WADHAM COLLEGE, UNIVERSITY OF OXFORD

B cell responses to conjugate and polysaccharide meningococcal vaccines

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Dr Maheshi N Ramasamy

Trinity 2012

Supervisors: Professor Andrew J Pollard and Dr Dominic F Kelly, Department of Paediatrics

B cell responses to conjugate and polysaccharide meningococcal vaccines

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Dr Maheshi N Ramasamy
Wadham College and the Department of Paediatrics
University of Oxford
Trinity 2012

Abstract

The primary approach to the control of meningococcal disease remains effective vaccination programmes in susceptible populations. Vaccines against serogroups A, C, W and Y offer broad protection against meningococci and both polysaccharide and conjugate quadrivalent vaccines are licensed for use in the UK. Previous studies have assessed the antibody response to meningococcal polysaccharide and conjugate vaccines, but there is limited information on the nature of the B cell response to these antigens. As part of a clinical trial using both polysaccharide (MenACWY-PS) and conjugate (MenACWY-CRM) vaccines in adult volunteers, this DPhil reports the analysis of subsets of antigen specific B-cells produced in response to either vaccine. Prior MenACWY-PS impaired the response to a subsequent dose of MenACWY-CRM. This may be due to MenACWY-PS driving terminal differentiation of antigen specific cells into plasma cells, without replenishment of the memory B cell pool. In addition, despite prior data indicating that it may act as a thymus dependent antigen, the serogroup A polysaccharide component of MenACWY-PS appears to behave in the same way as serogroup C, W & Y polysaccharide components.

Antibody molecules recognise and bind to a multitude of conformational epitopes. This variability is enabled by the complexities of immunoglobulin variable domain gene recombination which can generate a vast potential repertoire of unique antibody molecules. However, the diversity of the antibody repertoire is more restricted against specific antigens and within defined B cell subsets. In this DPhil, 'next generation' sequencing technologies were used to investigate the diversity of the B cell variable domain before and after vaccination of adult volunteers. Individuals at baseline were found to have distinct antibody repertoires. Vaccination with a *Haemophilus influenzae* type b (Hib) conjugate vaccine resulted in an oligoclonal antibody response, with enrichment for Hib specific canonical antibody sequences.

Acknowledgements

I am indebted to Professor Andrew Pollard, for the opportunity to broaden my horizons by pursuing this DPhil at the Oxford Vaccine Group, where I have been exposed to a fantastic scope of scientific experience. I am very grateful for his patient guidance and support through the various highs and lows of the last three and a half years. I would also like to thank Dr Dominic Kelly for his enduring good humour, optimism and common sense as we peeked into the 'black box' of 454 sequencing.

This thesis is based on the results of two clinical studies, and I am very grateful to all the study volunteers who were so generous with their time. I could not have undertaken the clinical studies without the support and hard work of all those at the Oxford Vaccine Group and beyond who were involved in the study conduct. I wish to acknowledge the contribution of the following people:

- Professor Andrew Pollard who designed both studies.
- Dr Geraldine Blanchard-Rohner and Dr Chaam Klinger who drafted the initial study protocols for the ACWY study and the Hib reference study respectively
- Mrs Amanda Sibley who completed the initial regulatory and ethic submissions for the studies
- Dr Elizabeth Clutterbuck, Mrs Jaclyn Bowman, Mrs Amber Thompson, Miss Elizabeth Kibwana and Dr Will Crane who provided technical assistance with laboratory assays
- Miss Kathryn Haworth and Mrs Mushiya Mpelembue who provided nursing assistance during study visits
- Dr Theresa Nickells who provided medical assistance during study visits
- Mrs Rebecca Beckley, Mrs Emma Plestead and Mrs Shirley Ashmore who offered administrative support

- Miss Merryn Voysey, of the Centre of Statistics of Medicine, who performed the ANCOVA analyses
- Novartis Vaccines and Diagnostics, Sienna, who performed the serum bactericidal assays

I would also like to express my gratitude to Dr Matthew Snape for his unwearied support and advice with the complexities of protocol writing and regulatory body applications. I particularly wish to thank Dr Elizabeth Clutterbuck for her infinite patience in explaining the mysteries of immunology, her constant encouragement and her wry sense of humour.

I am grateful to Professor Julian Parkhill and Mr Richard Rance of the Wellcome Trust Sanger Institute, Hinxton, for performing the 454 library preparation and sequencing.

I have been lucky enough to do my DPhil with some inspiring fellow students. Mrs Amanda Sibley provided much-appreciated non-immunological conversations during my time at the OVG. Dr Johannes Trück has also been a good friend and I will always be slightly in awe of his 'bubble plots' on R which he kindly created for me.

The love and support of my family have ensured I have kept my perspective over the last few years. My daughters Apsara and Amrita have been understanding and patient throughout my various absences and the arrival of my son Nibodh resulted in a very happy, albeit completely unproductive, year of my DPhil. My husband Ketan has been the constant star to my wandering bark; I could not have completed this thesis without him.

Finally, I would like to dedicate this thesis to the memory of my mother, Professor Manthri Samaranayake-Ramasamy, who would have been proud.

Table of Contents

List of figures

List of tables

Abbreviations

AE	Adverse event
ANCOVA	Analysis of covariance
bp	Base pair
C (μ,γ,α,δ,ε)	Constant domain (IgM, IgG, IgA, IgD, IgE)
CDR	Complementarity determining region
CI	Confidence interval
D	Diversity gene segment
ELISA	Enzyme linked immunosorbant assay
ELISpot	Enzyme linked immunosorbant spot assay
FACS	Fluorescent activated cell sorting
FAM	Fluorescence activated molecule
FDC	Follicular dendritic cells
FOB	Follicular B cells
FR	Framework region
GMC	Geometric mean count
GMT	Geometric mean titre
GSP	Gene specific primers
Hib	*Haemophilus influenzae* type b
HIV	Human immunodeficiency virus
hSBA	Human complement source serum bactericidal activity
Ig(G,M,A,D)	Immunoglobulin (G,M,A,D)
IGMT®	International Immunogenetics Information System® http://www.imgt.org
IM	Intramuscularly
ITT	Intention to treat
J	Joining gene segment
LOS	Lipooligosaccharide
MACS	Magnetic activated cell sorting
Men(A,C,W,Y)	Meningococcal serogroup (A,C,W,Y)
MenACWY-CRM	Protein–polysaccharide conjugate meningococcal A, C, W-135 and Y vaccine
MenACWY-PS	Plain polysaccharide meningococcal A, C, W-135 and Y vaccine
MID	Multiplex identifier
min	Minutes
mL	Millilitre
NRT	No reverse transcription
NT	No template
PCV	Pneumococcal conjugate vaccine
PP	Per-protocol
PPV	Pneumococcal polysaccharide vaccine
PRP	Polyribositol phosphate
q-PCR	Quantitative polymerase chain reaction
RAG (1,2)	Recombinase activating gene
rpm	Revolutions per minute
rSBA	Baby rabbit complement source serum bactericidal activity
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds

SAE	Serious adverse event
SBA	Serum bactericidal activity
SC	Subcutaneously
SD	Standard deviation
Siglec	Sialic acid binding Ig-like lectin
TD	Thymus dependent
TdT	Terminal deoxynucliotidyl transferase
TI	Thymus independent
TLR	Toll-like receptors
V	Variable gene segment
V(H,λ,κ)	Variable domain of (heavy, lambda, kappa) chain

Chapter 1 – Introduction

Meningitis is an inflammation of the protective membranes encasing the central nervous system, the dura mater, arachnoid mater and pia mater, collectively termed the meninges. Infectious meningitis is largely caused by bacteria, viruses or fungi. This thesis concerns vaccines against meningitis caused by *Neisseria meningitidis*, also called the meningococcus, which is unique among causes of bacterial meningitis in its ability to cause epidemic disease. Clinically, the disease presents with a fever, headache, neck stiffness and altered consciousness, and additionally in the case of meningococcal meningitis, a purpuric rash.

1.1.Microbiology of *Neisseria meningitidis*

1.1.1. The first descriptions of meningitis

The word 'meningitis' was first used by the army surgeon Herpin in 1803 [1] to describe an inflammation of the meninges which he observed to occur some days after an open skull fracture. In 1806, the Swiss physician Vieusseux gave the first clear account of an epidemic of meningitis in Geneva [2]. The disease was marked by the sudden onset of headache, vomiting and delirium and in many instances was fatal within 24 hours. Most cases occurred within the same household and were in children or young adults. In the same year, Danielson and Mann described a similar outbreak of disease in Medfield, Massachusetts, which was termed a 'spotted fever' [3].

Following the widespread acknowledgement of Koch's postulates in 1884 [4], several physicians attempted to describe the causal agent of epidemic meningitis. However, it was not until 1887 that Weichselbaum accurately identified what was then termed the *Diplococcus intracellularis* from the cerebrospinal fluid of 6 out 8 patients who had died of meningitis [5]. In 1906, during an epidemic of meningitis in New York, the American

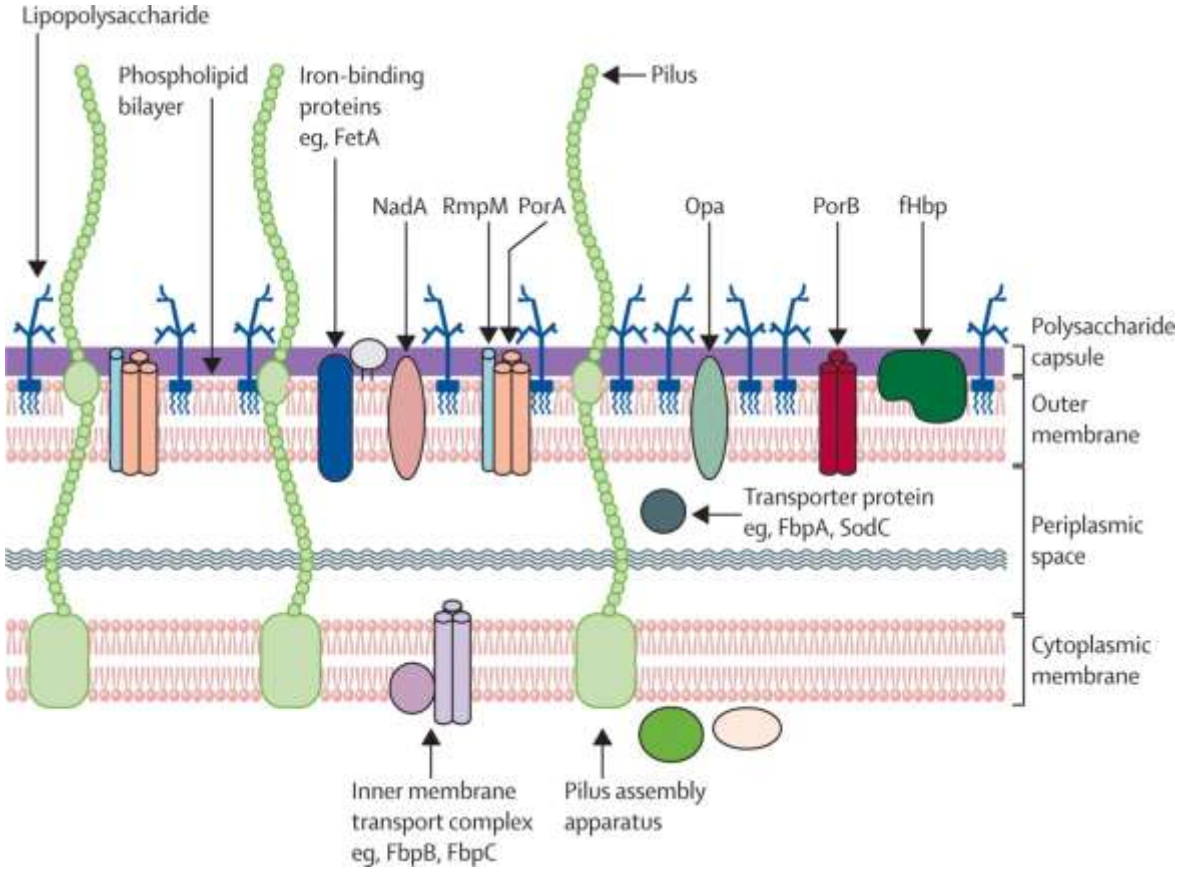
physician Flexner demonstrated the protective effect of equine meningococcal anti-serum in a monkey challenge model of meningitis and noticed that this was associated with phagocytosis of the bacteria by neutrophils [6]. He went on to demonstrate that intrathecal antiserum had protective efficacy in humans, reducing the mortality from meningitis from 80% to 20% [7].

1.1.2. *Neisseria meningitidis* is a Gram negative diplococcus

Diplococcus intracellularis was subsequently classified as a member of the *Neisseria* genus, and renamed *Neisseria meningitidis*. The genus was named after the German venereologist Albert Neisser, who identified the closely related *N gonorrhoeae* as the causative agent of gonorrhoea. *N. meningitidis* is a Gram negative, encapsulated, oxidase positive, aerobic diplococcus [8], which exclusively colonises the nasopharyngeal passages of humans and is spread by airborne droplets [9]. In a minority of cases *N. meningitidis* invades and causes disease [10].

In common with other Gram negative organisms, the meningococcus has a thin peptidoglycan cell wall sandwiched between inner and outer cell membranes. The outer membrane is an asymmetric bilayer, containing lipopolysaccharide (LPS) or lipooligosaccharide (LOS) in the outer layer and phospholipids in the inner layer. The outer membrane proteins (OMPs) play important roles in immune evasion (*e.g.* factor H binding protein or fHbp), adhesion to host cells (*e.g.* pili) and transport of small molecules into the cytoplasm (*e.g.* porins such as PorA and PorB). The polysaccharide capsule surrounds the outer membrane as shown in **Figure 1**.

Figure 1 Surface structures of *N. meningitidis*. Reproduced from Sadarangani & Pollard, [11] with permission from Elsevier.



1.1.3. *N. meningitidis* is classified in terms of surface molecules and genetic sequence

The structural components of the meningococcal surface allow the classification of the organisms using specific monoclonal antibodies. The serogroup of a meningococcal strain is determined by the biochemical composition of the polysaccharide capsule as shown in Table 1. There are 13 diverse polysaccharide capsules but A, B, C, W (previously known as W-135) and Y cause the majority of disease.

Table 1 Important meningococcal serogroups and capsular structures, adapted from Hill *et al*, 2010[12]

Serogroup	Structure		
A	Non-sialic acid capsule	Homopolymers of *N*-acetyl-D-mannosamine-1-p	(α1→6)-linked-*N*-acetyl-D-mannosamine-1-phosphate
B	Sialic acid capsule (5-*N*-acetyl-neuraminic-acid)	Homopolymers of sialic acid	(α2→8)-linked-5-*N*-acetyl-neuraminic-acid
C			(α2→9)-linked-5-*N*-acetyl-neuraminic-acid
W		Heteropolymers of sialic acid containing disaccharides	(α2→6)-linked-6-D-Gal(α1→4)-*N*-acetyl-neuraminic-acid
Y			(α2→6)-linked-6-D-Glc(α1→4)-*N*-acetyl-neuraminic-acid

The polysaccharide capsule is an important virulent determinant [13, 14], which protects the meningococcus from phagocytosis and down regulates the activation of the alternative complement pathway [15]. Invasive meningococcal disease is almost exclusively caused by encapsulated strains, with a few rare exceptions [16], and most unencapsulated strains are harmlessly carried in the nasopharynx [17]. Indeed, anti-capsular antibodies are associated with protection against invasive disease [18]. Due to the resulting selection pressure on the capsular polysaccharide, meningococci have evolved mechanisms to alter the type of capsule produced from one serogroup to another, termed 'capsule switching'. This involves the homologous recombination of capsular biosynthesis genes obtained by horizontal transfer between meningococci of different serogroups [19].

The meningococcus is classified into serotypes based on the PorB molecule and subserotypes based on the PorA molecule. The outer membrane lipooligosaccharide (LOS) structure allows further classification into immunotypes. The classification nomenclature follows the pattern serogroup:serotype:subserotype:immunotype. For example, the strain responsible for the meningitis epidemic in New Zealand in the mid-nineties is B:4:P1.7b:4 [20].

For epidemiological purposes, the genetic links between strains were previously classified into enzyme types (ET) using multi-locus enzyme electrophoresis (MLEE), which compared the electrophoretic migration patterns of 11 relatively conserved housekeeping proteins. However, as this method was prone to inter-laboratory variation, Maiden *et al.* developed a system based on sequencing of the housekeeping genes themselves, termed multi locus sequence typing (MLST), which has since replaced MLEE as a definitive way of characterising the genetic lineage of invasive strains [21]. This characterises the alleles of each housekeeping gene, based on the assumption that housekeeping genes are less subject to selective pressures than genes encoding immunogenic antigens and is particularly useful to track disease isolates given the ability of meningococci to undergo capsule switching. Sequences are aligned against a global MLST database [22] and a sequence type (ST) assigned to a given strain. Closely related strains are grouped into clonal complexes. Most invasive disease is caused by meningococci from a few defined hypervirulent lineages or clonal complexes [23] as shown in Table 2. More recently, a classification system based on variations of the 53 bacterial ribosome protein subunit genes (ribosomal multi locus sequence typing or rMLST) has been proposed [24]. An open access database of the whole genome sequences of all the meningococcal isolates that caused disease in the UK in 2010/2011 is also being established (the Meningitis Research Foundation Meningococcal Genome Library) [25]. These developments will allow much finer mapping of meningococcal phylogeny.

Table 2 Clonal complexes of hypervirulent N meningitidis strains, adapted from Harrison et al[20].

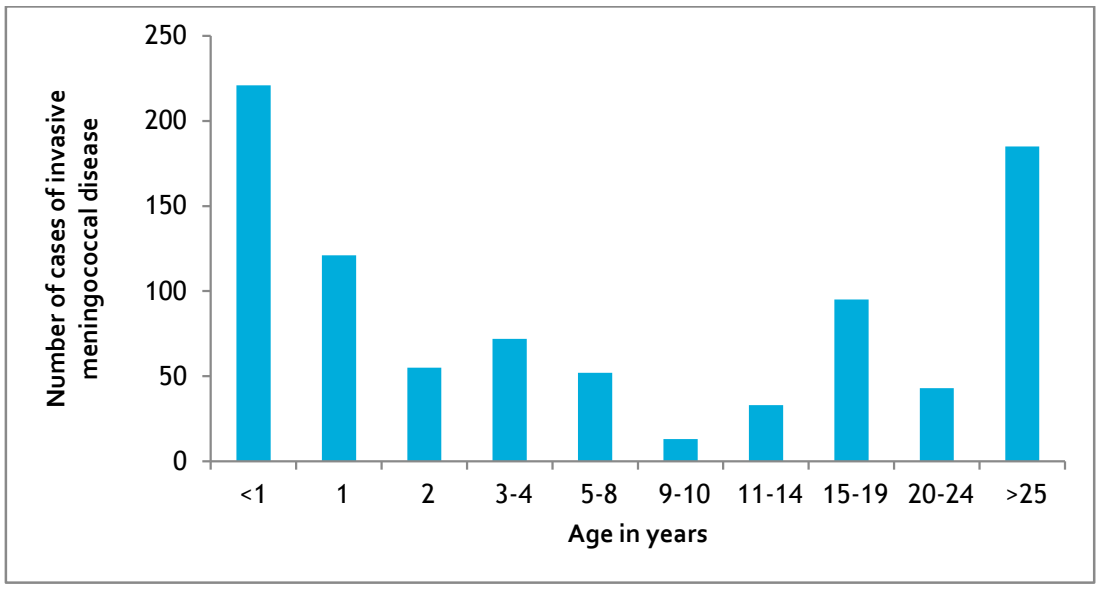
Serogroup	Clonal complex
A	ST-5
B	ST-32, ST-41
C	ST-11
W	ST-11
Y	ST-23

1.2. The epidemiology of meningococcal disease

1.2.1. Invasive meningococcal disease is rare but nasopharyngeal carriage is common

There are approximately 1000 cases of culture confirmed invasive meningococcal disease a year in the United Kingdom [26].The highest incidence of meningococcal disease occurs between the ages of 6 months and 2 years, which reflects the waning of protective maternal antibody in the first few months after birth [18, 27]. A second peak of disease occurs in young adults between 15-24 years of age. This is probably related to behavioural practices in this age group which increase the rate of meningococcal transmission *e.g.* smoking, frequenting of enclosed environments such as pubs and bars, intimate kissing and university dormitory attendance [28-30] .

