 1.00)	0.89 (0.76, 0.96)
4	0.22 (0.12, 0.36)	0.42 (0.29, 0.57)	0.0284	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
5 [†]	0.96 (0.87, 1.00)	0.83 (0.70, 0.92)	0.0279	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
6A [†]	1.00 (0.93, 1.00)	0.96 (0.87, 1.00)	0.1573	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
6B	0.98 (0.90, 1.00)	0.98 (0.90, 1.00)	0.9889	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
7F [†]	0.71 (0.56, 0.83)	0.39 (0.25, 0.54)	0.0014	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
9V	0.78 (0.65, 0.89)	0.83 (0.70, 0.92)	0.5847	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
14	0.76 (0.63, 0.87)	0.78 (0.65, 0.89)	0.8127	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
18C	0.31 (0.19, 0.46)	0.38 (0.25, 0.53)	0.4506	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
19A [†]	1.00 (0.93, 1.00)	0.98 (0.90, 1.00)	0.3196	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
19F	0.92 (0.81, 0.98)	0.94 (0.84, 0.99)	0.6759	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)
23F	0.84 (0.71, 0.93)	0.92 (0.81, 0.98)	0.2061	1.00 (0.93, 1.00)	1.00 (0.92, 1.00)

* Binomial exact confidence interval; ** P Value from chi-square test of proportions at 40 months, no comparisons were made at 41 months as almost all proportions were 1; [†] Serotypes only present in PCV-13

Table 10.3: Proportion of participants with serotype-specific OPA titres ≥ 8 .

Serotype	PRE-BOOSTER (40 MONTHS)		POSTBOOSTER (41 MONTHS)	
	Proportion OPA ≥ 8 (95% CI) *		Proportion OPA ≥ 8 (95% CI) *	
	PCV-13	PCV-7	PCV-13	PCV-7
1 [†]	0.27 (0.15, 0.42)	0.04 (0.00, 0.13)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
3 [†]	0.85 (0.72, 0.94)	0.45 (0.31, 0.60)	1.00 (0.93, 1.00)	1.00 (0.91, 1.00)
4	0.30 (0.16, 0.47)	0.29 (0.16, 0.44)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
5 [†]	0.35 (0.21, 0.50)	0.0 (0.0, 0.08)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
6A [†]	0.74 (0.58, 0.86)	0.49 (0.33, 0.65)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
6B	0.78 (0.62, 0.89)	0.71 (0.56, 0.84)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
7F [†]	0.91 (0.78, 0.97)	0.76 (0.60, 0.89)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
9V	0.86 (0.73, 0.95)	0.84 (0.71, 0.94)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
14	0.82 (0.67, 0.92)	0.83 (0.70, 0.93)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
18C	0.52 (0.37, 0.68)	0.57 (0.42, 0.72)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)
19A [†]	0.94 (0.83, 0.99)	0.69 (0.53, 0.82)	1.00 (0.93, 1.00)	1.00 (0.91, 1.00)
19F	0.63 (0.47, 0.77)	0.72 (0.57, 0.84)	1.00 (0.91, 1.00)	1.00 (0.91, 1.00)
23F	0.72 (0.56, 0.85)	0.83 (0.69, 0.92)	1.00 (0.92, 1.00)	1.00 (0.91, 1.00)

* Binomial exact confidence interval; ** P Value from chi-square test of proportions at 40 months, no comparisons were made at 41 months as almost all proportions were 1; [†] Serotypes only present in PCV-13

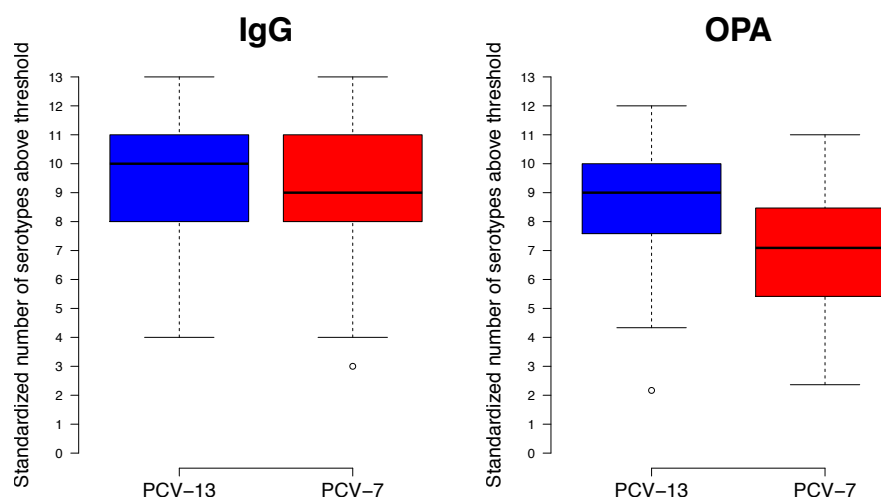


Figure 10.3: Standardized number of serotypes above thresholds. Box plots of standardised number of serotypes above thresholds (IgG ≥ 0.35 $\mu\text{g/ml}$ and OPA titres ≥ 8) at 3.5 years in each of the groups [standardised to 13 serotypes]. The median is shown as a line across the box with the box representing the lower and upper quartiles. Whiskers extend to the maximum or minimum values within 1.5 times the IQR above and below the 3rd and 1st quartile, respectively. Points outside this range are represented as dots.

for 4/7 shared serotypes, the exceptions being serotypes 4, 18C and 19F (Figure 10.4). For the 6 additional serotypes OPA titres ≥ 8 were observed in $\geq 74\%$ of PCV-13 group participants for serotypes 3, 6A, 7F and 19A, but were lower for serotypes 1 and 5 (Figure 10.4). In the PCV-7 group $\geq 45\%$ of participants had OPA titres ≥ 8 for serotypes 3, 6A, 7F and 19A and very few participants had detectable OPA titres against serotypes 1 and 5 (Table 10.3). OPA geometric mean titre (GMT)s at 40 months of age before the PCV-13 booster were similar between the groups for the shared serotypes. As for the PCV-13 serotypes, significant differences were observed for serotypes 1, 3, 5, 6A and 19A but not for serotype 7F, for which there were very high OPA titres in both groups (Table 10.5 and Figure 10.5).

Immunogenicity of the PCV-13 pre-school booster

Following the PCV-13 booster all participants from both groups had IgG concentrations ≥ 0.35 $\mu\text{g/ml}$ for all serotypes with the exception of serotype 3 (PCV-13 group: 96% and PCV-7 group: 89%; Figure 10.4). Post-booster antibody GMCs were significantly higher in the PCV-13 group for 5/6 of the additional serotypes only included in PCV-13, except for serotype 3 (Table 10.4 and Figure 10.5). Following the PCV-13 booster, OPA titres rose significantly in both groups and all participants had OPA titres ≥ 8 for all serotypes. Significant differences

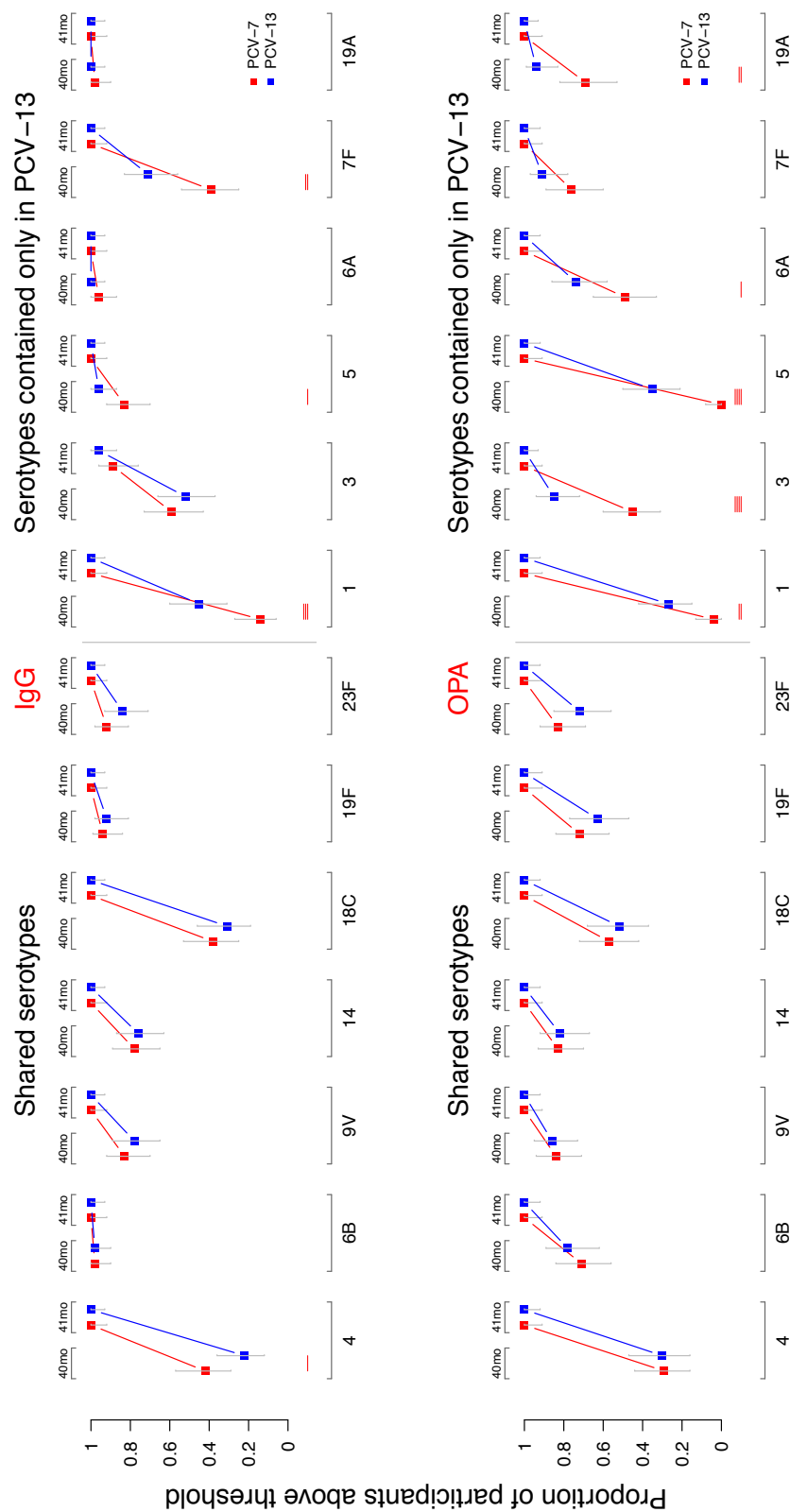


Figure 10.4: Proportion of participants with serotype-specific IgG concentrations and OPA titres above thresholds. Proportion of participants with serotype-specific IgG concentrations and OPA titres equal or above a threshold of 0.35 $\mu\text{g/ml}$ and 8, respectively, for each of the serotypes before (40 months, 40mo) and after (41 months, 41mo) the PCV-13 booster. Shown are mean values along with their 95% confidence intervals. Red lines are shown at the bottom if p-values from chi-square test of proportions between the groups were significant (p-values ≤ 0.05 , ≤ 0.01 , ≤ 0.001 , and ≤ 0.0001 are represented by 1, 2, 3, and 4 lines).

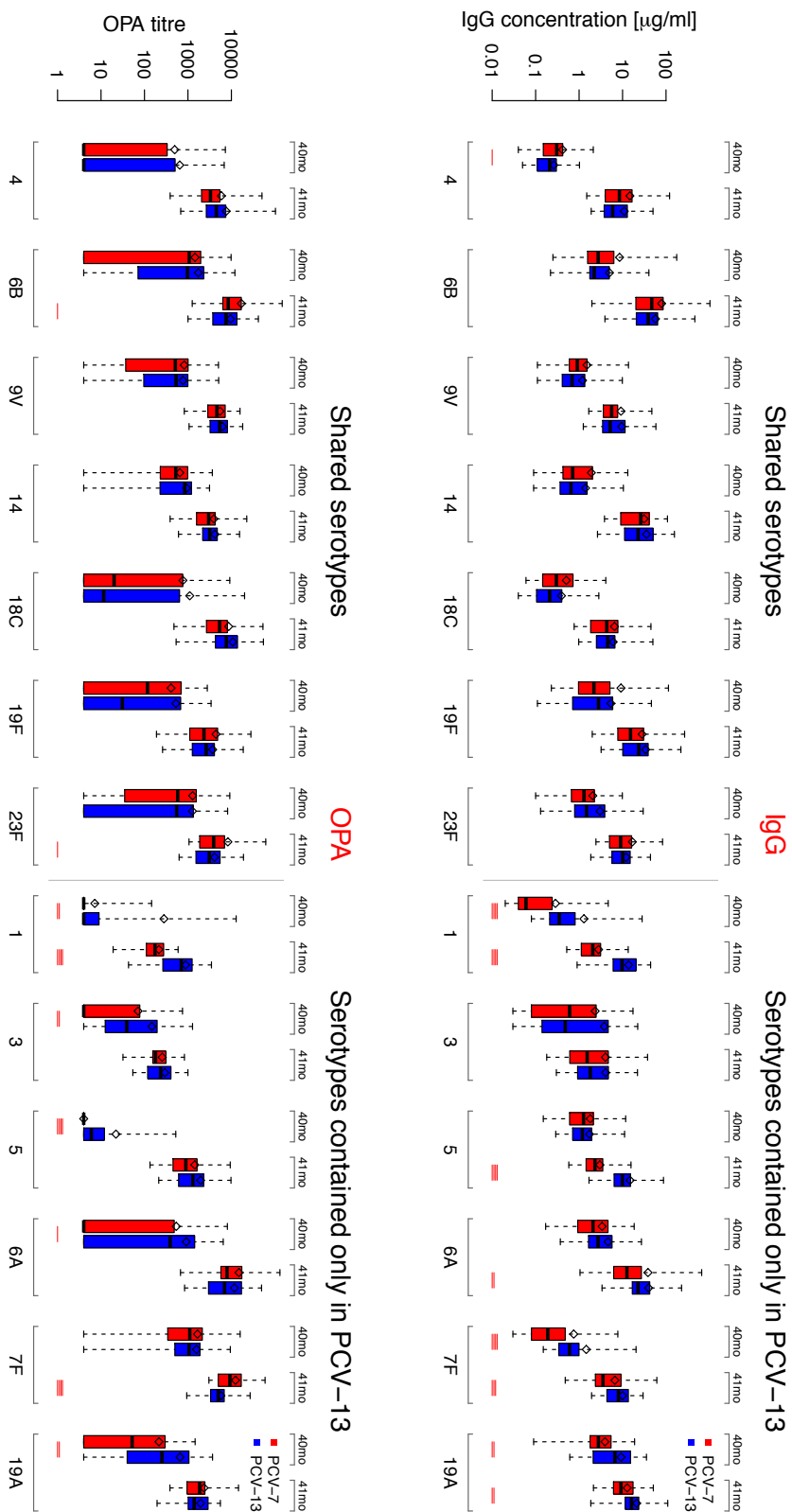


Figure 10.5: Serotype-specific IgG GMTs and OPA GMTs. Box plots of IgG GMTs and OPA GMTs for each of the serotypes before (40 months, 40mo) and after (41 months, 41mo) the PCV-13 booster. The median is shown as a line across the box with the box extending to the lower and upper quartiles, respectively. The whiskers extend to the maximum or minimum values within 1.5 times the IQR above and below the 3rd and 1st quartile, respectively. Points outside this range are represented as dots. Means are shown as a diamonds and t-test were performed for each serotype at each time point between the groups with p-values being represented as red lines if significant (1, 2, 3, and 4 lines if $p \leq 0.05$, ≤ 0.01 , ≤ 0.001 , and ≤ 0.0001 , respectively).

Table 10.4: Serotype-specific geometric mean concentrations before and after the booster at 40 and 41 months of age with t-tests.

Serotype	PRE-BOOSTER (40 MONTHS)			POSTBOOSTER (41 MONTHS)		
	GMC (95% CI)		P value ^{**}	GMC (95% CI)		P value ^{**}
	PCV-13	PCV-7		PCV-13	PCV-7	
1 [†]	0.45 (0.32, 0.62)	0.10 (0.07, 0.15)	<.0001	10.16 (8.04, 12.84)	2.03 (1.61, 2.56)	<.0001
3 [†]	0.71 (0.40, 1.28)	0.56 (0.32, 0.99)	0.5552	2.18 (1.59, 2.98)	1.71 (1.16, 2.52)	0.3357
4	0.20 (0.16, 0.24)	0.28 (0.22, 0.36)	0.0267	7.37 (5.80, 9.37)	8.72 (6.48, 11.73)	0.3744
5 [†]	1.22 (0.97, 1.53)	1.11 (0.84, 1.48)	0.6168	10.46 (8.34, 13.13)	2.28 (1.84, 2.81)	<.0001
6A [†]	3.04 (2.34, 3.94)	2.14 (1.63, 2.83)	0.0684	26.81 (21.01, 34.20)	13.55 (9.27, 19.80)	0.0032
6B	2.79 (2.06, 3.78)	3.28 (2.35, 4.57)	0.4779	37.82 (29.57, 48.37)	40.25 (28.78, 56.28)	0.7616
7F [†]	0.67 (0.51, 0.90)	0.25 (0.17, 0.37)	<.0001	7.82 (6.38, 9.59)	4.32 (3.33, 5.61)	0.0004
9V	0.76 (0.59, 0.98)	0.91 (0.69, 1.19)	0.3532	6.43 (5.06, 8.16)	6.23 (4.89, 7.94)	0.8546
14	0.75 (0.54, 1.02)	0.91 (0.65, 1.28)	0.3800	22.51 (17.00, 29.82)	21.51 (16.41, 28.18)	0.8145
18C	0.22 (0.16, 0.30)	0.31 (0.23, 0.41)	0.1051	4.33 (3.48, 5.41)	4.25 (3.25, 5.55)	0.9054
19A [†]	5.34 (3.84, 7.42)	2.75 (2.11, 3.58)	0.0020	15.70 (12.62, 19.54)	9.49 (7.54, 11.94)	0.0019
19F	2.30 (1.55, 3.42)	2.65 (1.78, 3.94)	0.6135	22.18 (17.05, 28.87)	15.29 (11.16, 20.96)	0.0701
23F	1.48 (1.05, 2.10)	1.31 (0.99, 1.72)	0.5646	9.10 (7.33, 11.32)	10.25 (7.84, 13.39)	0.4897

^{**}P value from independent samples t-test using Satterthwaites method for unequal variances where appropriate; [†]Serotypes only present in PCV-13

Table 10.5: Serotype-specific geometric mean OPA titres before and after the booster at 40 and 41 months of age with t-tests.
PRE-BOOSTER (40 MONTHS) POSTBOOSTER (41 MONTHS)

Serotype	GMT (95% CI)		P value**	GMT (95% CI)		P value**
	PCV-13	PCV-7		PCV-13	PCV-7	
1 ⁺	8.02 (5.34, 12.04)	4.45 (3.80, 5.21)	0.0086	588.2 (435.8, 793.9)	175.2 (140.6, 218.3)	<.0001
3 ⁺	46.37 (28.61, 75.13)	16.16 (9.86, 26.50)	0.0028	227.1 (183.3, 281.4)	209.9 (171.3, 257.2)	0.5965
4	22.98 (9.13, 57.82)	19.47 (8.99, 42.19)	0.7797	4453 (3407, 5821)	3320 (2373, 4644)	0.1671
5 ⁺	8.00 (5.93, 10.79)	4.00 (4.00, 4.00)	<.0001	1296 (976.5, 1719)	921.6 (683.8, 1242)	0.0978
6A ⁺	172.4 (78.84, 376.8)	45.43 (19.84, 104.1)	0.0204	7023 (5072, 9724)	8814 (6351, 12232)	0.3251
6B	338.6 (145.3, 789.3)	241.1 (103.7, 560.5)	0.5690	6605 (5013, 8702)	9962 (7313, 13571)	0.0478
7F ⁺	677.2 (380.0, 1207)	354.3 (148.7, 844.5)	0.2134	4696 (3866, 5704)	9313 (7287, 11902)	<.0001
9V	240.5 (126.2, 458.4)	229.7 (118.7, 444.3)	0.9200	5069 (4149, 6193)	4433 (3547, 5540)	0.3675
14	337.3 (171.7, 662.5)	264.3 (147.8, 472.6)	0.5868	3179 (2551, 3961)	2886 (2244, 3711)	0.5593
18C	50.37 (21.32, 119.0)	56.72 (25.52, 126.1)	0.8386	7530 (5780, 9809)	5119 (3713, 7058)	0.0624
19A ⁺	187.0 (105.7, 330.8)	50.25 (27.27, 92.60)	0.0021	1557 (1261, 1923)	1649 (1276, 2131)	0.7274
19F	59.72 (27.45, 130.0)	73.30 (37.42, 143.6)	0.6877	2249 (1612, 3140)	2496 (1743, 3576)	0.6682
23F	179.4 (76.38, 421.3)	291.7 (142.5, 597.2)	0.3792	2972 (2316, 3815)	4466 (3236, 6162)	0.0452

** P value from independent samples t-test using Satterthwaites method for unequal variances where appropriate; ⁺ Serotypes only present in PCV-13

in post-booster GMTs were observed for serotypes 6B and 23F from the shared serotypes (higher in the PCV-7 group) and for serotypes 1 (higher in the PCV-13 group) and 7F (higher in the PCV-7 group) – Table 10.5 and Figure 10.5.

Reactogenicity

No serious adverse events were recorded following the PCV-13 booster at 3.5 years of age. Local reactions such as redness, hardness and swelling were either absent or mild in most cases. However, moderate or severe tenderness was experienced by 18% of all participants (Table 10.6). Decreased appetite and irritability were recorded in 18% and 41% of participants, respectively, which were moderate or severe in 4% and 13% of children. Low-grade fever (38–39 °C) was noted in 3% of the participants and none of the children had a temperature >39 °C (Table 10.6). The reactogenicity profile of the PCV-13 booster was similar regardless of whether participants had previously been immunised with PCV-7 or PCV-13.

10.3.1 Discussion

This is to our knowledge the first clinical trial investigating the persistence of total and opsonophagocytic serotype-specific antibodies up to the age of 3.5 years following a CRM₁₉₇-based 7- or 13-valent PCV schedule. We have shown that sustained effectiveness of both direct and indirect effects can be anticipated on a population level if based on the currently accepted “protective thresholds”. For the PCV-7 serotypes, this finding is consistent with IPD effectiveness data demonstrating almost complete disappearance of vaccine serotypes over a period of approximately 5–7 years [28, 238, 239] so that, at the population level, these responses appear to be adequate for effective disease control.

Only 2 other studies looked at the persistence of pneumococcal antibody beyond the second year of life. Silfverdal et al. [240] assessed the immune response in children aged 36–46 months previously vaccinated with the 10-valent PCV (PCV-10) in a 2+1 or 3+1 schedule compared to an unvaccinated control group of children of the same age. Although the focus of their work was to demonstrate induction of memory by PCV-10, they report information on the baseline antibody GMC and OPA GMT, which were generally lower for the majority of the vaccine serotypes even in the group of children who were vaccinated in a 3+1 schedule compared with our study. In a further study by Madhi et al. [241] in South Africa, trial participants who had received 3 doses of PCV-9 at approximately 6, 11 and 16 weeks of age were followed to approximately 6 years and compared with unvaccinated controls for age. Efficacy against vaccine-type IPD remained

Table 10.6: Local and systemic reactions following the pre-school PCV-13 booster at 40 months of age.

Level	PCV-13 (N=55)						Both groups (N=104; 103 for T°)	
	PCV-13 (N=55)			PCV-7 (N=49; 48 for T°)				
	N	Proportion		N	Proportion	P value**	N	Proportion
Redness	Any [severe ≥ 5 cm]	26 [3]	47% [6%]	20 [1]	41% [2%]	0.5566	46 [4]	44% [4%]
Swelling	Any [severe ≥ 5 cm]	8 [1]	15% [2%]	14 [1]	29% [2%]	0.0959	22 [2]	21% [2%]
Hardness	Any [severe ≥ 5 cm]	10 [0]	18% [0%]	16 [0]	33% [0%]	0.1136	26 [0]	25% [0%]
Tenderness	Any	32	58%	28	57%	1.0000	60	58%
	Discomfort during routine activities	9	16%	6	12%		15	14%
Poor appetite	Interfering with limb movement	2	4%	2	4%		4	4%
	Appetite as usual	45	82%	40	82%	0.2322	19	18%
	Eating less/ no effect on normal activity	6	11%	9	18%		15	14%
	Interfering with normal activity	3	6%	0	0%		3	3%
Irritability	Not eating at all	1	2%	0	0%		1	1%
	Behaviour as usual	27	49%	34	70%	0.1925	43	41%
	More irritable/ no effect on normal activity	18	33%	11	22%		29	28%
Axillary Temperature	Interfering with normal activity	6	11%	2	4%		8	8%
	Preventing normal activity	4	7%	2	4%		6	6%
	<38.0°C	55	100%	45	94%	0.0978	100	97%
	38.0-38.4°C	0	0%	2	4%		2	2%
	38.5-38.9°C	0	0%	1	2%		1	1%

** Fisher's Exact Test

high (78%) in HIV uninfected children, which was associated with a high proportion of participants ($\geq 75\%$) maintaining IgG concentrations ≥ 0.35 $\mu\text{g/ml}$ against 5/7 PCV-7 serotypes. Lower proportions of participants reached the threshold against serotypes 4 and 18C, which is similar to our study. No functional antibody measurements by OPA were performed in that study.

There are data available on persistence of antibody against other polysaccharide-encapsulated bacteria following immunisation with glycoconjugate vaccines in infancy [231]. In a recent study, the proportion of individuals with functional antibodies against serogroup C meningococci six years after vaccine priming was low (12-16%) after 2 or 3 doses in infancy [242]. In contrast, persistence of antibodies after infant vaccination with Hib conjugate was somewhat better with a high proportion of vaccinees (86-99%) having antibodies above the protective threshold at age 3-4 years [243, 244].

As shown in previous studies there is variation of immunogenicity and persistence between different pneumococcal serotypes. In the present study, we evaluated the persistence of total and functional antibody against all PCV-13 serotypes in children who had received PCV-13 and PCV-7 in a 2+1 schedule. Proportions of participants with serotype-specific IgG concentrations ≥ 0.35 $\mu\text{g/ml}$ and absolute antibody concentrations were not different between the groups for the shared serotypes, except serotype 4 for which higher responses were found in the PCV-7 group at 40 months. Data from the original study showed a similar picture for serotype 4 before the toddler dose at 12 months [234], although GMTs were similar at 40 months. The lower antibody levels seen for serotypes 4 and 18C may be acceptable in populations with high PCV uptake that establishes herd immunity, as shown by large decreases in invasive disease cases both in vaccinated and unvaccinated individuals [31]. Participants in both groups showed a very good immune response to all 7 shared serotypes following the pre-school booster with PCV-13 suggesting PCV-7 and PCV-13 have comparable priming ability for the shared serotypes. Overall similar immunogenicity and persistence for these 7 serotypes induced by the two vaccines is consistent with early PCV-13 post-licensure data from the UK showing equivalent effectiveness against these serotypes [154].

Marked differences were, however, present between the groups for the serotypes contained only in PCV-13 both before and after the pre-school booster. We found low proportions of participants with OPA ≥ 8 and lower GMTs in the PCV-7 group at baseline and highly significant differences in GMT between the groups for serotypes 1 and 5. (Figure 10.4 and 10.5). Both serotypes were very rarely found in carriage studies both before and after introduction of PCV-7 into the UK routine immunisation schedule [9, 27, 245], which may account for the low antibody levels seen in the PCV-7 group until the pre-school

age compared with the other serotypes contained only in [PCV-13](#). The significance of lower antibody persistence for serotypes 1 and 5 is currently unknown although there is some evidence that herd immunity is rapidly accumulating for most of the 6 additional serotypes [154], which implies that transmission at least in part can be blocked. Following the pre-school booster, significant differences between the groups remained for serotype 1, but not for serotype 5, when measured by [OPA](#) suggesting that priming with serotype 1 is important (Table 10.5). This seems to be less critical for serotype 5 for which vaccine unprimed individuals mounted a very good immune response (when measured by [OPA](#)) suggesting that at least for this serotype age may be a more important factor than the number of previous vaccine doses in determining the amount of functional antibody generated, as previously shown for other polysaccharide-containing vaccines [242, 244]. Post-licensure surveillance data from the UK show that children aged <5 years vaccinated with [PCV-7](#) are not protected against serotypes 1 and 5 [31], which is consistent with low functional antibody titres in our study in the [PCV-7](#) group (Figure 10.5).