Figure 2 Number of cases of invasive meningococcal disease in England and Wales 2009-2010 (y axis) by age (x axis). Data from the Health Protection Authority [31].

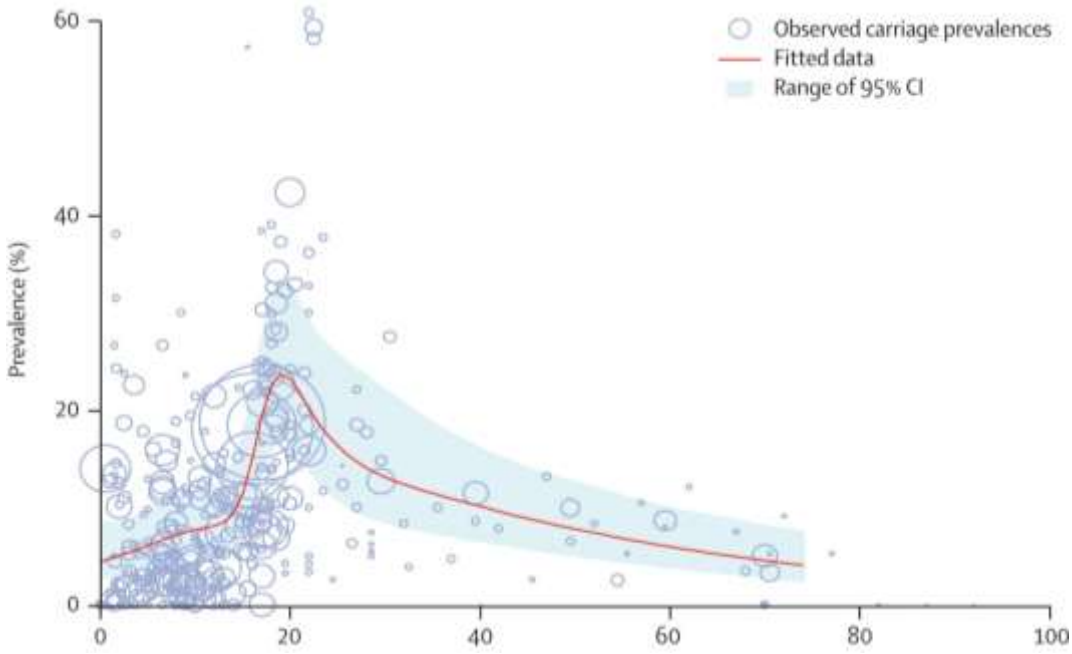


Invasive disease is a relatively rare outcome of meningococcal infection. The likelihood that a colonising organism will invade through the mucosa and cause disease is dependent on both the strain and on host factors. Colonising strains are genetically diverse, while invasive strains tend to be limited to a few hypervirulent clones [20, 23, 32]. Recent viral

upper respiratory tract infection, smoking and certain immunodeficiencies have been shown to be associated with an increased risk of invasive meningococcal disease as discussed in Section 1.3.1 . In most cases, nasopharyngeal colonisation leads to the development of natural immunity and clearance of the organism[32]. Episodes of carriage may be transient or last several months [33, 34], but invasive disease usually occurs in the first 2 weeks after acquisition of a strain, before immunity has developed [35].

Estimates of meningococcal nasopharyngeal carriage vary widely according to age. A meta-analysis of 89 carriage studies performed in Europe revealed that 4.5% of infants carry meningococci, rising to peak of 23.7% by the end of the teens and subsequently declining to 7.8% in 50 year olds [9] as shown in **Figure 3**. Carriage is important in predicting the efficacy of a vaccine at population level, as newer conjugate meningococcal vaccines provide herd immunity by reducing nasopharyngeal carriage.

Figure 3 Prevalence of nasopharyngeal carriage of *N. meningitidis* (y axis) by age in years (x axis). Reproduced from Christensen et al [9], with permission from Elsevier.

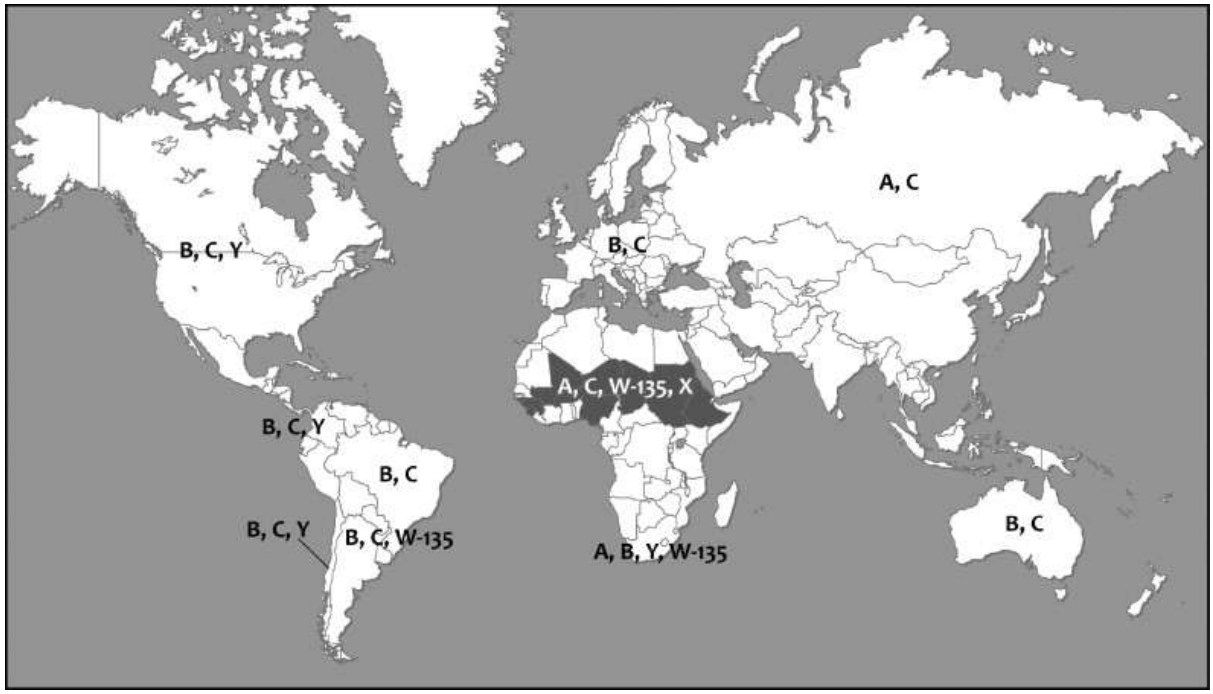


1.2.2. There are global variations in invasive meningococcal disease

The description of the global epidemiology of meningococcal disease is dependent on the accuracy of disease surveillance. The gold standard of active surveillance with laboratory confirmation of suspected clinical cases and strain identification using molecular diagnostics is not feasible in all countries. Within these constraints, the incidence of invasive meningococcal disease is ranges from approximately 1/100,000 in Europe to up to 1,000/100,000 in Africa during epidemic years [36].

The serogroup distribution of meningococcal disease also shows considerable geographical variation. Serogroup C is responsible for >70% of meningococcal disease in North America, serogroups B & C are responsible for the vast majority of disease in Europe and serogroup A causes cyclical meningitis epidemics in the African meningitis belt, including the recent outbreak in 2009/2010 [reviewed 36, 37].

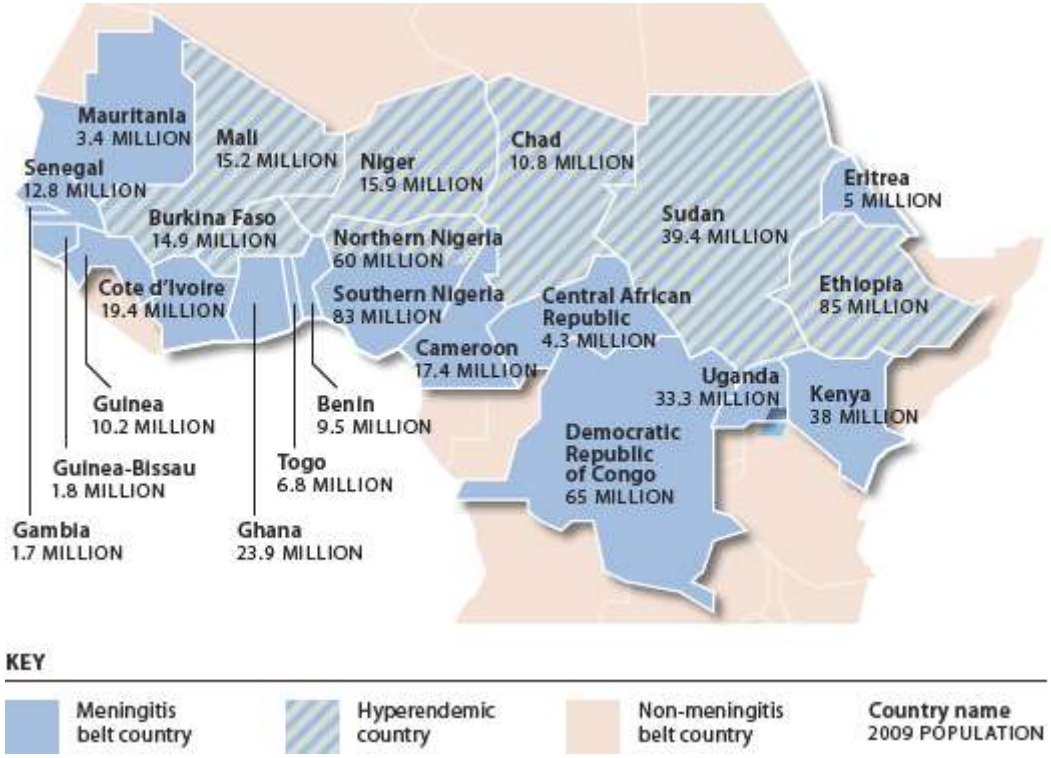
Figure 4 The global distribution of meningococcal disease. Reproduced from Harrison *et al.*, [20], with permission from Elsevier.



1.2.3. Serogroup A meningococci cause epidemic meningitis

Epidemic meningitis due to *N. meningitidis* was first described by the colonial army doctor Horn in West Africa in 1908 with at least 8000 deaths reported in Ghana alone [38]. The cyclical nature of these epidemics was recognised by the French colonial doctor Lapeyssonnie in 1963 [39]. He documented the epidemiological features of the disease, in particular that it was restricted to a geographically distinct area, which has come to be called the 'African meningitis belt' (Figure 5). He also noted that affected demographic was slightly older than in Europe or North America, with the majority of cases occurring between the ages of 2 and 30 years of age.

Figure 5 Map of the African meningitis belt. Reproduced from the PATH website at www.path.org



Within the meningitis belt, epidemics occur with a periodicity of 5-10 years, lasting 2-3 years. The epidemics are invariably associated with the dry season, and cease at the beginning of the rainy season. It has been suggested that the low humidity aids droplet transmission and the heat and dusty winds of this season damage the nasopharyngeal

mucosa increasing susceptibility to invasive disease [40]. These epidemics affect extremely large numbers of people – in the most recent outbreak in 2009, almost 90,000 cases were reported, with over 5000 deaths [41]. Within individual communities, attack rates as high as 1:10 have been reported [40]. Figures such as these are often under-estimates, as reporting systems frequently break-down during major epidemics.

Serogroup A meningococcus has been responsible for most of the epidemic disease in the African meningitis belt [36, 42], but serogroups X and W have been associated with smaller epidemics in the region since 2002 [43]. Unlike the sialic acid based capsule of serogroups B, C, W & Y, serogroup A polysaccharide consists of n-acetyl mannosamine phosphate as shown in Table 1. It has been suggested that the structural differences in the capsular polysaccharide may underlie the differences in the epidemiology of serogroup A disease. For example, the relative lack of serogroup A disease in Europe and North America in the last 30 years may be due to immunity induced by other mucosal organisms with similar cross-reactive capsules [44, 45].

1.3. Innate immunity to meningococci

Non-specific or innate mechanisms of resistance to invasive meningococcal disease are particularly important in early life, before the development of adaptive immunity [46].

1.3.1. The environment of the nasopharynx discourages colonisation by meningococci

The epithelial cells of the nasopharyngeal cavity reduce surface colonisation by airborne organisms such as meningococci, by mucous production and ciliary removal and production of bactericidal compounds such as lactoferrin and hydrogen peroxide [47]. Laminar airflow over the surface of the nasopharynx and the cough reflex are also thought to reduce nasopharyngeal carriage and thus the risk of invasion [47]. The

importance of an intact mucosal barrier is highlighted by studies showing the increased risk of invasive disease with factors that damage the nasopharyngeal epithelium such as smoking [28, 48] and recent upper respiratory tract infections [29, 49]. However, these observations may be partly confounded by an increased aerosol transmission of meningococci between individuals with coughing.

1.3.2. Innate effector cells recognise meningococci via pattern recognition receptors and antibody receptors

As well as acting as a physical barrier, the mucosal epithelium contains specialised innate mediator cells, including neutrophils, macrophages and dendritic cells. These cells recognise meningococci and other bacteria via a set of germline-encoded pattern recognition receptors (PRPs) which recognise relatively conserved products of microbial metabolism or pathogen associated molecular patterns (PAMPs). The best characterised PRPs are the toll-like receptor (TLR) family, which recognise a variety of microbial products [50]. Stimulation of TLRs activate intra-cellular signalling pathways which upregulate innate killing mechanisms and the production of inflammatory cytokines.

Of the 10 known members of the family, TLR-4, TLR-2 and TLR-9 have been implicated in protection from meningococci [51, 52]. TLR-4 is a cell-surface PRP which recognises the outer membrane LOS of gram negative bacteria [53]. In meningococci, this recognition is specific to the lipid A component [54]. However, LPS-deficient meningococci stimulate the production of pro-inflammatory cytokines [55], which is independent of TLR-4 and instead involves signalling via TLR-2 [56] . The meningococcal PAMP recognised by TLR-2 has since been shown to be the outer membrane porin, PorB [57]. TLR-9 is an endosome-associated PRP which recognises PAMPs derived from phagocytosed organisms. Its specific ligand is unmethylated CpG oligonucleotide sequences, which are common in bacterial DNA but are rarely found in mammalian DNA

[58]. Purified meningococcal DNA induces inflammatory responses in TLR-9 expressing cell lines [52].

The mannose receptor, found exclusively on macrophages and dendritic cells is a cell membrane bound C-type lectin which binds mannose residues such as those on meningococcal capsules and the outer membrane LOS [59]. Scavenger receptors are a heterogeneous group of phagocytic receptors, found largely on macrophages, which bind negatively charged ligands such as bacterial cell wall lipotechoic acid [60].

Neutrophils, macrophages and dendritic cells also recognise pathogens that have bound antibody using receptors for the antibody constant domains (see 1.4.3) called Fc receptors (FcR). The aggregation of antibody on bacterial surfaces cross-links FcR on the phagocyte, promoting phagocytosis and destruction of the bacteria. In contrast, free immunoglobulin molecules bind FcR with low avidity and do not cross-link FcR [reviewed 61].

Figure 6 Properties of human FcR. Adapted from Woof & Burton [61].

Receptor	Order of binding affinity	Cellular distribution	Effect of ligation
FcγRI	IgG1=IgG3>IgG4	Macrophages, dendritic cells, neutrophils, eosinophils	Uptake, stimulation, activation of respiratory burst
FcγRIIa	IgG3≥IgG1>IgG2	Macrophages, neutrophils, platelets Langerhans cells	Uptake
FcγRIIb	IgG3≥IgG1>>IgG2>IgG4	Macrophages, B cells	Inhibition of stimulation
FcγRIIc	Not determined	Macrophages, neutrophils, B cells	Inhibition of stimulation
FcγRIII	IgG1=IgG3>>IgG2=IgG4	Macrophages, natural killer cells	Induction of killing
FcεRI	IgE	Mast cells, basophils, Langerhans cells,	Secretion of granules
FcαRI	IgA1=IgA2	Neutrophils, some macrophages and dendritic cells	Uptake

1.3.3. Innate phagocytic cells play a key role in early meningococcal infection

Neutrophils are the most common cell type in the cerebrospinal fluid of patients with meningococcal meningitis. Neutrophil activation has been observed within the first 24 hours of invasive meningococcal disease, before adaptive responses have had time to evolve [62]. The role of neutrophils in protection from invasive disease was demonstrated in a rodent model, where depletion of neutrophils with monoclonal antibodies led to 100% mortality following intra-peritoneal challenge with meningococci, even in vaccinated animals [63]. Neutrophils phagocytose bacteria via opsonin dependent and opsonin independent mechanisms [64]. Neutrophil opsonins may be adaptive, *i.e.* specific antibody (section1.4.3), or innate, *i.e.* the C3b component of complement (section 1.3.4) or the secreted PRP C-reactive protein (CRP) which binds to phosphorylcholine residues in bacterial cell membranes [65]. In meningococcal disease, opsonin independent mechanisms of phagocytosis involve the recognition of LOS and the outer membrane Opa protein [66], by TLR and carcinoembryonic antigen-related cell adhesion molecule (CECAM) receptors respectively [67].