Serotypes 6A and 19A are usually referred to as “cross-reactive” serotypes because antibodies induced by serotypes 6B and 19F cross-react in vitro with those 2 serotypes, respectively. We have shown in this study that [PCV-7](#) induces high total and functional antibodies against 6A [234], which also persist at high levels up to the pre-school years (Figure 10.4 and 10.5). There is some evidence from both the US and the UK that cross-reactive antibodies against serotype 6A are effective in preventing carriage and disease [27, 31, 239, 245, 246], although serotype 6C isolates were initially misclassified as 6A, and changes in [IPD](#) rates caused by 6A may also depend on other factors than vaccination [247]. A completely different picture can be seen for serotype 19A. Although less pronounced compared with serotype 6A, [PCV-7](#) induces a good [234] and persistent immune response against serotype 19A (Figure 10.4 and 10.5). Although in vitro these cross-reactive antibodies appear to be functional, [PCV-7](#) vaccinated children are, in fact, at increased risk of serotype 19A disease following widespread [PCV-7](#) immunisation, compared with the pre-vaccine era [31, 239], most likely as a result of an increase in 19A carriage rates [27, 245, 248]. There are several potential reasons for this discrepancy between the in vitro and in vivo data: (a) [OPA](#) measures functional antibodies using one particular laboratory strain, which may differ significantly from disease isolates; (b) higher antibody concentrations/titres may be necessary to protect against [IPD](#) compared with other serotypes, or antibody-mediated opsonophagocytosis may not be the main mechanism of killing for this serotype; early data from the UK, however, indicate that [PCV-13](#) is highly effective against serotype 19A disease [249]; (c) there is a theoretical possibility that a small percentage of individuals who are not able to generate cross-reactive

antibodies, along with increased carriage rates in PCV-7 vaccinated populations, may be responsible for replacement disease.

In the present study, antibody concentrations and functional OPA titres against serotypes 3 and 7F in the PCV-7 group increased over time irrespective of vaccination [234], so that GMC/GMT were relatively high at 40 months of age (Figure 10.5). This increase in (functional) antibody appeared despite low carriage rates in young children both before [246] and after [27, 245] routine PCV-7 immunisation. These in vitro functional antibodies, however, don't appear to be effective in preventing IPD caused by serotypes 3 and 7F as shown by post-licensure effectiveness studies [31, 239]. It is again unclear how these antibodies differ from those generated by PCV-13, for which early vaccine effectiveness against serotype 7F has been demonstrated [249]. Serotype 3 may behave differently from other serotypes both in terms of immunogenicity and vaccine efficacy [250, 251] and it is possible that higher thresholds are required to prevent disease caused by serotype 3 [249].

Limitations and strengths of the study

There are several potential limitations to our study. This study only allows a snapshot of the antibody concentrations and OPA titres at a certain time point. Differences in serotype epidemiology due to forces related and unrelated to routine immunisation mean that serology is likely to be different in other populations at different times. Nevertheless the children studied here, alongside routine use of PCV-7 in the general population, permit interpretation of our findings together with UK post-licensure surveillance data.

10.4 CONCLUSION

We have shown that following PCV-13 immunisation at 2, 4 and 12 months of age, antibody persistence against most serotypes can be expected until the age of 3.5 years, which is consistent with post-licensure IPD effectiveness and carriage data [28, 238, 239]. However, this might not be the case either in situations with lower vaccine coverage [252] or natural acquired (functional) antibodies as shown in the present study for serotypes 3, 7F and 19A. Accordingly, serological studies of this kind are important alongside population surveillance of invasive disease, carriage studies and information on vaccine coverage to obtain information on the relationships between vaccine responses and population-wide effectiveness.

A pre-school booster with PCV-13 is well tolerated with low rates of local and systemic side effects after priming with either PCV-7 or PCV-13.

MEMORY B CELL RESPONSE TO A PCV-13 PRE-SCHOOL BOOSTER

11.1 BACKGROUND

S. pneumoniae is a significant cause of mortality and morbidity in both children and older adults, with infection resulting in invasive disease, pneumonia and otitis media [45, 46]. Vaccine-induced protection against pneumococcal infection is mediated by the generation of serotype-specific functional antibodies and antigen-specific B_{MEM}, the latter capable of generating a rapid and marked secondary antibody response on re-exposure to antigen [129]. Although many studies have investigated the immunogenicity of pneumococcal vaccines in different age groups by measuring serotype-specific antibodies, there is more limited information about the B cells underlying such an immune response. Investigating the cellular basis of humoral immunity (B cell immunity) is important in understanding the origin of the long-term antibody production that is induced by these vaccines. PCVs have had a major impact on disease incidence in countries that have implemented a routine vaccination program [31, 239]. Conjugating serotype-specific polysaccharides to carrier protein creates TD antigens, which are, in contrast to pure polysaccharides, highly immunogenic in very young children and induce immune memory. Although it is still a matter of debate how exactly conjugate vaccines are processed in the immune system [108, 111] generation of B_{MEM} are thought to be a hallmark of the immune response to PCV [253]. Evidence of induction of memory also forms part of the three immunological criteria for licensure of a new PCV as recommended by the WHO [235]. In addition to generation of specific antibodies by vaccination, immunological memory may also be an important mechanism of protection, particularly after antibody levels have waned [231], and may also be involved in maintenance of serum antibodies. We studied the B_{MEM} response to a booster dose of a 13-valent pneumococcal conjugate vaccine in preschool children who had been primed with 2 different pneumococcal vaccines covering 7 or 13 serotypes.

11.2 METHODS

Participants, design and study objectives

A follow-on, open label, phase IV clinical trial was conducted from May to December 2010 in four sites across the United Kingdom in-

volving 108 healthy children as described in chapter 10. Briefly, study participants who had completed the parent infant trial (NCT00384059) were recruited at around 3.5 years of age at which time a PCV-13 booster was given. Blood was taken before and 1 month after vaccination. Here we report only on participants who were enrolled at 2 sites in Oxford and Bristol and had their B_{MEM} tested using the cultured ELISpot assay ($n=35$ and $n=27$ in the PCV-13 and PCV-7 group, respectively). Laboratory staff remained blinded to vaccine group allocation throughout the study. The study was approved by the Oxfordshire Research Ethics Committee C (Reference Number 10/Ho606/09). The primary objective of the study (see 10), was to determine the persistence of serotype-specific antibody at pre-school age as determined by proportion of participants immunised with PCV-13 in infancy. B_{MEM} responses to the PCV-13 booster are shown in this report and presented as a descriptive secondary objective.

Laboratory measurements

The cultured ELISpot assay to detect antigen-specific B_{MEM} was performed as described in methods sections 7.2 and 7.4. Briefly, PBMCs were isolated from fresh whole blood by density gradient centrifugation and after a further wash step were cultured at a concentration of 2×10^5 cells/well in a mixture of SAC, CpG and PWM for 5-6 days at 37 °C, 5% CO₂ and 95% humidity. Cells were then harvested, washed and seeded at 2×10^5 (viable) cells/well onto a 96-well plate with a PVDF-membrane (Millipore, UK), pre-coated with the different antigens. Following overnight incubation, the plate was washed before the wells were incubated with a Goat anti-human IgG alkaline phosphatase conjugate (Calbiochem, UK) to detect bound antibody. The antibody was visualised using the alkaline phosphatase substrate kit (Bio-Rad, UK), the reaction was stopped with distilled water and the plate dried over-night in a drying-oven before to be read using an automated ELISpot reader (AID ELRo3, AID Diagnostika, Strassberg, Germany). Spots were manually checked to exclude artefacts and multiplied by five to obtain B_{MEM} frequencies per million PBMCs.

Statistical analysis

Data were log₁₀-transformed prior to analysis to obtain normality. Geometric means with their 95% confidence intervals are reported but untransformed B_{MEM} frequencies with median values are reported graphically. Groups were compared using independent samples t-tests using log-transformed data. To compare B_{MEM} frequencies with total and functional antibody data, Pearson correlation coefficients were calculated on log-transformed data. As a large number of secondary endpoint comparisons were conducted in this study, no single

Table 11.1: Summary of study participants' characteristics.

	PCV-13 (N=35)	PCV-7 (N=27)
PROPORTION OF FEMALE STUDY PARTICIPANTS	17 (48%)	15 (56%)
PROPORTION OF WHITE CAUCASIAN STUDY PARTICIPANTS	34 (97%)	25 (93%)
MEAN AGE AT VISIT 1 IN YEARS (RANGE)	3.46 (3.16 – 3.75)	3.42 (3.15 – 3.76)
MEAN TIME SINCE 12-MONTH BOOSTER TO VISIT 1 IN YEARS (RANGE)	2.45 (2.13 – 2.77)	2.42 (2.15 – 2.76)
MEAN TIME INTERVAL BETWEEN VISIT 1 - VISIT 2 IN DAYS (RANGE)	34.8 (26.5 – 49.4)	34.8 (27.6 – 42.5)

result was considered in isolation. No formal adjustment of any p-values to account for multiplicity was made. Data were analysed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) and the R [237].

11.3 RESULTS

At visit 1, the age and time since last pneumococcal conjugate vaccination at 12 months of age did not differ significantly between the two study groups. Similarly, the proportion of female and white caucasian participants (the dominant ethnicity) and the time interval between visit 1 and visit 2 were similar in both groups (Table 11.1). None of these parameters differed substantially from the study population of the main study (see chapter 10).

Memory B cells determined by cultured B cell ELISpot assay

In total 98 blood samples were analysed by the B_{MEM} ELISpot assay. B_{MEM} frequencies specific for diphtheria toxoid (representing the carrier protein CRM₁₉₇) were significantly higher 1 month after the booster, whereas B_{MEM} frequencies specific for tetanus toxoid (not included in the booster vaccine) remained unchanged (Figure 11.1). Numbers of B_{MEM} specific for PCV-7/13 'shared' serotypes were low at baseline and did not differ between the groups for serotypes 4 and 23F but were significantly higher in the PCV-13 group for serotype 14 (Figure 11.2 and Table 11.2). After the booster vaccination at 40 months of age, B_{MEM} numbers increased for these three serotypes with no differences between the groups. For serotypes included only in PCV-13 (1, 3, 19A) baseline B_{MEM} numbers were lower in the PCV-7 (unprimed) group for all 3 serotypes (geometric mean 4.2, 0.6, and 4.7 vs. 7.4, 2.7, and 7.0 B_{MEM}/10⁶ PBMC, significant only for serotype 3; p<0.01) and increased for all 3 serotypes 1 month after the booster. Post-booster B_{MEM} frequencies were similar for serotypes 1, 3 and

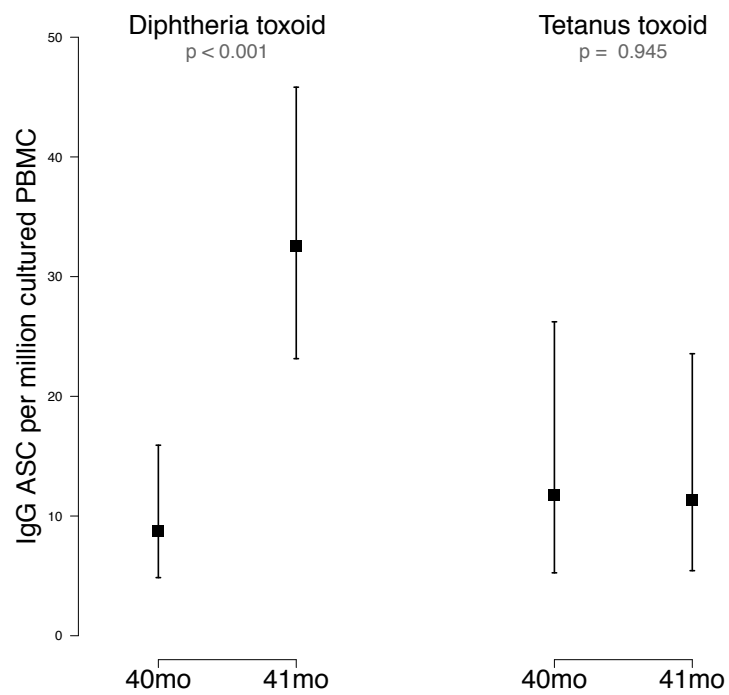


Figure 11.1: Geometric mean frequencies (along with 95% CI) of B_{MEM} specific for diphtheria toxoid were found to be significantly higher 1 month after the booster, whereas B_{MEM} numbers specific for tetanus toxoid remained unchanged.

19A in both groups (geometric mean $13\text{--}37 B_{\text{MEM}}/10^6\text{cells}$; $p=\text{NS}$ for group comparisons). B_{MEM} counts are summarised in Table 11.2 and in Figure 11.2.

Correlation between B_{MEM} frequencies and IgG antibody concentrations / OPA titres

A consistent correlation between IgG concentrations, OPA titres and B_{MEM} frequencies was only found for serotype 3 (Figure 11.3). For this serotype the ability to produce B_{MEM} 1 month after the PCV-13 booster was diminished by the amount of pre-existing antibody at baseline. This was true for both the PCV-13 primed group (data not shown) as well as using data from both groups (Figure 11.4).

11.4 DISCUSSION

In the present study, we have shown for the first time that the level of pre-existing serotype 3-specific antibody is negatively correlated with, and may directly impair the B_{MEM} response to a booster dose of PCV-13 containing serotype 3 glycoconjugate. The immune response to serotype 3 pneumococcal polysaccharide is known to be different from other serotypes both in vitro [250, 251] and in vivo, resulting in reduced booster responses [251] and lack of protection against acute otitis media in a large efficacy trial [166, 254]. Although antibody responses against serotype 3 were generally found to be well above the presumed protective thresholds in previous immunogenicity studies, they were still lower than other serotypes [234]. In addition, previous studies using serotype 3-containing conjugate vaccines also showed low or absent booster antibody responses [251]. However, this is the first report demonstrating a correlation between production of B_{MEM} and antibody, unique to serotype 3 in this study. The reason for this distinct immunological relationship remains unclear, but might have an impact on antibody persistence, as it has been shown for children primed and boosted with a group C meningococcal conjugate vaccine [173]. Hence, it may be a contributing factor to the limited vaccine effectiveness for serotype 3 invasive disease observed in early UK surveillance data [249].

Overall, there was a trend to B_{MEM} frequencies being lower in children primed with PCV-7 compared to those primed with PCV-13 with significant differences for only serotype 3 and 14. While this difference might have been expected for serotype 3, which is only included in PCV-13, the finding that serotype 14 B_{MEM} frequency was also significantly lower in the PCV-7 group is surprising since it is common to both PCV-7 and PCV-13. Although both vaccines have serotypes in common, their formulation differs both in the quantity of carrier protein and the polysaccharide/carrier protein ratio with the quantity of

Table 11.2: Geometric mean memory B cell count per 10⁶ cultured PBMC with 95% confidence intervals.

GMC (95% CI)*				GMC (95% CI)*			
N (N MISS)				N (N MISS)			
SEROTYPE	PCV-13 40 MONTHS	PCV-7 40 MONTHS	P VALUE 40 MONTHS	PCV-13 41 MONTHS	PCV-7 41 MONTHS	P VALUE 41 MONTHS	
1†	7.42 (4.04, 13.62) 25 (10)	4.21 (2.15, 8.24) 25 (2)	0.202	36.44 (26.97, 49.24) 27 (8)	22.28 (11.33, 43.80) 21 (6)	0.177*	
3†	2.69 (1.35, 5.37) 24 (11)	0.61 (0.29, 1.28) 25 (2)	0.004	15.41 (8.99, 26.40) 27 (8)	12.90 (6.21, 26.81) 21 (6)	0.681	
4	6.28 (2.72, 14.49) 20 (15)	3.47 (1.51, 7.98) 19 (8)	0.300	26.66 (17.04, 41.69) 16 (19)	24.69 (15.46, 39.42) 14 (13)	0.802	
14	6.46 (3.14, 13.29) 15 (20)	1.64 (0.52, 5.16) 13 (14)	0.033	9.66 (3.73, 25.03) 12 (23)	12.31 (7.18, 21.10) 14 (13)	0.620	
19A†	6.96 (3.65, 13.30) 24 (11)	4.73 (2.23, 10.05) 25 (2)	0.427	16.68 (10.55, 26.38) 25 (10)	12.55 (7.35, 21.42) 20 (7)	0.403	
23F	5.96 (2.59, 13.73) 17 (18)	5.41 (2.24, 13.06) 16 (11)	0.865	22.66 (16.49, 31.14) 14 (21)	20.25 (10.06, 40.76) 14 (13)	0.756*	

* SATTERTHWAITE'S METHOD FOR UNEQUAL VARIANCES; ** INDEPENDENT SAMPLES T-TESTS COMPARING PCV-13 TO PCV-7 GROUPS; † SEROTYPES ONLY PRESENT IN PCV-13

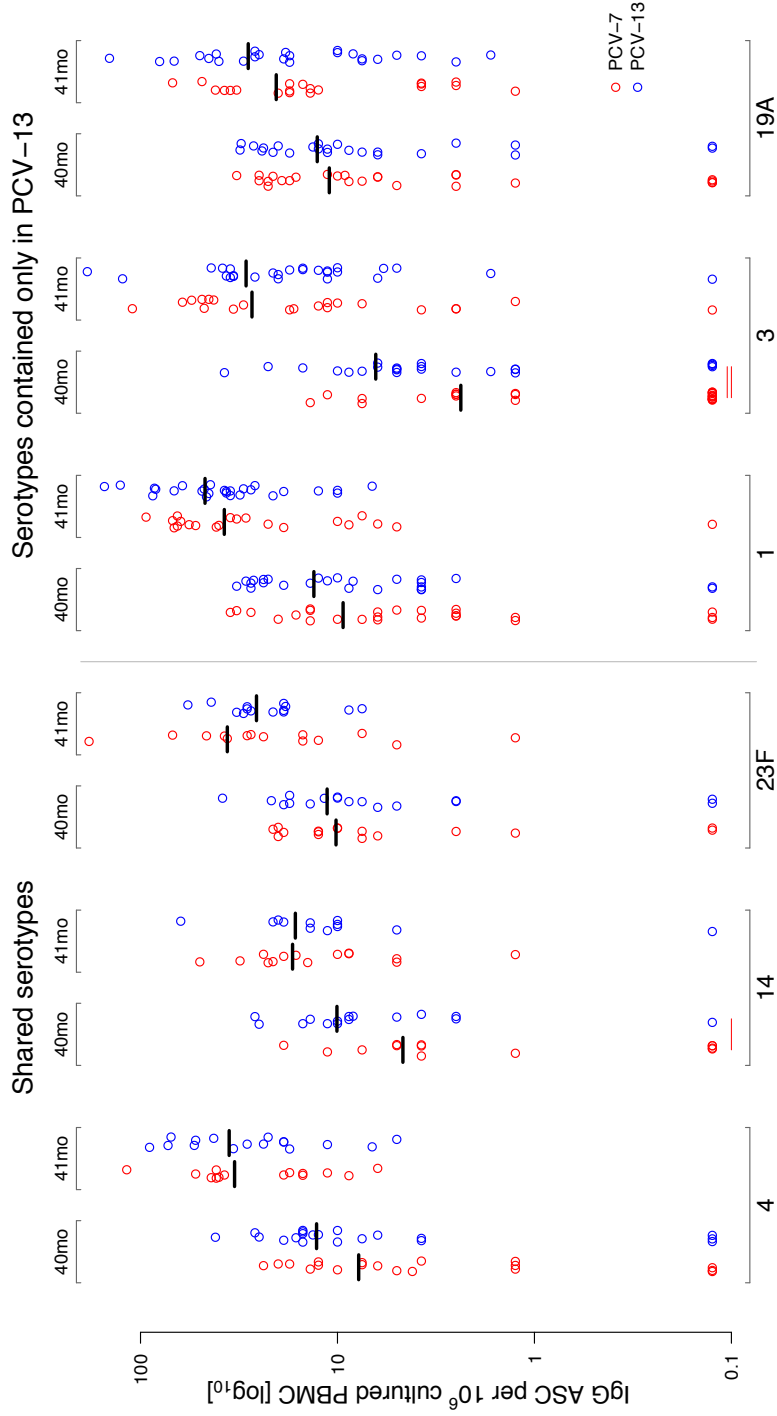


Figure 11.2: Before the booster, B_{MEM} numbers were significantly lower in the [PCV-7](#) group for serotype 14 and serotype 3. Post-booster B_{MEM} numbers were higher for all serotypes than pre-booster with no differences between the groups. Shown are the geometric mean values along with their 95% confidence intervals of IgG ASC per 10^6 cultured PBMC for each of the serotypes measured by group and time point. Red lines are shown at the bottom if p-values from t-tests between the groups were significant (p-values ≤ 0.05 , ≤ 0.01 , ≤ 0.001 , and ≤ 0.0001 are represented by 1, 2, 3, and 4 lines).

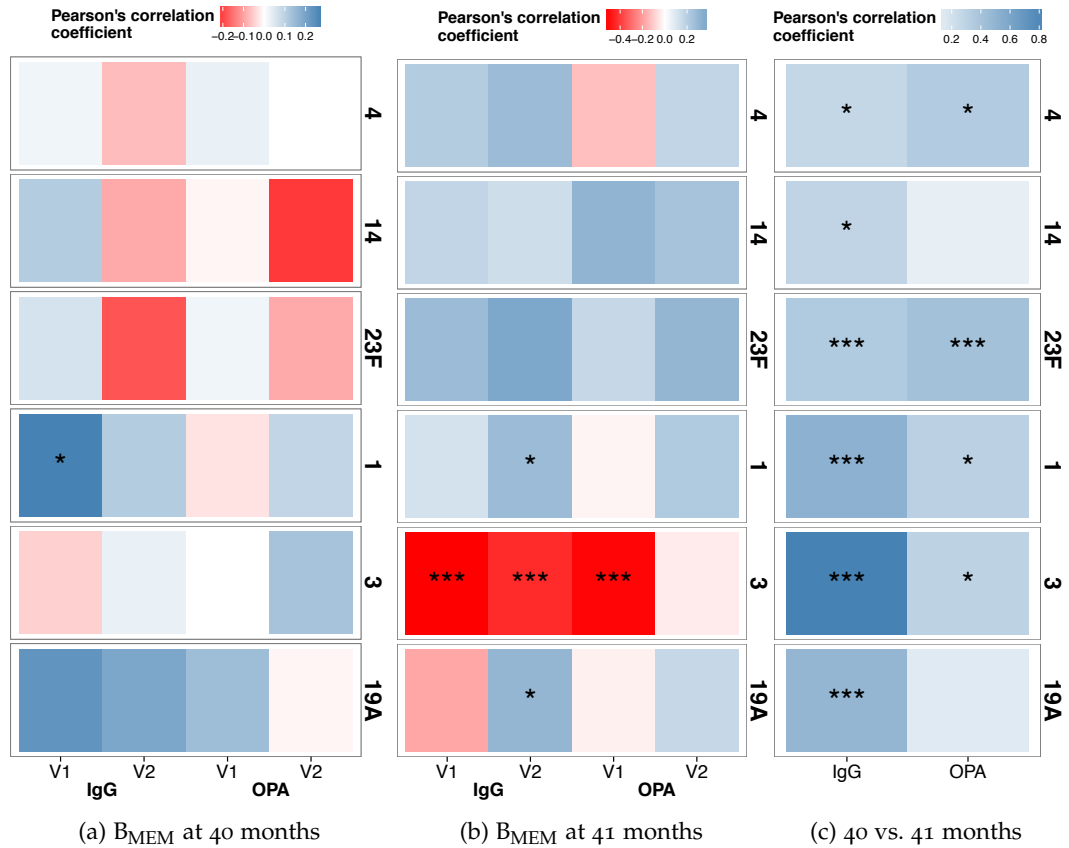


Figure 11.3: Correlations between IgG or OPA at both visits and B_{MEM} frequency at 40 months (a) or 41 months (b). Correlation between IgG and OPA between 40 and 41 months is shown in (c). From all the correlations that were calculated between IgG antibody concentrations, OPA titres and B_{MEM} frequencies, only serotype 3 stands out with a consistent and strong negative correlation for antibody (IgG & OPA) at baseline and B_{MEM} numbers following the PCV-13 booster. Shown are Pearson coefficients of correlations including data from both groups; stars indicate the associated p-value (***) $<.001$; ** $<.01$; * $<.05$).

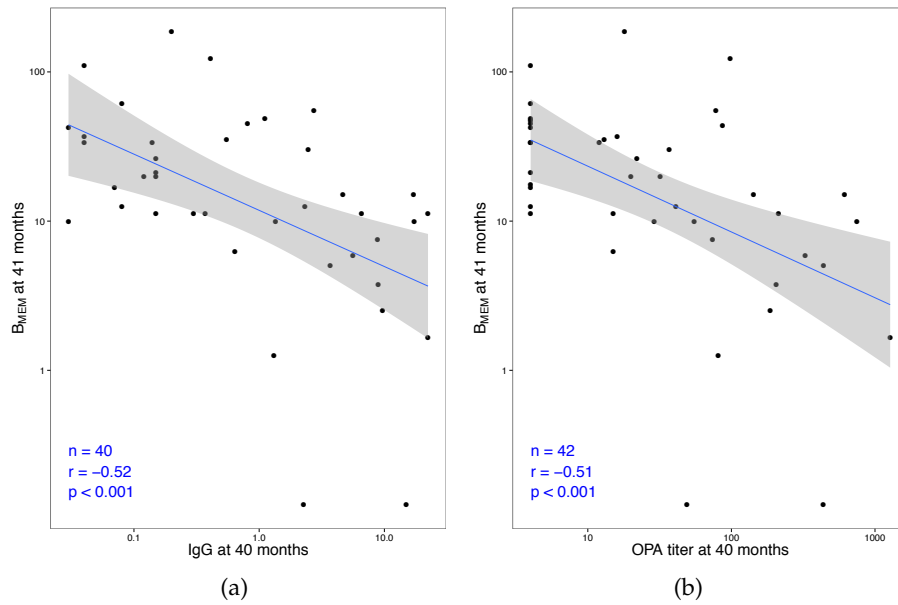


Figure 11.4: Correlation between serotype-specific antibody at baseline and numbers of B_{MEM} generated one month following a PCV-13 booster. An inverse correlation is shown for B_{MEM} frequency post vaccination compared to both serotype 3-specific (a) IgG antibody concentration and (b) OPA titres at baseline. Both axes are on a log scale. N indicates the number of paired measurements, p the associated p-value and r the Pearson correlation coefficient.

polysaccharide being higher in PCV-13 (e.g. serotype 14: 2 µg in PCV-7 vs. 2.2 µg in PCV-13; CRM₁₉₇: 20µg in PCV-7 vs. 34µg in PCV-13), which may be a possible explanation for the observation of lower baseline B cell frequencies for antigens shared by both vaccines. Post-booster B_{MEM} frequencies for the PCV-7/13 common serotypes were similar in both groups implying that the 2 vaccines are similar in their capacity to prime the immune system. Interestingly, similar B_{MEM} numbers have also been found for the PCV-13 only serotypes and also for serotypes with significantly lower B_{MEM} frequencies at baseline in the PCV-7 group (serotype 3 and 14). This is a surprising finding, which might be due to the maturity of the immune system at this age being able to generate as many B_{MEM} after primary vaccination as after a booster dose. Another possibility is that children in the PCV-7 group were primed through carriage of serotypes 1, 3 and 19A (or exposure to cross-reacting antigens). As the original study was undertaken several years before implementation of PCV-13 into the routine immunisation schedule, carriage rates for the PCV-13 additional serotypes including the three that have been looked at in this study (1, 3, and 19A) are likely to have been at least as high as prior to implementation of PCV into the routine immunisation schedule and even increasing throughout the study due to serotype replacement [27, 245]. It is not possible to exclude an impact of carriage on the B_{MEM} numbers. Similarities between groups in terms of antigen specific B_{MEM} may be in part determined by carriage of serotypes 1, 3 and 19A, a parameter not included in the study. In addition, B_{MEM} frequencies detected as specific for serotype 19A might have been confounded by cross-reactive antibodies against serotype 19F, which is included in both vaccines, as has been suggested by findings of the initial part of this follow-on study [234].