Dendritic cells are phagocytic cells that are essential in linking the innate and adaptive immune responses [68]. Located in the submucosa, immature dendritic cells recognise and internalise bacteria via TLRs and scavenger receptors. The specific meningococcal ligands recognised by the latter class of receptor have not been well characterised, but may be LOS [51]. Dendritic cells response to these signals by expressing the chemokine receptor CCR7, which directs migration of the cells to the draining secondary lymph nodes, and by processing of pathogen-derived proteins. Signalling through CCR7 also drives maturation of the dendritic cell and upregulates expression of the co-stimulatory molecules B7 and MHC class I and I molecules, facilitating antigen presentation and activation of naïve T cells. Dendritic cells also release the pro-inflammatory cytokines

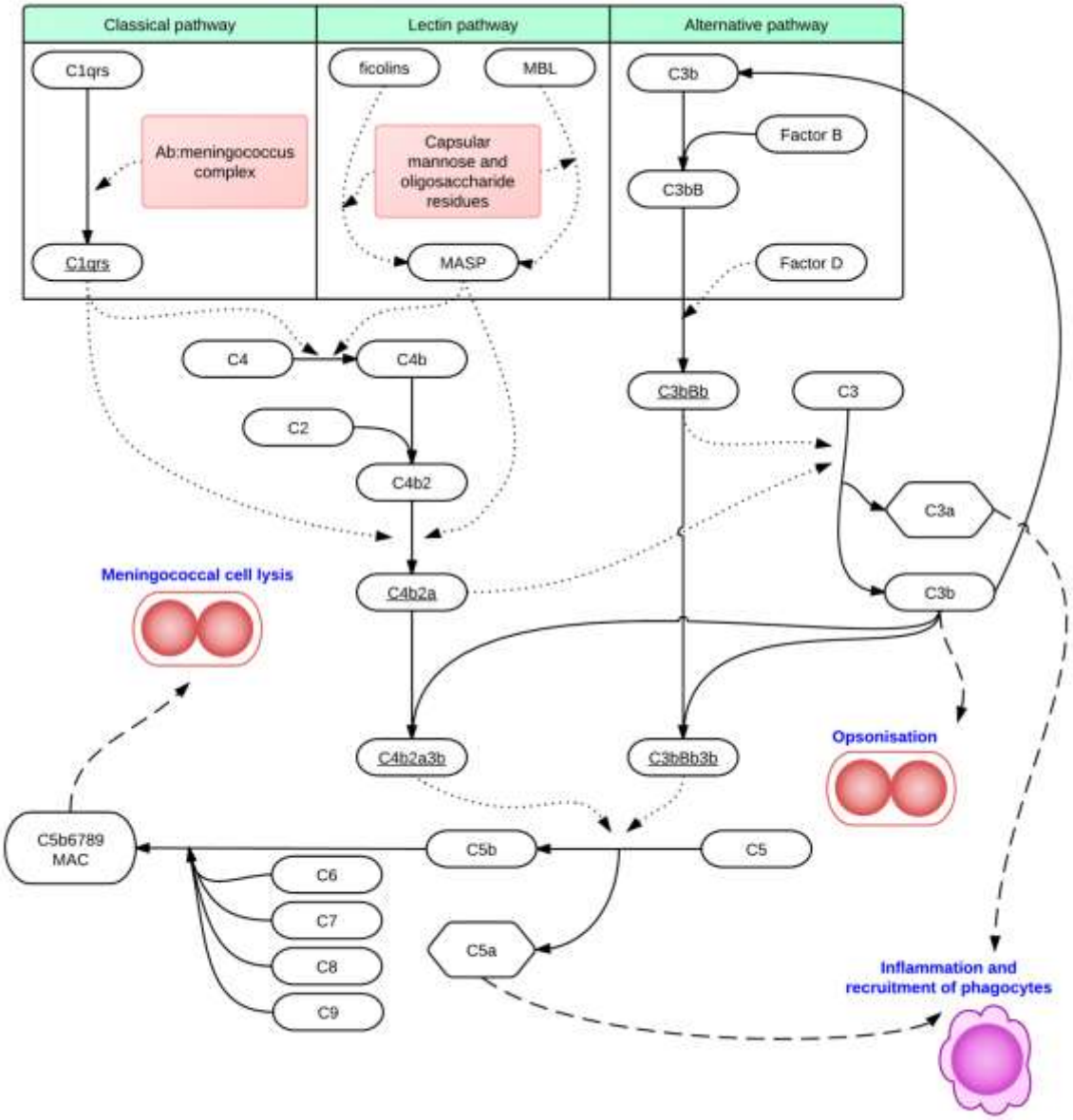
TNFα, IL-6 and IL-8 on internalisation of meningococci [69], high levels of which are associated with the severity of meningococcal disease [70].

Tissue macrophages, like dendritic cells, have several PRPs including TLRs, scavenger receptors, complement receptors and mannose receptors which can bind to and internalise bacteria. Again, signalling via these PRPs leads to upregulation of MHC II and CD80/B7. Although macrophages have not been demonstrated to activate naïve T cells directly, they may play a role in promoting immune responses already initiated by dendritic cells.

1.3.4. The complement cascade provides a link between innate and adaptive immune mechanisms

The complement system is a set of plasma proteins that act in concert to identify and destroy extra-cellular pathogens. Complement activation can occur by specific antibody or CRP binding to meningococci (classical pathway), activation by capsular oligosaccharides (lectin pathway), or by spontaneously by the hydrolysis of serum C3 on meningococcal cell surfaces which lack endogenous complement inhibitors (alternative pathway). These pathways converge in the production of C3b by the C3 convertases C4b2a and C3bBb (see Figure 7). C3b deposits on meningococcal surfaces and acts an opsonin, facilitating uptake by phagocytic cells via complement receptors CR1, CR3 and CR4 which recognise C3b or its inactivated products. C3b is also a component of the C5 convertases C4b2a3b and C3bBb3b which mediate C5-9 membrane attack complex (MAC) insertion into meningococcal outer membranes. The MAC forms a transmembrane channel permeable to electrolytes and water, thus directing lysis of the meningococcus.

Figure 7 Activation of the complement cascade by meningococci



CRP - C reactive protein, MAC- membrane arrack complex, MBL- mannose binding lectin, MASP-MBL associated serine proteases

The importance of meningococcal cell lysis by the MAC in protective immunity is demonstrated by the recurrent episodes of invasive meningococcal disease suffered by individuals with deficiencies in the terminal C5-9 components [71-73]. The increased risk

of meningococcal disease in infancy may in part be due the relatively low levels of C9 at this age and thus diminished bactericidal activity [74].

Less is known about the role of complement-mediated opsonophagocytosis via C3b deposition. *In vitro* studies using serum depleted of a single terminal complement component show that neutrophil opsonophagocytosis of meningococci does occur [75, 76]. Additionally, vaccination of individuals with terminal complement component deficits reduces the frequency of invasive meningococcal disease [77], which suggests that opsonophagocytosis, the only available mechanism of meningococcal killing in these individuals, must mediate a degree of protection.

1.4. Adaptive Immune responses develop later in the course of an infection

Adaptive immune responses, unlike innate responses, are antigen specific. This specificity arises from complex mechanisms for generating diversity in the antigen receptor molecules on the cells of the adaptive immune system – namely the secreted or surface antibody molecules of B lymphocytes or the cell surface receptor of T lymphocytes.

1.4.1. Serum antibody is critical for protection from meningococcal disease

The protective role of serum was first demonstrated in humans by Flexner in 1913 who reduced the mortality from invasive meningococcal disease by administering intrathecal horse serum [7]. In a series of experiments in rodents and rabbits, Matsunami and Kolmer showed that the resistance of animals to intra-peritoneal meningococcal challenge was related to the bactericidal activity of whole blood, and observed that in humans, this bactericidal activity increased with age [78]. This latter observation was confirmed by the epidemiological studies of Goldschneider *et al.*, who first established the inverse correlation between serum bactericidal activity (SBA) and the incidence of meningococcal

disease at population level [27]. The importance of SBA in protection against meningococci was confirmed by prospective studies in military recruits which showed that individuals with low SBA titres were more likely to develop disease that those with high titres [27]. The target of bactericidal antibodies is largely the polysaccharide capsule in serogroups A and C, but subcapsular protein epitopes are more likely to be important in immunity against serogroup B [79].

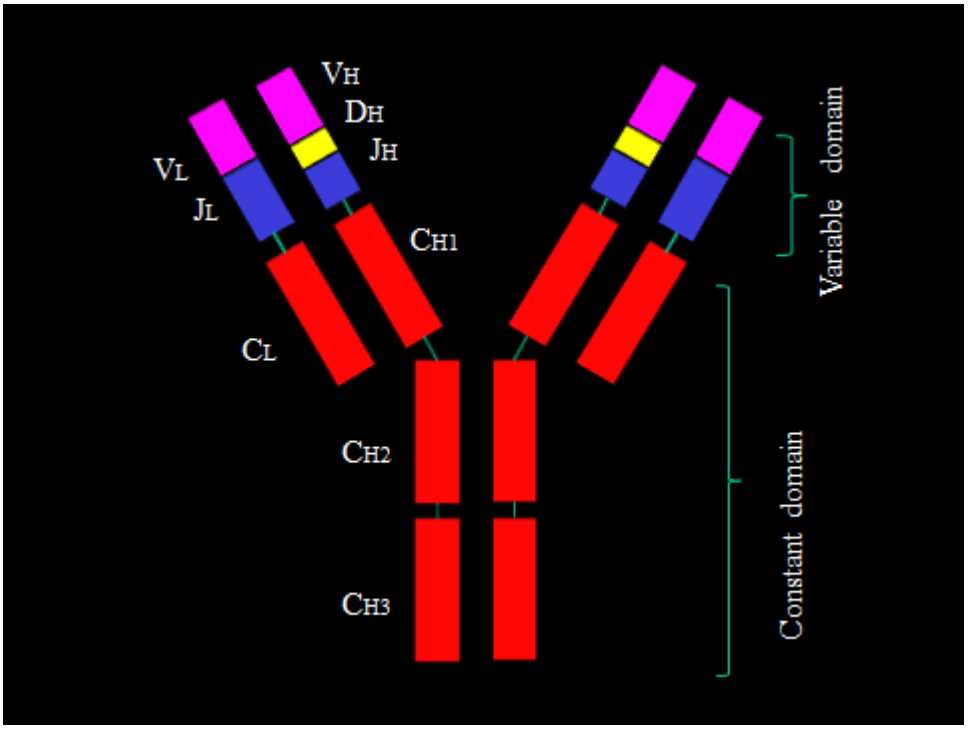
1.4.2. The acquisition of protective antibody

Protection from meningococcal disease in the first 6 months of life derives from transplacentally acquired maternal IgG [18]. As passive antibody wanes, it is replaced acquired antibody during early childhood. However, meningococcal carriage under the age of 5 years is relatively rare [9], and it possible that protective antibody is instead acquired due to cross-reactive epitopes from the closely related but non-pathogenic *Neisseria lactamica*, which is much more commonly carried in this age group [80, 81]. Carriage of *N. lactamica* is associated with the development of bactericidal antibodies against meningococci [81]. The identity of the cross-reactive antigens involved in this protective immunity is unclear. *N. lactamica* is unencapsulated, but shares LOS epitopes with N. meningitidis [82]. Immunisation with killed *N. lactamica* protects mice from meningococcal challenge, but this may be mediated by outer membrane proteins rather than by LOS [83]. Other encapsulated non-neisserial bacteria may also play a role in inducing cross-protection during early life. For example, the capsules of several enteric Gram negative bacteria are both structurally and immunologically similar to the capsules of serogroup A and C meningococci [44, 45].

1.4.3. Anti-meningococcal antibody subclasses and their function

Antibody classes and subclasses fulfil diverse roles in the immune system, and this can be ascribed to differences in structure of their constant domains (see Figure 8). These differences are summarised in Table 3.

Figure 8 Structure of an antibody molecule



V_H=variable, D_H=diversity, J_H=junctional segments of variable heavy domain
V_L=variable, J_L=junctional segments of variable light domain
C_H1-3 =constant heavy domains 1-3
C_L=constant light domain

Table 3 Physical properties of human antibody isotypes. Adapted from Immunobiology, Garland Press [84].

Class	Principal molecular form	Adult serum level (mg/mL)	Serum half life (days)	Classical pathway activation	Placental transfer	Binding to phagocyte Fc receptors
IgM	Pentamer	1.5	10	++++	-	-
IgG1	Monomer	9	21	++	+++	+
IgG2	Monomer	3	20	+	+	-
IgG3	Monomer	1	7	+++	++	+
IgG4	Monomer	0.5	21	-	-	-
IgA1	Dimer	3.0	6	-	-	+
IgA2	Dimer	0.5	6	-	-	+
IgD	Monomer	0.03	3	-	-	-
IgE	Monomer	5×10^{-5}	2	-	-	+

Meningococcal infection in humans results in the production of IgG, IgM and IgA antibody classes [85-87]. Post-infection IgM, though a potent activator of complement, does not contribute to phagocyte-mediated killing in the absence of complement [88].

Convalescent sera from children contains IgG1 and IgG3 subclasses [89] while adults produce IgG1, IgG2 and IgG3 [90]. The role of these subclasses was elucidated in experiments using chimeric antibodies with identical antigen-binding specificities but varying constant domains. These suggested that, as expected by studies of hapten-induced antibodies, IgG1 and igG33 directed against PorA mediate complement mediated lysis and phagocytic uptake of meningococci [91].

Anti-PorA IgA does not directly activate complement, and modestly promotes phagocytosis, but is a potent stimulator of the phagocyte respiratory burst [91]. IgA has been documented to impair the complement-mediated lysis of meningococci by IgG and IgM, possibly by binding to and blocking antigenic epitopes [91, 92]. There are few data

on the role of mucosal IgA in preventing nasopharyngeal colonisation or invasion by meningococci. However, *in vitro* studies have shown that secretory IgA can block the adherence of meningococci to human buccal epithelial cells [93]. In addition, meningococcal vaccination generates mucosal IgA, suggesting that it does play some part in protection [94, 95].

1.4.4. Individual subsets of B cells play specific roles in protection from meningococcal disease

As the cells that produce antibody, B lymphocytes are the principal cellular determinants of protection against meningococcal disease. B cells are classified into B1 cells, (which are further subdivided into B1a and B1 b subsets) and B2 cells (which are subdivided into follicular and marginal zone B cells) based on their developmental origin during lymphopoiesis, surface marker phenotype and the nature of antibody they produce [96] as shown in Table 4 below. However, it is likely that *in vivo*, there is a spectrum of functionality across the different subsets and that they are not as clearly defined as traditional classification schemes suggest.

Table 4 Properties of B1 and B2 B cell subsets in mice and humans

	T independent			T dependent	
	B1		B2		
	B1a (mice)	B1b (mice)	Marginal zone/IgM memory	Follicular	
				Naïve	Switched memory
Surface markers (mice)	$CD19^{+}$ $CD20^{+}$ $CD27^{-}$ IgM^{hi} $CD23^{lo}$ $CD21^{lo}$ CD5+	$CD19^{+}$ $CD20^{+}$ $CD27^{-}$ IgM^{hi} $CD23^{lo}$ $CD21^{lo}$ CD5-	$CD19^{+}$ $CD20^{+}$ $CD27^{+}$ IgM^{hi} $CD23^{lo}$ $CD21^{hi}$	$CD19^{+}$ $CD20^{+}$ $CD27^{-}$ IgM^{lo} $CD23^{hi}$ $CD21^{lo\text{-}neg}$	$CD19^{+}$ $CD20^{+}$ $CD27^{+}$ IgM^{lo} $CD23^{hi}$ $CD21^{lo\text{-}neg}$
Activation mechanisms	Recognition of PAMPs	BCR cross-linking		Recognition of specific antibody via BCR	
Antibody produced	Low affinity IgM, IgA Polyspecific	Low affinity IgM, IgA, IgG3 (IgG2 in humans) Specific		High affinity IgM Highly specific	Higher affinity, IgG1,2,3,4, IgA, IgE Highly specific
Antibody gene repertoire	Pre-diversified	Minimal somatic hypermutation		Somatic hypermutation	Extensive somatic hypermutation and class switch recombination
Age related changes	Declines with age		Poor function <2years of age, declines with age	Memory populations established with age and Ag exposure Clonal expansions of memory populations in elderly, which may impair naïve responses	
Nature of antibody response	Rapid but short lived No previous Ag encounter required	Longer lasting response Previous Ag encounter required	Rapid response Some improvement in Ab quality	Long lasting response Previous Ag encounter required, Improved Ab with each encounter	

Ag = antigen, Ab= antibody, BCR = B cell receptor

1.4.5. The production of antibody by B cells occurs with or without T cell help

Antibody responses to soluble protein or peptide antigens require costimulatory signals from CD4+ T helper cells, and as such these antigens are termed T dependent (TD). However, animals that lack T cells are still capable of producing antibodies to certain bacterial non-protein antigens termed T independent (TI) antigens. There are 2 classes of TI antigens which activate B cells by different mechanisms.

TI-1 antigens are polyclonal activators of B cells which cause B cell proliferation and differentiation of B cell regardless of their antigenic specificity and are often referred to as B cell mitogens. Bacterial LPS is the best described TI-1 antigen, and binds to B cells via TLR4.

TI-2 antigens consist of molecules with highly repetitive structures such as meningococcal capsular polysaccharides, which contain no intrinsic B cell stimulating ability[97]. TI-2 antigens are thought to simultaneously cross-link multiple BCR on an antigen specific B cell, generating a first signal for activation of that cell. A second signal is provided by engagement of C3b with the CD19/CD21 B cell complex[98], which drives differentiation into IgM secreting plasma cells. C3b may be generated by the direct activation of the lectin or alternative complement pathways by polysaccharide, which then remains associated with it. Dendritic cells in secondary lymphoid tissue may play a role in displaying the polysaccharide to antigen specific B cells and delivering this second signal [68]. The relatively low levels of C3 and reduced expression of B cell CD21 observed in infancy [98] may also contribute to the susceptibility of this age group to invasive meningococcal disease. Responses to TI-2 antigens are thus both antigen specific and, since they do not require T cell help, rapid.

An exception to this paradigm are certain zwitterionic polysaccharides, such as the *Bacteroides fragilis* capsular polysaccharide or the pneumococcal polysaccharide serotype 1 polysaccharide, which may be processed and presented via MHC class II to polysaccharide specific CD4+ T helper cells [99].

As discussed in the following sections, TD and TI-2 responses are orchestrated by specific subsets of B cells. The characteristics of TD and TI antigens are summarised in below.

Table 5 Characteristics of T dependent and T independent antigens.

	TD	TI-1	TI-2
Antibody response in infants	Yes	Yes	No
Antibody response in the absence of T cells	No	Yes	No
Primes T cells	Yes	No	No
Polyclonal B activation	No	Yes	No
Requires repeating epitopes	No	No	Yes
Examples	Diphtheria toxin Viral haemaglutinin	Bacterial LPS	Meningococcal capsular polysaccharide

LPS = lipopolysaccharide

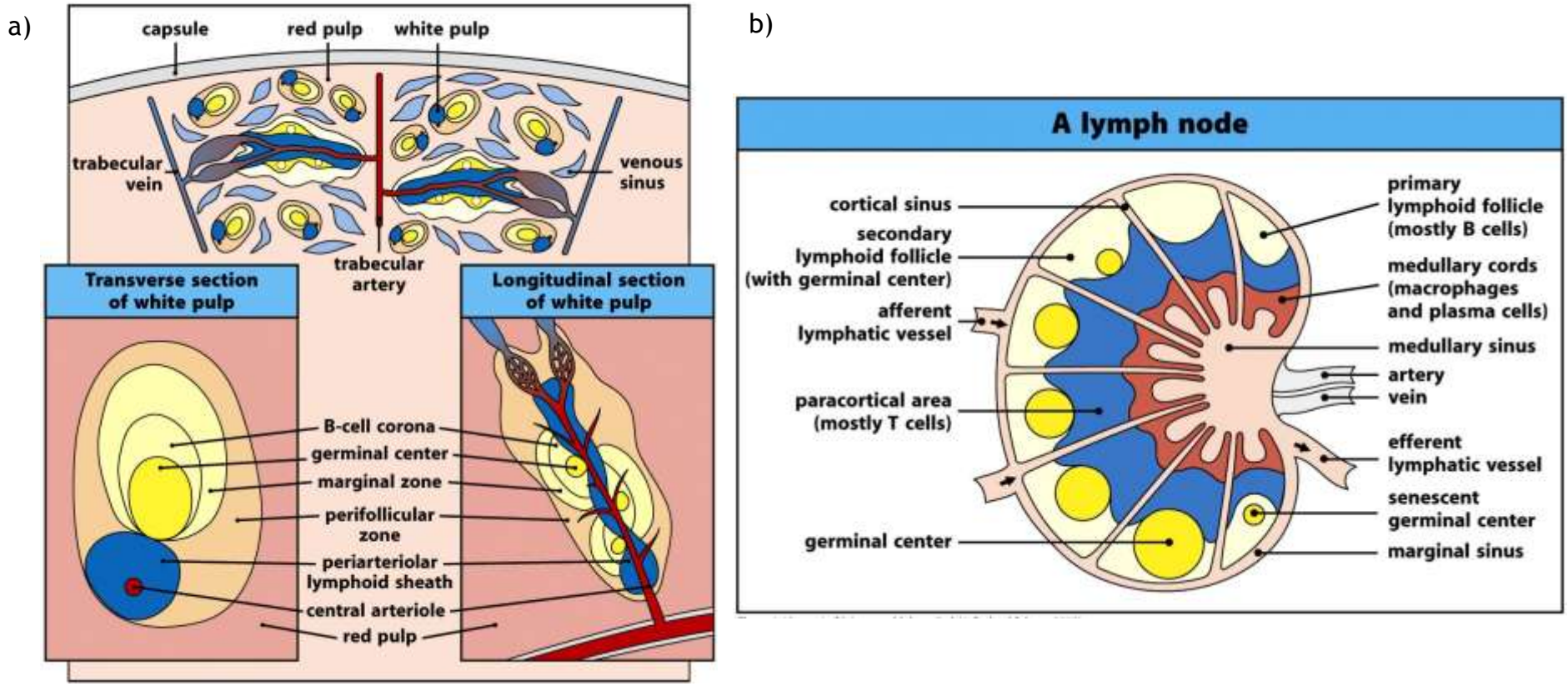
1.4.6. Follicular B cells mediate T cell dependent responses

Follicular B cells (FOB) form the majority of circulating B cells in the blood and mediate TD humoral immune responses [100]. Specific FOB recognition of microbial protein bound to its cell surface B cell receptor (BCR) results in migration to the lymph nodes. The microbial protein is internalised, processed and peptides derived from it are presented via the Major Histocompatibility Complex class II (MHC II) molecule to cognate $CD4^+$ T-cells within lymph nodes previously primed by the same peptides

presented by dendritic cells. This interaction promotes the generation of antibody-secreting plasma cells in 2 ways.