Due to the design of the study, potential differences in the kinetics of the B_{MEM} response between the groups could not been addressed. It is possible that children who had been primed with PCV-13 experience a faster increase in B_{MEM} specific for serotypes 1, 3 and 19A when challenged with a further dose of vaccine than do children who had not been primed with a vaccine containing these serotypes. Previous studies have demonstrated that the peak level of polysaccharide and protein-specific B_{MEM} occurs earlier in the peripheral blood at day 6-8 after a booster dose compared to day 8-15 in a primary response [171–173]. In contrast, for antibody persistence, assessing B_{MEM} numbers 1 month after vaccination might be a good time point as has been suggested for group C meningococcal polysaccharide in infants [173]. However, the contribution of B_{MEM} to maintenance of antibody levels still needs to be defined. In the current study, the observed similarity in B_{MEM} numbers for serotype 1, 3 and 19A in children irrespective of priming with PCV-13 nevertheless indicates that

pre-school vaccination might be sufficient to generate a strong and long-lasting immune response in pre-school children.

11.5 CONCLUSION

In summary, these data show that a [PCV-13](#) pre-school booster results in similar post-booster B_{MEM} frequencies in individuals irrespective of prior priming with [PCV-7](#) or [PCV-13](#) for serotypes 1, 3 and 19A despite lower pre-booster B_{MEM} frequencies in the unprimed group. Further, a strong negative correlation was found between pre-existing serotype 3-specific antibodies and the generation of B_{MEM} formation suggesting a unique immune response mechanism for this serotype compared to other serotypes.

DETAILED B CELL RESPONSE TO PNEUMOCOCCAL VACCINATION

12.1 BACKGROUND

Vaccination induces long-lasting immunity through PC_{MEM} and B_{MEM} [255]. Plasma cells secrete antibodies and are either short- or long-lived [256, 257]. On the other hand, B_{MEM} that are generated during the primary immune response to a foreign antigen, proliferate and differentiate into plasma cells after re-exposure with the same antigen. Although different B cell subsets have been defined, their specific roles in an immune response are still incompletely defined [88, 110, 118, 129]. In particular the contribution of different B cell subsets in the response to unconjugated as compared to protein conjugated polysaccharide vaccines in humans is yet to be definitively established. A small number of studies have used flow cytometry to evaluate the kinetics of B cells in humans following vaccination specific for the hepatitis B surface antigen (HBsAg) [258], the meningococcal serogroup C (MenC) polysaccharide [259], a digoxigenated recombinant C-fragment of tetanus toxin [182, 260], and pneumococcal polysaccharides 14 and 23F [261–263].

Ward et al. used HBsAg-conjugated microbeads to enrich for HBsAg-positive B_{MEM} ($CD19^+CD27^+$) and found that in previously vaccinated healthy individuals, HBsAg-specific memory B cells can be detected in the peripheral blood, although at very low levels [258]. In the same study, frequencies of $HBsAg^+ B_{MEM}$ did not correlate with HBsAg serum antibody concentrations or HBsAg-specific plasma cell frequencies as detected by ELISpot. Henneken et al. directly labeled MenC polysaccharide and TT with a fluorescent dye in order to detect antigen-specific B cells by flow cytometry based on the binding to fluorescently labeled antigen [259]. They used this method to determine the frequencies of MenC- and TT-specific B cells in the peripheral blood in children and adults several months after immunisation with a MenC-TT conjugate vaccine. MenC-specific B cells were found at low levels (0.01 - 0.78% of $CD19^+$ B cells) and their frequencies were not correlated with the age at which the study participants had been vaccinated. Frolich et al. [182] assessed the kinetics of tetanus toxoid-specific plasma ($CD19^+CD27^{++}$) and ($CD19^+CD27^+$) memory B cells in response to a TT booster vaccination in 3 healthy adults on days 0, 2, 5, 6, 7, 8, 9, 12, 14, and 20 and additionally for one donor on day 41 after vaccination. A peak of TT-specific plasma cells was observed at days 6-7 following vaccination. TT-specific memory B cells

increased to their maximum levels on day 14 following booster immunisation. The Westerink laboratory have recently investigated the phenotype of pneumococcal polysaccharide-specific B cells, again by use of fluorescently labeled antigen that is bound by B cells bearing antigen-specific BCRs and can then be visualised by FACS. Applying this method to PBMCs taken from adults who had been given a dose of PPV-23, Khaskhely et al. [261] demonstrated that peripheral blood B cells that bind serotype 14 or 23F polysaccharide 7 days after vaccination are predominantly IgM B_{MEM} (CD19⁺CD27⁺IgM⁺ and CD19⁺CD20⁺CD3⁻CD70⁻CD27⁺IgM⁺CD43⁺CD5^{+/-} [263], respectively). The percentage of serotype 14 and 23F positive B cells out of all CD19⁺ B cells were around 0.5% at baseline and 2.5% 7 days after PPV-23 immunisation. Interestingly, the proportions of switched B_{MEM} were not different before and after vaccination. However, a further study undertaken in elderly adults by the same group showed that the majority of serotype 14 and 23F positive peripheral blood B cells were switched B_{MEM} (CD19⁺CD27⁺IgM⁺) [262].

That an understanding of the B cell subsets involved in the immune response is essential has been suggested in a recent mouse experiment [264]. Pape et al. demonstrated that different B_{MEM} populations (IgM B_{MEM} and switched B_{MEM}) have specific kinetics after vaccination with IgM B_{MEM} being longer lived. In addition, in their model, the secondary response was dominated by activation of switched B_{MEM} as the activation of IgM B_{MEM} seem to be inhibited by specific neutralising serum immunoglobulin.

In the present study, the immune response to pneumococcal vaccines given to healthy adults using a variety of laboratory methods was investigated in detail. An understanding of the immunobiology of the B cell subsets involved in the response to the two vaccine types will enhance the understanding of the B cell subsets involved in antibody production and the potential link between memory B cells and persistent antibody production by long-lived plasma cells. The purpose of the study was also to find the optimal time points for measurement of antigen-specific B cells by flow cytometry in the peripheral blood in order to assess these B cell frequencies on a larger scale in future clinical trials.

12.2 METHODS

Four healthy adults were randomly assigned to receive either PPV-23 or PCV-13. Following vaccination the immune response was measured repeatedly using a variety of methods covering antibody concentrations, B cell frequencies and RNA expression. The design of the study including the time points at which blood was taken is summarised in Figure 12.1. The main aim of this study was to investigate in detail the frequency versus time after immunisation of the different B cell sub-

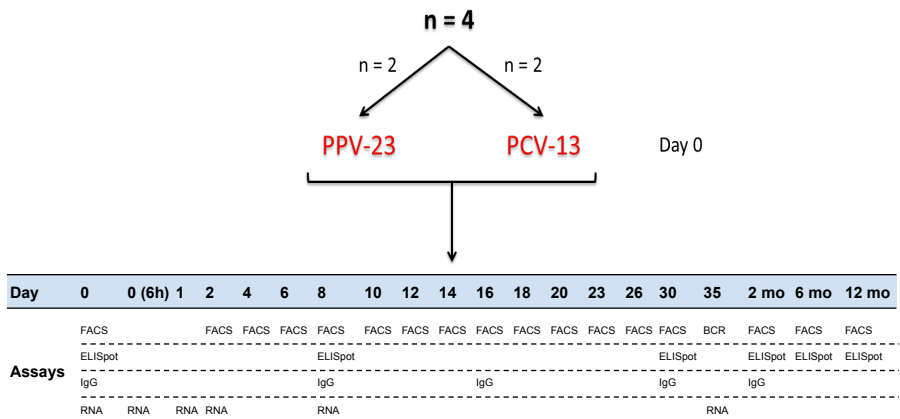


Figure 12.1: Study design with time points of study visits. Four healthy adults were randomly assigned to receive either PPV-23 or PCV-13. Blood was taken before and at several time points following vaccination and subsequently analysed by flow cytometry, the cultured ELISpot, pneumococcal serotype-specific antibody concentrations and gene expression at the shown time points.

sets in the peripheral blood using flow cytometry. Antigen-specific B cells were detected by positive staining with fluorescently labelled antigen as described in chapter 8. The gating strategies to determine B cells and B cell subsets are shown in Figures 12.2 and 12.3.

In addition to flow cytometry, antigen-specific B_{MEM} were also enumerated at days 0, 8, 30, and 60 following immunisation using the cultured ELISpot technique as detailed in chapter 7.

Anti-pneumococcal IgG concentrations were performed at days 0, 8, 16, 30, and 60 after vaccination specific for the PCV-13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) as described in section 7.7.

Genome wide analysis of gene expression was performed utilising Illumina’s Human-HT-12-v4 Expression BeadChip. The supplied RNA was quantified by nanodrop reading and quality controlled using Bioanalyser, then converted into labelled cRNA and used for hybridisation. The hybridised and washed chips were then scanned using Illumina IScan Scanner. A gene expression project was created using Illumina’s Genomestudio software to carry out quality control checks of the procedure and to provide final reports. Data were subsequently analysed and displayed using the statistical software R [237].

12.3 RESULTS

B cell subsets as determined by flow cytometry

Plasma cells / blasts were identified as being CD19⁺CD20⁻CD27⁺⁺ as shown in Figure 12.2 and calculated as a percentage of all CD19⁺ B cells. At baseline, the percentage of plasma cells ranged from 2 to 7%

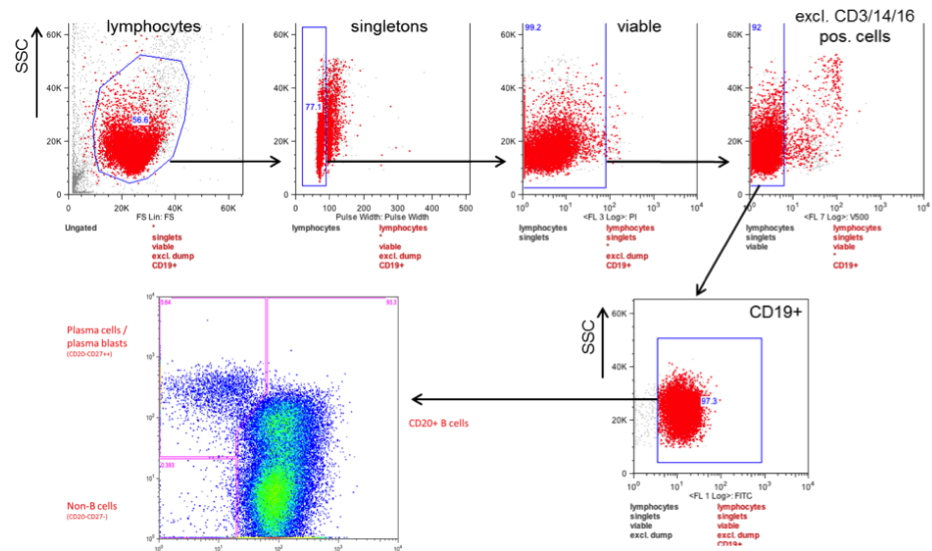


Figure 12.2: Gating strategy for determination of major B cell subsets. Lymphocytes were selected based on forward (FCS, x-axis) and side scatter (SSC, y-axis) light and singletons by excluding cells with greater pulse width. Dead cells and non-B cells were excluded using a propidium iodide (PI) gate followed by a CD3/14/16 gate. Finally, B cells were positively selected by gating on CD19⁺ cells. Plasma cells were defined as CD19⁺CD20⁻CD27⁺⁺ and the CD19⁺CD20⁺ B cell gate was used for definition of further B cell subsets as shown in Figure 12.3.

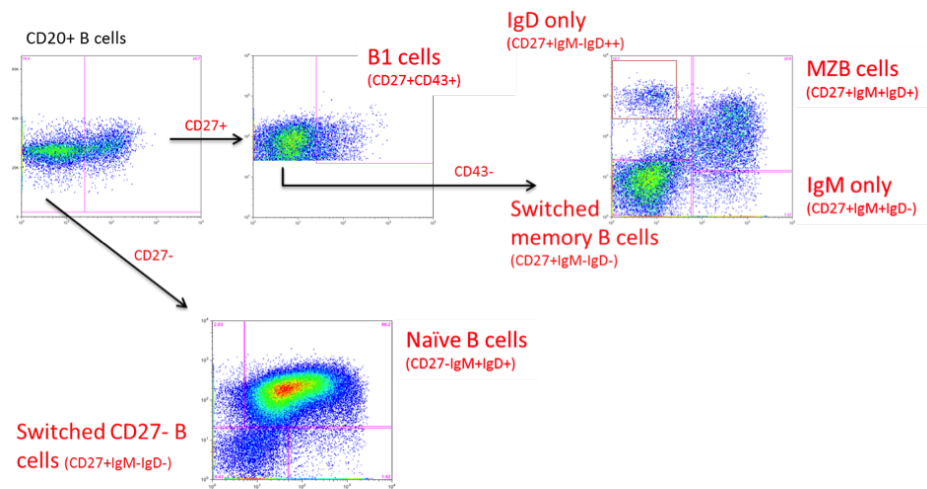


Figure 12.3: Gating strategy for determination of further B cell subsets. First, B cells were divided based on the CD27 (memory) marker. The B1 subset was defined as CD20⁺CD27⁺CD43⁺ [89]. Using markers for IgM (x-axis) and IgD (y-axis), switched memory B cells were identified as CD20⁺CD27⁺CD43⁻IgM⁺IgD⁻, IgM only B cells as CD20⁺CD27⁺CD43⁻IgM⁺IgD⁺, marginal zone B cells as CD20⁺CD27⁺CD43⁻IgM⁺IgD⁺, and IgD only B cells as CD20⁺CD27⁺CD43⁻IgM⁺IgD⁺. Naïve B cells were defined as CD20⁺CD27⁻IgM⁺IgD⁺ and switched CD27⁻ B cells as CD20⁺CD27⁻IgM⁺IgD⁻.

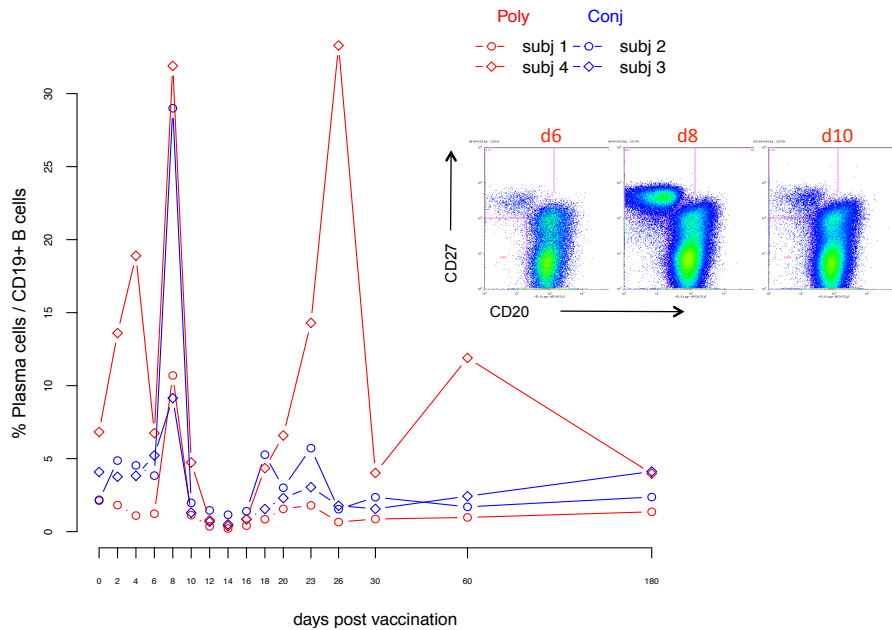


Figure 12.4: Kinetics of plasma cells / plasmablasts expressed as a proportion of all CD19⁺ B cells. The superimposed FACS plots show the distribution of the major B cell subsets (CD20 vs. CD27) at days 6, 8 and 10 from participant 3.

and stayed at a similar level over the first 6 days with the exception of participant number 4, who experienced a peak at day 4 following immunisation with 18.9% of all B cells expressing plasma cell markers. All participants showed a distinct plasma cell peak at day 8 following vaccination with percentages ranging from 9 - 31% and a smaller relative increase of this subset around day 20-23 (Figure 12.4). It was again participant 4 who showed a different plasma cell kinetic compared with the other study participants with peaks at day 26 (33%) and day 60 (12%). The lowest percentages of plasma cells in the peripheral blood were noted between day 12 and 16 post-vaccination ranging from 0.2 to 1.4 % of all B cells (Figure 12.4).

The analysis of other B cell subsets such as MZB and IgM memory B cells revealed distinct patterns of responses following vaccination in all of the participants irrespective of the vaccine these individuals had received. The percentages of MZB cells ranged from 2.5 to 12.1% at baseline, increased over the first 10 days following vaccination with a peak at day 8 and stayed higher than baseline for around 3 weeks. A relative nadir of this B cell subset was seen at day 30 following vaccination with percentages ranging from 4.6 to 13.1% followed by a continuous increase until 6 months post-vaccination (Figure 12.5). Over the whole observation period, percentages of MZB cells were higher in the 2 participants who had received PPV-23 compared with the two PCV-13 recipients although the kinetics between the groups were similar.

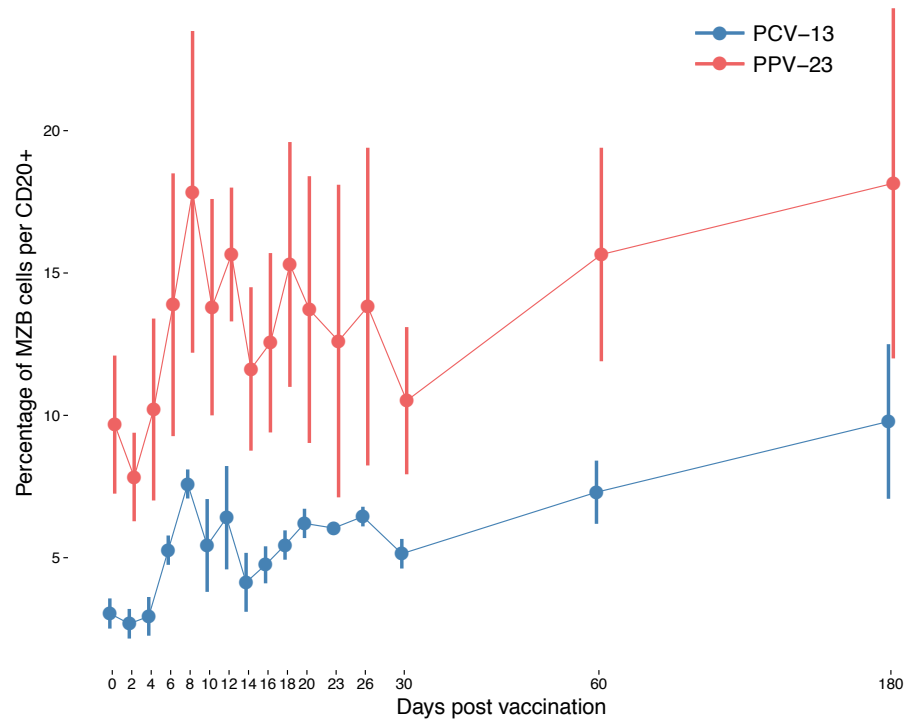


Figure 12.5: Kinetics of marginal zone B cells expressed as a proportion of CD20⁺ B cells.

The relative frequency of IgM B_{MEM} was low at baseline (1.1 - 2.9% of CD20⁺ B cells), continuously increased over the first month following vaccination and reached again baseline values at 6 months (Figure 12.6). There were no differences in the kinetics or percentages between the vaccine groups.

A different pattern was seen for switched B_{MEM} for the kinetics between the vaccine groups. At baseline, percentages of this B cell subset ranged between 13.9 and 17.5% and these relative frequencies dropped to 9 - 12% at day 12 and day 18, for the two different study participants in the PCV-13 group, respectively, before increasing to a maximum of 17 - 20% at day 23. In the PPV-23 group, however, the percentages of switched memory B cells remained more constant over the observed time period with a mild increase in the relative frequency of this subset between 1 and 6 months post-vaccination (Figure 12.7).

Quantification and kinetics of antigen-specific B cell subsets

At baseline, the mean percentages of CD19⁺ antigen-specific B cells ranged between 3.4 and 8.9% of all CD19⁺ B cells for pneumococcal serotypes 1, 3, 9V, 18C and 23F and was lower with percentages ranging between 3.3 and 3.9% for diphtheria and tetanus toxoid positive B cells, respectively. The relative frequencies of these antigen-positive B cells continuously decreased over time with a potential moderate

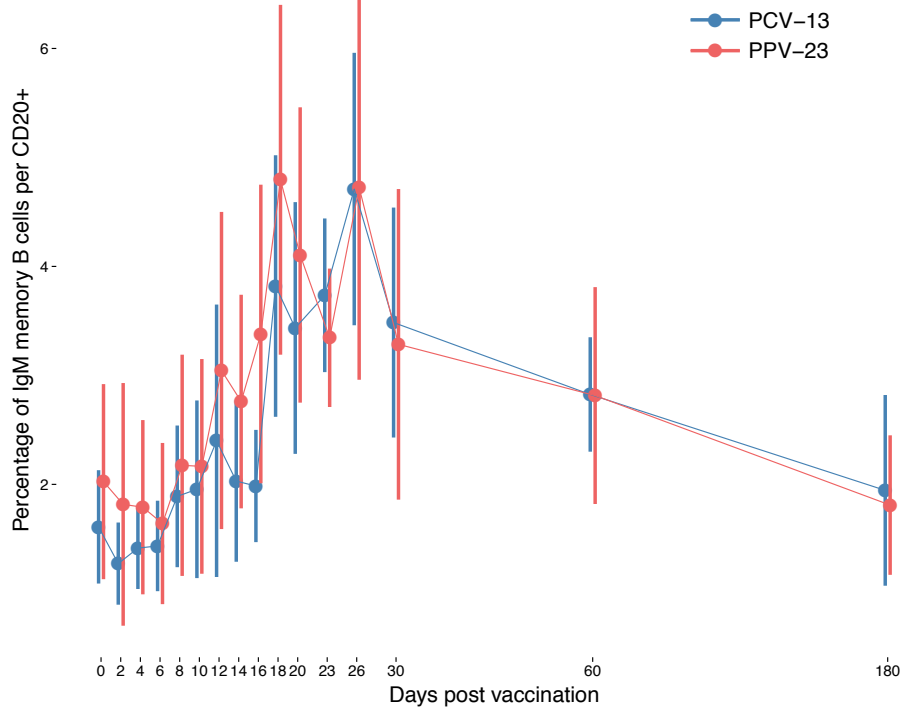


Figure 12.6: Kinetics of IgM memory B cells expressed as a proportion of CD20⁺ B cells.

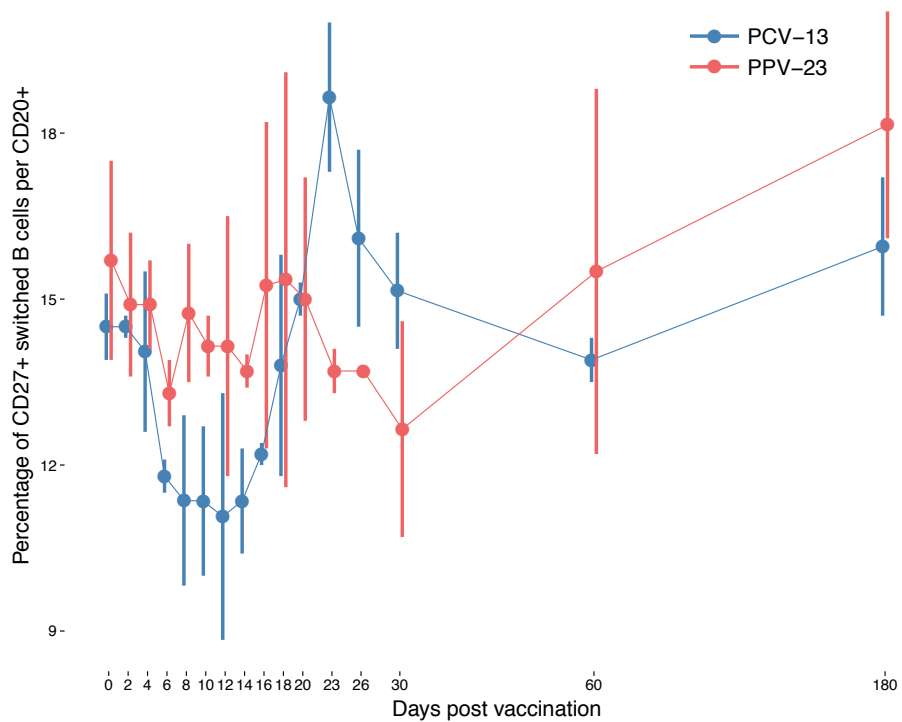


Figure 12.7: Kinetics of switched memory B cells expressed as a proportion of CD20⁺ B cells.

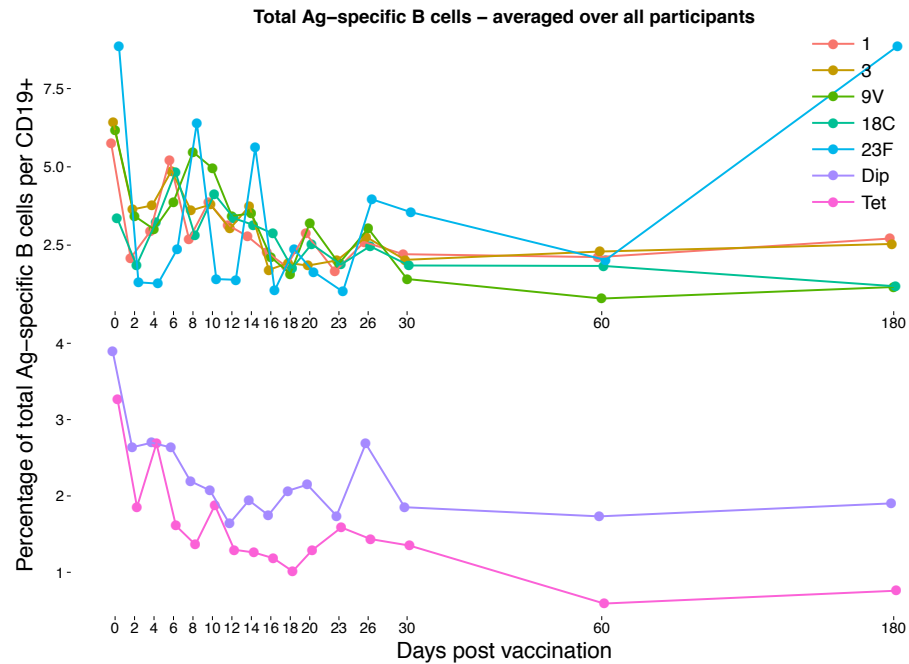


Figure 12.8: Kinetics of total antigen-specific B cells averaged over all participants and expressed as a proportion of CD19⁺ B cells.

peak in the 2nd week following vaccination (Figure 12.8). At 6 months post-vaccination, the mean percentage of antigen-specific B cells as detected by the labelling method was lower compared to baseline values for most of the serotypes (1.2 - 2.7%) with the exception of serotype 23F (8.9%). The mean frequencies for the protein antigens diphtheria and tetanus toxoid decreased as well and were around half of the baseline values (1.9 and 0.8% for diphtheria and tetanus toxoid positive B cells, respectively).

Antigen-specific plasma cell frequencies showed a similar pattern as total plasma cells for vaccine antigens, whereas relative B cell numbers directed against the non-vaccine antigen tetanus toxoid remained low (Figure 12.9). Again as for total plasma cells, a second peak was observed at around day 20 for cells stained with vaccine antigens (Figure 12.9).

The relative frequencies of antigen-specific B cell subsets followed kinetics similar to trends observed for the total B cell subsets as described above. The kinetics of serotype 9V and 18C positive MZB, IgM and switched B_{MEM} are shown in Figures 12.10, 12.11 and 12.12 as a percentage of CD20⁺ B cells.