Firstly, short-lived plasma cells with a half-life of 3-5 days are produced by the so-called 'extra-follicular' pathway, secreting germline encoded specific IgM [101, 102]. These rapidly formed extrafollicular plasma cells may play a role in early immunity to meningococci. Secondly, a slower 'follicular' pathway results in the formation of a histologically discrete structure, the germinal centre (GC), where intense B cell proliferation occurs. Here, under the influence of the enzyme activation-induced cytidine deaminase (AID), the FOB undergo somatic hypermutation of their antibody variable domains, which increases the affinity of the BCR to antigen, and class switching from IgM to IgG, IgA or IgE, which extends the breadth of antibody effector mechanisms available to clear the infection. Affinity-matured, isotype-switched B-cells differentiate into switched memory B-cells which provide long-term immune protection or long-lived PCs that migrate to the bone marrow and secrete antibody [100]. The mechanism for committing to a follicular or extra-follicular pathway is thought to depend on B lymphocyte induced maturational protein 1 (Blimp-1). Blimp-1 inhibits the transcription of the PAX5 gene family important in germinal centre formation, and Blimp-1 expression is upregulated in extrafollicular plasma cells [103].

Figure 9 Organisation of the lymphoid tissue of a) the spleen and b) a peripheral lymph node. Reproduced with permission from Adapted from Immunobiology, Garland Press [84]. .



In both the spleen and peripheral lymph nodes, B and T cell areas are largely segregated. In the unstimulated state, B cells aggregate in primary follicles composed of resting B cells within a meshwork of follicular dendritic cells. After antigenic challenge, they form secondary follicles which consist of a germinal centre consisting of rapidly proliferating B cells with a few associated T helper cells. The germinal centre is surrounded by a corona of marginal zone B cells.

1.4.7. Marginal zone B cells are innate-like cells with a prediversified antibody repertoire

Marginal zone B-cells (MZB) reside within the splenic marginal sinus. Unlike mouse MZB, which remain in the spleen, human MZB recirculate and correspond to the 'IgM memory' subset observed in peripheral blood [104]. They proliferate faster and at lower antigen concentrations than FOB, and rapidly differentiate into extra-follicular plasma cells within 3 days of antigen exposure [105]. MZB respond predominantly to T-cell-independent type II (TI-2) antigens such as bacterial capsular polysaccharides [106]. Together with their anatomical localisation near the highly vascular red pulp of the spleen, these observations suggest that they are important in the early part of the immune response to blood borne encapsulated pathogens, such as meningococci [107].

MZBs produce somatically hypermutated IgM antibodies [108]. Interestingly, although MZB have similar usage patterns of V,D and J genes to switched memory cells, they show considerably less hypermutation and distinct patterns of junctional diversity generation [109, 110]. This hypermutation occurs in individuals with 'Hyper-IgM' syndrome, who lack CD40 or CD40L mediated T cell co-signalling mechanisms, but not in individuals with deficiencies in AID [104, 111]. These observations suggest that T cell help is not required for MZB development, and that hypermutation occurs under the influence of AID in an extra-follicular process.

The diversification of the MZB antibody repertoire resultant from hypermutation exists even in infants as young as 8 months of age, implying it occurs before exposure to antigen, though when this occurs in B cell ontogeny is unclear [112]. However, responses to encapsulated bacteria which present TI antigens are impaired under the age of 2 years. This may be explained by histological studies that show the absence of a splenic marginal zone under the age of 8 months and a poorly disorganised marginal zone from the ages of 8 months to 2 years of age [113]. As the spleen is essential for the survival of MZBs [114],

this relative functional immaturity of infant splenic marginal zones may partly explain the relative susceptibility of infants to invasive meningococcal disease.

Despite their ability to differentiate quickly into antibody secreting cells without the requirement for T-cell help, MZB have a complex relationship with T cells. Murine experiments suggest that MZB's are capable of responding to TD type antigens [115] and may in fact be involved in both the activation and suppression of CD4 and CD8 T cells in infection [116].

1.4.8. B1 cells respond to TI antigens

B1 cells are innate-like B cells that are localised to the peritoneal and pleural cavities and respond to T independent antigens[117]. Much of what is known about B1 cells is based on rodent data. Murine B1 cells are identified by the surface expression phenotype IgM^{hi} $CD23^{lo}$ $CD21^{lo}$ and are further classified into B1a (CD5+) and B1b (CD5-) cells[117].

B1a cells constitutively produce 'natural IgM' - broadly specific circulating antibody that binds to PAMPs such as LOS and phosphorylcholine in the absence of antigenic stimulation[118]. Natural IgM also activates complement by the classical pathway and may promote phagocytosis of encapsulated organisms by initiating the deposition of the opsonin C3b [119]. B1a cells are relatively short-lived and are self-renewing [120]. It has been suggested that by virtue of their abilities to differentiate quickly into plasma cells on recognising conserved pathogenic motifs, B1a and MZB act in concert in early responses to encapsulated bacteria. [107]

Unlike B1a cells, B1b cells produce IgM in response to antigen specific stimulation and can be long-lived [121, 122]. Further, vaccination with specific antigen is protective against

subsequent bacterial challenge in CD 19-/- mice (who have B1b cells, but not FOB, MZB or B1a cells). This is reminiscent of a 'memory' type response, classically thought to involve T cells. B1b may also undergo limited class-switching, largely to secrete IgA and sequencing of the B1b V_H genes indicate that they undergo a process of somatic hypermutation [123]. As B1b cells are excluded from the GC pathway by virtue of their anatomical localisation, this may involve the generation of a pre-diversified antibody repertoire in a similar mechanisms to MZBs.

Thus B1a and B1b cells appear to have distinct functional characteristics, with the former representing a more non-specific innate-type response whereas the latter provide a more adaptive-type response with antigen specificity and 'memory'. A recent study which demonstrates the ability of murine B1 cells to phagocytose bacteria and present antigen to T cells confirms their role in linking the innate and adaptive arms of the immune response [124].

Identification of a human B1 population has been difficult as the marker CD5 is present on may populations of human lymphocytes. Griffin *et al.* recently identified a B1-like population in human umbilical cord blood and in peripheral blood that expresses the surface markers CD20+CD27+CD43+CD70- with some characteristics of mouse B1 cells including spontaneous IgM secretion [125, 126]. However, isolating this population is technically challenging and prone to error, suggesting that the functional correlation of these cells with murine B1 cells remains to be ascertained [127].

1.4.9. Memory B cells mediate anamnestic antibody responses to antigen

Immunological memory refers to the rapid, higher affinity specific response on secondary encounter with antigen and is a hallmark of the adaptive immune system.

Vaccination aims to establish immune memory to reduce the risk of subsequent infection. Memory B cells, are classically described as products of FOB activation by TD antigens in a germinal centre response, with surface the surface phenotype CD27+IgD- the latter suggesting that they have class-switched ('switched memory' B cells). However, circulating CD27+IgM+ or 'IgM memory' B cells (Section 1.4.7) have also been described, and the role played by these cells in long-term immune protection is unclear. It had been proposed that CD27+ IgM+ cells are not true memory cells as they derive from extra-follicular responses and exhibit limited repertoire pre-diversification [100]. In addition, CDR3 spectratyping [112] and high throughput analysis of the variable domain gene usage [128] suggest that CD27+IgM+ and CD27+IgM- memory B cells evolve through different BCR selection mechanisms. However, antigen specific CD27+IgM+ cells can persist in mice for up to 1 year after vaccination, exhibit clonal expansions suggestive of antigenic selection and are associated with germinal centres[129, 130]. Taken together, these findings suggest that there is considerable heterogeneity and overlap in function between the CD27+IgM+ and CD27+IgM- memory populations.

Several mechanisms underlie the rapid kinetics of the memory B cell response. Firstly, the frequency of antigen specific memory B cells as a population that by definition has undergone expansion, is greater than that of naïve B cells [100]. Secondly, memory B cells are strategically located in areas of frequent antigen encounter, such as the spleen, unlike naïve B cells which are located in lymph node primary follicles [131]. Thirdly, the somatic hypermutation and associated increase in BCR affinity of memory B cells means they bind antigen more efficiently. Finally, Memory B cells upregulate the expression of co-stimulatory molecules such as B7/CD80 and thus respond to proliferation signally via the BCR more rapidly [132, 133].

Memory B cells peak in the peripheral blood 28 days after a primary antigenic stimulus [134]. Once induced, memory B cells can persist for decades. For example, *Vaccinia* virus -specific memory B cells have been found in humans at low levels up to 50 years after immunisation against smallpox [135]. The mechanisms for the longevity of the memory B cell response remain unclear. Memory B cells in humans spontaneously turn over more rapidly than naïve B cells, so long term immunity may be due to a self-renewing population of antigen specific cells [136]. Experiments in CD4+ depleted mice suggest that though T helper cells are important in the initial germinal centre reaction that generates memory B cells, they are not required for their long term turnover [137]. In addition, *in vivo* BCR specificity switching experiments demonstrate that the persistence of specific antigen is not required for memory B cell proliferation [138]. However, it has been suggested that basal proliferation of memory B cells may be due to polyclonal activation by microbial PAMPs which are recognised by TLRs [139].

1.4.10. Plasma cells continuously secrete protective antibody

Plasma cells are terminally differentiated B cells which produce antibody in the absence of further antigenic stimulation. Antigen specific B cells differentiate into plasma cells following an encounter with antigen, which subsequently emigrate from the lymph nodes to the bone marrow 6-7 days afterwards [134, 140].

Given the rapid progression from acquisition of a virulent strain of meningococcus to invasion, persistent antibody is critical in protection from disease. As the half-life of free antibody is less than 3 weeks [141], persistent antibody must be due to continuous secretion by plasma cells. As plasma cells themselves are unable to proliferate, long lasting antibody may be produced by memory B cells which constantly differentiate into plasma cells or alternatively, by long-lived plasma cells.

Radioactive-thymidine incorporation experiments in mice confirm the existence of 2 distinct populations of plasma cells – a short-lived population with a lifespan of 3-5 days located predominantly in the lymph nodes and spleen and a longer-lived population restricted to the bone marrow [101]. It has been suggested that short-lived plasma cells arise from low-affinity, TI antigen mediated extra-follicular responses and that long-lived plasma cells arise from TD antigen mediated, germinal centre responses [142, 143]. Intriguingly, a recently published study showed that T deficient mice were indeed able to generate long-lived IgM plasma cells against LPS, a TI antigen, suggesting that a component of this immunity may be germinal centre independent [144]. This finding may explain previous observations of 'memory' type responses following immunisation of T deficient mice with TI *Borrelia* antigens [122].

Long-lived plasma cells have been show to survive in mice for up to 1 year [145, 146]. The mechanisms underlying the longevity of post-follicular plasma cells are unclear, although plasma cells require interactions with bone marrow stromal cells for their long-term survival [147]. As infant bone marrow stromal cells lack the signalling mechanisms required for promoting plasma cell survival, this may explain the poor persistence of antibody in this age group [148]. Single cell sequencing of bone marrow plasma cells confirms somatic hypermutation of their variable domain, indicating they have proceeded through the germinal centre and undergone affinity maturation [149]. Thus on the basis of a GC phase in development, of their extended life span and of the continued production of high-affinity antibody for protracted periods of time well after antigen clearance, long-lived plasma cells may be considered a part of the memory B cell compartment.

Evidence for the existence of long lived bone marrow plasma cells in humans is indirectly demonstrated using rituximab, an anti-CD20 monoclonal antibody which selectively depletes mature and naïve B cells but not plasma cells, which lose the surface

expression of CD20 during differentiation. Therapeutic rituximab induced B cell depletion in the treatment of rheumatoid arthritis results in an initial fall in anti=tetanus toxoid and pneumococcal polysaccharide specific antibodies which then plateaus, in some cases at protective levels, until B cell recovery up to 33 months later [150]. This suggests the constant production of antibody by CD20 negative plasma cells. Ex vivo tissue culture models of human tonsils and small intestine mucosal biopsies also show evidence for a non-proliferating population of cells which constitutively secrete antibody for up to 4 weeks in the absence of antigenic stimulation [151, 152]

1.4.11. T cells and meningococcal disease

The role of T cells in meningococcal disease remains to be fully elucidated. Cytokine release assays demonstrate that peripheral blood mononuclear cells (PBMCs), of children convalescing from meningitis respond to meningococcal OMPs [153]. The authors of this study suggest that balance of Th-1 and Th-2 immunity mediated by these T cells may play a role in the susceptibility to or severity of disease. Older children make a more Th-2 type response, whilst younger children make a more Th-1 type response. However, a more recent study in young adults suggests that carriage alone does not skew the Th-1/Th-2 response profile in circulating T cells [154]. Carriage also stimulates mucosal T cells. Tonsillar T cells of both naïve and memory phenotype proliferate in response to meningococcal OMPs [155], even though responses did not correlate with protective SBA titres.

1.5.Meningococcal vaccines

1.5.1. The first meningococcal vaccines were based on capsular polysaccharide

Dochez and Avery first identified a ‘soluble substance’ in clinical specimens from patients with invasive disease caused by another encapsulated bacterium that can cause

meningitis, *Streptococcus pneumoniae,* in 1917 [156]. This was subsequently characterised as a polysaccharide [157] and found to be type specific. The recognition that anti-polysaccharide immune responses were associated with bactericidal activity underpinned the development of capsular polysaccharide based vaccines. The first trial of a pneumococcal polysaccharide vaccine was conducted in over 8,000 army recruits by MacLeod *et al.* in 1945. This demonstrated type-specific protection from lobar pneumonia with purified pneumococcal capsular polysaccharide as well as reduced nasopharyngeal carriage of the pneumococcus [158].

Following on from this, Kabat *et al.* immunised humans with purified meningococcal capsular polysaccharide, but this was not found to be immunogenic [159], probably due to the low molecular weight of the polysaccharide used (<50 kDa). In the late 1960's Gotschlich *et al.* purified high molecular weight (>100 kDa), serogroup A and C capsular polysaccharides [160] and demonstrated their immunogenicity in adult volunteers [161]. The MenC polysaccharide was subsequently used as a vaccine in two pivotal efficacy trials involving nearly 30,000 American army recruits vaccines, which demonstrated an overall protective efficacy of 90% over a 8 week period [162, 163]. A similar efficacy was provided by the MenA polysaccharide in over 16,000 Finnish army recruits over a period of 9 months, during a regional epidemic of serogroup A meningitis [164]. Since these early studies, other monovalent and combination polysaccharide vaccines have been developed. In the United Kingdom (UK), the only licensed meningococcal polysaccharide vaccine is the quadrivalent MenACWY-PS (ACWYVax®, Glaxo-Smith-Kline) which contains 50 μg of each of the capsular polysaccharides of serogroups A, C, W and Y [165].

1.5.2. Polysaccharide meningococcal vaccines offer limited immune protection

A single dose of a meningococcal polysaccharide vaccine may provide bactericidal antibodies for up to 10 years [166], although the Department of Health guidelines recommend re-vaccination at 5 yearly intervals for those at on-going risk of meningococcal disease [165]. Dellicour and Greenwood performed a meta-analysis of 25 studies which examined the effect of polysaccharide vaccines on nasopharyngeal carriage [167]. They concluded that while polysaccharide vaccines may effect a short-term reduction in carriage in populations with high colonisation rates such as military recruits, little effect was seen on civilian populations with lower natural rates of meningococcal carriage [162, 164].

Capsular polysaccharide meningococcal vaccines are TI-2 antigens (Table 5), consisting of repetitive saccharide units which cross-link B cell surface antigen receptors. Thus due to the absence of T cell help during a polysaccharide vaccine response, memory B cell are not generated and affinity maturation does not occur. This explains the brevity of protection afforded by polysaccharide vaccines and the lower affinity of antibody produced [168]. TI responses are classically thought to mediated by marginal zone B cells [106], and the relative immaturity of the splenic marginal zone under the age of 2 years, explains the poor efficacy of most polysaccharide vaccines in this age group[104].

1.5.3. Polysaccharide vaccines can cause hyporesponsiveness

Vaccine-induced hyporesponsiveness is the inability to mount a booster response of at least the same magnitude as that produced to the priming dose. Previous vaccination with meningococcal polysaccharide vaccine has been shown to impair antibody responses to subsequent meningococcal polysaccharide [169, 170] or conjugate vaccines [171]. One theory to explain this is that plain polysaccharide vaccines drive antigen specific B cells to

terminally differentiate into plasma cells, without replenishment of the memory B cell pool, thus leaving few antigen specific cells available to respond to subsequent antigenic challenge [168]. Plain polysaccharide vaccine-induced hyporesponsiveness is considered further detail in Chapter 5.

1.5.4. Serogroup A capsular polysaccharide has unusual immunological properties

As shown in Table 1, serogroup A meningococcal capsules differ from those of serogroups B, C, W and Y, and are composed of N-acetyl mannosamine phosphate. Plain serogroup A polysaccharide vaccines show unexpected immunogenicity in young infants[172, 173]. In addition some studies suggest that MenA polysaccharide vaccine may not cause hyporesponsiveness as has been observed for MenC polysaccharide [169, 174]. These results suggest that perhaps due to its chemical structure, group A polysaccharide may not be handled by the immune system as a classic TI antigen. This is discussed further in Chapter 5.