Memory B cells determined by memory B cell ELISpot assay

In addition to the use of the labelling method in combination with flow cytometry, antigen-specific B_{MEM} were also identified by the cultured ELISpot at several time points following vaccination. Figure

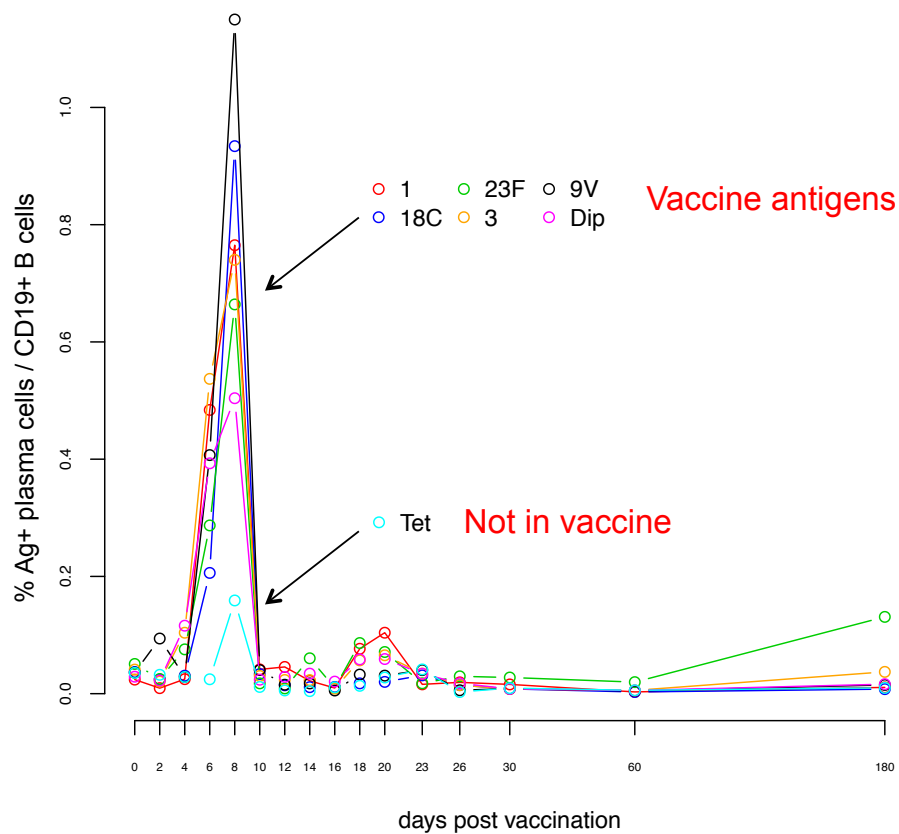


Figure 12.9: Kinetics of antigen-specific plasma cells / blasts expressed as a proportion of all CD19⁺ B cells from one study participant but similar cell kinetics were observed in the other participants.

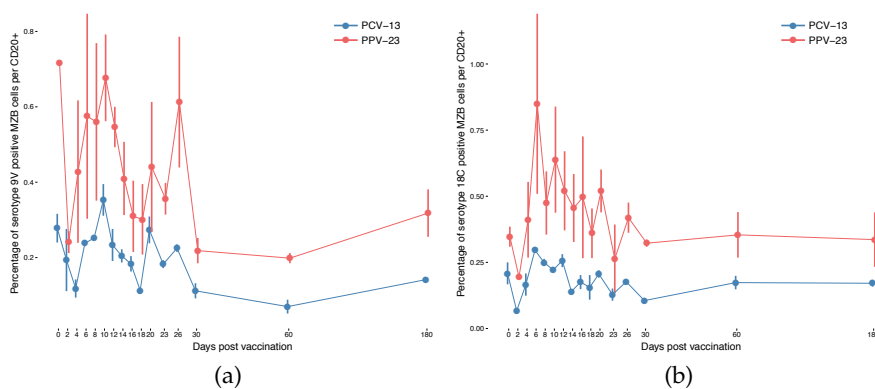


Figure 12.10: Kinetics of (a) 9V and (b) 18C positive MZB cells expressed as a proportion of CD20⁺ B cells.

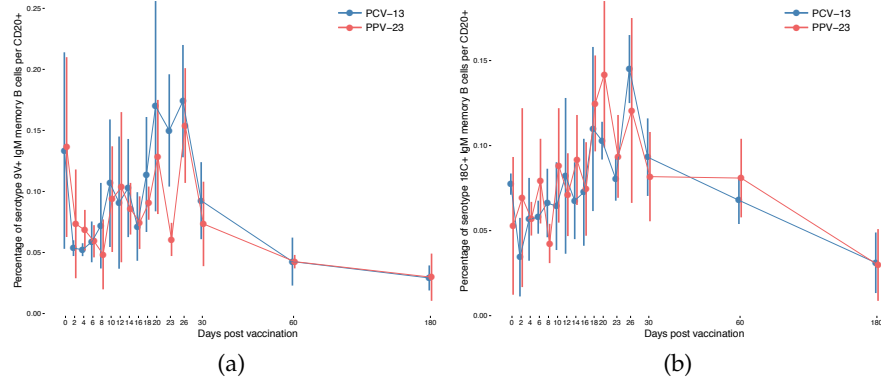


Figure 12.11: Kinetics of (a) 9V and (b) 18C positive IgM B_{MEM} expressed as a proportion of CD20⁺ B cells.

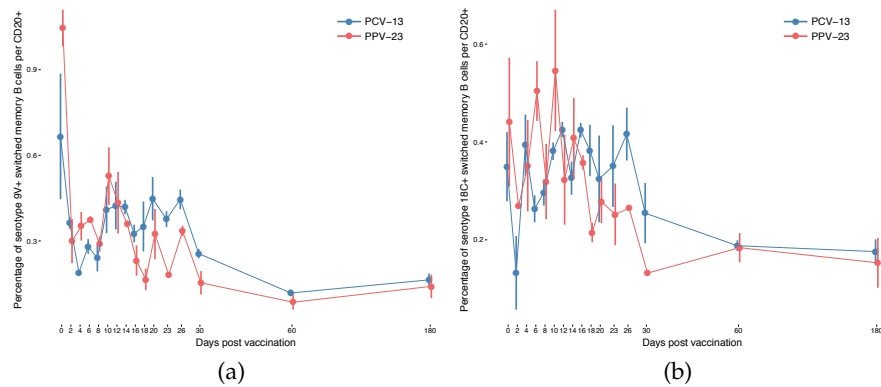


Figure 12.12: Kinetics of (a) 9V and (b) 18C positive switched B_{MEM} expressed as a proportion of CD20⁺ B cells.

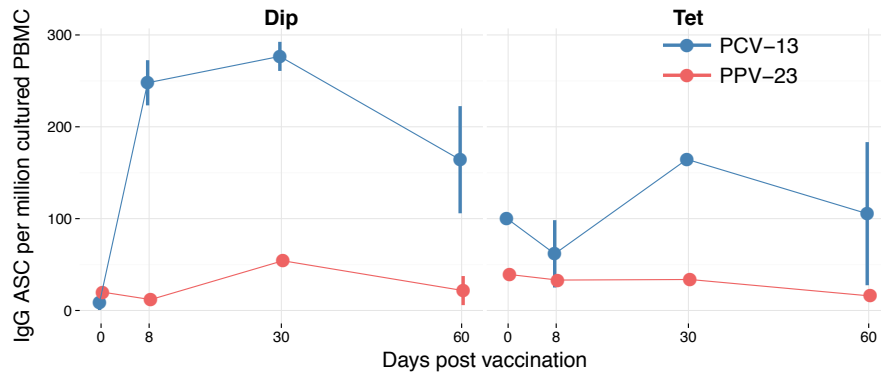


Figure 12.13: Frequencies of memory B cells specific for diphtheria toxoid (represent carrier protein CRM₁₉₇) and tetanus toxoid (not included in any of the vaccines) as determined by the cultured ELISpot method are shown for both vaccine groups at 4 time points post-vaccination.

12.13 shows the number of IgG secreting diphtheria and tetanus specific B_{MEM} at the 4 chosen study time points. In the study participants who had received PCV-13, B_{MEM} numbers specific for diphtheria toxoid, representing the carrier protein CRM₁₉₇, were low at baseline in both groups and increased significantly to a peak at day 30 post-vaccination. No change in the frequency of diphtheria toxoid IgG B_{MEM} was seen in the PPV-23 group. Tetanus toxoid specific IgG ASC frequencies were slightly higher at baseline and remained low in the PPV-23 group, whereas a moderate increase with a peak at day 30 was seen in the 2 study participants who had received PCV-13 (Figure 12.13).

Pneumococcal serotype-specific IgG ASC frequencies were low at baseline and increased following vaccination with a peak at day 30 for serotypes 1, 3, 9V and 18C in the PCV-13 group. In the PPV-23 group, maximum numbers of serotype-specific IgG ASC were in general lower and the kinetics was different with an earlier peak at day 8 at least for serotypes 1 and 18C. In both groups, B_{MEM} responses to serotype 23F showed a similar bimodal kinetic with IgG ASC frequencies peaking at day 8 and 60 (Figure 12.14).

Serotype-specific IgG concentrations

Antibody concentrations were measured against all serotypes contained in PCV-13. The kinetics of the antibody response is shown in Figure 12.15. In both groups, the kinetics of serotype-specific IgG concentrations were similar with a rise in the first 2 weeks post-vaccination and then remaining at a high level until day 60. In general, IgG concentrations tended to be equal or higher in the 2 participants who had received PPV-23 with the exception of antibody concentrations against

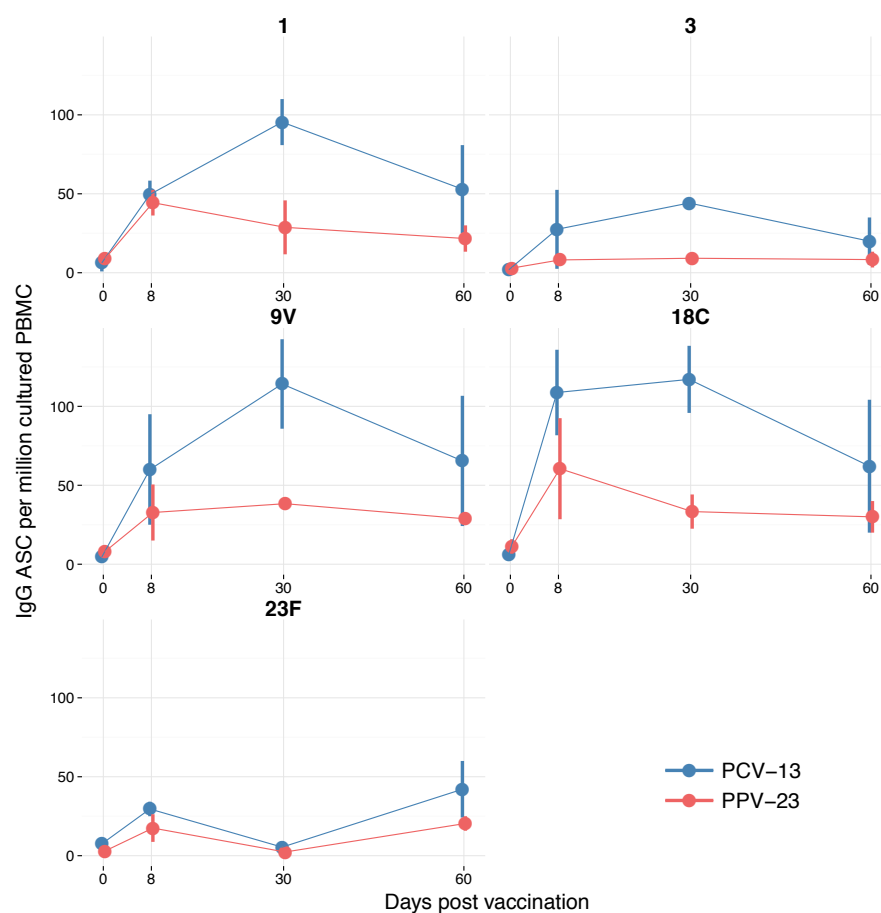


Figure 12.14: Memory B cell frequencies determined by the cultured ELISpot assay specific for serotypes 1, 3, 9V, 18C, and 23F (all serotypes contained in both vaccines) for each of the study groups at 4 time points post-vaccination.

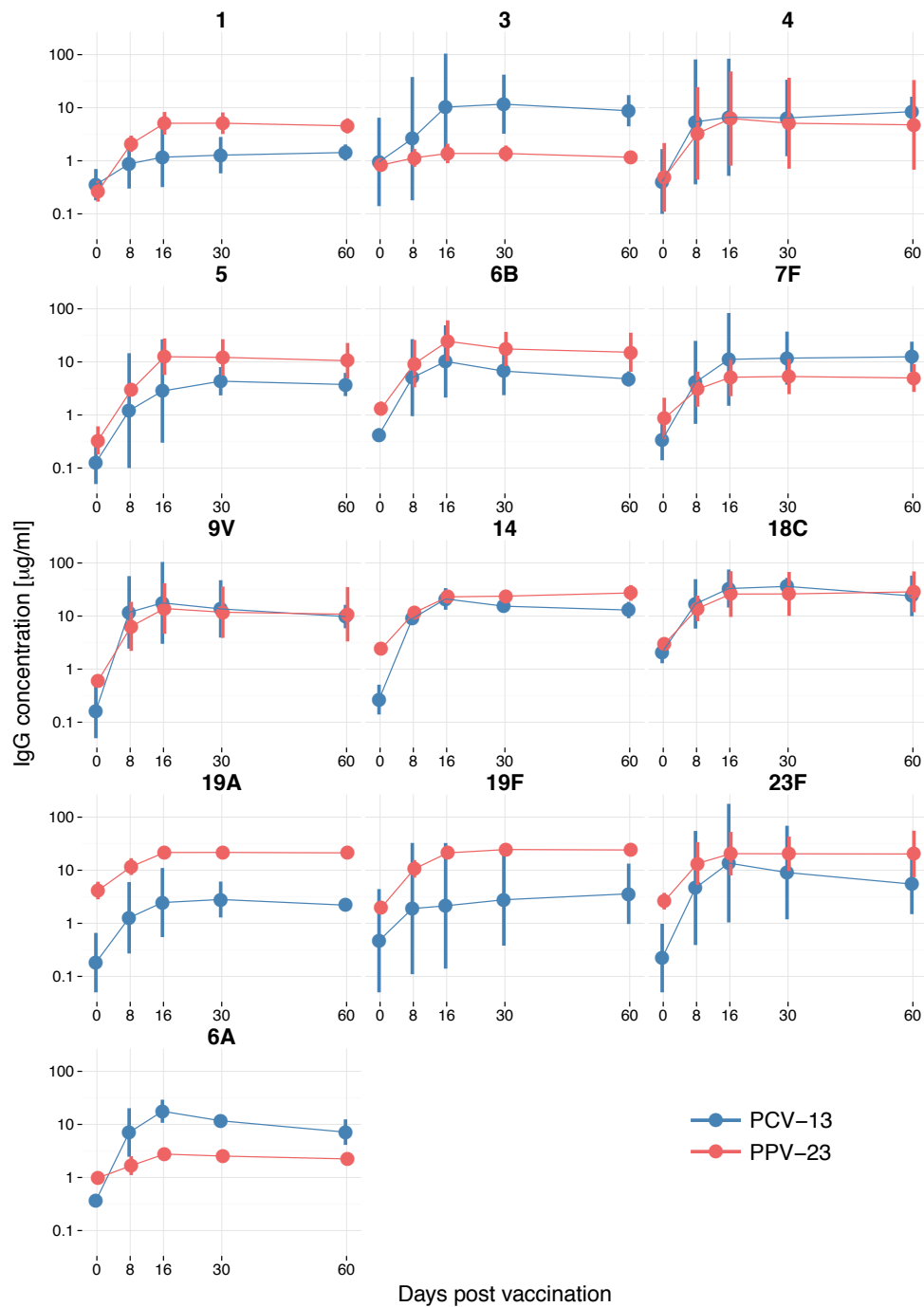


Figure 12.15: Kinetics of serotype-specific IgG concentrations following PCV-13 or PPV-23 vaccination. Shown are mean values (points) with the whiskers extending to the actual data points from the 2 study participants in each group. Serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F are contained in both vaccines and serotype 6A is only contained in PCV-13.

serotype 3 and 6A (only contained in [PCV-13](#)), which were higher in the [PCV-13](#) group.

Illumina gene expression profiling

The analysis of the large body of data generated by the gene expression profiling is ongoing and is beyond the scope of this thesis so that only results of a preliminary analysis are presented.

Using normalised and filtered data on all 4 participants at all 5 time points (6 and 24 hours, 2, 8, and 35 days following vaccination) and treating all participants as one group, a total of 238 genes at 24 hours and 428 genes at 8 days were differentially expressed compared to baseline. No genes were found to be differentially expressed with an adjusted p-value below 0.05 at 6 hours, 2 and 35 days post-vaccination (Figure [12.16](#)). The gene sets that were found by this approach were largely different between the two time points (Figure [12.17a](#)) with mainly innate immune pathways being overrepresented at 24 hours and adaptive immune pathways at day 8 (data not shown).

Splitting samples into the 2 groups revealed a different gene expression profile at day 8 following vaccination with, compared to baseline, many more genes differentially expressed in the [PCV-13](#) than the [PPV-23](#) group. Using this approach, (adjusted) statistical significance was not reached for any of the genes at any other time point (data not shown).

12.4 DISCUSSION

In this study, the appearance of B cell subsets and antigen-specific B cells in the peripheral blood was assessed at several time points following vaccination using a cultured [ELISpot](#) method as well as flow cytometry in combination with a labelling technique. For the first time, the kinetics of peripheral B cell subsets following vaccination were determined in such great detail.

Using a detailed gating strategy to identify B cell subsets by flow cytometry, it was shown in this study that several B cell subsets follow distinct patterns in the immune response to both vaccines. Using this technique and evaluating the relative frequencies of B cell subsets at several time points following vaccination, a previously unknown second plasma cell peak 20-26 days following vaccination was identified in the peripheral blood of all study participants irrespective of the type of vaccine they had received (Figure [12.4](#)). This secondary plasma cell peak may reflect re-circulating plasma cells that leave the lymph nodes and was also seen by gating on antigen-specific plasma cells (Figure [12.9](#)) further suggesting that it contains recently stimulated B cells. To our knowledge this is the first study performing a detailed analysis of plasma cells in the peripheral blood in the whole

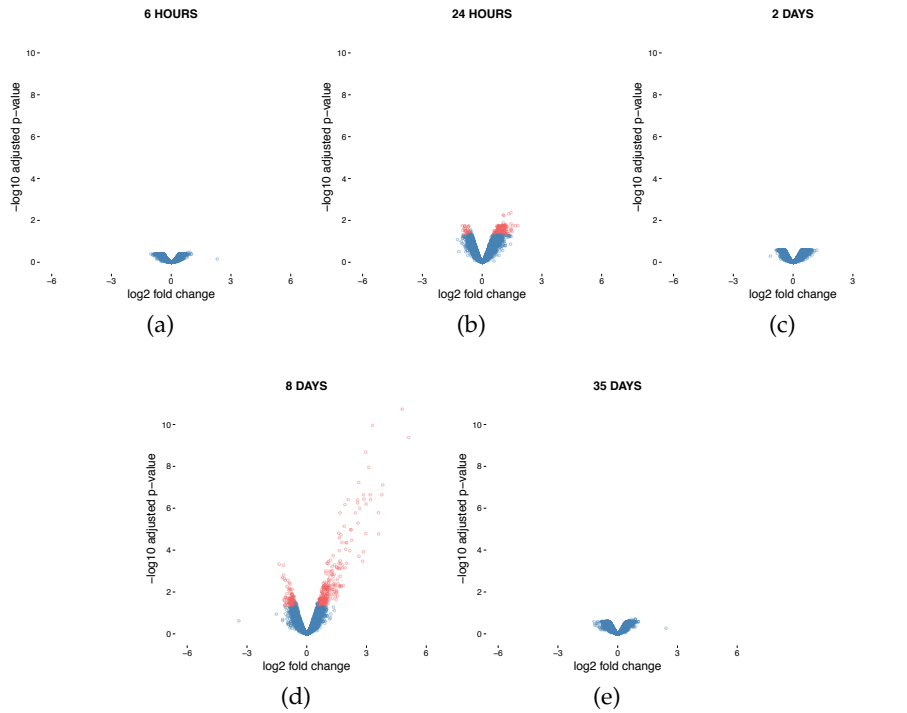
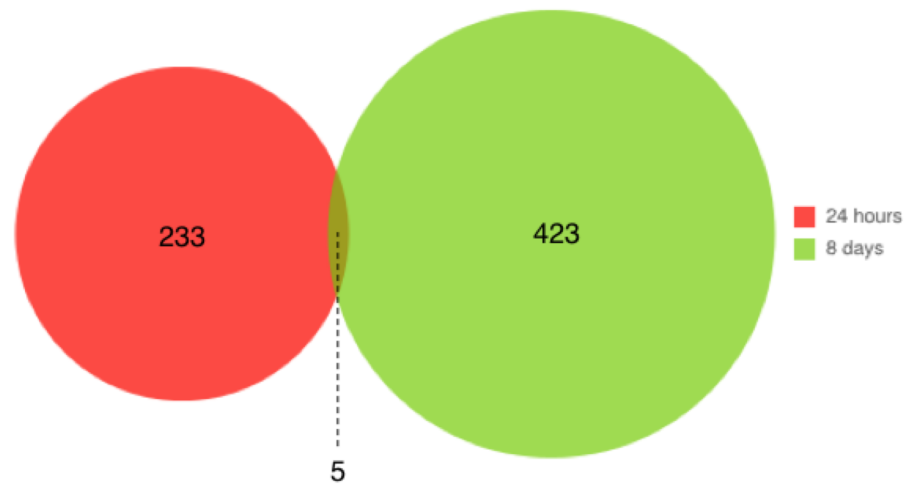


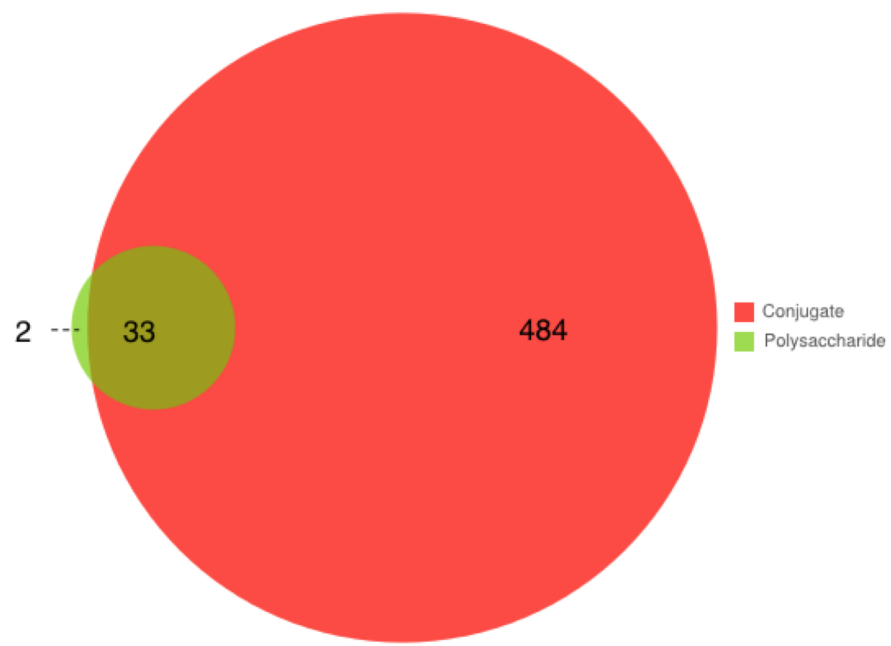
Figure 12.16: Volcano plots of all genes at different time points following vaccination. Genes that have an absolute fold change greater than 2 ($\log_2$ value > 0.58) and an adjusted p-value less than 0.05 are shown in red.

of the first month post-vaccination, which may be the reason for this second peak having remained undiscovered in the past. Frolich et al. performed similar investigations of tetanus toxoid-specific plasma cells by FACS and found a similar peak at day 7 but did not measure frequencies beyond 20 days post-vaccination [182]. Although not discussed in their manuscript, a second peak at day 14 was observed in 2 out of 3 study participants with levels returning to baseline at day 20 following vaccination. Only one study observed a (minimal) overall increase in plasma cell frequencies in the third week following the first dose of meningococcal C conjugate vaccine in infants [169].

Previous studies have investigated the plasma cell response to protein and (conjugated) polysaccharide vaccines by ELISpot and found antigen-specific cell frequencies to be highest 6-7 days post-immunisation [168, 171, 173, 207, 265]. Blanchard-Rohner et al. compared plasma cell responses to rabies vaccine as a not naturally occurring model protein antigen and showed that after a single dose of vaccine, in the naive group plasma cells appeared later (peak at day 10) than in the primed group (peak at day 7) and were of lower frequency [207]. Because both types of pneumococcal vaccines contain polysaccharide antigens of frequently colonising bacteria, such as *S. pneumoniae* and *N. meningitidis*, individuals receiving these vaccines are considered to



(a)



(b)

Figure 12.17: Venn diagrams showing number and overlap of differentially expressed genes (a) at 24 hours and 8 days post vaccination (all participants treated as one group), and (b) at day 8 following immunisation with either [PCV-13](#) (Conjugate) or [PPV-23](#) (Polysaccharide).

be primed consistent with the findings of an early antigen-specific plasma cell peak in the present study (Figures 12.4 and 12.9).

In the present study, several B_{MEM} subsets were followed over time. The relative frequencies of peripheral switched B_{MEM} ($CD27^+IgM^+IgD^-$) were different in the 2 vaccine groups and showed a decrease in the first two weeks following vaccination in the PCV-13 group, possibly due to relocation of this cell population in the lymphatic organs and therefore disappearance from the peripheral blood (Figure 12.7). This initial decrease of switched B_{MEM} was then followed by an increase in the peripheral blood in the third week after vaccination, probably due to post-GC output of this cell population. This kinetic (first decrease, then increase) was not seen in PPV-23 recipients consistent with the TI nature of the antigens contained in this vaccine. A similar picture was seen when antigen-specific switched B_{MEM} were graphically displayed albeight to a lesser degree (Figure 12.12). Of particular interest, IgM B_{MEM} ($CD27^+IgM^+IgD^-$) showed a continuous increase over the first month following vaccination (Figure 12.6), which is consistent with the findings of a recent study assessing antigen-specific B cells using a similar labelling technique [261]. This B cell subset is of interest also because its appearance in the peripheral blood as measure by FACS follows a similar kinetic to B_{MEM} as measured by ELISpot. Human MZB are thought to be the B cell subset mainly responding to TI-2 antigens such as pneumococcal polysaccharides [118, 119]. In the present study, relative frequencies of MZB showed yet another distinct kinetic peaking at 8 days following vaccination in the peripheral blood followed by a steady decline over the next 3 weeks (Figures 12.5 and 12.10). This early increase in MZB is consistent with the these cells responding to antigens in an extrafollicular manner [118].

Total antigen-specific B cell numbers (IgG^+) expressed as a percentage of all $CD19^+$ B cells decreased over time for all of the antigens when measured by the labelling method and flow cytometry (Figure 12.8). It is possible that the decreasing number of antigen-specific B cells in the peripheral blood following encounter with their antigen reflects the homing of these cells to secondary lymphoid tissues. The finding of decreasing antigen-specific B cells in the peripheral blood following vaccination contradicts the ELISpot data, which consistently show an increase of cultured antigen-specific B cells following vaccination. There is, however, a big difference between the two methods both measuring antigen-specific B cells. The number of cells found by ELISpot is very much confounded by the in-vitro viability and proliferation capacity of those cells and only reflects cells that have been stimulated and differentiated into antibody-secreting cells. On the other hand, identification of antigen-specific B cells with flow cytometry using the antigen-labelling technique may be confounded by binding of antigen to BCRs of a broad range of specificities.

Frequencies of diphtheria toxoid-specific B_{MEM} as determined by ELISpot demonstrated a clear separation between the vaccine groups with the participants who received PCV-13 showing an increase in dip-specific B_{MEM} following vaccination in contrast to PPV-23 recipients who did not mount a cellular immune response against diphtheria toxoid. Along with previous reports, this finding confirms that diphtheria toxoid B_{MEM} can serve as a marker for B_{MEM} directed against the common conjugate carrier protein CRM₁₉₇ [169, 171–173, 266] and 11. In the present study pneumococcal serotype-specific B_{MEM} frequencies as determined by ELISpot were higher in PCV-13 compared with PPV-23 recipients, which is in line with previous findings in adults and adolescents [168, 209, 250] and consistent with the TD and TI characteristics of PCV-13 and PPV-23, respectively. Similar to previous reports [168, 209], this difference in magnitude of response (PCV-13 higher than PPV-23) was most striking 1 month after vaccination (Figure 12.14).

Using gene expression profiling on whole blood samples collected at several time points following immunisation, it was demonstrated that a relatively big number of differentially expressed genes can be identified by using paired data from only 4 study participants (Figure 12.16). Using the preliminary results presented in this thesis, it also appears that the conjugate vaccine PCV-13 represents a much stronger stimulus to the immune system compared with the plain polysaccharide PPV-23 (Figure 12.17b). A more detailed analysis of the gene expression profile and the nature of differentially expressed genes may reveal underlying patterns of functional difference between the two vaccines.

12.5 CONCLUSION

This study showed that multi-colour flow cytometry in combination with a method of fluorescently labelling antigen-specific B cells may be used to gain further insight into the B cell response to vaccination. Using this approach, it was demonstrated that several B cell subsets follow distinct patterns in the immune response to both vaccines. In addition to an expected peak of plasma cells at day 8, a secondary peak in the third week post-vaccination was seen, possibly reflecting re-circulating plasma cells that leave the lymph nodes. Marginal zone B cells peaked early in the response at day 8 followed by a steady decline until 30 days following vaccination. IgM B_{MEM} showed a continuous increase over the first month following vaccination and followed a similar kinetic to serotype-specific ASC detected by the cultured memory ELISpot. Switched B_{MEM} followed different kinetics in the two groups. In the PCV-13 group, this cell population decreased in the first 3 weeks before reaching a relative maximum in

the fourth week post-vaccination whereas the relative frequency of switched B_{MEM} remained constant in the two PPV-23 recipients.

Two different gene expression profiles were found in response to vaccination showing early innate immune signals at 24 hours and adaptive signals coinciding with the plasma cell peak at day 8 but further work needs to be performed to unravel the large body of data collected and to integrate the different immunological data sets.

HIGH-THROUGHPUT ANALYSIS OF THE POST-VACCINATION ANTIBODY REPERTOIRE

13.1 INTRODUCTION

The human humoral response is anticipatory with a wide variety of antibody specificities even prior to antigen stimulation to account for the extensive range of potential antigens likely to be encountered in an individual's life-time. The basis for this diverse repertoire is the multiple variable (V), diversity (D) and junctional (J) B cell gene segments encoding the variable region of the antibody protein [267]. Distinct combinations of these gene segments are used in rearrangement events mediated by the enzymes RAG-1 and RAG-2. Further variation is created by combinatorial association, junctional diversity, and somatic hypermutation, leading to the creation of up to 10^{13} unique antibody molecules [105]. NGS technologies perform large scale DNA sequencing faster and at lower cost than traditional Sanger sequencing [268], allowing in-depth analysis of the antibody repertoire of the circulating B cell pool. The Roche 454 platform generates up to 1 million reads of 450 bp in a single sequencing run and is therefore suited to interrogate the entire recombined VDJ region. 454 sequencing of antibody variable regions has been used to obtain estimates of antibody repertoire diversity [269, 270], to detect and track clonal expansions in lymphoid malignancy [271] and to investigate the characteristics of different B cell lineages [198, 272, 273]. However it still remains challenging to understand the diversity of antibody repertoire in relation to the prime function of the antibody pool, antigen recognition. This is an important area to understand for diverse fields such as autoimmunity, protection against infectious diseases and immunisation. Studies of antibody repertoire generated in response to specific antigens such as bacterial polysaccharides [191, 274, 275], viral glycoproteins [276–278] and autoimmune antigens [279] have used small numbers of immortalised B cell lines and suggested that genetically diverse individuals utilised similar combinations of VDJ segments in response to a given antigen. However there is some evidence that global VDJ gene segment usage may be relatively independent of antigen specificity supported by the fact that entirely different BCR sequences can have the same VDJ usage. NGS approaches have the potential to advance our understanding of this area through access to vastly increased numbers of BCR sequences across a larger number of individuals. NGS sequencing of antigen specific B cells is possible but requires the development of antigen-specific staining and sorting

protocols to detect low frequency B cell populations. Several studies have utilised the relative enrichment for antigen specific B cells that occurs at day 7 following immunisation when plasma cells migrated from secondary lymphoid tissue to bone marrow. Whilst these have demonstrated changes in the large-scale structural features of the repertoire they have not investigated which features of BCR sequences indicate antigen specificity. We utilised a model antigen in the form of a vaccine in which Hib and serogroup C meningococcal polysaccharides are conjugated to tetanus toxoid to stimulate human B cell responses. A significant amount of BCR sequence data is already available for the Hib polysaccharide. This was first investigated by Insel, who demonstrated by isoelectric focusing of serum that the human antibody response to Hib polysaccharide vaccine was restricted to a limited number of clonotypes and that these clonotypes were similar between individuals [280]. Affinity chromatography purification of Hib-specific antibody and subsequent amino acid sequencing revealed a VH3 family dominance of the response to Hib polysaccharide vaccination [281]. More recently, B cells enriched in specificity for Hib polysaccharide from vaccinated infants were used to construct phage display antibody libraries [196]. Nucleotide sequencing of the antibodies expressed revealed usage of a single VH gene segment (V3-23) and only two JH and two VL and JL segments, consistent with findings in older studies using Hib specific-antibody secreting hybridomas [183]. The canonical Hib-specific antibody has a conserved CDR3 amino acid motif of Gly-Tyr-Gly-Phe/Met-Asp (GYGMD or GYGFD) [117]. For tetanus toxoid the data indicate that in the limited number of individuals studied to date there is a much more diverse repertoire with very few sequences that are shared. The use of antigens with known antigen-specific CDR3 region together with deep sequencing of B cell variable domains following antigen stimulation provides an opportunity to determine the features of BCR repertoire that indicate specificity for the stimulating antigen. To explore this possibility, we vaccinated individuals with a Hib polysaccharide vaccine and used 454 sequencing to delineate the B cell response to vaccination.

13.2 METHODS

Participants and vaccine

Antibody responses specific for Hib were studied as part of a single centre, open-label clinical study in healthy adult volunteers aged 18-65 years of age in Oxford, UK undertaken to provide a Hib reference serum. Written informed consent was obtained from the participants before enrolment in accordance with the Declaration of Helsinki. Ethical approval was obtained from the Oxfordshire Research Ethics Committee (OXREC Reference: 08/H0605/74) prior to study

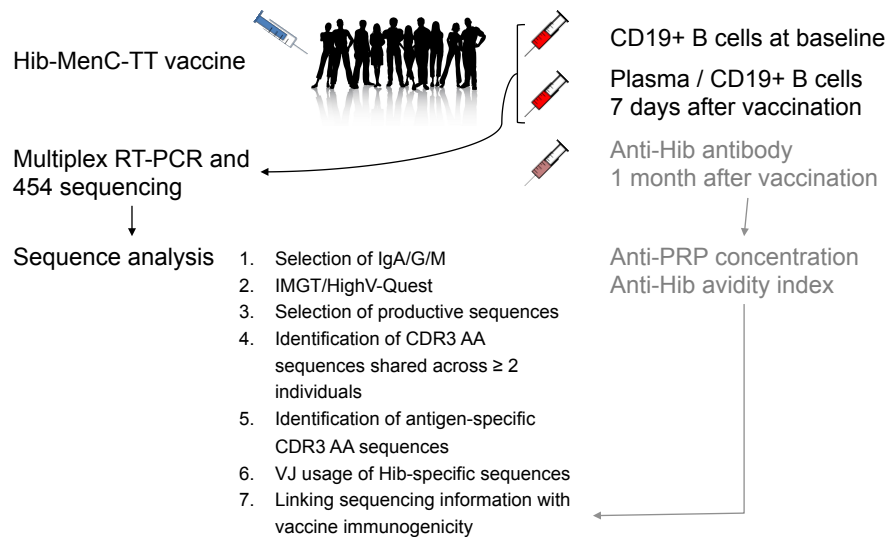


Figure 13.1: Workflow through the study.

commencement. Volunteers received a Hib-MenC vaccine (Menitorix®, GlaxoSmithKline), containing Hib polyribosyl ribitol phosphate (PRP, 5 µg) polysaccharide and meningococcal serogroup C polysaccharide (5 µg) individually conjugated to tetanus toxoid carrier protein (total 17.5 µg). Blood was taken from participants immediately prior to vaccination (baseline), and at 7 and 28 days after vaccination.

Anti-PRP antibody and avidity index

Anti-PRP IgG concentrations were measured from serum taken 28 days after vaccination. Antibody avidity was determined by elution using a 0.15M solution of the chaotrope sodium thiocyanate. Relative avidity (avidity index) was expressed as the percent reduction in the IgG concentration compared to the concentration in the absence of the chaotrope [282].

B cell isolation and cell sorting

Peripheral blood mononuclear cells were obtained by density gradient centrifugation over Lymphoprep (Axis-Shield) as described in section 7.2. CD19⁺ cells were separated at baseline from all participant samples and at day 7, either plasma or CD19⁺ cells were obtained. Enrichment of cell populations was performed by magnetic cell separation using CD19 MicroBeads or Plasma cell isolation Kit II and the AutoMacs™ (Miltenyi Biotec). The purity of sorted populations was checked using flow cytometry and was 94-99% on all samples subsequently used for sequencing.

cDNA synthesis and PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesised using random hexamers and Superscript III reverse transcriptase (Invitrogen). Reverse transcription was performed at 42 °C for 60 min followed by inactivation at 95 °C for 10 min. Ig genes were amplified using previously published primers (consensus forward primers for each of the families VH1 to VH6, and reverse primers enabling identification of IgM, IgA and IgG) [272]. Two rounds of PCR amplification were performed using Taq DNA polymerase (Qiagen). cDNA and first round PCR product were used as templates in the first and second rounds respectively. A simplified 'hot start' protocol was followed to reduce non-specific primer binding to template, whereby template DNA was the final addition to the reaction mix, and PCR tubes were kept on ice until placed in a thermocycler preheated to 94 °C. PCR conditions were as follows: an initial denaturation at 94 °C for 3 min; followed by 30 (first round) or 15 (second round) cycles of denaturation at 94 °C for 30s, annealing at 58 °C for 30s, extension at 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. To prepare sufficient DNA for 454 sequencing, while minimising PCR amplification, up to 8 replicates of each first round PCR sample were used as a template in a second round of PCR, and the products pooled.

Library preparation and 454 sequencing

The PCR amplicons were prepared for sequencing and blunt-end ligated to multiplex identifier (MID) tags using the Roche GS FLX Titanium Rapid Library Preparation Protocol. Emulsion PCR and pyrosequencing were performed on each library using the GS FLX Titanium XL+ sequencing platform according to the manufacturer's instructions.

Sequence analysis

Output files were converted into fasta files and sequences were assigned a sample and isotype identifier based on the terminal MID sequence. For two individuals at both time points, isotype-specific amplicons were sequenced without separate MID tags and sequences were therefore assigned an isotype by respective motifs of the constant region sequence. Sequences that could not be matched were discarded. Remaining sequences were analysed using the web-based IMGT/HighV-Quest tool allowing for insertions and deletions [283] and only productive sequences were further considered. For each sample, frequencies of unique VDJ combinations and unique CDR

amino acid (AA) sequences were calculated using R [237] and graphically displayed using the ggplot2 package for R [284]. Clonality was determined by calculating (a) the total number of sequences divided by the number of unique VDJ combinations and (b) the area under the curve (AUC) when graphically displayed as cumulative proportion of VDJ combination 13.3.

Sequences with identical CDR3 AA sequences, which were shared between at least 2 individuals were used to determine the 'public repertoire'. Of the day 7 shared CDR3 AA sequences, Hib-specific sequences were selected if they (a) contained either the string 'GYGMD' or 'GYGFD' [117] and (b) were exactly 10 AA long. The VJ combinations used for each of these Hib-specific CDR3 AA sequences and the relative abundances were calculated for each sample. Further, for each of the Hib-specific CDR3 AA sequences, the frequency of study participants using identical VJ combinations to create the same Hib-specific CDR3 AA sequence was also calculated. The software Geneious (version 6.1, Biomatters) was used to generate consensus Hib-specific CDR3 AA motifs.

CDR3 AA sequences were also compared to previously described sequences specific for Hib [117, 183, 192, 281], TT [176, 181, 182, 285], H1N1 influenza [178, 286], and MenC [287, 288] by searching for identical and closely related sequences in our dataset. Enrichment of these sequences was calculated as fold changes between sequence numbers at 7 days post vaccination divided by their frequency at baseline. For sequences, which were not present at baseline, fold change was calculated as 1.5 times frequency post vaccination.

In order to investigate whether the frequencies of presumptive Hib-specific sequences at day 7 post-vaccination were indicative of the antibody response 1 month following immunisation, proportions of Hib-specific per total number of sequences were calculated for each sample and their log-transformed values correlated with the log-transformed antibody concentration and avidity indices, respectively. The same analysis was performed to calculate the correlation between the frequencies of unique Hib-specific CDR3 AA sequences per sample. A schematic diagram of the strategy for the analysis of sequences is shown in Figure 13.1.

Isotype subclass information for IgA and IgG was determined by mapping constant region nucleotide sequences to all possible isotype (subclasses) using a modified version of Stampy [289] and VEGA constant region sequences as reference [290]. Number of V gene mutations were taken from IMGT output files and compared between sequences of different specificities and by isotypes subclass.

13.3 RESULTS

General overview

From 5 individuals blood was obtained at day 0 (CD19 B cells isolated) and day 7 (CD19 B cells from 2 individuals and plasma cells from 3 individuals). From each sample IgG, IgA and IgM BCR libraries were prepared with one library preparation failing (IgG from plasma cells) resulting in 29 samples. In total, 460,077 sequences were obtained (average 15,860 [range: 1,612 - 32,540] per sample), of which 184,844 (average 6,374 [1,342- 12,070] per sample) were considered as productive by IMGT (Table [13.1](#)).

Table 13.1: Characteristics of sequence data.

Participant	Isotype	B cell subset [day post- vaccination]	Total no. of sequences	No. of productive sequences	No. of Hib CDR3 AA sequences ^a	No. of unique Hib CDR3 AA sequences ^b	Anti-PRP Ab/avidity index [day 28]
1	IgA	CD19 [0]	3313	2527	0	0	49.0 µg/ml 59.83
	IgG		2277	1689	0	0	
	IgM		7843	5998	0	0	
	IgA	PC [7]	3750	3026	277	4	
	IgG		2165	1710	78	3	
	IgM		8694	6932	601	6	
2	IgA	CD19 [0]	5071	3997	0	0	45.89 µg/ml 343.29
	IgM		12802	10256	1	1	
	IgA		3899	3289	620	6	
	IgG	PC [7]	1612	1342	446	10	
	IgM		7131	5819	1221	13	
3	IgA	CD19 [0]	23857	9522	1	1	7.3 µg/ml 30.15
	IgG		19412	3763	0	0	
	IgM		20773	10879	1	1	
	IgA	PC [7]	24768	12073	143	4	
	IgG		15334	5350	97	8	
	IgM		27272	10156	71	5	
4	IgA	CD19 [0]	21952	7858	1	1	29.1 µg/ml 66.51
	IgG		32545	2391	0	0	
	IgM		24633	11237	0	0	
	IgA	CD19 [7]	22630	9080	624	10	
	IgG		20871	3723	614	10	
	IgM		23315	9279	399	8	
5	IgA	CD19 [0]	26016	9051	0	0	123.7 µg/ml 3.52
	IgG		18655	3227	0	0	
	IgM		23552	11774	0	0	
	IgA	CD19 [7]	19134	7804	50	3	
	IgG		16379	4885	5	2	
	IgM		20422	6207	1	1	

^aNumber of CDR3 AA sequences of length 10AA containing the string “GYGMD” or “GYGFD” [117].

^bNumber of different CDR3 AA sequences showing Hib-specific characteristics as in ^a.

Sequence clonality and CDR3 length distributions

Clonality was similar between the different isotypes at baseline but increased in plasma cell samples at day 7 post-vaccination (Figure 13.3). For CD19⁺ B cell samples, this increase in clonality was only seen for IgG and not IgA or IgM sequences (Figure 13.3). There were no differences in CDR3 length distributions between isotypes IgA, IgG and IgM at either time point and before and after vaccination (Fig-

ure 13.4). Graphical representation of repertoire also shows increase clonality (Figure 13.2).

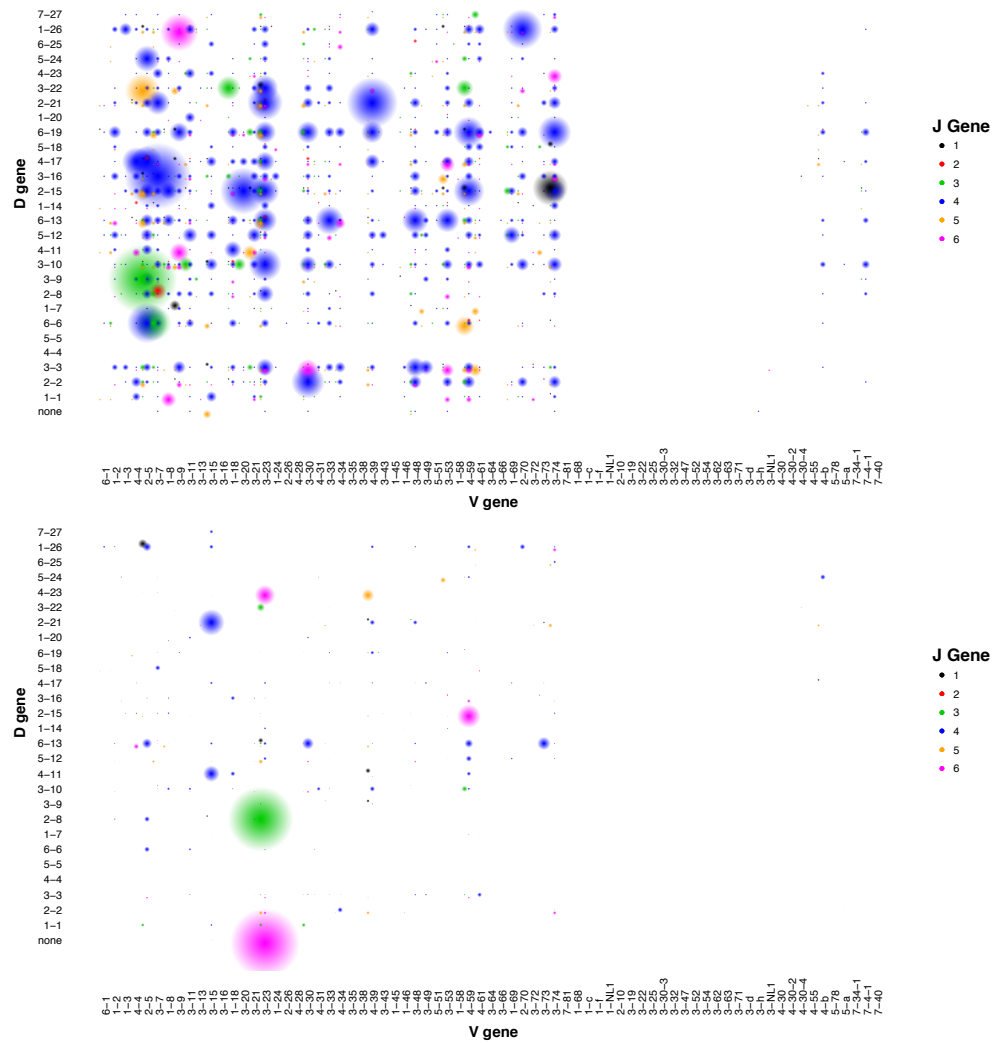


Figure 13.2: Distribution and frequency of VDJ combinations of a representative baseline (top) and day 7 (bottom) sample. The x- and y-axes indicate V genes and D genes, respectively. The color shows the J gene usage and the size of the bubble represents the frequencies of respective VDJ combinations.

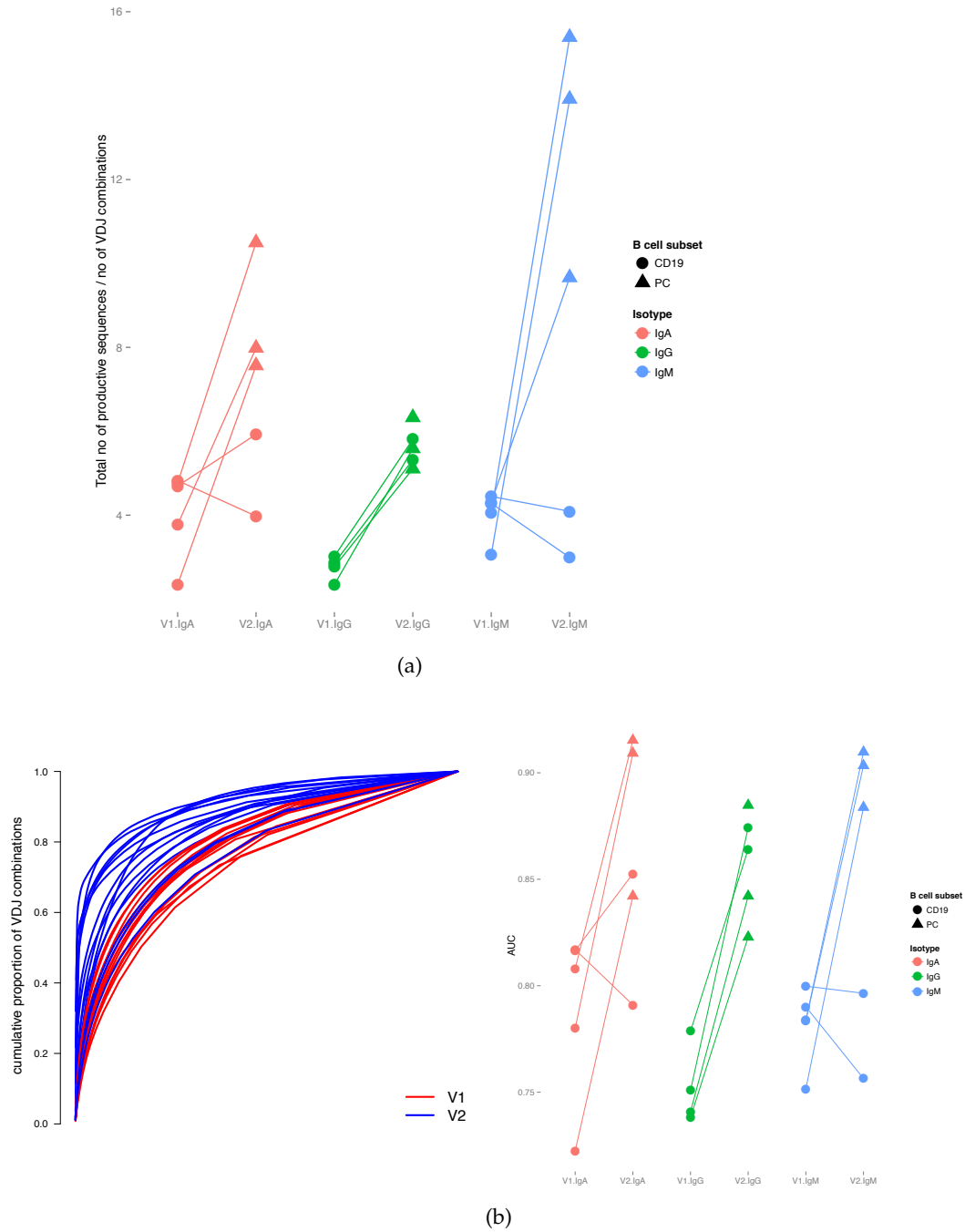


Figure 13.3: Day 7 sequences are more oligoclonal as indicated by (a) number of unique VDJ combinations per total number of sequences and by (b) cumulative proportion of VDJ combinations. At day 7, there were differences in the degree of clonality between plasma cell and total CD19 B cell samples for IgA and IgM whereas the type of sample did not make any difference for IgG sequences as indicated by the area under the curve (AUC).

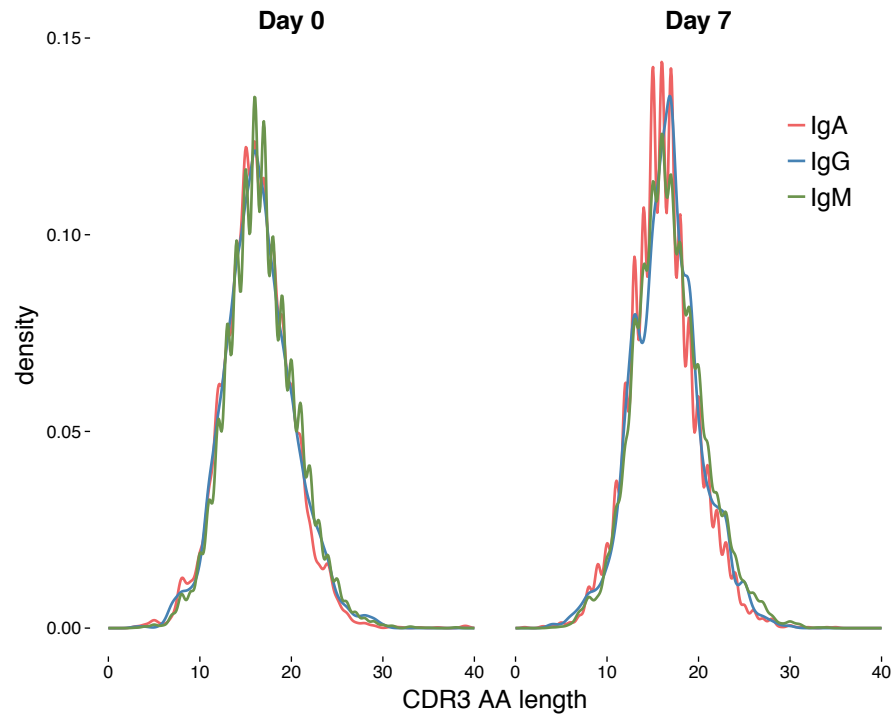


Figure 13.4: CDR3 AA length distribution of IgA, IgG and IgM sequences at baseline (V1) and 7 days following vaccination (V2).

The post-vaccination 'public repertoire' is dominated by Hib-specific sequences

A total of 47 CDR3 amino acid (AA) sequences were shared by at least two out of the 5 individuals 7 days following vaccination ('public repertoire'). More than 50% of these CDR3 AA sequences were exactly 10 AA long, which was considerably shorter than the average CDR3 AA sequence lengths at day 7 (Figure 13.5).

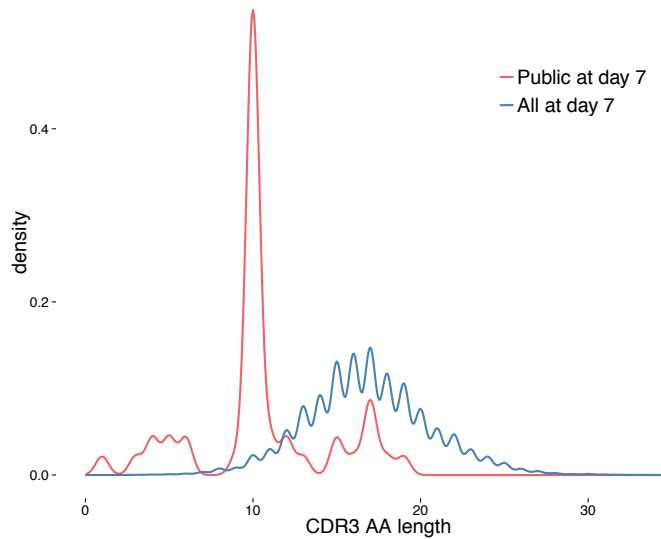
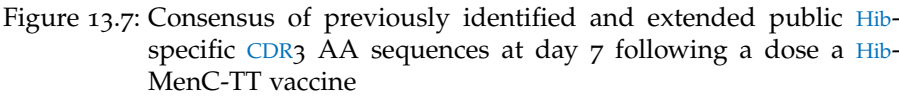
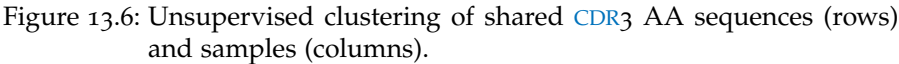


Figure 13.5: CDR3 AA length distribution of unique sequences shared by at least two individuals 7 days following vaccination ('Public at V2') compared with all day 7 sequences ('All at V2').

We further assessed the presence (or absence) of these 47 shared CDR3 AA clones in all samples. The result is represented as a unsupervised heatmap with dendrograms in Figure 13.6. A large proportion of the day 7 public CDR3 AA sequences (14/47, 30%) showed distinctive characteristics of previously identified Hib-specific motifs [117]. The frequency of sequences determined as Hib-specific were significantly enriched 7 days following vaccination in all of the isotypes tested although the frequency of those sequences differed considerably between individuals (Table 13.1). All of the top 3 CDR3 AA sequences, which were shared by all 5 individuals and present in 10-14 out of 15 samples, were Hib-specific sequences. Another 9 of the public CDR3 AA sequences at day 7 differed only slightly from previously described Hib-specific CDR3 AA motifs and were also 10 AA long (Figure 13.7). Hence, almost half (23/47) of the shared CDR3 AA sequences 7 days after vaccination showed characteristics of sequences targeting the Hib polysaccharide antigen.



In addition, we found at least one shared CDR3 AA sequence previously known as TT-specific with another sequence closely resembling this sequence.