1.5.5. Serogroup B capsular polysaccharide is poorly immunogenic

Since the successful introduction of a serogroup C conjugate vaccination campaign in 1999, most invasive disease in the UK is caused by serogroup B [31]. However, capsular polysaccharide from serogroup B in not immunogenic in humans [175]. This is thought to be due to structural similarities between the serogroup B polysaccharide and human neural cell adhesion molecules, resulting in immunological tolerance to the capsular polysaccharide [176]. Experimental serogroup B vaccines have therefore focussed on subcapsular antigens such as the OMPs, and a variety of different approaches have been attempted to formulate an effective vaccine [Reviewed 11]. However, there is as yet no licensed serogroup B vaccine.

1.5.6. Conjugate vaccines consist of capsular polysaccharide covalently linked to a protein carrier molecule

Avery and Goebel first demonstrated that pneumococcal polysaccharide elicits greater immune responses in rabbits if covalently linked to a horse globulin protein than if administered in its pure form [177]. The covalent linkage of capsular polysaccharide to a protein carrier molecule, such as tetanus or diphtheria toxoid, creates a conjugate vaccine. Conjugate vaccines have been developed for use against several encapsulated organisms including *H. influenzae and S. pneumonia*.

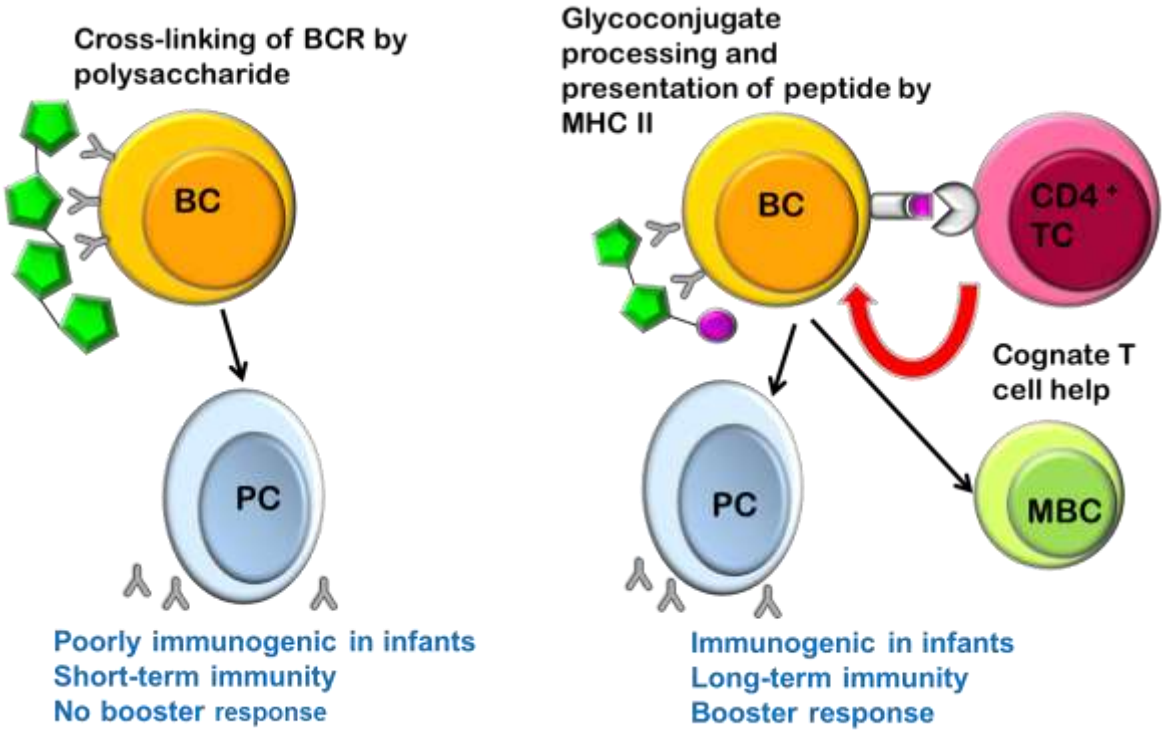
Conjugate serogroup C meningococcal vaccines were first used in the UK in 1999 as part of a nationwide vaccination campaign [178] and have been shown to be immunogenic from infancy through to adulthood [179-181]. Conjugate vaccination is highly effective over the first year post vaccination (efficacy 88-96%) [182] and to produce herd immunity [183]. This latter observation may largely be due to the reduction in nasopharyngeal acquisition subsequent to conjugate vaccination [167]. The mechanisms for reduced carriage remain to be elucidated. Both polysaccharide and conjugate vaccines induce mucosal IgA and IgG responses [95, 184], but evidence suggests that these responses are more short-lived after polysaccharide vaccination [94].

A conjugate quadrivalent ACWY meningococcal vaccine using a diphtheria toxoid carrier was first licensed for use in the USA in 2005. Two further quadrivalent vaccines using either CRM197, a mutant *Corynebacterium diphtheriae* toxoid carrier, or a tetanus toxoid carrier have since been licensed. The observed differences in immunogenicity between these conjugate vaccines [185, 186] may reflect differences in saccharide chain length [187] or the relative immunogenicity of the carrier proteins themselves [188].

1.5.7. The recruitment of T cells underlies the superior immunogenicity of conjugate vaccines

Conjugate vaccines act as TD antigens. Thus the polysaccharide moiety cross-links polysaccharide-specific B cell surface receptors prompting internalisation and processing of the whole conjugate molecule. Carrier-derived peptides are then presented on MHC class II to carrier-peptide specific CD4+ T helper cells, allowing cognate B-T interaction as shown in Figure 10. The T cell mediated follicular immune response allows the generation of memory cells to conjugate vaccines , even in young infants [189]. In theory, the establishment of a memory B cell pool allows a rapid response to subsequent infection, even in those in whom the levels of circulating antibody have waned after an initial immunisation.

Figure 10 B cell responses to polysaccharide and conjugate vaccines



PC – plasma cell, MBC – memory B cell

This generation of memory B cells underlies the ability of conjugate vaccines to 'prime' for potentiated antibody responses on subsequent exposure to antigen, as measured by SBA or enzyme linked immunosorbant assay (ELISA). This has been well documented for meningococcal conjugate vaccines in infants [174, 190] and toddlers [179]. However, whether conjugate vaccines can prime for immune memory in adults on the basis of antibody tires is unclear. Granoff *et al.* showed that adults vaccinated with a MenC conjugate vaccine mounted protective SBA responses when challenged with a polysaccharide vaccine 4 years later [191]. A subsequent study by Lakshman *et al.* compared the SBA responses to a bivalent MenAC conjugate vaccine in vaccine naïve individuals or in individuals primed with the same vaccine 1 year previously [192]. In this study, the SBA responses after a single dose of conjugate were similar to those after a second dose of conjugate, which is not suggestive of a memory response. Although these two studies are not directly comparable due to differences in antigenic content between the two conjugate vaccines used, in adults, alternative strategies to determine qualitative differences in antibody responses to polysaccharides and conjugates may be required.

1.5.8. Qualitative differences between polysaccharide and conjugate induced antibody

Antibody avidity, the strength with which multivalent antibody binds to antigen, increases over the course of an immune response due to somatic hypermutation of the variable domains, a process termed affinity maturation. As discussed in sections 1.4.6-8, although affinity maturation is classically thought to occur in TD immune responses, it may also occur to a limited extent in TI responses. Avidity is quantified experimentally by measuring the amount of a chaotrope required to disrupt antibody binding to antigen, generating an 'avidity index'. As memory responses are characterised by high avidity antibody, avidity indices have been used as a surrogate marker for priming [193, 194].

In infants, evidence suggests that avidity indices increase after conjugate, but not polysaccharide MenAC vaccination [190]. Furthermore, avidity continues to increase over a period of up to 6 months [190, 195]. In adults, the superiority of conjugate over polysaccharide is less certain. Goldblatt *et al.* showed that MenC avidity indices 1 month after MenAC polysaccharide vaccination are relatively high and similar to those 1 month after a MenC conjugate vaccination [196]. In contrast, Harris *et al.* showed that MenC avidity indices were higher 1 month after using a MenAC conjugate than 1 month after a MenAC polysaccharide[197]. Furthermore, the authors showed that post-conjugate serum conferred greater bactericidal activity in a rat intraperitoneal meningococcal challenge model compared to post-polysaccharide serum, suggesting differences in the functional activity of antibody induced by each type of vaccine. A confounding factor in interpreting the differences between these studies is the conjugate vaccine used. Both studies used the same polysaccharide comparator, but the Harris study used a MenAC-diphtheria toxoid conjugate vaccine with a lower MenC polysaccharide content than the MenC-CRM conjugate used by the Goldblatt group.

An alternative method of looking at qualitative differences in immune responses to polysaccharide and conjugate vaccines is to examine the DNA sequence encoding the V_H domain of antibody produced after vaccination. Baxendale *et al.* established pneumococcal antibody secreting hybridomas 7 days after vaccination with either a pneumococcal conjugate or polysaccharide vaccine and sequenced the V_H domain of these cells [198]. The authors showed that both polysaccharide and conjugate vaccine-induced antibody showed features of a memory response being isotype switched and hypermutated. These findings are consistent with a study by Hougs *et al.*, who showed that in a vaccine naïve individual, a *Haemophilus influenzae b* (Hib) conjugate vaccine induced a clonally selected B cell response at 7 days with somatically mutated V_H domains. Taken together, these findings suggest that both polysaccharide and conjugate vaccines

may in fact stimulate memory B cells formed by natural priming in adults, as a result of prior nasopharyngeal carriage.

1.6. The generation of antibody diversity

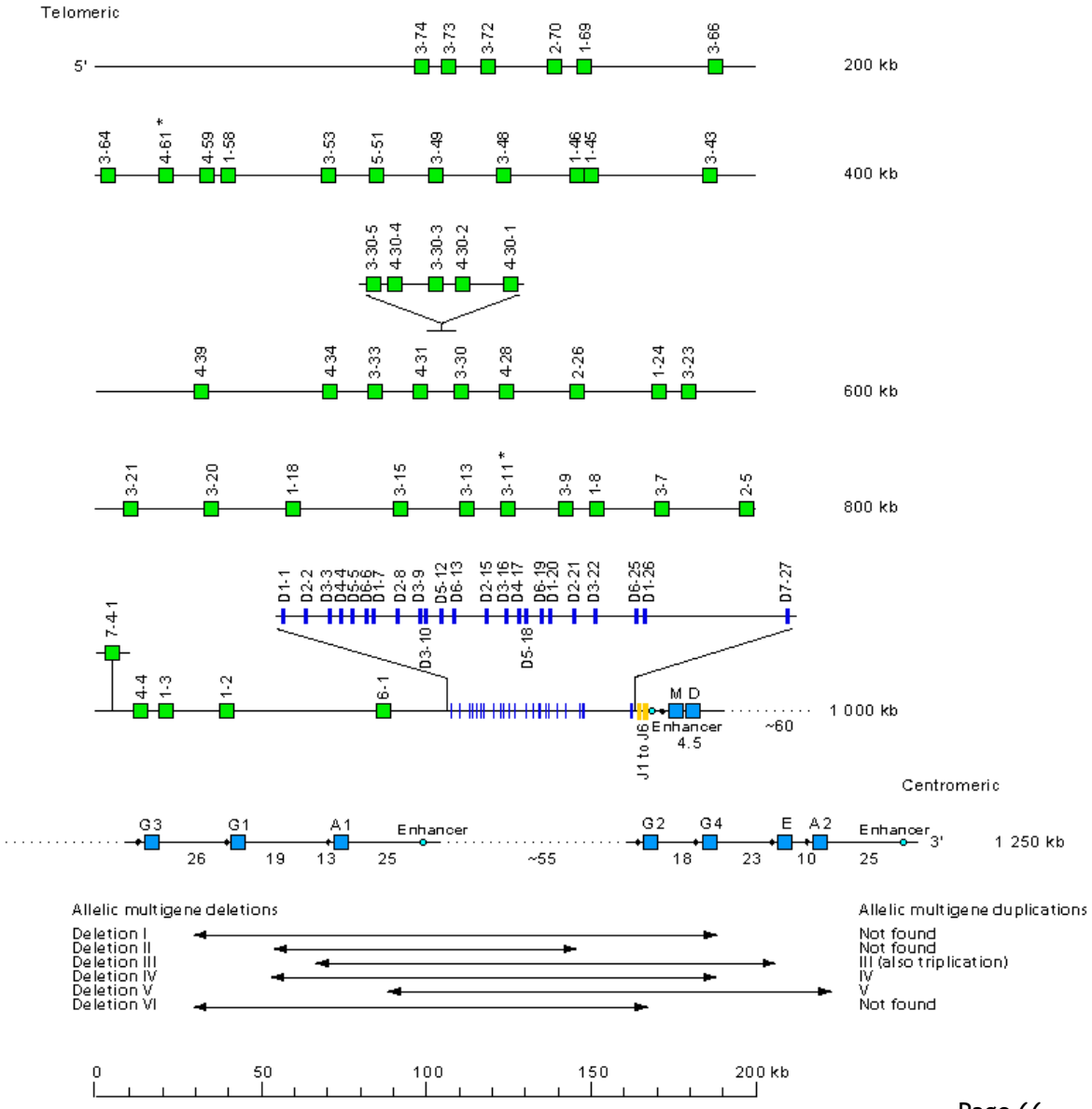
Much of the information on the clonality and affinity of the antibody response to conjugate or polysaccharide vaccines derives from studies of Hib vaccines and there are few data on the molecular basis of antibody responses to meningococcal vaccines. The remainder of this chapter describes the mechanisms responsible for generating the diversity of the antibody response and suggests novel methods for elucidating differences in the quality of antibody produced by polysaccharide and conjugate vaccines.

1.6.1. Four mechanisms underpin antibody diversity

The human humoral response is anticipatory; potential specific antibody exists prior to contact with the extensive collection of antigen encountered over the lifetime of an individual. The basis for this diverse repertoire is the multiple gene segments comprising the variable region of the antibody molecule, different combinations of which can be used in rearrangement events, mediated by the enzymes RAG-1 and RAG-2 [199]. The heavy chain locus on chromosome 14 (Figure 11) contains approximately 40 variable (V) segments, 23 diversity (D) segments and 6 junctional (J) segments [200]. The κ and λ light chain loci (on chromosomes 22 and 2 respectively) both contain approximately 50 V segments and 4 or 5 J segments [201, 202]. The potential for each V, D and J of the heavy chain locus or each V and J segment of the light chain loci to combine to create a unique variable domain introduces combinatorial diversity to the repertoire. The nucleotide

sequences of each of these gene segments and corresponding alleles is uniquely numbered and recorded at the International ImMunoGeneTics Information System® (IMGT®, http://www.imgt.org), the reference database for immunogenetics.

Figure 11 Representation of the human heavy chain locus, indicating the multiplicity of functional gene segments. V segments in green, D segments in dark blue, J segments in yellow and regulatory elements in light blue. Reproduced from IMGT®, the international ImMunoGeneTics information system® http://www.imgt.org (founder and director: Marie-Paule Lefranc, Montpellier, France)



During rearrangement, further nucleotides not encoded by the gene segments may be added to ('*n* nucleotides') the junction of the gene segments by the enzyme terminal deoxynucleotidyl transferase (TdT). In addition, unequal nicking of DNA hairpins formed at the joining ends of rearranged V, D and J segments yield further short additional palindromic nucleotides, termed '*p* nucleotides'. Nucleotides can also be deleted from gene segment junctions by exonucleases. Together, these sequence modifications result in termed junctional diversity. As the total number of nucleotides added to a junction is variable, it can disrupt the reading frame of the coding sequence beyond the joint. This leads to a non-productive rearrangement which cannot generate functional immunoglobulin, thus arresting further development of the B cell. Thus diversity is achieved at the expense of B cell loss.

Each unique heavy chain can potentially associate with either a κ or a λ chain, with many different possible combinations of heavy and light chain pairing. This combinatorial association introduces further diversity to the repertoire. These four mechanisms create a potential repertoire of up to 10^{13} antibody molecules as summarised in Table 6. Furthermore, during maturation of the antibody response, somatic hypermutation of the variable region occurs during B cell proliferation in the germinal centres, introducing point mutations into the coding sequence. This underlies the increase in antibody affinity in secondary responses to antigen.

Table 6 Diversity in immunoglobulin genes. Adapted from Immunobiology, Garland Science.

Diversity element		Heavy chain	Light chain	
			κ	λ
Multiplicity of segments	Variable segments (V)	38-46	34-38	29-33
	Diversity segments (D)	23	0	0
	Joining segments (J)	6	5	4
Combinatorial diversity	Unique VDJ combinations	6000	200	120
Junctional diversity	Joints with *n* and *p* nucleotides, exonuclease trimming	2	0	
	Junctional diversity	~3 x 10^7		
Combinatorial association	Number of V gene pairs	1.9 x10^6		
Total repertoire	**~5 x 10^{13} unique antibody molecules**			

It is unclear what fraction of the potential antibody repertoire is expressed at any one time in individuals or in response to specific antigenic stimuli. Up to 1 billion B cells are present in any given individual, with a daily turnover of around 10 million B cells. Thus not every potential unique antibody molecule can be used at any one time. Conventional estimates of the extent of the functional B cell repertoire have involved analysis of cDNA libraries [203] or Ig genes expressed in small samples such as lymphoblastic cell lines [198] or single sorted B cells [204]. These techniques are limited in their scope by the capacity of traditional chain-termination sequencing methods and able only to evaluate a fraction of the potential breadth of the repertoire. In particular, they lack the sensitivity to identify smaller populations of B cells and less frequent VDJ recombinations.

1.6.2. Complementarity determining regions (CDR) of antibody molecules exhibit the greatest sequence diversity

Within the variable domain of the heavy chain, there are 3 regions of particularly high sequence variation, termed the complementarity determining regions (CDR), which encode the amino acid loops that interact with the antigen at the antigen binding site, and are particularly susceptible to somatic hypermutation [205]. In between these regions are more conserved regions, termed the framework regions (FR) which encode structural components of the variable domain. The nucleotide sequences for CDR1 and CDR2 are located within the coding section of the V gene. However, CDR3 sequence spans the V-D joint to the D-J joint with their associated junctional diversity mechanisms discussed in Section 1.6. As it is composed of several components, this is therefore the most variable part of the V_H gene and the most closely studied in relation to antigen specificity and somatic hypermutation.

The stereochemical antigen-binding properties of the CDR3 amino acid loop are thus determined by nucleotide sequence and length. The lengths of the CDR1 and CDR2 sequences, however, are relatively constant, lacking the scope for addition or deletion of extra nucleotides, thus nucleotide sequence alone defines the antigen binding. B cells appear to select for short CDR3 lengths during the course of an immune response. Antibodies comprising VDJ sequences that have undergone somatic hypermutation have shorter CDR3 lengths than unmutated antibodies [206]. Antigen-experienced B cell populations also have shorter CDR3 lengths than naïve populations [128, 207]. This may be due to selective use of shorter J_H gene segments [208], but may also be due to differential TdT and exonuclease activity [206]. It has been suggested that long CDR3 amino acid loops fill the antigen binding pocket, limiting the access of epitopes to CDR1

and 2 loops. Thus, short CDR3 nucleotide sequences that encode small CDR3 amino acid loops, creating a binding pocket which allows antigen access to CDR1 and CDR2. In addition, it has been observed that antigen experienced B cells tend to have a more hydrophilic amino acids loops [128, 207] . The significance of this is unclear.

1.7. 'Next generation' sequencing technologies

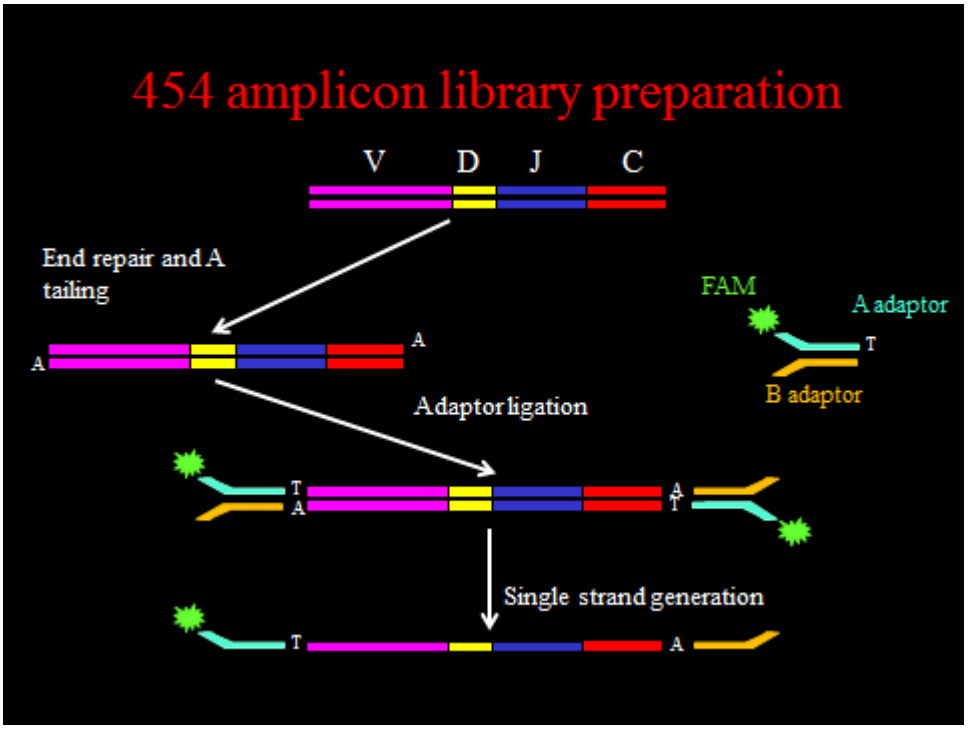
The development of 'next-generation' sequencing technology as part of the drive towards more accessible whole genome sequencing, presents a powerful tool to perform large scale DNA sequencing much faster and at lower cost than traditional Sanger sequencing [209]. Several platforms exist with the capability to sequence the great depth required to investigate the potential diversity encoded by the variable domains, each of which utilise different sequencing technologies and yield different numbers and lengths of sequenced reads. Of these, the Applied Biosystems SoLiD system generates up to 10 million 100 base pair (bp) reads which are too short to span the potentially 400bp length of the recombined VDJ sequence. Illumina technology generates up to 15 million individual 200bp paired end reads, which has been used to sequence the variable CDR3 region in depth[210]. However, the Roche 454 platform can generates up to 1 million reads of 450 bp in a single sequencing run and is therefore best suited to interrogate the whole of the recombined VDJ gene.

1.7.1. 454 Library preparation

The DNA target used in 454 sequencing may be a short sequence generated by PCR (amplicon sequencing) or by fragmentation of a much longer sequence, for example during whole genome sequencing (shotgun sequencing). The 454 system relies on the attachment of 'A' and 'B' sequencing adaptors to either end of the DNA sequence of

interest, either by incorporation using modified primers during PCR, or by ligation to create a sequencing library [211]. If the adaptors are added by ligation, the target DNA is first Klenow filled to yield a blunt end, and then the 3' strand is adenylated or 'A-tailed'. The B adaptor has a thymidine overhang, thus ensuring that the adaptors bind correctly at each end as shown in Figure 12. This also reduces the possibility of 2 adaptors binding to each other. The adaptors have a fluorescence activated molecule (FAM), which allows quantification of the library against a standard fluorescent curve. A unique 10 bp multiplex identifier (MID) sequence may also be attached to a single library during the adaptor ligation step, which allows up to 12 libraries to be sequenced in the same pool for maximum efficiency. The library is also checked for quality using an electropherogram on an Agilent Bioanalyser. Once the library is quantified by PCR, a working dilution containing 2 x 107 molecules of DNA/μL is created for the next step of emulsion PCR.

Figure 12 Double stranded PCR amplicons of the V_H domain are blunt end ligated to 454 sequencing adaptors A & B. A single stranded library containing A and B adaptors at either end is generated for subsequent emulsion PCR.



V-variable gene segment, D – diversity gene segment, J- joining gene segment, C-constant domain, FAM- fluorescence activated marker

1.7.2. Emulsion PCR

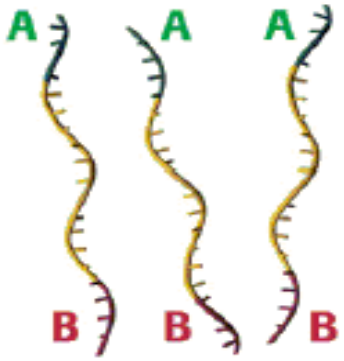
The prepared library is then denatured to yield single stranded DNA, each of which have a single A and B adaptor at each end [212]. This is added to a suspension of microbeads, titrated such that a single strand of DNA binds to a single microbead, the association mediated by the A adaptor. The microbeads are placed in an oil-in-water emulsion, with a single microbead suspended in a single droplet containing all the reagents required for PCR. Within this droplet, multiple rounds of PCR occur using the B primer for initiation, such that each microbead eventually has several million identical copies of the target sequence linked to it. The emulsion is broken and the amplified DNA is denatured yielding microbeads with clonally amplified single strands ready for pyrosequencing.

1.7.3. Pyrosequencing

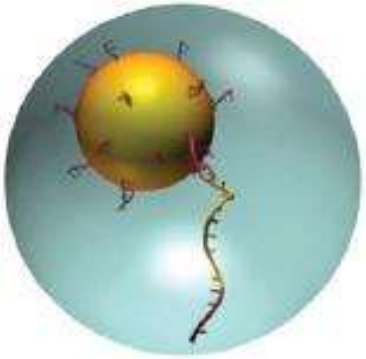
The microbeads are individually placed in the wells of a microreactor plate and covered with smaller spheres that contain the enzymes required for pyrophosphate sequencing [213]. Successive washes of nucleotides are passed over the plate – incorporation of a nucleotide is manifested by the generation of inorganic pyrophosphate and the emission of photons, which is detected by a light sensing diode. Reads are expressed in FASTA file format for bioinformatics analysis.

Figure 13 An overview of 454 sequencing. Reproduced from www.roche-applied-science.com.

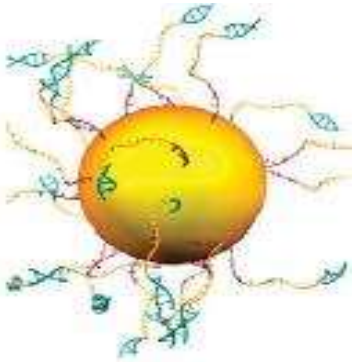
A) Single strand library



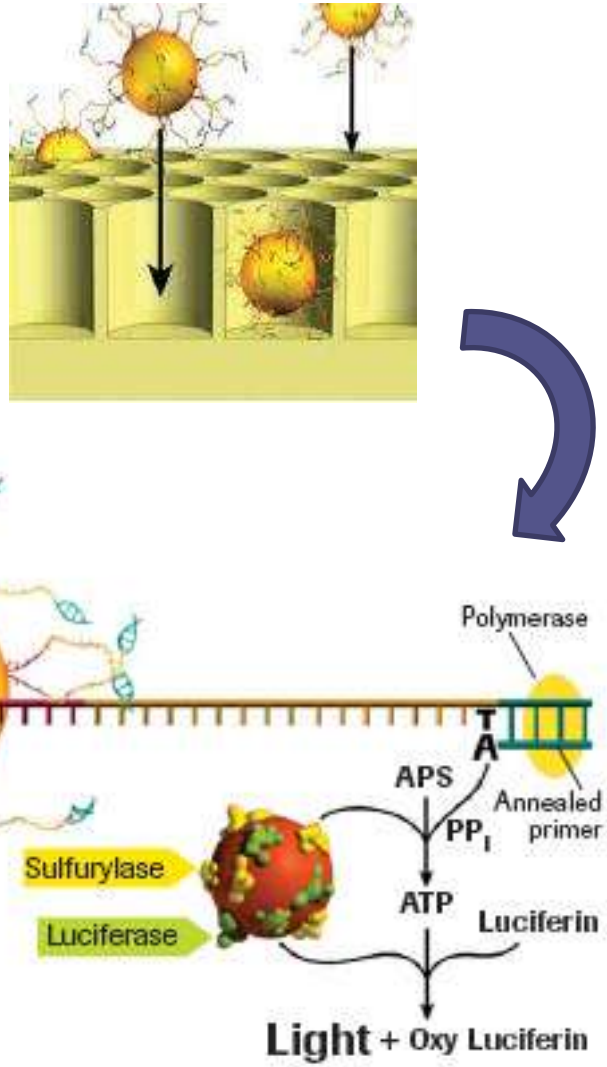
B) Microbead pre amplification



C) Clonal amplification



D) Pyrosequencing



A) A library of single stranded amplicons is generated with an A and B adaptor ligated at either end. B) A single amplicon attached to a microbead via the A adaptor and is placed in an oil-in-water emulsion. C) Clonal amplification of the amplicon sequence occurs. D) Beads with the attached clonally amplified sequences are placed in individual wells of a microtitre plate and pyrosequenced.

1.8.Aims and objectives of the thesis

This thesis aims to detail the B cell and antibody responses to quadrivalent meningococcal vaccines through the following objectives:

- To compare the immunogenicity of conjugate and polysaccharide quadrivalent meningococcal vaccines;
- To characterise the antigen specific memory B cell and plasma cell responses following immunisation with conjugate or polysaccharide quadrivalent meningococcal vaccines;
- To examine the phenomenon of polysaccharide vaccine induced hyporesponsiveness;
- To investigate whether serogroup A polysaccharide acts as a T-dependent antigen;
- To develop techniques for isolating RNA and amplifying variable domain gene sequences from small populations of antigen-specific B cells and distinct B cell subsets;
- To use novel 'next generation' sequencing techniques to describe the antibody repertoire before and after vaccination.

Chapter 2 - General Methods

2.1.Buffers

2.1.1. 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)/50% hydrochloric acid (HCl; Sigma-Aldrich; S814-8). The solution was autoclaved and kept for up to 6 months at room temperature.

2.1.2. PBS + 0.25% Tween /Elispot wash

Two point five millilitres of Tween detergent (Sigma-Aldrich; P1754) was added to 1 l of PBS. The solution was kept for up to 6 months at room temperature.

2.1.3. PBS + 2mM Ethylenediamine tetra-acetic acid (EDTA) /PBS-EDTA/ rinse buffer

Five PBS tablets and 0.744 mg of Ethylenediamine tetra-acetic acid disodium salt (EDTA; ICN Biochemicals, Aurora, USA; 195173) were dissolved in 1000 ml sterile, pyrogen free water. The pH was adjusted to 7.2 with 10 M NaOH/50% HCl. The solution was autoclaved and kept for up to 6 months at room temperature.

2.1.4. PBS-EDTA + 0.5% new born bovine serum (NBBS) /running buffer

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

2.1.5. RPMI + l-glutamine + penicillin + streptomycin/R0/complete medium

To 500 ml RPMI–1640 with phenol red indicator and 25mM hepes modification (Sigma-Adrich; R5886) was added 5 ml L-glutamine at 2mM (Sigma-Aldrich; G7513) and 5 ml of penicillin-streptomycin solution containing 50 U/ml and 0.05 g/ml of each respectively (Sigma-Aldrich; P4458). The medium was stored at 4°C.

2.1.6. R0 + 5% NBBS/ R10

Fifty millilitres of heat inactivated NBBS was added to 450 ml R0. The medium was stored at 4°C.

2.1.7. Tris-acetate-EDTA/TAE

A stock solution of 50x TAE buffer was prepared consisting of 242.2g Tris Base (Sigma-Aldrich; T1503) and 37.2 g EDTA disodium salt, titrated to pH 8.0 with glacial acetic acid (BDH, Lutterworth, UK; 100001CU), made up to 1 L with distilled water and stored at room temperature for up to 6 months. When required, 50 mL of stock solution was diluted with 450 mL distilled water to make a 1x TAE solution.

2.2.Reagents

2.2.1. 70% v/v Ethanol

300 mL of distilled water was added to 700 mL 100% ethanol (Sigma-Aldrich; E7023) and stored at room temperature.

2.2.2. 10 mM Deoxyribonucleotide triphosphates/dNTP mix

20 µL each of 100 mM deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxythymidine triphosphate (all Qiagen, Paisley, UK; 201900) were added to 160 µL distilled water and stored at -20 °C.

2.2.3. Antibodies for flow cytometry

Table 7 Fluorescent labelled monoclonal antibodies used in flow cytometry

Antibody & flurochrome	Volume added to 200µL cell suspension	Cytometer channel
CD27-FITC	12	FL1
CD5-PE	4	FL2
CD38-PE	1	FL2
IgM-PECy5	1	FL3
CD19-PECy5	1	FL3
CD19-APC	2	FL4
CD20-APC	3	FL4

FITC = fluorescein isothiocyanate, PE = phycoerythin, PerCP = peridinin chlorophyll protein, PECy5 = R-phycoerythin + cyanine 5 tandem conjugate, APC = allophycocyanin
All antibodies were manufactured by Miltenyi Biotech, Gladbach, Germany.

2.2.4. 6x DNA loading buffer

A stock solution of 6x loading buffer was prepared consisting of 25 mg bromophenol blue (Sigma-Aldrich; B5525), 25 mg xylene cyanol (Sigma-Aldrich; X4126) 25 mg orange G (Sigma-Aldrich; O1625) and 25 mg Ficoll 400 (Sigma-Aldrich; F4375) made up to 10 mls with EDTA (60 mM, pH 8.0). This was stored at room temperature until required.

2.3.Laboratory methods

2.3.1. Sample collection and handling

The required volume of venous blood was obtained using a sterile 22G butterfly needle from the anterior cubital fossa of adult volunteers. All collected blood samples were labelled with a participant number before they were transferred to the laboratory. A second randomised laboratory number was created for each study participant by the study statistician. This information was maintained on a password-controlled spread sheet to ensure that all study staff involved in processing and analysing blood samples were blinded to the vaccines received by the participant. Blood tubes were re-labelled by two

members of the laboratory team, who ensured that the correct laboratory numbers was assigned to each sample.

2.3.2. Serum bactericidal assay (SBA)

Two millilitres of blood from each participant were collected in a serum tube and allowed to clot. Serum was separated within 24 h of sampling by centrifugation, divided into 3 aliquots and was stored at −80°C until required. Serum bactericidal assays (human complement source serum bactericidal activity [hSBA]) for meningococcus serogroups A, C, W-135, and Y were performed at the laboratories of Novartis Vaccines, Marburg, Germany according to methods described previously [214]. The reference strains used for the relevant serogroups were serogroup A, F8238; C, C 11; W-135, M01-240070; and Y, 860800. hSBA titres were expressed as interpolated titres according to the reciprocal serum dilution's yielding 50% or greater killing of the target strain (as determined the by number of colony forming units/millilitre) after 60 minutes of incubation compared with growth at time 0.

2.3.3. Preparation of peripheral blood mononuclear cells (PBMCs)

Up to 5 ml of heparinised blood was diluted 1:2 with Ro. The PBMCs were then separated by density gradient centrifugation over Lymphoprep (Axis-Shield, Dundee, UK; NYC-1114545). PBMCs were then washed once in R0 before further preparation for cell culture or ex-vivo ELISpot assays.

2.3.4. Cell Counts

PBMCs or separated B cells were counted prior to use in ELISpot assays or MACS. 50 µL of cell suspension were mixed with 50 µL each of PBS and 0.4% trypan blue (Sigma; T6146). 10 µL of the resulting suspension was added to a haemocytometer (VWR

International, Lutterworth, UK; 720-0104). The number of cells in the original solution was determined using the equation below.

$$\text{Concentration of cells in original mixture (cells/ml)} = \left(\frac{\text{number of cells counted}}{\text{proportion of chamber counted x volume of chamber counted}}\right) \times \left(\frac{\text{volume of sample dilution}}{\text{volume of original mixture in sample}}\right)$$

2.3.5. Preparation of plates for Enzyme-linked immunosorbant spot assay (ELISpot)

ELISpot plates (96-well PVDF membrane) (Millipore, Darmstadt, Germany; MAIPS4510) were coated with a volume of 100 µl/well of either 5 µg/ml serogroup A, C, W-135 or Y meningococcal polysaccharides (NIBSC, Potters Bar, UK; 98/722, 08/214, 01/428 and 01/426 respectively) conjugated to 5 µg/ml methylated human albumin (NIBSC; 04/142), or 10 µg/ml diphtheria toxoid (Statens Serum Institut, Copenhagen, Denmark;), or 10 µg/ml goat anti-human Ig (Caltag Laboratories, Carlsbad, USA; CB1560499) in sterile PBS. PBS alone was added to the Ag blank wells. Multiple wells were used for each antigen. Up to 2 study samples could be loaded onto each 96 well plate as shown in Table 8 to maximise sensitivity. The coated plates were stored at 4 °C until use (no longer than 1 month). To block nonspecific binding, coated ELISpot plates were washed with PBS and blocked with R10 for a minimum of 30 min at 37 °C in 5% CO_2 prior to adding cells.

Table 8 Antigen coating plate layout with control cell suspension dilutions

	1	2	3	4	5	6	7	8	9	10	11	12
A	MenA	MenC	MenW	MenY	Dip	Ig 1:100	MenA	MenC	MenW	MenY	Dip	Ig 1:100
B	MenA	MenC	MenW	MenY	Dip	Ig 1:100	MenA	MenC	MenW	MenY	Dip	Ig 1:100
C	MenA	MenC	MenW	MenY	Dip	Ig 1:100	MenA	MenC	MenW	MenY	Dip	Ig 1:100
D	MenA	MenC	MenW	MenY	Dip	Ig 1:1000	MenA	MenC	MenW	MenY	Dip	Ig 1:1000
E	MenA	MenC	MenW	MenY	Dip	Ig 1:1000	MenA	MenC	MenW	MenY	Dip	Ig 1:1000
F	MenA	MenC	MenW	MenY	Dip	Ig 1:1000	MenA	MenC	MenW	MenY	Dip	Ig 1:1000
G	MenA	MenC	MenW	MenY	Dip	PBS	MenA	MenC	MenW	MenY	Dip	PBS
H	MenA	MenC	MenW	MenY	Dip	PBS	MenA	MenC	MenW	MenY	Dip	PBS

Dip = diphtheria toxoid, Ig= immunoglobulin control, 1:100/1000= cell dilution from 2x10^6 cell/ml stock, PBS = phosphate buffered saline.