VJ usage of Hib-specific sequences

Sequences containing known Hib-specific CDR3 AA motifs were further analysed. These sequences were dominated by gene rearrangements consisting of IGHV3-23 and IGHJ6 in all isotypes. The most frequently occurring and most shared CDR3 AA sequences showed a broader range of VJ usage. In general less diversity was observed in the IgG samples than in IgM and IgA samples (Figure 13.8). On the other hand a participant-specific signature of VJ usage was seen across the different isotypes (Figure 13.10). We also assessed how many participants used the same VJ combination to create similar Hib-specific CDR3 AA sequences and found that VJ usage of some of the Hib-specific CDR3 AA sequences is very diverse whereas for other CDR3 AA sequences a particular VJ combination seems to be needed (Figure 13.9).

IgA and IgG isotype subclasses of constant region sequences

IgA and IgG subclasses were determined by mapping the constant region sequences against VEGA reference sequences. 7,094 out of 61,023 (11.6%) IgA constant region sequences were too short for identification of an IgA subclass and 110 sequences (0.2%) were assigned a different isotype to the original PCR amplified isotype. For IgG, 2,781 out of 25,280 (11%) sequences a subclass could not be identified and 19 sequences (0.08%) were assigned a different isotype to the original isotype (data not shown). Before vaccination, IgA sequences were dominated by IgA₁ and IgG₁ was most common amongst the IgG sequences with IgG₂, IgG₃ and IgG₄ representing second, third and fourth most common IgG subclasses. After vaccination, there was a relative increase in IgA₁ and IgG₂ sequences (Figure 13.11).

Tetanus toxoid-specific antibody repertoire

In addition to Hib-specific sequences dominating the shared repertoire at day 7 post vaccination, we found one shared CDR3 AA sequence previously known as TT-specific ("CASGSTLDYW") [181] with another sequence of similar length closely resembling this sequence ("CTSGSTFDYW"). However, by comparing our dataset to previously described sequences specific for Hib, TT, H1N1 influenza and MenC, we identified several other TT sequences that were enriched and shared between individuals 7 days post vaccination (Table 13.2).

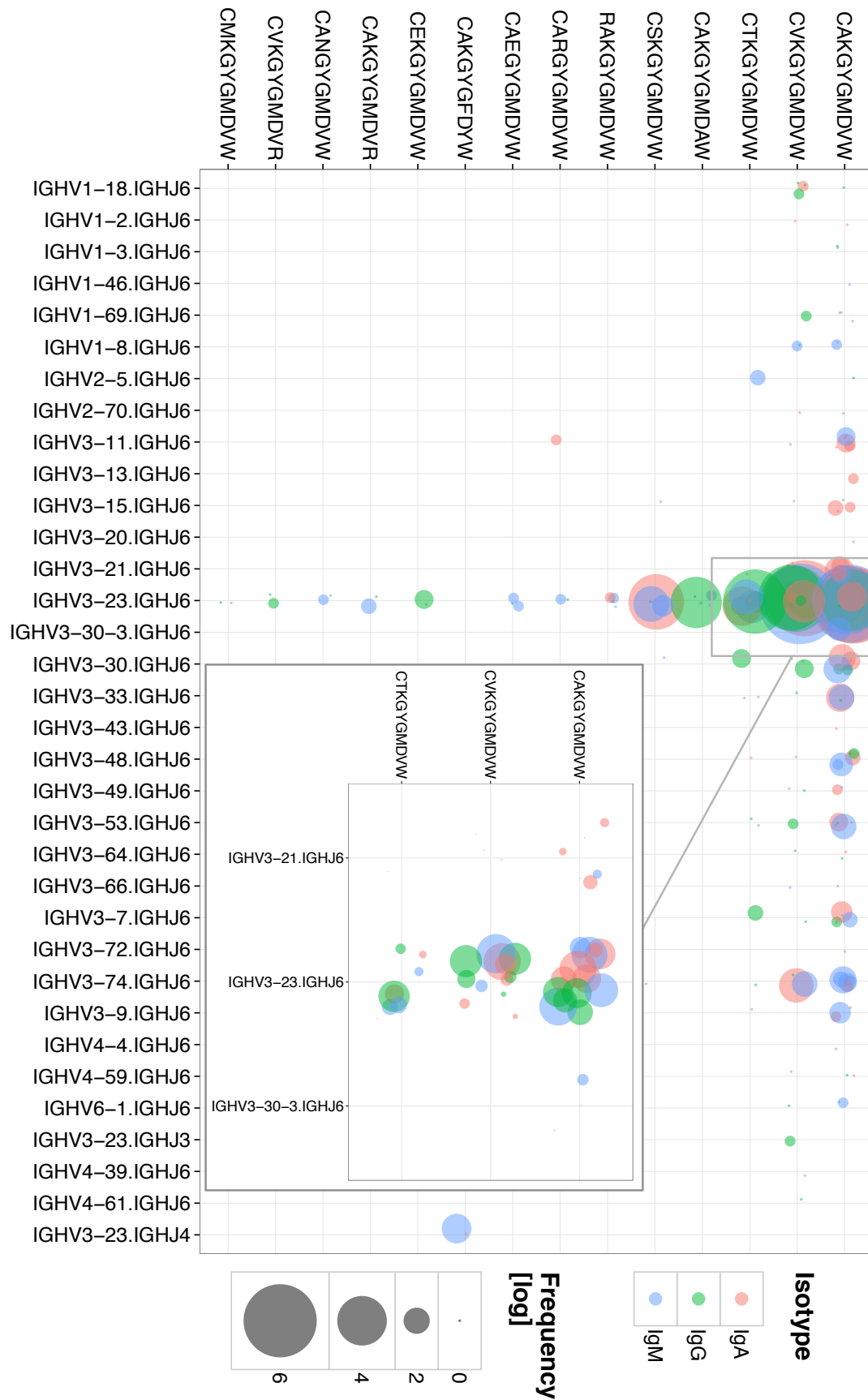


Figure 13.8: VJ usage and frequencies (log) of sequences with Hib-specific CDR3 AA motif

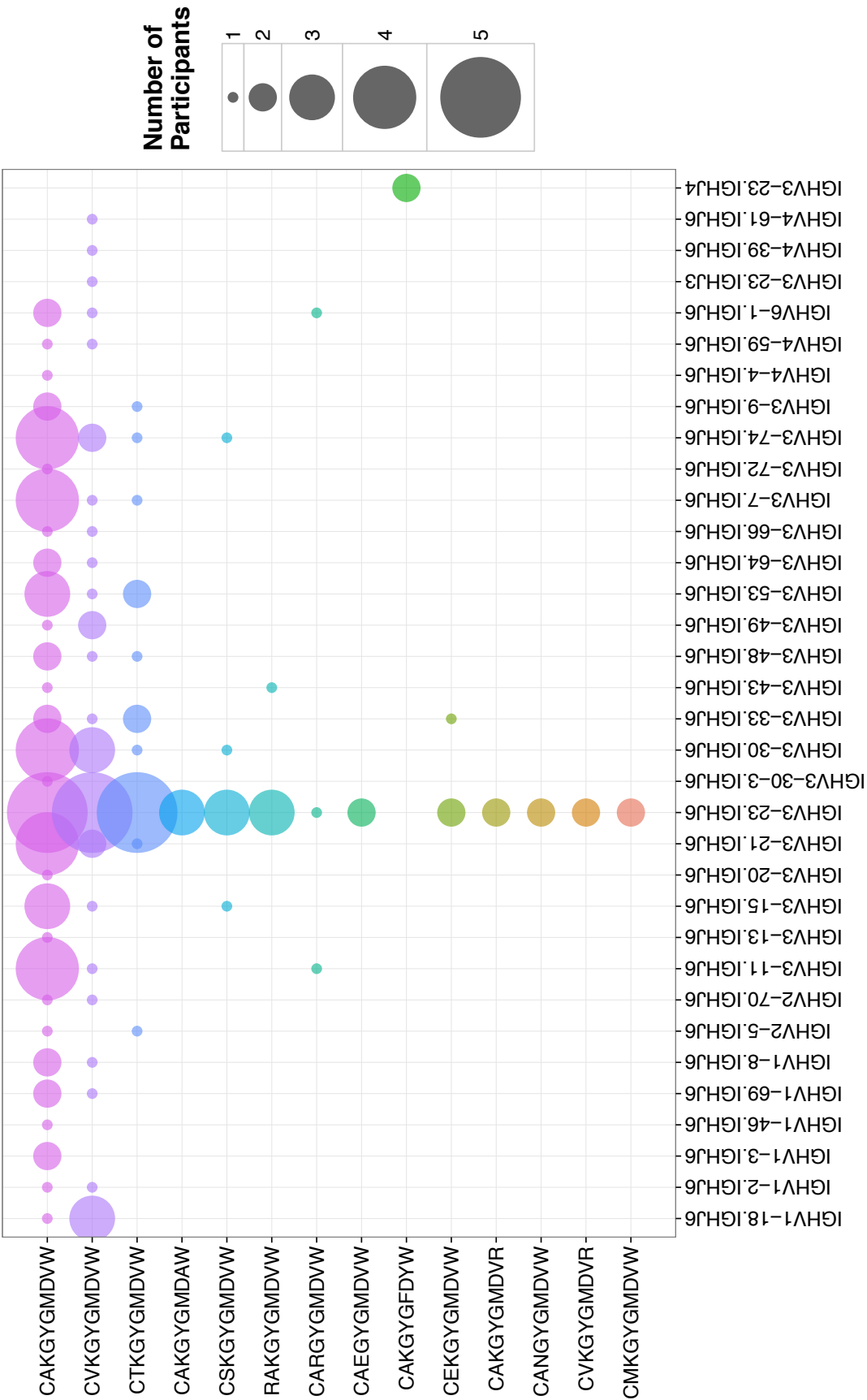


Figure 13.9: Number of participants sharing the same VJ combination for production of identical Hib-specific CDR3 AA

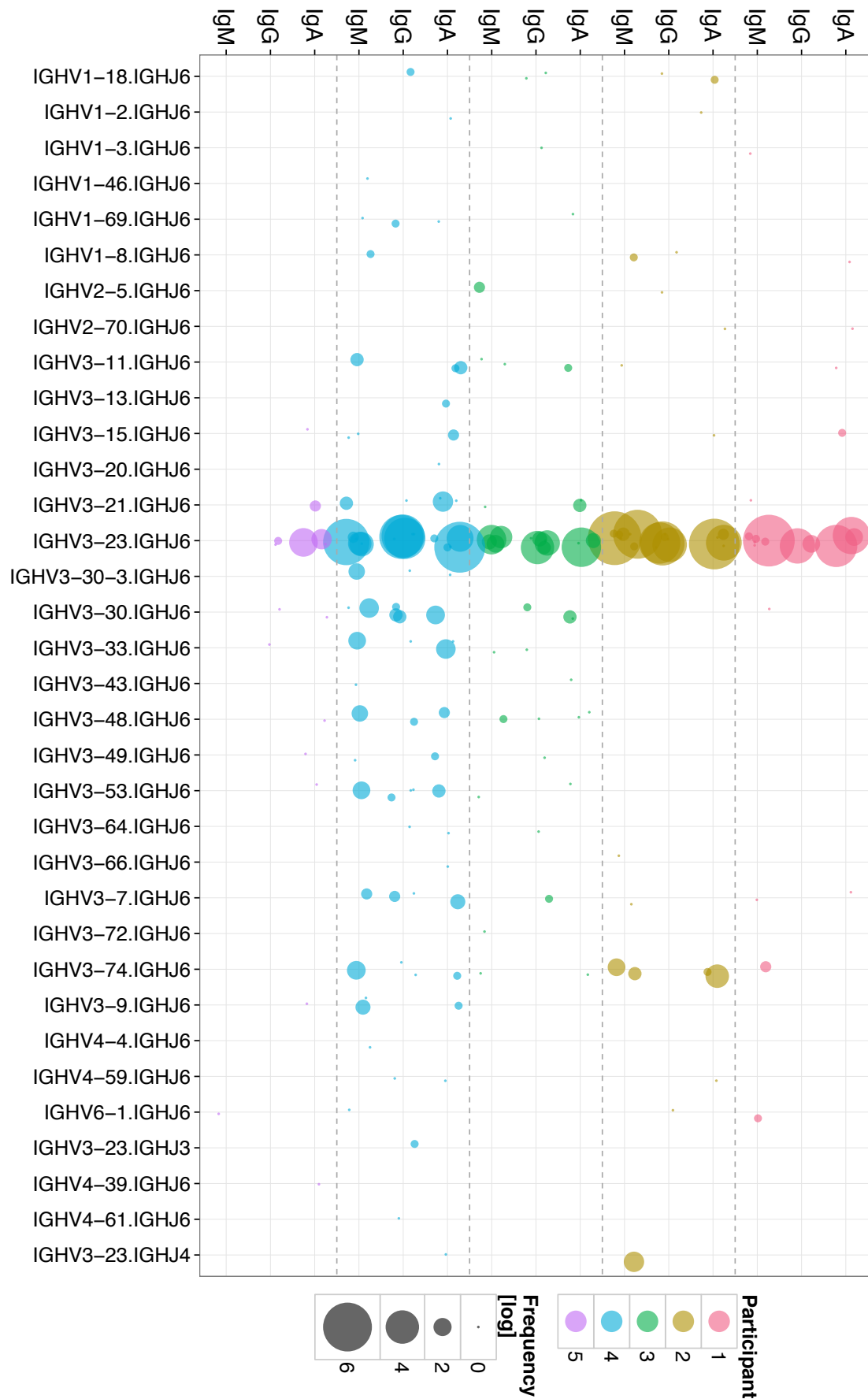


Figure 13.10: VJ usage of Hib-specific CDR3 AA sequence is similar between isotypes within each participant.

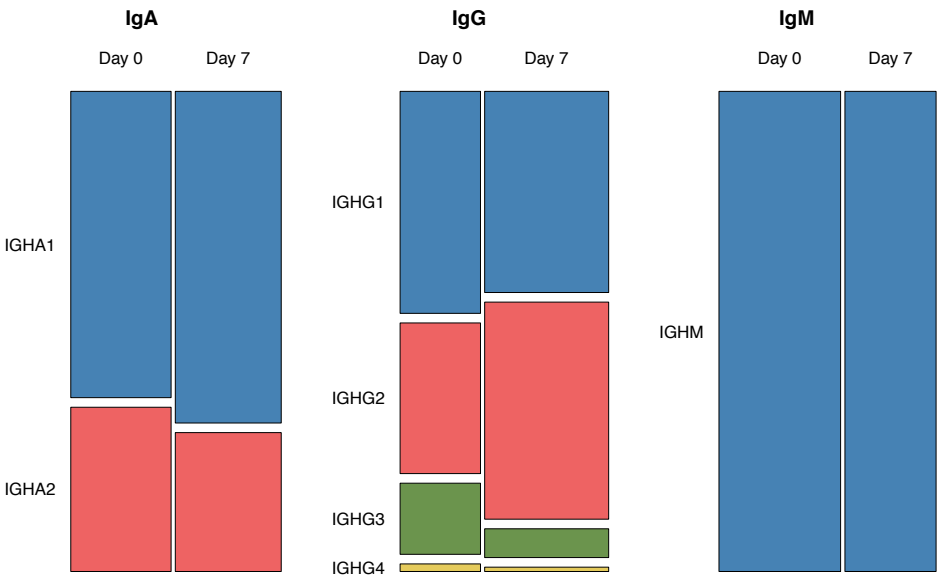


Figure 13.11: Isotype and isotype subclass distribution in samples taken at baseline and 7 days post-vaccination. The surface of each rectangle represents the relative proportion of sequences.

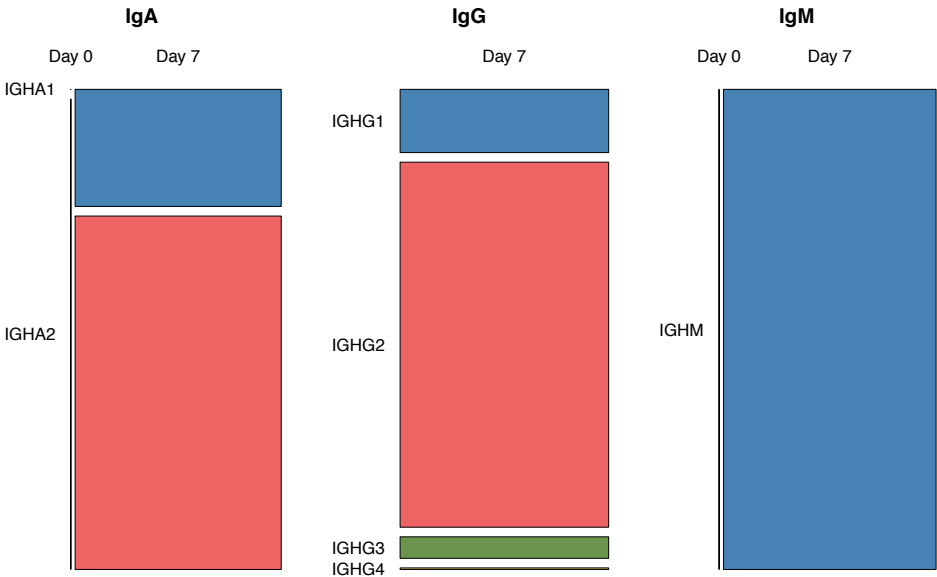


Figure 13.12: Post-vaccination Hib-specific sequences were most frequently of subclasses IgA₂ and IgG₂.

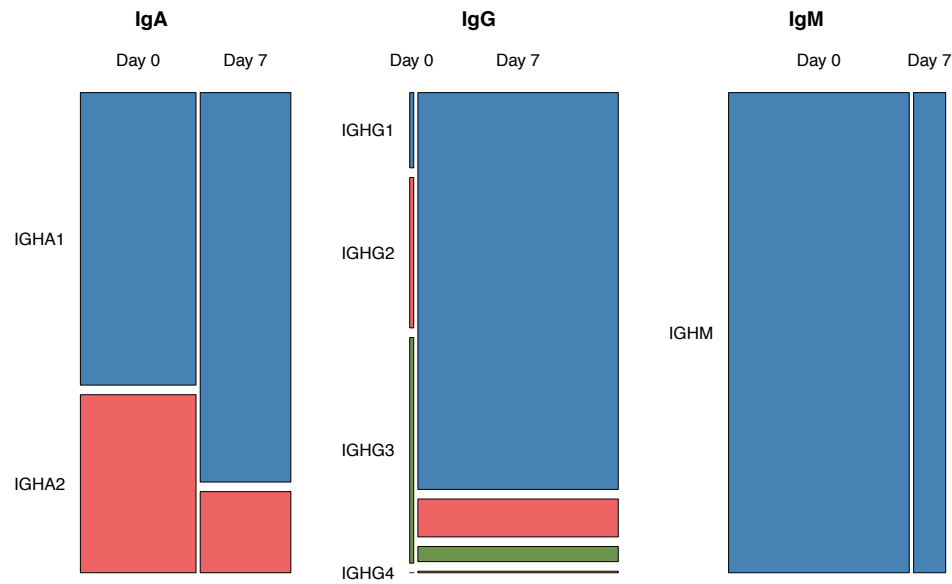


Figure 13.13: Post-vaccination **TT**-specific sequences were most frequently of subclasses IgA₁ and IgG₁.

H. influenzae-specific **CDR3** AA frequency and diversity are correlated with functional anti-**Hib** antibody and show increased mutational rate post vaccination

The proportions of **Hib**-specific **CDR3** AA sequences per sample at day 7 and isotype correlated very well with anti-**Hib** avidity indices 1 month following vaccination (Figure 13.14, Pearson's $r = 0.8-0.94$, significant for IgG and IgM) but not with anti-PRP antibody concentrations (Figure 13.15). Similarly, the number of unique **CDR3** AA sequences per sample (diversity of **Hib**-specific sequences) correlated with avidity indices (Figure 13.14, $r = 0.68-0.93$, significant for IgM) but not with antibody concentrations (Figure 13.15).

Frequencies of nucleotide (nt) mutations in V genes (V_{MUT}) differed between isotypes with IgM sequences having much fewer mutations both before and after vaccination. V_{MUT} increased significantly for IgM and IgG sequences but not for IgA sequences (data not shown). At baseline, sequences identified as **Hib** or **TT** had similar V_{MUT} compared with sequences of unknown specificity although numbers of antigen-specific sequences were low. Seven days post-vaccination, V_{MUT} differed significantly between sequences of **Hib**, **TT** and unknown specificity (**TT** >> **Hib** >> unknown, $p < 10^{-16}$ for all comparisons; Figure 13.16).

Hib-specific sequences were dominated by the subclasses IgA₂ and IgG₂ (Figure 13.12). For sequences related to known **TT**-sequences a relative increase for subclasses IgA₁ and IgG₁ was seen (Figure 13.13).

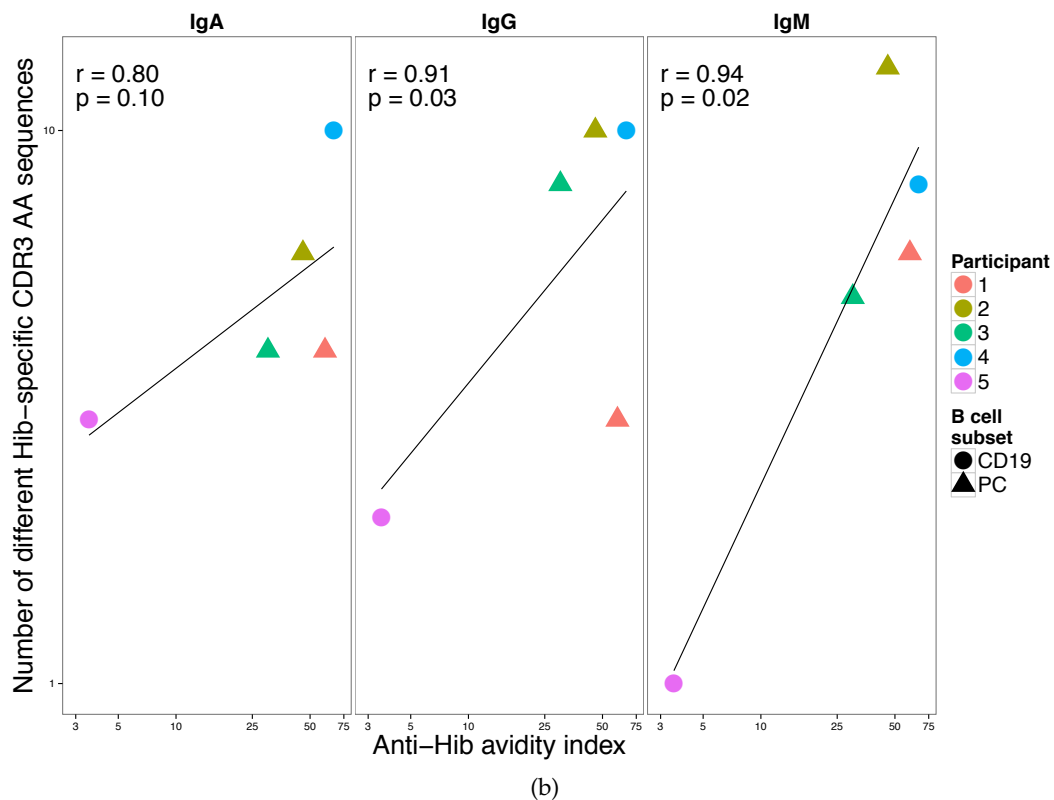
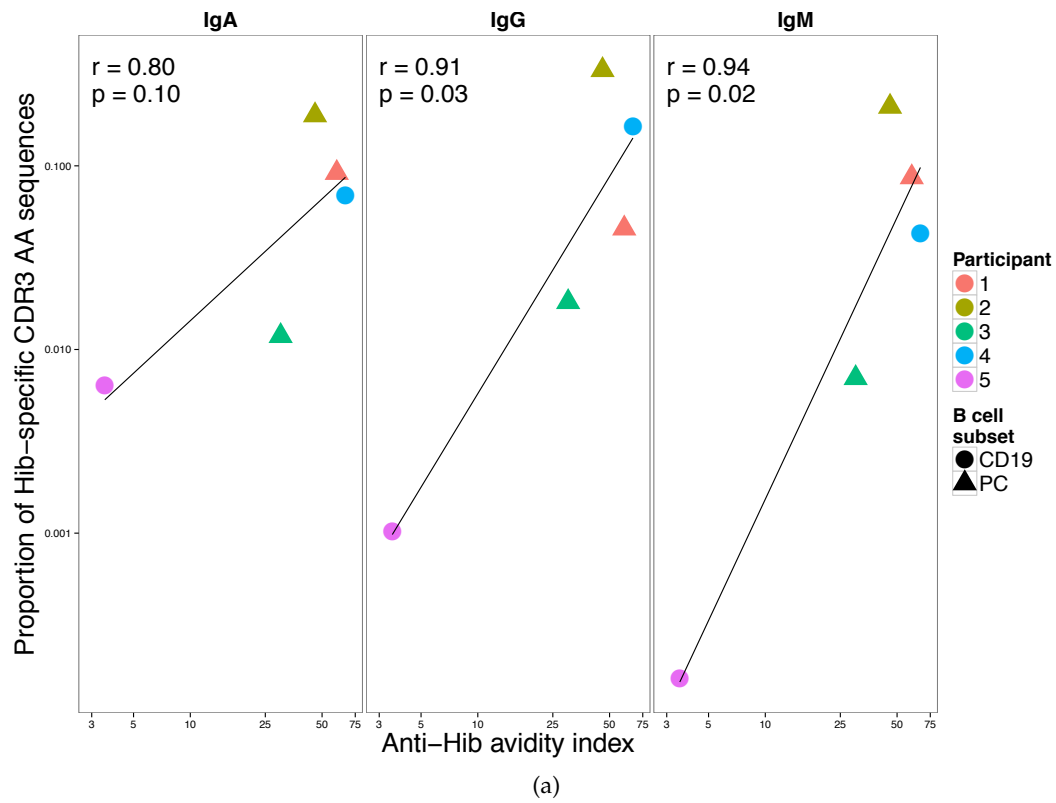


Figure 13.14: Correlation between serum anti-Hib avidity indices (log) 1 month after vaccination and (a) proportion and (b) number of different Hib-specific CDR3 AA sequences at day 7 (log).