2.3.6. Determination of ex-vivo antigen-specific plasma cells by ELISpot assay

The ex-vivo ELSpot assay was adapted from a previously published method [215]. Briefly, PBMCs prepared from peripheral blood were washed 3 times in R10, counted and resuspended in R10 to a final concentration of 2 x 10^6 PBMC/mL. One hundred microliters per well of the suspension (containing 2 x 10^5 PBMCs) was added to ELISpot plates pre-coated with meningococcal polysaccharides as described, and incubated overnight at 37°C in 5% CO2. Antibody-secreting cells (ASCs) were detected with a 1:5000 dilution of goat anti–human immunoglobulin G (IgG) -chain–specific alkaline phosphatase conjugate (Calbiochem-Novabiochem, Nottingham, UK; #401902), in R10 followed by 5-bromo-4-chloro-3-indolyl phosphate in nitroblue tetrazolium dissolved in aqueous dimethylformamide (Bio-Rad Laboratories, Hercules, USA; 170-6432). Spots were allowed to develop until the background showed signs of darkening. The reaction was then stopped with 200µl/well of water. Plates were then placed in a drying oven. Antigen specific plasma cells were not measured at baseline (i.e. day 0 and day 28 of the trial); their frequency at this time point was likely to be below the level of detection of the assay [134].

2.3.7. Determination of antigen-specific memory B cells by ELISpot assay

The cultured memory B cell ELISpot assay was based on previously published methods [215]. Briefly, PBMCs prepared from peripheral blood were diluted in R10 at a final concentration of 2 × 10^6 PBMCs/ml and added (100 µl/well) to 96-well round bottom culture plates (Fisher-Scientific, Loughborough, UK; DPS-100-080N) in a final volume of 200 µl of medium, containing 1/10,000 *Staphylococcus aureus* Cowan strain protein A [SAC, (Calbiochem, San Diego, USA; 539202)], 41.5 ng/ml pokeweed mitogen [PWM, (Sigma-Aldrich; L8777)] and 1.25 µg/ml CpG oligonucleotide [ODN-2006, (Invitrogen, Paisley, UK; tlr-2006)]. Cultures were incubated at 37 °C in 5% CO2 for 6 days. Cultured cells were plated onto pre-coated ELISpot plates at 2 x10^5 cells/well and then were incubated and developed as described for the ex-vivo ELISpot assay. The frequency of Ag-specific memory B-cells was expressed per 10^6 PBMCs added to the original well for culture.

2.3.8. ELISpot counting

Spots were counted using an AID ELISpot Reader System comprising an optical reader device (AID ELR03, serial number ELR030408215) and image analysis programme [AID ELIspot version 4.0 (Autoimmun Diagnostika)]. Identical settings were used for all plates and antigens as shown in Table 9 below, based on prior studies using these antigens [215, 216]. Well images were edited to remove artefacts (eg cracks in the well membrane) and the operator was blinded to the sample being counted.

Table 9 Spot count settings for OVG lab AID ELISpot reader

Antigen	Intensity		Size		Gradient		Emphasis
	Min	Max	Min	Max	Min	Max	(target spot size)
ACWY+PBS	15	255	30	5000	1	90	Tiny
Diphtheria	15	255	30	5000	1	90	Big
Ig	5	255	30	5000	1	90	small

Dip = diphtheria toxoid, Ig= immunoglobulin control, PBS = phosphate buffered saline.

2.3.9. Separation of B cell subsets

PBMCs were separated as described above with the exceptions that all wash steps were performed with PBS-EDTA (rinse buffer) and a total of three washes were performed after density gradient centrifugation. For B cell separation, PBMCs were resuspended in 0.5% BSA (Sigma-Aldrich; A8806) and incubated with CD19 MicroBeads and FcR blocker (both Miltenyi Biotech; 130-050-301 and 130-059-901 respectively) at 4°C for 20 minutes. Cells were washed again in 0.5% BSA, run on the Possel_s setting on a magnetic cell separator (AutoMACS; Miltenyi Biotech) and the positive fraction collected.

A plasma cell isolation kit (Miltenyi Biotech; 130-092-262) was used according to the manufacturer's instructions to obtain plasma cells. Briefly, PBMCs were incubated with a non-plasma cell depletion cocktail containing biotin-conjugated antibodies against CD2, CD3, CD14, CD15, CD22, CD34, CD56, CD123 and CD235a in addition to anti-biotin MicroBeads. The negative fraction was collected on a Depl_025 AutoMACS setting. The un-labelled pre-enriched plasma cell fraction was further incubated with CD38 MicroBeads and the positive fraction collected on a Possel_d2 setting. Separated cells were pelleted, snap frozen and stored at -80 °C until required.

2.3.10. Antibody labelling of cells

Separated CD19+ B cells or plasma cells separated as described in section 2.3.9 were labelled using surface expression markers for the assessment of purity or for B cell subset sorting. A standard labelling procedure was used for labelling the cells (OVGL SOP 014). Briefly, pelleted cells were resuspended in the appropriate volume of PBS-EDTA and FcR block as shown in Table 10 and incubated at 4 °C for 15 min to reduce non-specific binding, followed by 2 washes with PBS-EDTA at 1800 rpm for 15 min.

Table 10 FcR and PBS-EDTA volumes for FcR blocking prior to antibody labelling

Total cell number	FcR block (µL)	PBS-EDTA (µL)
1×10^6	20	60
$1 \times 10^{7*}$	20	60
2.5×10^7	50	150

* if cell counts were higher than $1x10^7$, reagent volumes were multiplied by the cell count.

Cells were resuspended in 200 µL PBS-EDTA, then incubated with pre-determined volumes of fluorochrome-labelled antibodies as shown in Table 7 for 30 min on ice, protected from light. Labelled cells were subsequently washed twice with PBS-EDTA at 1800 rpm for 15 min prior to resuspending in 500 µL PBS-EDTA for analysis by flow cytometry. If the cells were to be stored for longer than 1-2 hours prior to phenotyping by flow cytometric analysis, they were fixed by adding 100 µL 1% formaldehyde (BD Bioscience, Franklin Lakes, USA; 340181) and stored for up to 12 h in the dark at 4 °C. Labelled cells for B cell subset sorting and antibody repertoire analysis were not fixed but sorted as quickly as possible to reduce cell death.

2.3.11. Flow cytometry

Phenotyping was performed using a four colour flow cytometer (FACSCalibur™; BD Bioscience) which detected mean wavelengths of 530nm, 585nm, 670 nm and 661 nm (channels FL1-4 respectively). Cells were kept on ice and protected from light throughout. Data were collected from up to 10^5 events and analysed using CellQuest Pro™ software. B cell subset sorting for repertoire analysis was performed by a trained technician using a high speed cell sorter (MoFlo™; Beckman Coulter, Brea, USA).

2.3.12. Agarose gel electrophoresis

The ends of a gel tray (Bio-Rad, Hemel Hempstead, UK) were sealed with masking tape and one or more combs inserted as required. A 2% w/v solution of agarose (Bethesda Research Labs, Gaithersburg, USA; 5510UB) in 1x TAE buffer was prepared and heated gently in a microwave oven. Ethidium bromide (ICN Biochemicals; 802511) was added to the molten gel at a final concentration of 0.5 μg/mL before pouring into a gel tray. The gel tray was allowed to solidify at room temperature and the combs removed prior to submerging the gel in 1x TAE in a gel tank to cover it to a depth of approximately 5 mm.

Table 11 Characteristics of agarose gels

Gel tray dimensions (cm)	Gel volume (mL)	Gel thickness (cm)
7 x 10	45	0.75
15 x 10	90	0.75
15 x 25	270	0.75

DNA was mixed with 6x loading buffer in a 5:1 ration before loading into the wells of the submerged gel. DNA size and concentration were determined by comparison to a DNA ladder (Bioline Reagents, London, UK; Hyperladder I/Hyperladder IV, BIO-33025/BIO-33029), 5 μL of which was loaded into adjacent wells. A voltage of 100 V was applied across the gel until DNA fragments had separated sufficiently. DNA was visualised by examination under ultraviolet light at a wavelength of 302nm using a transilluminator.

2.3.13. DNA extraction from agarose gels

The QIAquick gel extraction kit (Qiagen; 28706) was used according to the manufacturer's instructions to extract amplified DNA from agarose gels by binding to a silica membrane. DNA was eluted with 50 μL EB elution buffer and stored at -20 °C until required.

2.3.14. Quantification of DNA and RNA

Amplified DNA was approximately quantified by comparison to bands in a DNA ladder (Bioline Reagents; BIO-33025/BIO-33029) during agarose gel electrophoresis. Precise quantification of purified DNA or RNA was performed with a spectrophotometer (Nanodrop 2000; Thermo Scientific, Wilmington, USA), using distilled water or elution buffer for blank measurements as appropriate.

2.4. Statistical Methods

2.4.1. Sample size calculation for MenACWY-CRM/MenACWY-PS clinical vaccine study

75 participants were randomised to each group. Allowing for a 10% drop out rate, this enabled 67 participants in each group. As outlined in Table 12 below (based on the SBA GMTs observed following administration of MenACWY as a comparator arm for a previous study of MenACWY [217]), this provided 80% power to demonstrate a 30% difference in the serogroup A specific SBA GMTs at day 7 following administration of MenACWY or MenACWY PS at a 1% level of significance. For serogroups C this sample size provided at least 90% power to demonstrate a 30% difference in SBA GMTs between the 2 groups at a 1% level of significance.

Table 12 Study sample size calculation

Serogroup	Level of significance	Power	Mean log_{10} (sd) for PS vaccine	Estimated difference	Sample size per group
A	1%	90%	1.447 (0.7256)	30%	85
		80%			67
	5%	90%			60
		80%			45
C	1%	90%	2.3483 (0.8115)	30%	41
		80%			33
	5%	90%			29
		80%			22

2.4.2. General considerations

Statistical analyses were performed using STATA (StataCorp LP, College Station, USA), Prism (GraphPad Software, La Jolla, USA) and Excel 2010 (Microsoft, Washington, USA). Skewed data was log_{10} transformed prior to analysis.

Substitutions were made in the following way where data were unable to be log-transformed:

- Zero ASC counts were replaced by 0.25 prior to taking logs.
- SBA values which were below the lower limit detectable by the assay were replaced by a value half the lower limit of detection (*i.e.* titre of 1:2).

2.4.3. Analysis to address Primary and Secondary Objectives

The intention to treat (ITT) population includes all patients randomised.

The per-protocol (PP) population is a subset of the ITT population. Patients excluded from the PP population include those with the following expected protocol violations;

- Participants who were subsequently found to have received a meningococcal vaccine prior to enrolment in the study.
- Participants who had visits outside the time frames specified in the protocol (ie Visit 2 = day 7 +/-1, Visit 3= day 28 +/-5, Visit 4= day 35 +/-1, Visit 5= day 56 +/-5).

The primary analysis was conducted on the ITT population. All other analyses were conducted on the PP population.

2.4.4. Statistical tests

The results of group comparisons on log scales are presented as geometric means in each group, as well as the relative difference, 95% confidence interval (CI) and associated *p* value for the group comparison. Comparisons between memory B cell numbers and serum bactericidal titres at varying time points were made using the analysis of covariance (ANCOVA) with adjustment for baseline values prior to vaccination. Comparisons of plasma cell numbers were made using independent 2 sample t–tests. All statistical tests were 2-sided and *p* values less than 0.05 were considered significant.

2.4.5. Multiple Testing

A large number of secondary endpoint comparisons were conducted in this study which increases the chance of observing false positive results. However, all comparisons are important in giving the full clinical picture surrounding the immunological response to the different vaccine regimes. No single result was considered in isolation and there was no formal adjustment of any *p* values to account for multiplicity.

2.4.6. Interim analysis

An interim analysis was performed after 60 participants completed visit 5. The primary purpose of this analysis was to aid in the rationalisation of laboratory procedures, specifically plasma and memory cell enumeration via the Elispot assay. Analysis of secondary endpoints for plasma cell and memory B cells was conducted. No statistical adjustments were made to account for this interim analysis as it did not involve data for the primary endpoint of the study.

Chapter 3 – A clinical study comparing quadrivalent conjugate and polysaccharide meningococcal vaccines in healthy adult volunteers

3.1. Introduction

Quadrivalent ACWY meningococcal vaccines are available as plain polysaccharide vaccines and protein-polysaccharide conjugate vaccines. These vaccines are largely used in the UK as travel vaccines or in the immunocompromised and are not part of the routine immunisation of individuals against meningococcal disease. However, the recent increase in serogroup Y disease in the UK suggests that an ongoing careful consideration of the need for broader protection in the general population is required. The MenACWY-CRM/MenACWY-PS[1] clinical study was designed to compare the immunogenicity of a novel quadrivalent conjugate vaccine with the existing licensed quadrivalent polysaccharide, and to specifically investigate the phenomenon of polysaccharide induced hyporesponsiveness. In this chapter, the clinical study design, objectives, recruitment and safety data will be described.

3.1.1. ACWY disease in the UK

In November 1999, the Departments of Health of the UK introduced a campaign of MenC conjugate vaccination for all children and adolescents under the age of 18 which resulted in a sharp decline in MenC disease[182, 218]. The majority of UK meningococcal disease is now caused by serogroup B[26] as shown by data from the Health Protection Authority in Figure 1 below. However other serogroups continue to cause disease albeit at low rates.

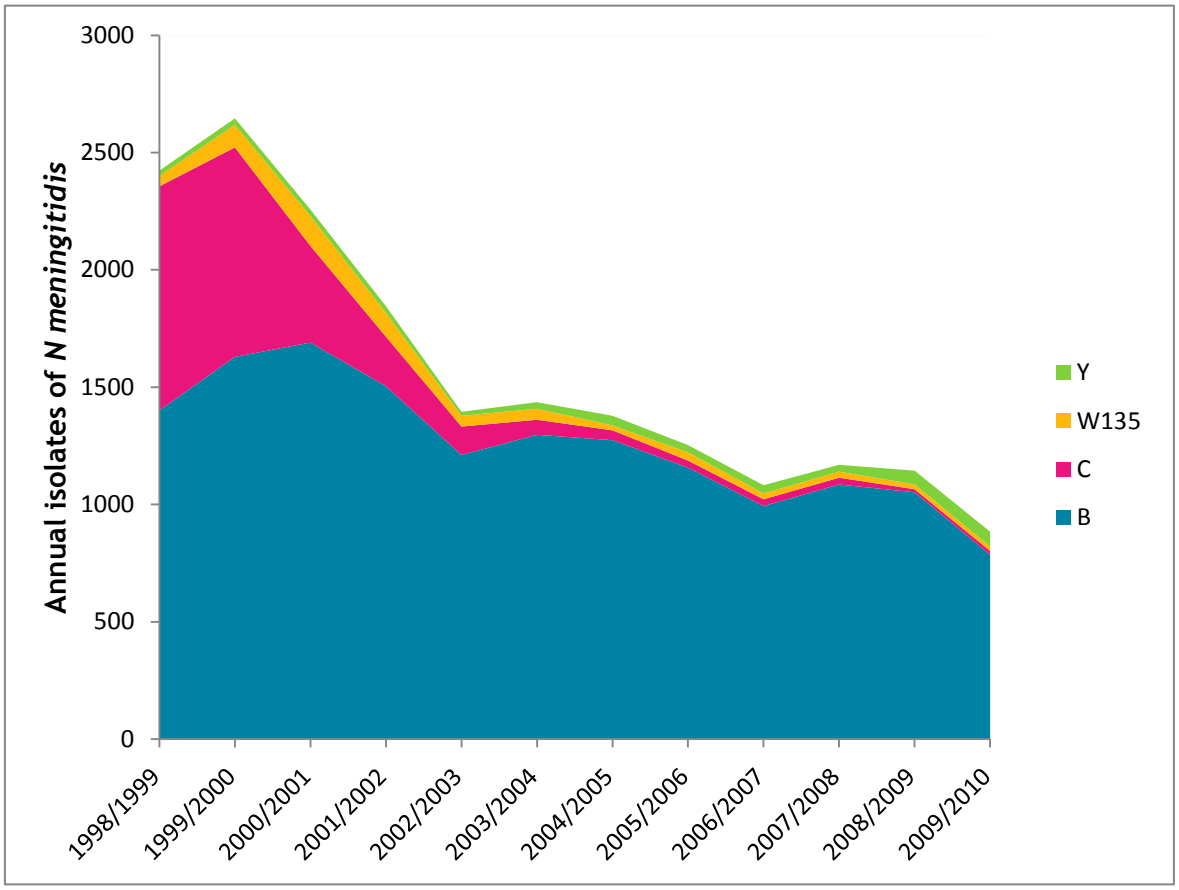
[1] Full study title 'A single centre, open-label, randomised clinical trial to investigate meningococcal serogroup a, c, w-135 and y saccharide specific B cell responses to a primary and a booster dose of the meningococcal ACWY conjugate vaccine and to a primary dose of the ACWY polysaccharide vaccine followed by a booster dose of the meningococcal ACWY conjugate vaccine administered to adult volunteers'

Clusters of serogroup A disease in the UK were previously associated with large outbreaks of disease in the Hajj pilgrimages in the late 1980's. Further outbreaks were successfully prevented by the introduction of A/C polysaccharide vaccination as a requirement for obtaining a Saudi Arabian visa. Serogroup A meningococci have not been found in clinical isolates in the UK in the past 5 years[26].

In 2000, an outbreak of serogroup W135 disease occurred in the UK, thought to be linked to pilgrims returning from Hajj or Umrah pilgrimages to Mecca or their close contacts[219, 220]. The Departments of Health recommended a change to quadrivalent ACWY vaccines in travellers in 2001, and in 2002, proof of immunisation with a quadrivalent ACWY vaccine became a requirement for obtaining a visa to Saudi Arabia. The incidence of disease caused by serogroup W-135 has since fallen in the UK.

Serogroup Y causes over a third of disease in the US but has previously been unusual in the UK. However, a rise in the incidence of serogroup Y invasive disease has been observed in adults in the UK since 2009[221]. The UK serogroup Y disease isolates belong mainly to the ST-23 clonal complex, a hyperinvasive lineage associated with both invasive meningococcal disease and carriage[222-224]. Carriage studies in university students in 2009 have demonstrated that serogroup Y meningococci represent up to 50% of nasopharyngeal isolates[225], as compared to approximately 5% of isolates in 15-19 year olds in 2001[226]. Despite the apparent increase in both carriage and invasive disease, protective immunity against serogroup Y remains low[227].

Figure 14 The number of *N. meningitidis* isolates in England & Wales (y axis) by serogroup and year (x axis) [26]



This year on year increase in serogroup Y disease is reminiscent of the pattern of disease in the USA. There, the proportion of disease caused by serogroup Y meningococci increased from 2% in 1989[228] to 10.6% in 1992 and further increased to 32.6% in 1996[229]. It is difficult to predict whether the current rise in serogroup Y will continue, ongoing surveillance of meningococcal disease is required to determine whether the broader protection afforded by quadrivalent vaccines may be needed in the UK.

3.1.2. Quadrivalent polysaccharide and conjugate vaccines are licensed for use in the UK

The MenACWY-PS polysaccharide vaccine (ACWYVax®, GSK) has been licensed in the UK since 2001 for use in children over the age of 5 and adults. The MenACWY-CRM conjugate vaccine (Menveo®, Novartis Vaccines) has been licensed in the UK since 2010 in

children over the age of 11 and adults. The Departments of Health currently recommend vaccination with the quadrivalent vaccine for pilgrims and seasonal workers travelling to Saudi Arabia and long-stay or high-risk visitors to sub-Saharan Africa, for example those who will be living or working closely with local people, or those who are backpacking[230].