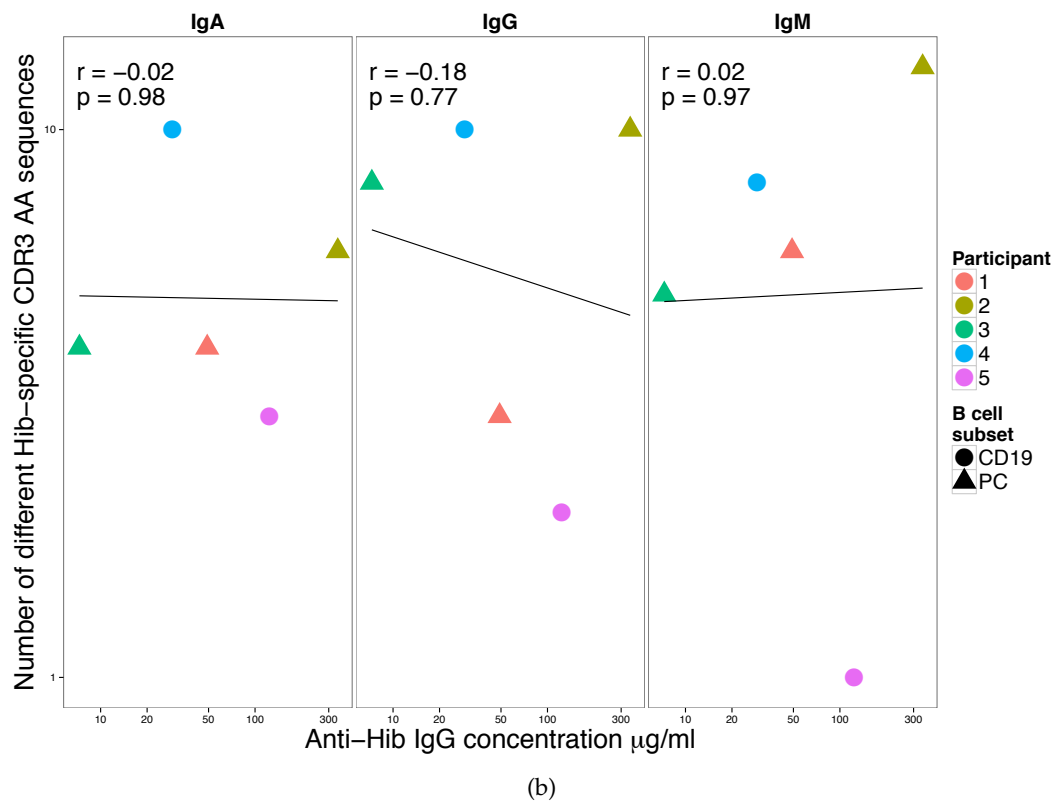
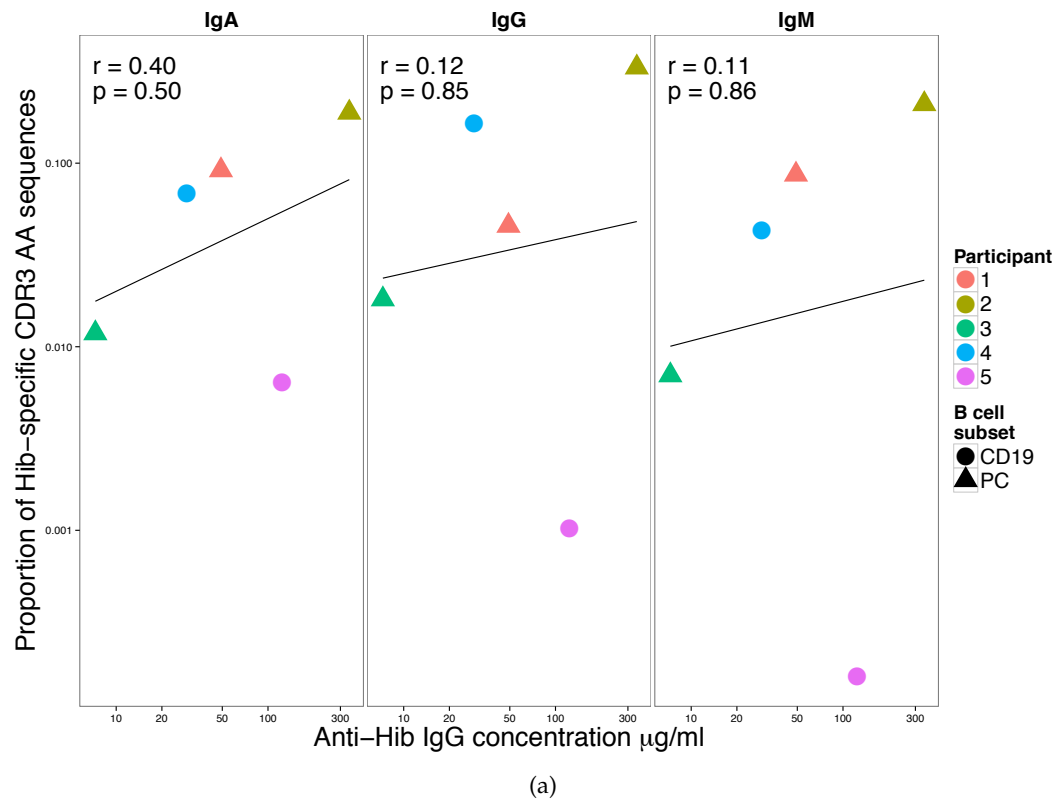


Figure 13.15: Correlation between serum anti-Hib antibody concentrations (log) 1 month after vaccination and (a) proportion and (b) number of different Hib-specific CDR3 AA sequences at day 7 (log).

By comparing publicly available [Hib](#)- and [TT](#)-specific sequences with our dataset we were able to identify related sequences, which were greatly enriched and shared across participants at 7 days post vaccination. Sequences previously identified as influenza (H1N1) specific were not enriched post vaccination (Figure [13.17](#)).

13.4 DISCUSSION

The features that define antigen-specific sequences in antigen-stimulated [BCR](#) repertoires are not well defined. In this paper we have demonstrated that analysis of the public 'repertoire' specifically identifies antigen specific sequences 7 days after vaccination. In this study, the B cell repertoire in the peripheral blood was sequenced and compared before and after vaccination with a [Hib](#)-MenC conjugate vaccine. We found that sequences differed in their clonality with post-vaccination samples being more oligoclonal than baseline samples (Figure [13.3](#)). In contrast to previous work [[198](#), [272](#)], we were unable to detect a difference between the clonality of IgA, IgG and IgM sequences when adjusted for the total number of sequences per sample (Figure [13.3](#)). Briney et al. calculated the contribution of the 50 most common VDJ combinations for the overall repertoire in 3 different B cell subsets, which had been isolated by flow cytometry [[198](#)] but did not adjust for total number of sequences observed in each sample, which may have biased this result. Wu et al. [[272](#)] similarly reported on clone size distributions of different B cell subsets and found that especially switched memory B cells showed bigger clone sizes [[272](#)]. Relatively few sequences per sample were considered in the latter study and their approach differed considerably from ours by first sorting B cell subsets by flow cytometry. We performed cDNA synthesis and PCR amplification straight from bulk cell populations such as total B cells or plasma cells. In the present study we had the advantage of comparing sequences before and after a dose of a very immunogenic polysaccharide conjugate vaccine. Post-vaccination sequences were in general more oligoclonal compared with baseline sequences for all isotypes. IgA and IgM repertoires obtained from total B cells were similar between the 2 sampling time points whereas clonality of plasma cell samples was increased compared with CD19 B cell baseline samples for IgA and IgM sequences. Interestingly, this difference in clonality between plasma and B cell samples at day 7 was not found in IgG sequences indicating that the IgG sequence pool of CD19⁺ B cells is dominated by newly generated (oligoclonal) antigen-experienced sequences.

Post-vaccination sequences, which were shared between study participants, had an unusual [CDR3](#) AA length distribution and were dominated by [CDR3](#) sequences with a length of 10 AA (>50% were 10 AA long; Figure [13.5](#)). A large proportion of shared sequences (14 out of

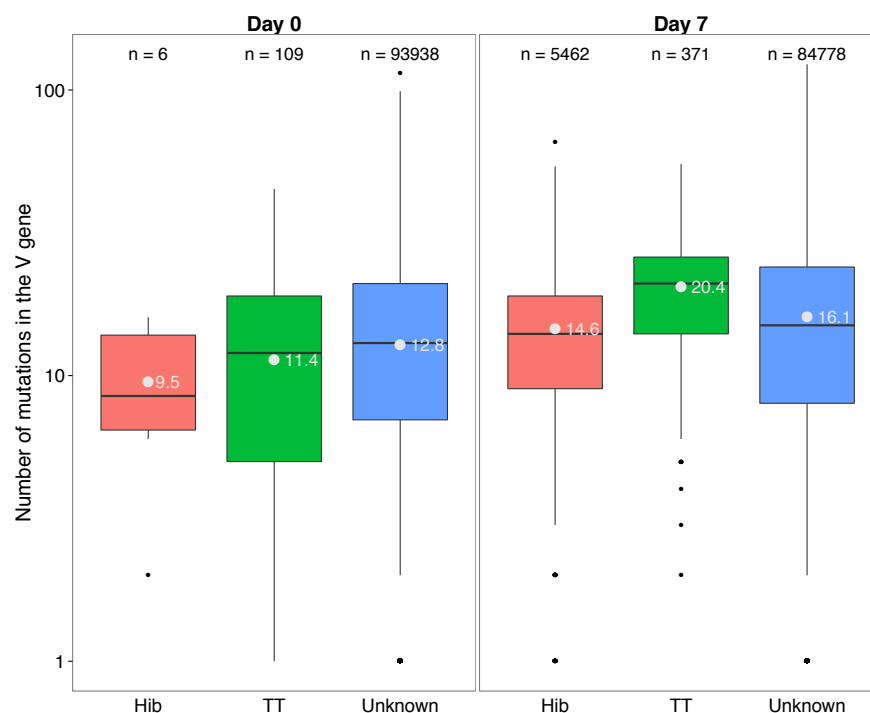


Figure 13.16: Frequency of V gene mutations by antigen specificity.

47, corresponding to 5,206 out of 8,099 (64%) sequences) showed an AA motif previously characterised by single-cell PCR to be specific for the capsular polysaccharide of Hib [117]. Unsupervised clustering of samples containing day 7 shared CDR3 AA sequences distinguished baseline and day 7 samples further indicating that these CDR3 AA sequences were produced in response to vaccination (Figure 13.6).

In addition to previous work, we identified another 9/47 (14%) CDR3 AA motifs closely resembling known Hib motifs, were enriched in post-vaccination samples and showed the characteristic CDR3 length of 10 AA (Figure 13.7). Sequences containing those additional motifs were much rarer in the present study (72/8099 shared sequences) and it is therefore likely that previous attempts using low-resolution techniques may have missed these sequences. It has been previously suggested both in infants [196] and in adults [117] that the majority of Hib-specific sequences are encoded by VH3-23 and JH6. In the current study using a deep-sequencing approach, we were able to show the whole breadth of V (and J) genes utilised in the response to challenge with Hib antigen (Figures 13.8 and 13.9).

We used the Hib-model as proof-of-concept to evaluate approaches for data analysis. For many vaccines, alternative immunogenicity outcomes are desirable, either because laboratory tests are too variable, too difficult or time consuming to perform on a large scale or are not available. We have explored this possibility by using high-throughput B cell receptor sequencing data in combination with a functional im-

Table 13.2: Known antigen-specific CDR3 AA sequences that are found with minor changes in the day 7 samples, are shared by at least 2 participants.

CDR3 AA sequence	Antigen	Reference	AA length	No. of (related) sequences at V1	No. of (related) sequences at V2	No. of participants with (related) sequences at V1	No. of participants with (related) sequences at V2
CTTGGGVG	Hib	[183]	8	3	107	2	3
CTTGGAAG	Hib	[183]	8	2	3	2	3
CVKGYGMDV	Hib	[183]	9	3	5144	3	3
CAKYGFD	Hib	[183]	8	8	3007	3	3
CAKYGMD	Hib	[183]	8	15	5337	3	3
CAKYGMDVW	Hib	[183]	10	6	5309	3	3
CTKDYGMDVW	Hib	[192]	10	0	276	0	3
GYGFD	Hib	[117]	6	41	42	3	3
GYGMDV	Hib	[117]	6	6236	9907	3	3
GYGMD	Hib	[117]	5	6619	10427	3	3
GYGFD	Hib	[117]	5	3294	8294	3	3
ARHADNWFDP	TT	[182]	10	1	4	1	3
ARQADNWFDP	TT	[182]	10	2	71	2	3
AFTADNWFDP	TT	[182]	10	0	3	0	2
CATGVTLDYW	TT	[176]	10	0	34	0	2
CVTGVTLDDYW	TT	[176]	10	0	10	0	2
CATGFTLDYW	TT	[176]	10	0	34	0	2
CATGVTPDYW	TT	[176]	10	0	10	0	2
CATGITLDYW	TT	[176]	10	0	41	0	2
CARDYFGSGSVYYFDYW	TT	[181]	17	0	22	0	3
CARDYFGSGSIYYFDYW	TT	[181]	17	0	30	0	3
CASGNTLDYW	TT	[181]	10	0	17	0	2
CATGRILDYW	TT	[181]	10	0	35	0	2
CARSVPATRAFDW	TT	[181]	15	0	2	0	2
CARQTDNWFDPW	TT	[181]	12	10	80	2	3
CARDYFGSGPIYYFDHW	TT	[181]	17	0	5	0	2
CARDYYGSGSHYYFDYW	TT	[181]	17	0	14	0	3
CASGSTDYW	TT	[181]	10	0	21	0	2
CATGNTLDYW	TT	[181]	10	0	46	0	2
CTSGVTFDYW	TT	[181]	10	2	10	1	2
CARRHYCSSTSCYDAFDIW	TT	[181]	19	0	3	0	3

Hib, *H. influenzae* type b; TT, tetanus toxoid

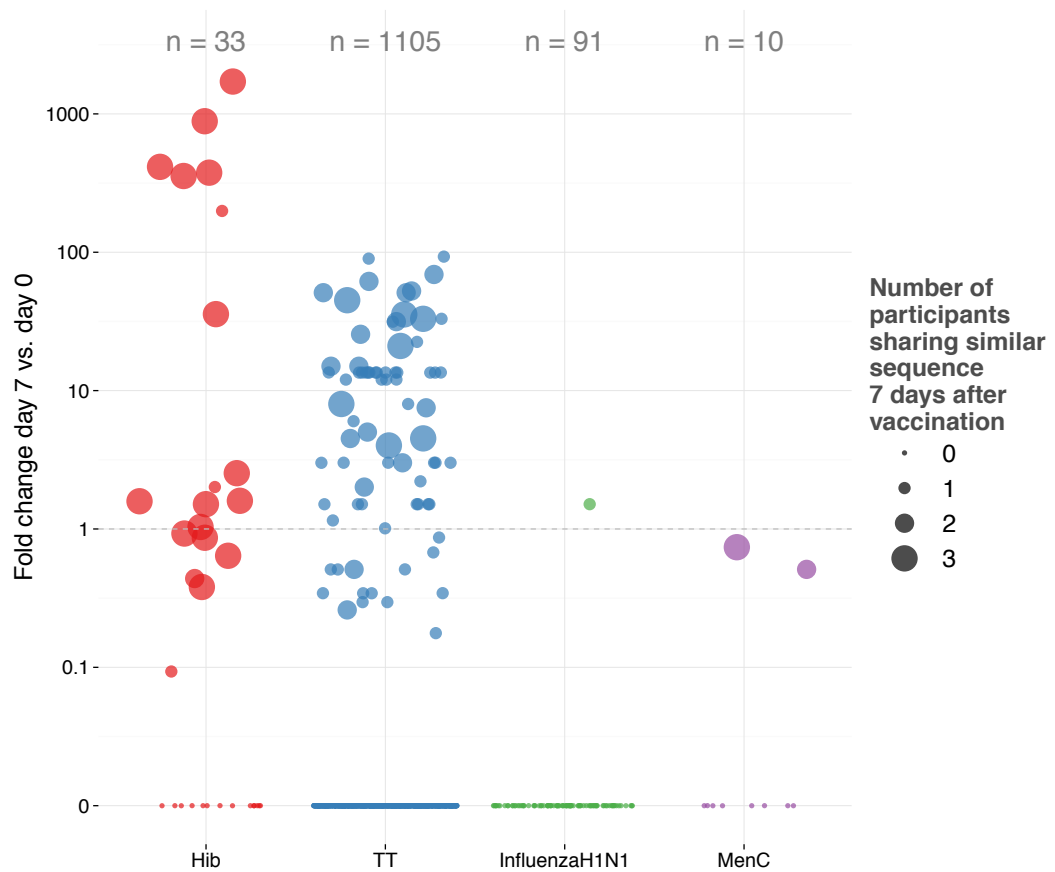


Figure 13.17: Fold change in frequencies at day 7 compared to baseline for sequences that were closely related to previously described to be specific for Hib, TT, H1N1 influenza and MenC and number of participants sharing these sequences.

munogenicity outcome of the response to Hib vaccination. We found that the proportion of Hib-specific sequences within in each sample correlates very well with anti-Hib avidity indices but not with total anti-PRP antibody concentration (Figures 13.14 and 13.15). This meant that it would potentially be possible to predict vaccine immunogenicity 1 month after immunisation by obtaining the proportion of antigen-specific sequences 1 week after vaccination. For IgG sequences there was no difference between plasma cell samples and total B cell samples so this approach may be possible by using total B cells as the starting material and PCR amplification of IgG sequences. Interestingly, not only the relative number of Hib-specific sequences but also the number of different Hib-specific CDR3 AA sequences correlated well with the anti-Hib avidity index 1 month post-vaccination. Thus, the expansion of closely related sequences sharing a similar length (in this case 10 AA) seems to be a feature of a more pronounced immune response and may therefore serve in the future as a further characteristic to identify 'good responders'.

Hib-specific sequences were mainly IgA₂ and IgG₂ sequences (Figure 13.11), which is consistent with previous work demonstrating that in IgG₂ anti-Hib antibodies are the predominant IgG subclass in the adult sera [291]. Sequences that were identified as TT-specific by being related to previously known TT sequences were enriched for IgA₁ and IgG₁ sequences post-vaccination (Figure 13.13), which is in line with published data [292–294] further confirming the validity of this approach.

In the present study, we expanded our sequence analysis aiming to identify and characterise sequences targeting the tetanus toxoid protein contained in the vaccine given to study participants. Only 1 of 47 shared CDR3 AA sequences were identical to known TT-specific sequences. However, by comparing our dataset to 3 different published sources [176, 181, 182], we identified many more sequences closely resembling TT-specific sequences (Table 13.2). Comparing to another dataset of 91 H1N1 influenza-specific sequences [178, 286], almost none of our sequences were detected, either before and after vaccination (Figure 13.17).

Mutational analysis of Hib- and TT-specific sequences within our dataset revealed that in general TT sequences are more mutated than Hib sequences. This may be the reason for the much more dominant Hib-response with Hib sequences being closer to germline rearrangement and therefore being potentially also generated from naive B cells. V gene mutations increased significantly for all specificity between baseline and post-vaccination samples further indicating that these sequences are in fact generated by vaccine-containing antigens.

13.5 CONCLUSION

Using high-throughput sequencing of the heavy chain B cell repertoire in the peripheral blood of participants following immunisation with a Hib-MenC-TT glycoconjugate vaccine we were able to identify Hib- and TT-specific sequences as demonstrated by (1) convergence of CDR3 AA sequences, and (2) similarity of CDR3 AA sequences to previously published work. We linked Hib-specific CDR3 AA frequencies to functional anti-Hib antibody data. Further characterisation of Hib-specific sequences showed that diversity of immunoglobulin gene rearrangements is much greater than previously recognised and confirmed that only a limited number of mutations are needed for successful generation of functional antibody [184]. Future work will be needed to directly confirm the structure and functionality of identified antigen-specific sequences and to link frequency of antigen-specific clones with immunogenicity in a larger population.

Part IV

RESEARCH SUMMARY

DISCUSSION

The aims of the work presented in this thesis were to investigate in detail the immune response to pneumococcal vaccines given to children and adults focussing on B cells. Although in wide-spread use, it is still not entirely understood how (pneumococcal) conjugate and polysaccharide vaccines stimulate the immune system, which B cell subsets are involved and important in the response to these vaccines and what determines an effective and long-lasting immune response. It is important to deepen our understanding of the biology underlying vaccine response in order to identify factors that predict and determine strong and long-lasting immunity to those vaccines and design the next generation of vaccines. In this thesis there is also an exploration of methods to analyse the antibody repertoire of B cells by use of next-generation sequencing methodologies. For the large sequencing datasets to be analysed, a solid bioinformatic knowledge had to be acquired and this thesis was also used to develop alternative ways of analysing pre- and post-vaccination antibody repertoires and visualisation techniques for the complex datasets.

THE MEMORY B CELL ELISPOT IS A ROBUST ASSAY

The [ELISpot](#) assay is used in vaccine studies for the quantification of antigen-specific B_{MEM} , and may be carried out using cryopreserved samples. The effects of cryopreservation on B_{MEM} responses and the repeatability of the cultured [ELISpot](#) assay when carried out by different operators or laboratories are unknown. In this study, blood was taken from healthy volunteers, and a cultured [ELISpot](#) assay was used to measure the frequency of B_{MEM} specific to two routine vaccine antigens (diphtheria and tetanus toxoid). Samples were processed by operators from two different laboratories, and results were assessed for intra- and inter-operator variability, inter-laboratory variation and the effects of cryopreservation. B_{MEM} frequencies were highly correlated when comparing fresh and frozen samples processed by the same operator, and were also well correlated when comparing two operators in the same laboratory. Results were more variable when samples were processed by operators from different laboratories, however repeatability was still acceptable. Following problems with transportation, cell viability was reduced in some cryopreserved samples; however B_{MEM} frequency could still be reliably measured. These results demonstrate repeatability of the [ELISpot](#) assay across operators and

laboratories, and support the use of cryopreserved samples in B_{MEM} studies.

However, for the B_{MEM} ELISpot assay to be used more routinely in multi-centre studies, several challenges remain. Only few clinical study sites have access to a laboratory with the required equipment and skilled staff for PBMC separation, which also needs to be performed in a timely manner after the blood has been taken further complicating the timing and logistics of study visits. In addition, if frozen cells were to be transported to a centralised facility for all testing to be carried out in a single laboratory, the samples would have to be stored in liquid nitrogen, which is again not frequently available at many study sites. Given these remaining challenges, use of the B_{MEM} ELISpot on a large scale and especially in paediatric multi-centre studies will probably still be limited to few occasions and research groups with an interest in B cell biology.

PRE-SCHOOL CHILDREN APPEAR TO MAINTAIN PROTECTIVE PNEUMOCOCCAL SEROTYPE-SPECIFIC (FUNCTIONAL) ANTIBODY

Young children frequently carry pneumococci in the nasopharynx [10] and are thought to be the main transmitters of pneumococci throughout the population including to other young children and the elderly [231]. This in turn may result in disease in these populations. Maintaining adequate (functional) antibody levels in pre-school children is the key to sustained direct protection of these vaccinated individuals, as well as blocking transmission of the organism to achieve herd immunity. Both are presumed to be mediated by antibody. Pneumococcal conjugate vaccines are highly effective in preventing carriage of the serotypes contained in the vaccine and thus disease [27]. Previous immunogenicity studies showed that with the currently used infant immunisation schedules, immunity to pneumococcal serotypes wanes rapidly during the 2nd year of life [232, 233]. In a previous UK multi-centre study children were randomised to receive three doses of PCV-7 or PCV-13 at 2, 4 and 12 months of age. At 13 months of age, most of these children had pneumococcal serotype-specific IgG concentrations ≥ 0.35 $\mu\text{g/ml}$ and OPA titres ≥ 8 . Children who had participated in the original study were enrolled again at 3.5 years of age. Persistence of immunity following infant immunisation with either PCV-7 or PCV-13 and the immune response to a PCV-13 booster at pre-school age were investigated. In total, 108 children were followed-up to the age of 3.5 years and received a PCV-13 booster at this age. For the 7 serotypes shared by both vaccines, most of the children in both groups had IgG concentrations and OPA titres above the presumed protective thresholds for serotypes 6B, 9V, 14, 19F, and 23F. For serotypes 4 and 18C, antibody persistence was lower. A large proportion of the PCV-13 recipients preserved (functional) an-

tibodies against 3, 6A, 7F, and 19A whereas in this group, persistence was lower for serotypes 1 and 5. Interestingly, a high proportion of participants who had not been vaccinated with these additional serotypes (PCV-7 group), had not only developed protective (functional) antibodies against the 2 cross-reactive serotypes 6A and 19A but also against serotype 7F and, to some extent, serotype 3 (Figure 10.4).

The aim of this study was to assess the persistence of serotype-specific (functional) antibodies up to the age of 3.5 years following routine infant immunisation. Although these data suggest that despite some decline in antibody from 13 months of age, a majority of pre-school children maintain protective serotype-specific antibody concentrations, several questions remain. In addition to generation of antibody by vaccination, serotype-specific immunity is confounded by colonisation events frequently and repeatably occurring in young children. The serotype distribution of circulating pneumococci undergoes temporal changes influenced by various factors including vaccine introduction, antibiotics prescribing as well as other unknown factors [295]. Thus, with pneumococcal colonisation affecting anti-pneumococcal antibody concentrations, changes in carriage rates occurring following widespread use of multivalent vaccines will also affect antibody persistence. There are no studies reporting on antibody persistence before and after routine implementation of pneumococcal conjugate vaccination into the national immunisation schedule. Preliminary data from our own group suggests that antibody persistence is indeed reduced following routine PCV vaccination. Following PCV-13 immunisation at 2, and 4 months of age, persistence of serotype-specific IgG concentrations against PCV-13 serotypes was assessed in children recruited to the study described in chapter 10 and was then compared to the same measurements in children who were recruited in 2011/12 at 12 months of age. Although possibly confounded by the different laboratories performing the assays, a marked reduction of the proportion of participants with IgG concentrations ≥ 0.35 $\mu\text{g/ml}$ against the majority of the tested serotypes was seen in the later study (Figure 14.1).

As pneumococcal disease incidence is linked to circulation of disease-causing strains [29], this decrease in antibody persistence may not be relevant on a population scale in order to achieve vaccine effectiveness although outbreaks may occur through exposure to vaccine strains from outside of the vaccinated population. Direct protection of the individual, however, is likely to be reduced, which may be again more relevant when vaccinated and unvaccinated populations are mixed, e.g. a child from the UK travelling into a country without a pneumococcal vaccine programme.

Another interesting piece of information obtained in this study is the kinetics of functional antibodies in PCV-7 recipients against serotypes that are only included in PCV-13. As already discussed in chapter

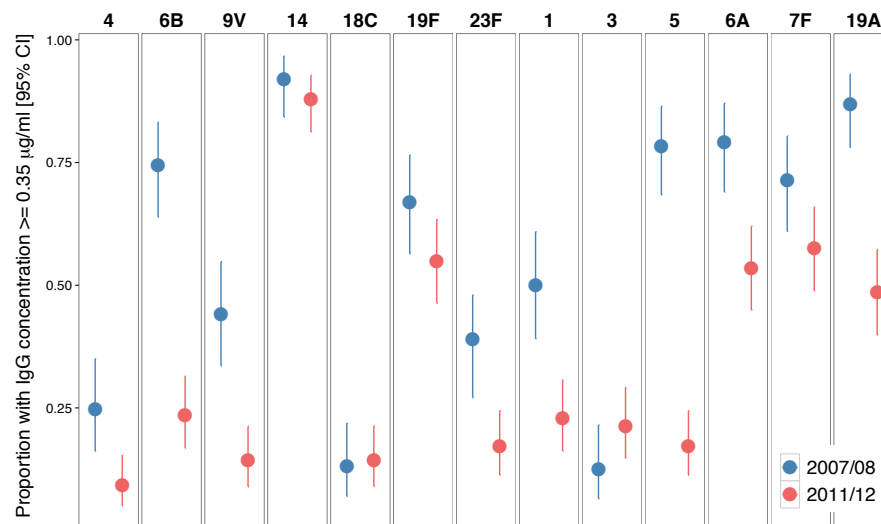


Figure 14.1: Proportion of participants with serotype-specific IgG concentrations equal or above $0.35 \mu\text{g/ml}$ following infant immunisation with PCV-13 at 2, 4 and 12 months of age measured in different calendar years. Shown are data of participants in the study described by Snape et al. (samples collected in 2007-08) [234] in blue and preliminary data from a study undertaken in 2011-12 (J. Trück, unpublished) in red.

10, OPA titres against serotypes 1 and 5 follow a coherent pattern with very low titres throughout early childhood until the pre-school PCV-13 booster is given (Figure 14.2). This picture is consistent with low carriage rates of both serotypes before and after PCV-7 introduction in the UK [9, 245] and lack of protection against these two serotypes in PCV-7 recipients [31].

OPA titres against the two cross-reactive serotypes 6A and 19A follow a different pattern. Including the data from the earlier study time points of our study demonstrates that the immune response against these 2 serotypes was greatly boosted by the PCV-7 dose given at 12 months of age (Figure 14.3). These serotype-specific antibody titres were possibly enhanced and maintained by high carriage rates that are usually found before and after PCV-7 introduction [245, 248]. Intriguingly, despite these relatively high and persistent functional antibodies, PCV-7 vaccinated children are at increased risk for serotype 19A carriage and disease compared to the pre-vaccine era [239, 248]. Several reasons may account for this discrepancy between *in vitro* and *in vivo* data as discussed in section 10.3.1.

A very interesting information arising from the analysis of this study are the kinetics of antibody directed against serotypes 3 and 7F. As shown in Figure 14.4, antibodies against these 2 serotypes increased over time irrespective of vaccination leading to similar antibody titres at 40 months of age in PCV-7 and PCV-13 recipients. This increase of functional antibodies appeared despite low carriage rates

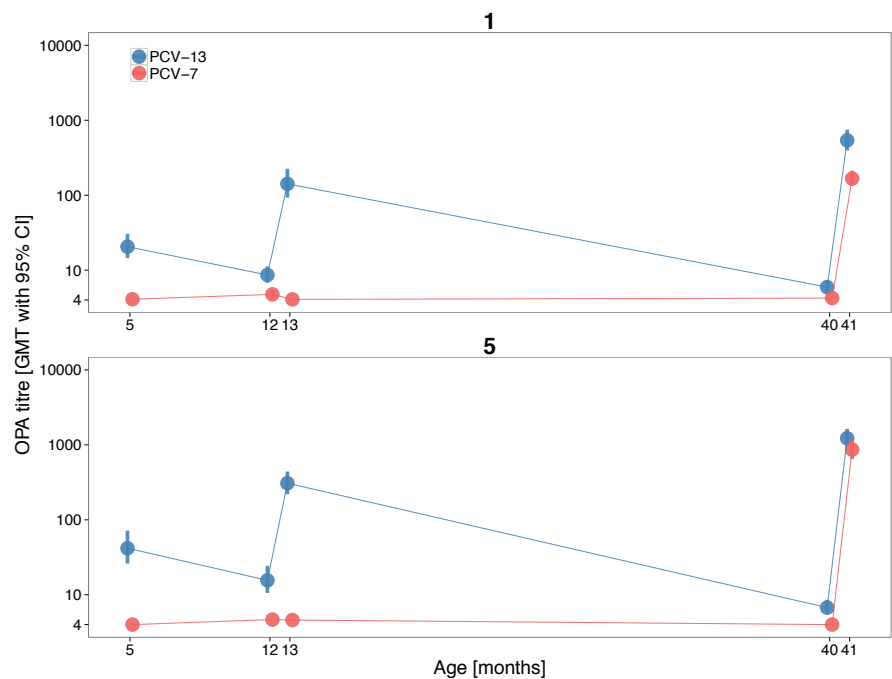


Figure 14.2: Kinetics of OPA GMTs against serotypes 1 and 5 throughout early childhood in children who participated in the follow-on study.

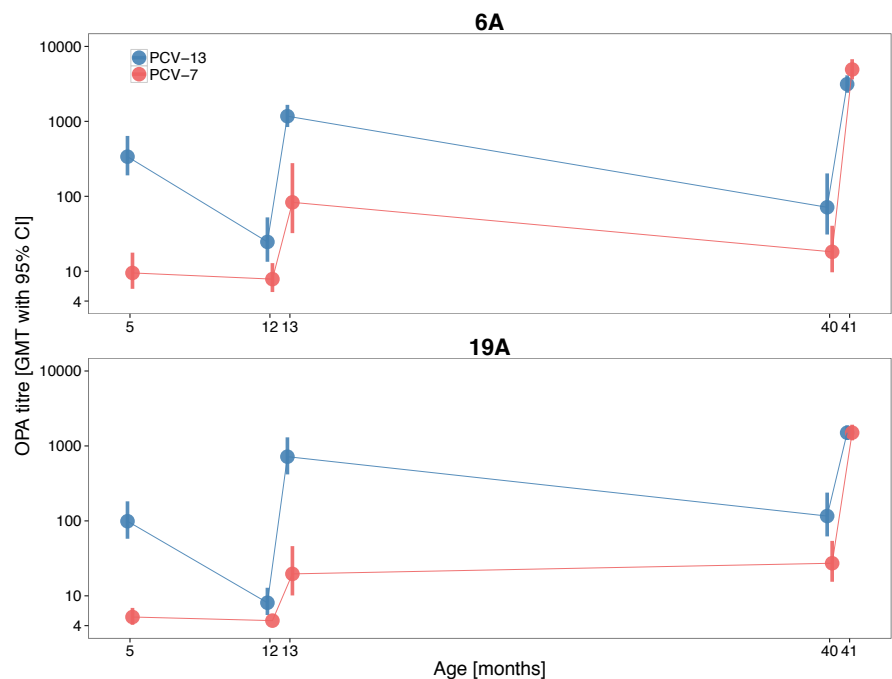


Figure 14.3: Kinetics of OPA GMTs against the cross-reactive serotypes 6A and 19A throughout early childhood in children who participated in the follow-on study.

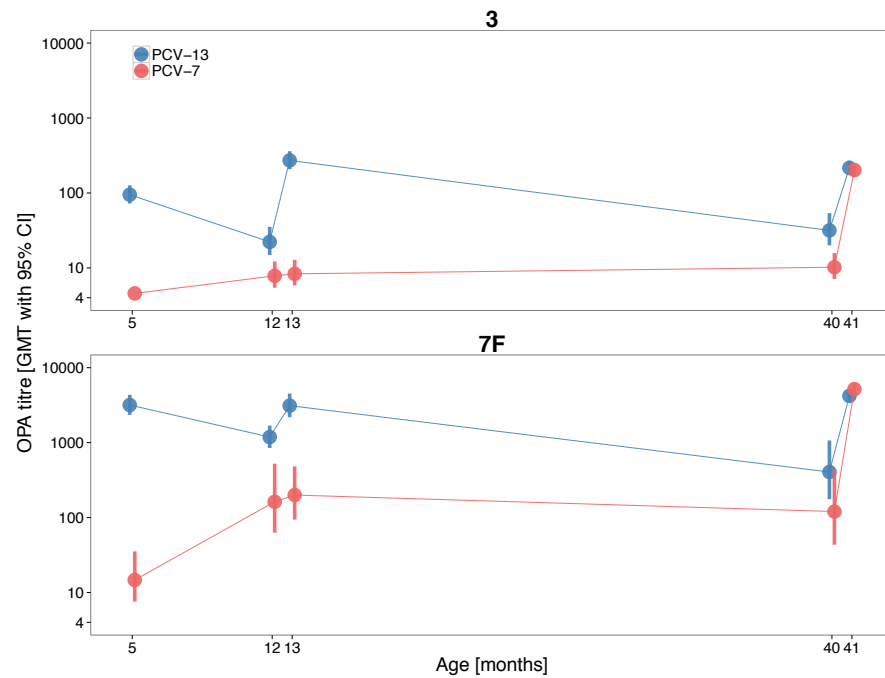


Figure 14.4: Kinetics of OPA GMTs against serotypes 3 and 7F throughout early childhood in children who participated in the follow-on study.

before and after routine PCV-7 immunisation [245]. Although the excellent boostability of PCV-7 vaccinated participants at pre-school age suggests that these individuals were primed with the polysaccharide antigens of serotypes 3 and 7F through natural exposure, these *in vitro* functional antibodies don't appear to be effective in preventing disease [31, 239].

These data show that cross-reactive or naturally acquired antibodies appear to be functional *in vitro* as measured by opsonophagocytosis but don't seem to be protective *in vivo*. Further, OPA responses to a pre-school PCV-13 booster are very similar irrespective of the vaccine these children were primed with. More work needs to be done to understand the link between disease susceptibility, carriage and vaccination at different ages and using different vaccine schedules in order to determine the risk of pneumococcal disease both on a population and an individual level. This knowledge may then be used to define the optimal and most cost-effective vaccination strategies.

STRONG AND RAPID ACTIVATION OF PNEUMOCOCCAL SEROTYPE-SPECIFIC MEMORY B CELLS IN PRESCHOOL CHILDREN FOLLOWING PNEUMOCOCCAL CONJUGATE VACCINATION

Direct vaccine-induced host immunity to pneumococcal disease is not only determined by anti-polysaccharide antibodies but also by the in-

duction of immune memory. There is limited information about the role of B_{MEM} in maintaining antibody levels following infant vaccination. In the same study of healthy preschool children who had participated in a previous randomised trial (see 10), we studied antigen-specific B_{MEM} in children 2.5 years after infant immunisation with PCV-7 or PCV-13 and following a booster dose of a PCV-13. Blood was taken before and 1 month after the booster dose. B_{MEM} were quantified using a cultured ELISpot assay for serotypes 1, 3, 4, 14, 19A, 23F and diphtheria and tetanus toxoid as controls. In total, 98 blood samples were available for analysis. Numbers of B_{MEM} specific for serotypes included in both the PCV-7 and PCV-13 (4, 14, 23F) were low at baseline and did not differ between the groups (except for serotype 14: higher in the PCV-13 group). B_{MEM} numbers post-booster were higher than baseline for these 3 serotypes with no differences between the groups. For serotypes only included in PCV-13 (1, 3, 19A), baseline B_{MEM} numbers were lower in the PCV-13 group for all 3 serotypes (significant only for serotype 3). However, post-booster B_{MEM} frequencies were similar for serotypes 1, 3, 19A in both groups. A strong inverse correlation was found between B_{MEM} at baseline and antibody concentrations / OPA titres following booster vaccination for serotype 3 but not for other serotypes. A PCV-13 pre-school booster results in similar post-booster B_{MEM} frequencies in individuals irrespective of prior priming with PCV-7 or PCV-13 for serotypes 1, 3 and 19A despite lower pre-booster B_{MEM} frequencies in the unprimed group. A single dose of PCV-13 given at preschool age is likely to be sufficient to induce immune memory to pneumococcal serotypes in individuals, who were previously not vaccinated against these antigens.

Although the cultured ELISpot is a robust assay and can be reliably applied to clinical trials in children by using small blood volumes, it suffers from the limitation that B_{MEM} can only be easily measured in the peripheral blood. In contrast to antibodies, which are secreted into the serum as their site of action, B_{MEM} as measured by the cultured ELISpot assay only pass the blood on their way to the secondary lymphoid tissues where they reside and meet their antigen. Hence, there is a smaller window of opportunity to measure B_{MEM} compared with antibody concentrations (see e.g. Figures 12.14 and 12.15) and the number of B_{MEM} measured at a given time point may not be representative of the total B_{MEM} output generated by vaccination.

In humans, it may never be possible to correlate B_{MEM} frequencies with vaccine efficacy, therefore B_{MEM} numbers as e.g. measured by the cultured ELISpot have always be related to another surrogate or correlate marker of protection such as antibody concentration. As an alternative outcome to infer protection without the need for undertaking large-scale efficacy studies, it may be helpful to determine B_{MEM} frequencies in combination with assessing pneumococcal carriage rates in future studies.

SEVERAL B CELL SUBSETS FOLLOW DISTINCT PATTERNS IN RESPONSE TO PNEUMOCOCCAL VACCINATION

Although immune responses in animals (mainly mice) have been investigated in detail, there are conflicting views on how vaccine antigens are processed and which cell types are involved in the immune response to vaccines in humans [111, 296]. Being a more controlled and 'cleaner' system, mice are genetically more similar to each other, have not been exposed to a variety of antigens at various time points in the past, and have a much shorter life span. In addition, only female mice are generally used in research as male mice are too difficult and expensive to be kept in cages. All these factors may contribute to the observed differences in immune responses in human vs. mice and emphasises the need for an in-depth study of human immune responses. There are, however, several obstacles to perform research in humans. The peripheral blood is usually the only site from which immune cells can be obtained whereas differentiation, proliferation and mutation of e.g. B cells occur in secondary lymphoid organs. The kinetics of vaccine immune responses in humans have also been rarely studied due to inherent difficulties in obtaining sequential blood samples.

I tried to tackle some of these questions in a study presented in this thesis by investigating in detail the kinetics of various B cell subsets in response to vaccination with either a conjugate or a polysaccharide vaccine. This study showed that several B cell subsets follow distinct patterns in the immune response to both vaccines without major differences between the participants and groups studied. Plasma cells ($CD19^+CD20^-CD27^{++}$) peaked at day 8 following vaccination with a previously unknown secondary plasma cell peak at around day 18-26 potentially reflecting re-circulating plasma cells that leave the lymph nodes. Marginal zone B cells are thought to play an important role in anti-polysaccharide TI responses in humans [118]. In this study this B cell subset showed a biphasic course with peaks at day 8 and in the 4th week after vaccination in both vaccine groups (Figure 12.5) indicating that MZB indeed are being stimulated by the polysaccharides contained in both vaccines, acting as TI antigens. In contrast to the MZB kinetics, there were divergent kinetics of switched B_{MEM} in the first month after vaccination. In the PCV-13 group, this cell population decreased in the first 3 weeks and then showed a peak in the fourth week post-vaccination whereas the relative frequency of switched B_{MEM} remained constant in the 2 individuals who had received PPV-23 (Figure 12.7). Finally, the kinetics of IgM B_{MEM} were again not different between the two vaccine groups but showed a very distinctive relative increase after vaccination peaking at around day 20-26 in all study participants (Figure 12.6). This finding is consistent with the results of a recent study investigating antigen-specific

B cells using a similar labelling technique [261]. IgM B_{MEM} also follow a similar kinetic to B_{MEM} as measured by ELISpot, it is therefore possible that a substantial proportion of cells measured in the ELISpot assay are derived from this B cell subset and express and secrete IgG antibodies following class-switching in culture.

There is a remarkable consistency in the kinetics of some of the B cell subsets with only little variation between individuals. Given the different ages, the timing and extend of exposure the 4 study participants must have had to the vaccine antigens, it is somewhat surprising to find such a uniform plasma cell peak at day 8 post-vaccination. Also, the type of vaccine had no impact on the timing of this peak suggesting that the stimulation, proliferation and circulation in the peripheral blood of plasma cells is 'programmed' rather than it being dependent on other factors such as the way the vaccine antigens are presented and processed (TI vs. TD).

A novel antigen-labelling technique was used in this study and worked well especially for plasma cells / blasts (Figure 12.9) confirming the findings seen in the total plasma cell kinetics. Similar to previous work in children using the same labelling method (J. Trück, data not shown), total antigen-specific B cells decreased over time following vaccination (Figure 12.8). Although it is possible that disappearance of these cells from the peripheral blood represent homing to secondary lymphoid tissues, methodological issues have to be considered and need to be explored in the future. Although specificity of the labelling technique to polysaccharide and protein antigens has been demonstrated to some extent (see chapter 8), there is likely to be non-specific binding of B cells either by binding of the labelled antigen through broadly specific B cell receptors or by binding to other receptors on the B cell surface. At baseline, the proportion of antigen-binding B cells was 3-7% for most of the antigens tested (Figure 12.8), which seems far too high for an unstimulated B cell population. In addition, in B cell subsets that showed consistent relative changes following vaccination (such as MZB or IgM B_{MEM}), the proportion of antigen-binding cells decreased over time similar to the kinetics seen in total antigen-binding cells further suggesting that the number of B cells that bind non-specifically is greater than the number of 'real' antigen-specific B cells binding with their antigen-specific BCR. For plasma cells / plasmablasts, a different pattern was seen. At baseline, very few plasma cells were positively labelled and the percentage of antigen-binding cells increased greatly after vaccination with a clearly defined positive population in the FACS plots (Figure 14.5). Figure 14.6 shows the percentage of plasma cells that were identified as positive for serotypes 1, 3, 9V, 18C and 23F as well as diphtheria and tetanus toxoid. Of note, the percentage of plasma cells that were positive for diphtheria and tetanus toxoid remained low in individuals who had not been immunised with a diphtheria and tetanus toxoid-containing

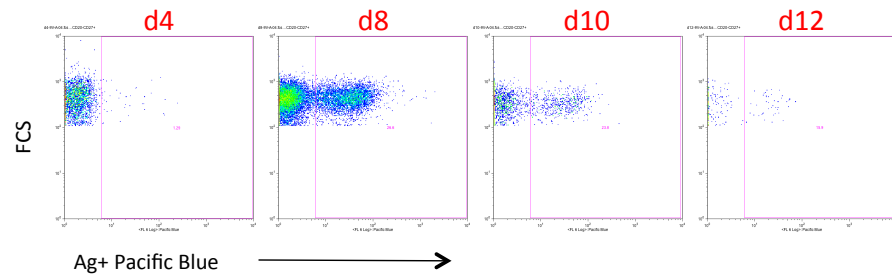


Figure 14.5: Examples of FACS plots showing the frequency of 9V-positive CD19⁺CD20⁻CD27⁺⁺ plasma cells (x-axis) vs. forward scatter (y-axis) at days 4, 8, 12, and 12 following vaccination.

vaccine (tetanus toxoid for both groups and diphtheria toxoid for the two PPV-23 recipients, respectively, Figure 14.6). The peak of the percentages of antigen-binding cells was generally on day 6 rather than day 8 following vaccination and therefore different to the actual number of total and antigen-positive plasma cells as shown in Figures 12.4 and 12.9.

Overall, these data suggest that the labelling method is most helpful in B cells with a lower density of B cell receptors (such as plasma cells / blasts) and probably also in B cell populations in which the peak to background ratio of antigen-positive cells is highest. Further works needs to be done in future studies including a greater number of participants in order to reduce inter-individual variation.

PNEUMOCOCCAL CONJUGATE OR POLYSACCHARIDE VACCINATION RESULT IN DIVERGENT GENE EXPRESSION PROFILES

Previous work has shown that pneumococcal conjugate vaccines have superior immunogenicity over PPV-23 and that in adults, administration of PPV-23 prior to PCV-7 resulted in significantly attenuated antibody concentrations compared with PCV-7 alone [297]. It has further been demonstrated that PCV-7 induces strong serotype-specific B_{MEM} responses in peripheral blood whereas immunisation with PPV-23 results in a decrease in B_{MEM} frequency [209]. These observations are consistent with plasma cells being rapidly generated by the TI polysaccharide vaccine via the extra-follicular pathway, while both B_{MEM} and plasma cells are generated through a germinal centre pathway by the TD conjugate vaccine highlighting the different ways these two vaccine stimulate the human immune system. Beyond work performed in mice, there are few data available investigating the immune responses to polysaccharide vs. conjugate vaccines in humans. As examined by genome wide analysis of gene expression (see chapter 12), two different time-dependent signatures were found in response to vaccination showing early innate immune signals at 24 hours and adaptive signals coinciding with the plasma cell peak at day 8 (Figure

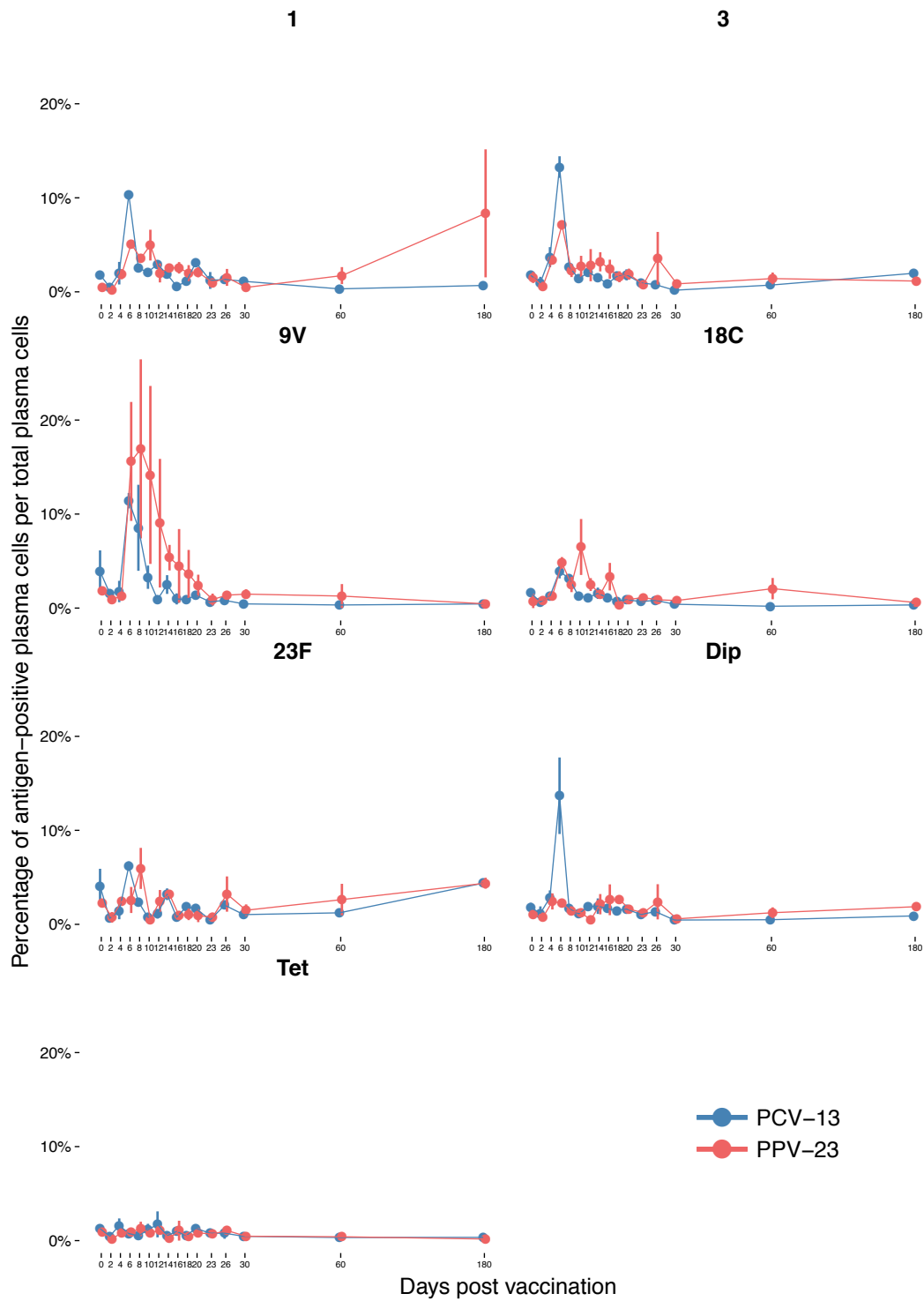


Figure 14.6: Kinetics of antigen-positive plasma cells as a percentage of total plasma cells for all tested antigens. Dip, diphtheria toxoid; Tet, tetanus toxoid

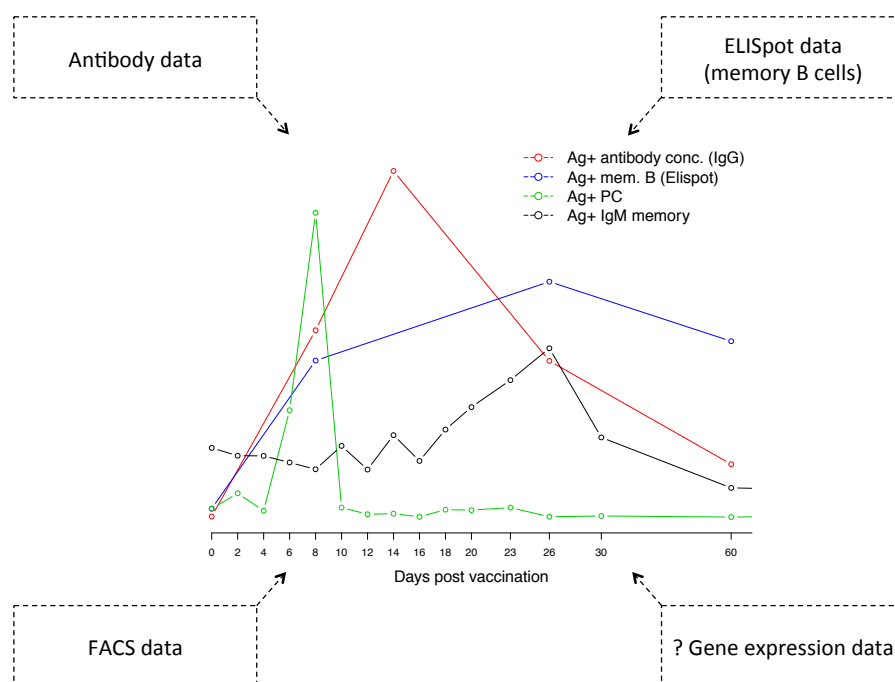


Figure 14.7: An example of how integration of different datasets may help to interpret common characteristics.

12.17). In addition, at day 8, marked differences in numbers of differentially expressed genes were seen between the two types of vaccines. Further characterisation of these distinct responses may be possible by examining the pathways of the divergent transcriptional profiles elicited by each vaccine.

INTEGRATION OF DIFFERENT IMMUNOLOGICAL DATASETS

Comparisons of the data obtained in the B cell kinetics study (see chapter 12) including the kinetics of serotype-specific antibody concentrations, B cell subsets (determined by ELISpot and FACS) and gene expression are planned. This approach of combining low- and high-throughput technologies for systems-wide measurements, networks and predictive modelling is also referred to as 'systems vaccinology' [298, 299]. Data integration of the different data types represents one of the major challenges of this approach with including data from several time points before and after vaccination further adding to the complexity of the data analysis (Figure 14.7).

HIGH-THROUGHPUT SEQUENCING ALLOWS IDENTIFICATION OF ANTIGEN-SPECIFIC B CELL RECEPTOR SEQUENCES

High-throughput sequencing of the antibody repertoire in the peripheral blood following vaccination has the potential to provide anti-

gen-specific sequences and may therefore be suitable as an alternative (and probably more accurate) measure of immunogenicity as well as giving in-depth insight into the B cell biology that underlies the immune responses. We explored this possibility by sequencing B cell receptor transcripts before and after vaccination with a vaccine containing capsular polysaccharides from Hib and MenC, individually conjugated to TT carrier protein. We identified a number of known and presumptive novel antigen-specific sequences by searching for heavy chain CDR3 AA sequences that were shared between participants and by comparing sequences with a database of publicly available antigen-specific sequences within the pool of post-vaccination sequences. We further determined the VJ usage, frequency of V gene mutations and isotype subclass information of these sequences and compared these measures between sequence populations. The anti-Hib avidity index (a measure of antibody function) that was measured in the same individuals 1 month after vaccination was strongly correlated with the relative frequency of Hib-specific sequences indicating that investigating the public post-vaccination antibody repertoire may indeed be useful as a measure of vaccine immunogenicity and has the potential to be used on a larger scale in clinical vaccine trials. Our approach of searching for CDR3 AA sequences that are shared between individuals having undergone the same intervention helps enormously in analysing these complex datasets and is of potential use in many other circumstances in which pre- and post-interventional samples are available, or a group of patients with a similar condition can be compared to healthy individuals.

Several studies have assessed the antibody repertoire before and after vaccination by using next-generation sequencing technology (Table 5.2). Despite the increasing number of studies of the antibody repertoire, there is little mention of the application of these data, with most publications instead focusing on methods development [176, 300]. Perhaps the most obvious use of antibody sequencing data is the generation of monoclonal antibodies by identification and cloning of BCR sequences of recently generated plasma cells [180, 181, 190]. In addition, antibody sequencing technology could potentially be used for multiple aspects of vaccine development, including measurement of vaccine response, predicting vaccine safety, and guiding the development of more effective vaccines by increasing the understanding of B cell and GC immunology. However, before it can be used as a routine tool, it is necessary to address the confounding effects of genotype, antigen exposure history, and age that may also potentially impact the repertoire, and how it responds to vaccination.

Laboratory and bioinformatic techniques available for studying the antibody repertoire are constantly improving. Major advances in the last year now allow high-throughput identification of paired heavy and light chains [176], quantification of PCR-related bias and muta-

tion [175], and identification of putatively antigen-specific sequences through repertoire convergence [189]. As repertoire sequencing is increasingly used by groups working on vaccine development, it is important that these different methods are synthesised, and a 'gold standard' sequencing protocol agreed so that data generated from different laboratories can be reliably compared. To make full use of available data, the creation of an open-access database for the accumulation of repertoire data needs to be considered. Researchers can then concentrate on how to reliably interpret and make practical use of the resulting data, in the face of the potential confounding factors. Analysis of the post-vaccination antibody repertoire is providing a fascinating view of the B cell response to vaccines. There is a compelling case that the practical application of these data will have a profound affect on the development of new vaccines.

SUMMARY

In this thesis, the immune response to pneumococcal vaccines using a variety of laboratory methods was investigated in detail. By examining the variability of a B_{MEM} ELISpot method, it was shown that this assay is robust and reproducible and can be performed on fresh or frozen samples and in different laboratories. Using this technique, in a study of pre-school children who have been primed with 3 doses of either PCV-7 or PCV-13, it was demonstrated that a PCV-13 pre-school booster results in similar post-booster B_{MEM} frequencies in individuals irrespective of prior priming with PCV-7 or PCV-13 for serotypes only contained in PCV-13 (serotypes 1, 3 and 19A). In the same study, it was shown that antibody persistence against most vaccine serotypes can be expected until the age of 3.5 years and that a pre-school booster with PCV-13 is well tolerated and very immunogenic. A novel antigen-labelling technique was used in a detailed kinetics study of the antigen-specific B cell subsets in response to either PCV-13 or PPV-23 in adults. The results of this study revealed distinct B cell subset response patterns that were observed in all study participants indicating that IgM memory seem to play a major role in the immune response to pneumococcal vaccines. In addition, in the same study, genome wide analysis of gene expression was performed and it was shown that vaccination with either a pneumococcal conjugate or polysaccharide vaccine results in a marked difference in numbers of differentially expressed genes 8 days following vaccination. Finally, analysis of a large dataset of high-throughput B cell receptor sequences revealed convergence of antigen-specific CDR3 AA sequences following vaccination. It was further demonstrated that for sequences directed against the *H. influenzae* type b polysaccharide, diversity of immunoglobulin gene rearrangements is much greater than previously recognised. Frequencies of Hib-specific CDR3 AA sequences were linked with anti-Hib avidity indices highlighting the potential of this method as an alternative (functional) measure of vaccine immunogenicity.

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DECLARATION

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research (unless otherwise specified, also see [Acknowledgments](#)).

University of Oxford, January 2014

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