Prior to the licensure of the MenACWY-CRM conjugate vaccine, MenACWY-PS was the only vaccine available for travellers to high risk countries. In view of the known short term protection afforded by polysaccharide vaccines in general, individuals at ongoing risk of disease were advised by the Departments of Health to have booster doses of MenACWY-PS every 3-5 years[161]. However, studies in Saudi adults who were required to have 3-yearly vaccination with MenA/C polysaccharide vaccine showed evidence of hyporesponsiveness to MenC following multiple vaccination with polysaccharide [169]. Several other trials have demonstrated hyporesponsiveness due to repeated doses of meningococcal polysaccharide in adults and children [231, 232]. Due to concerns over hyporesponsiveness and duration of protection afforded by polysaccharide vaccines, MenACWY-CRM has been recommended since its licensure by the Departments of Health in preference to MenACWY-PS for travellers over 11 years of age. In addition, it is suggested that MenACWY-CRM be used 'off label' in under 11s in preference to MenACWY-PS[161].

3.1.3. Rationale for the design of the study

A recent licensure study of MenACWY-CRM in Argentine and Columbian adults compared it with a different quadrivalent polysaccharide vaccine, MPSV4 (Menomune®, Sanofi-Pasteur) which is used routinely in North and South America where serogroup Y disease is common [233]. In common with MenACWY-PS, MPSV4 contains 50 μg of each of serogroups A, C, W and Y capsular polysaccharides. This South American study showed

higher serum bactericidal assay (SBA) geometric mean titres (GMTs) and a higher proportion of seroresponders in individuals vaccinated with MenACWY-CRM than MPVS4. The trial described in this thesis is the first study to directly compare MenACWY-CRM with MenACWY-PS, the only quadrivalent polysaccharide vaccine currently licensed in the UK.

Volunteers in each arm of the study received either 2 doses of MenACWY-CRM one month apart (Group 1) or a dose of MenACWY-PS followed by MenACWY-CRM one month later (Group 2), allowing several different inter-group and intra-group comparisons to be made. An overview of the study design is shown in Table 13. The immunogenicity of the MenACWY-CRM conjugate vaccine was compared with that of the MenACWY-PS polysaccharide vaccine at 7 and 28 days. Group 1 was designed to investigate the effect of priming and boosting with conjugate vaccine. Group 2 was designed to investigate the effect of prior vaccination with polysaccharide on subsequent responses to conjugate – *i.e.* polysaccharide induced hyporesponsiveness.

3.2. Methods

3.2.1. Participants and recruitment to clinical trial

A phase 3, open-label, randomised, parallel trial was conducted in Oxford, UK, involving adults 18–70 years of age. Exclusion criteria were as follows: previous anaphylactic reaction to a vaccine component, previous meningococcal vaccination or disease, HIV or immune dysfunction, receipt of blood products within the previous 3 months, pregnancy, breast-feeding, prolonged bleeding time and current participation in another clinical trial. Determination of previous meningococcal vaccination was based on the information provided by the participant but was confirmed after enrolment by contacting the participant's general practitioner. Written informed consent was obtained from the participants before enrolment. Ethical approval was obtained from the

Oxfordshire Research Ethics Committee C 09/H0606/20, Eudract number: 2007-001349-17. The study was fully compliant with ICH guidelines on Good Clinical Practice[234].

3.2.2. Study Objectives

Primary Objective

To determine whether meningococcal serogroup A specific SBA GMTs were significantly higher at 7 days after immunisation with MenACWY-CRM than 7 days after immunisation with MenACWY-PS.

Secondary Objectives

- To determine whether meningococcal serogroup C, W-135 and Y specific SBA GMTs were significantly higher at 7 days after immunisation with MenACWY-CRM than 7 days after immunisation with MenACWY-PS.

- To determine if response to the booster dose of MenACWY-CRM was greater than the response to a priming dose of MenACWY-CRM conjugate vaccine by comparing antigen specific plasma cells, memory B cells and SBA GMTs 7 or 28 days after the initial dose with results 7 or 28 days after the booster dose (*i.e.* comparing Day 7 with Day 35 or Day 28 with Day 56 within Group 1 MenACWY-CRM conjugate arm only).

- To compare the difference in response to a single dose of MenACWY-CRM conjugate vaccine in those who have received a previous dose of MenACWY-PS compared to those who had previously received no vaccine. (*e.g.* comparing Day 7 in the MenACWY-CRM conjugate group to Day 35 in the MenACWY-PS group and comparing Day 28 in the MenACWY-CRM conjugate group to Day 56 in the MenACWY-PS group).

- To compare the results of giving a booster dose of MenACWY-CRM conjugate vaccine to adults previously vaccinated with MenACWY-PS compared to those previously vaccinated with MenACWY-CRM conjugate. (*i.e.* comparing Day 35 in both groups, and Day 56 in both groups)

- To determine whether the treatment effect (difference between MenACWY-CRM and MenACWY-PS) differs according to serogroup – specifically whether the serogroup A component of the vaccine behaves differently to that of the other serogroups (C, W and Y) 7 and 28 days after treatment.

3.2.3. Study Design

Participants were randomized to receive one of two vaccination schedules: two doses of the conjugate vaccine MenACWY-CRM (Group 1) or a single dose of the polysaccharide vaccine MenACWY-PS followed by a single dose of MenACWY-CRM (Group 2). The randomisation list was produced by the study statistician. Allocation to groups was on a 1:1 basis generated by a computer-randomisation scheme. Group allocations were concealed in envelopes that were opened by the study doctor once the participant had been enrolled in the study. The randomisation envelopes were prepared by members of the OVG who were not involved in the study. The vaccines were given one month apart. Blood samples were obtained at 0, 7 and 28 days after each vaccination. Antigen-specific memory B cells were enumerated at Days 0, 28 and 56 and antigen-specific plasma cells at Days 7 and 36. SBAs for functional antibody were performed on samples at all 5 time points.

Table 13 Overview of the design of the MenACWY-CRM/MenACWY-PS study conducted in healthy adult volunteers.

	V1 Day 0	V2 Day 7	V3 Day 28	V4 Day 35	V5 Day 56
Group 1 n=75	**MenACWY-CRM conjugate**		**MenACWY-CRM conjugate**		
Group 2 n=75	**MenACWY-PS polysaccharide**		**MenACWY-CRM conjugate**		
Assays	SBA Memory B cells	SBA Plasma cells	SBA Memory B cells	SBA Plasma cells	SBA Memory B cells

3.2.4. Randomisation

Prior to study commencement, a randomization list was prepared by the study statistician, according to which cards indicating the designated study group were placed in 150 sequentially numbered opaque envelopes by a member of the Oxford Vaccine Group not associated with recruitment of participants to this study. If the participant was in good health and suitable for inclusion in the study, the next sequential participant number was assigned to them after obtaining informed consent.

Randomisation was designated to occur at the point that the opaque envelope corresponding to the designated study number was opened, thus revealing the details of the randomisation group. If the study participant declined to participate after randomisation had occurred this randomisation result was discarded and a new envelope assigned to the next participant.

The study was open labelled, so the study participant was informed of their randomisation group. Although the clinical team and the participants were not blinded to

the type of vaccine they received, the laboratory staff were blinded in order to assure objectivity of analysis.

3.2.5. Vaccines

MenACWY-CRM (Menveo; Novartis Vaccines; batch numbers: X79P45I1E, X79P45I1V) consisted of capsular oligosaccharides derived from *N. meningitidis* serogroups A, C, W-135 and Y individually conjugated to a CRM197 carrier protein. After reconstitution, the vaccine was given as a 0.5ml solution intramuscularly with a 21G 25 mm needle.

Table 14 Contents of a single 0.5 mL dose of MenACWY-CRM quadrivalent meningococcal conjugate vaccine.

Constituent	Amount per dose
MenA-CRM_{197} conjugate	10 µg MenA, 12.5 – 33 µg CRM_{197}
MenC-CRM_{197} conjugate	5 µg MenC, 6.5 – 12.5 µg CRM_{197}
MenW-CRM_{197} conjugate	5 µg MenW, 3.3 – 10 µg CRM_{197}
MenY-CRM_{197} conjugate	5 µg MenY, 3.3 – 10 µg CRM_{197}
Sodium chloride	4.5 mg
Sucrose	12.5 mg
Sodium phosphate buffer	10 mM
Potassium dihydrogen phosphate	5 mM
Water for injection	0.5 mL

MenACWY-PS (ACWYVax; Glaxo Smith Kline; batch number: A83CA066A) consisted of capsular polysaccharides derived from *N. meningitidis* serogroups A, C, W-135 and Y (50 µg of each serotype). After reconstitution, the vaccine was given as 0.5ml solution subcutaneously with a 23G 25mm needle.

Table 15 Contents of a single 0.5 mL dose of MenACWY-PS quadrivalent meningococcal polysaccharide vaccine.

Constituent	Amount per dose
MenA purified polysaccharide	50 µg
MenC purified polysaccharide	50 µg
MenW purified polysaccharide	50 µg
MenY purified polysaccharide	50 µg

Vaccines were labelled as required under the European Good Manufacturing Practice (GMP) legislation 2003/94/EC Article 12 and 2001/83/EC regarding the guidelines of Good Manufacturing Practice for Medicinal Products for Human Use and the requirements of the Clinical Trials Directive 2001/20/EC.

After each vaccination, participants were asked to remain at the study centre for 15 min in the event of an anaphylactic reaction. Any serious adverse event (SAE) occurring during the study was recorded. The relationships of adverse events (AE) to the study vaccine were determined according to criteria of temporal relationship and biological plausibility.

3.2.6. Primary and Secondary Endpoints/Outcome Measures

Primary endpoint

Meningococcal serogroup A specific hSBA GMTs were measured at Day 7 (Visit 2) following the initial immunisation with MenACWY and MenACWY PS.

Secondary endpoints

- Meningococcal serogroup C, W and Y SBAs were measured at days 0, 7, 28, 35 and 56 following the initial immunisation with MenACWY and MenACWY PS.
- Meningococcal serogroup A, C, W and Y specific memory B cells were measured on days 0, 7, 28, 35 and 56.

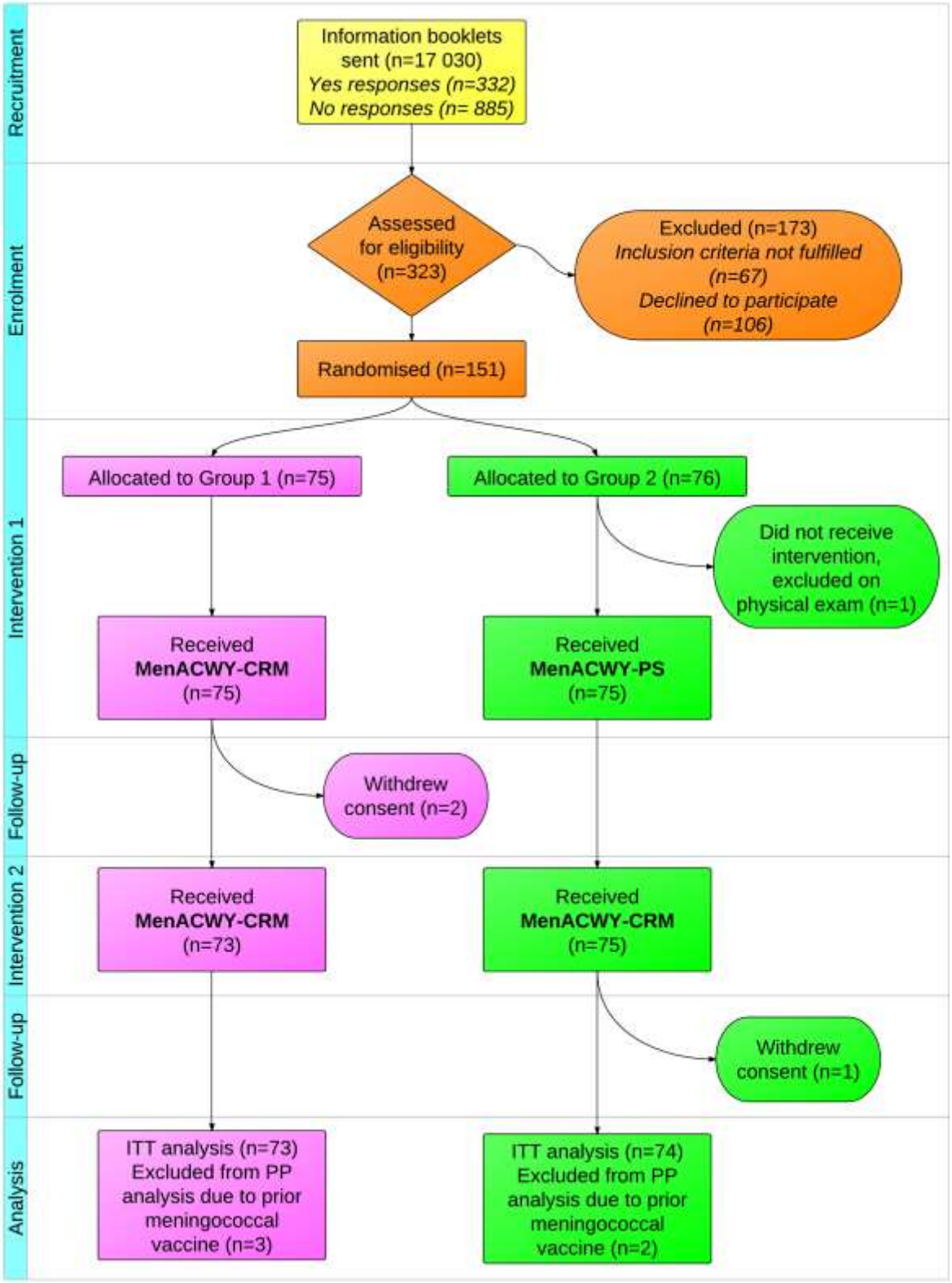
- Meningococcal serogroup A, C, W and Y specific plasma cells were measured on days 7 and 35 only.

3.3. Results

3.3.1. Recruitment

Recruitment for the study occurred between June 2009 and October 2010. The positive response rate was 2% and the exclusion rate was 21%. The main reason for exclusion was prior receipt of a meningococcal vaccine. The flow of participants through the study is shown in Figure 15.

Figure 15 CONSORT diagram indicating flow of participants through the MenACWY-CRM/MenACWY-PS quadrivalent meningococcal vaccine study in healthy adult volunteers.



3.3.2. Participants

The baseline characteristics of the 150 enrolled participants of the MenACWY-CRM/MenACWY-PS clinical study are shown in Table 16.

Table 16 Baseline characteristics of the participants of the MenACWY-CRM/MenACWY-PS clinical study.

	Group 1	Group 2
Mean age in years (range)	50.9 (28-70)	49.5 (23-70)
Sex %female	64%	49%
Number of participants	75	75

3.3.3. Safety

There were 9 AEs in Group 1 and 11 in Group 2, which are listed in Table 17. There was a single SAE in Group 2; a diagnosis of prostate cancer made in a participant who was known to have had a raised prostate specific antigen prior to enrolment in the study.

Table 17 Adverse events (AEs) and serious adverse events (SAEs) recorded during the MenACWY-CRM/MenACWY-PS clinical study listed by group allocation.

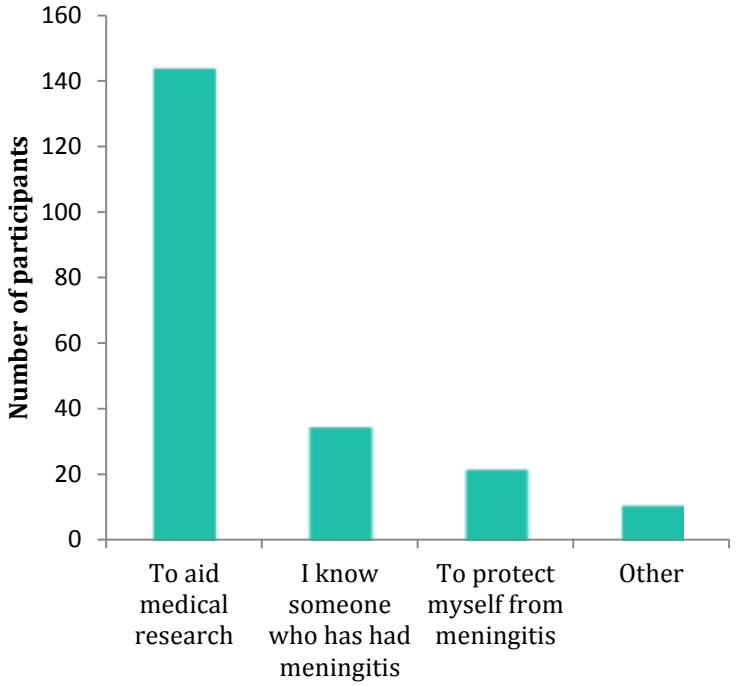
Group 1	Group 2
Haematuria	Gynaecomastia
Exacerbation of asthma	Urinary tract infection
Plantar fasciitis	Fracture fibula
Influenza	Coryzal symptoms
Mastalgia	Swollen fingers
High fasting blood sugar	Tiredness
Hypoglycaemia	Eye infection
Urinary tract infection	Tiredness
Diverticultis	Shingles
	Osteoarthritis exacerbation
	Lip haemangioma
	Prostate cancer (SAE)

NB Group 1 received 2 doses of MenACWY-CRM one month apart and Group 2 received a dose of MenACWY-PS followed by a dose of MenACWY-CRM one month later

3.3.4. Reasons for participation

Participants were asked to complete a feedback questionnaire at the end of the study to improve study planning and to assess participant satisfaction with the trial process. Part of the questionnaire asked why the participants had chosen to volunteer for the study. The results are summarised in Figure 16.

Figure 16 Reasons given by volunteers for participating in the MenACWY-CRM/MenACWY-PS clinical study.



3.4. Discussion

The recruitment of participants to a clinical trial can be challenging. Participants for this trial were largely recruited via direct mail correspondence, using addresses obtained from the UK Electoral Roll, targeted at individuals of the appropriate age living within the vicinity of the Oxford. Seven participants out of a total of 150 were recruited by posters displayed in the local hospitals, GP surgeries or Oxford University colleges and departments. Advertisements in local Oxford newspapers yielded no responses.

The initial age range targeted by the study was 18-50, with the aim to assess responses in those most likely to require quadrivalent meningococcal vaccines for travel purposes. Prior meningococcal vaccination was an exclusion criterion to avoid complicating the assessment of the immune response due to pre-existing memory. However, due to the success of the implementation of the national MenC vaccination campaign in 1999, the majority of volunteers under the age of 30 who expressed interest in the study would have been under 18 years of age in 1999 and therefore had received a MenC vaccine. Although

there is evidence that vaccines may be less efficacious in the elderly than in young adults (reviewed by Zhang et al. [95]), in November 2009 it was decided to increase the upper age limit of participation to 70 years to allow enrolment of the full cohort of 150 participants required by the study power calculation (see section 2.4.1). Raising the upper age limit of participation increased the number volunteers able to commit to participating in the study – many of the older cohort of volunteers were retired and were therefore able to attend all of the 5 study visits.

One concern with the increasing the age range of recruitment was the increasing likelihood of comorbidities. The initial study visit involved obtaining a medical history and physical examination by a study physician. Three potential participants were excluded due to potential comorbidities on examination (one diagnosis of chronic liver disease, one new diagnosis of a cardiac murmur and a new diagnosis of hypertension).

The mean age of participants was similar in each group. However, there were more female participants randomised to Group 1 than to Group 2, which reflects the true random nature of the 1:1 Group allocation process after enrolment. There is evidence of sex differences in humoral responses to hepatitis B vaccine and influenza vaccines [235, 236]. However the available published data on gender specific antibody responses to meningococcal vaccines does not suggest any significant differences between males and females [237].

None of the AEs nor the SAE were judged to be related to the study vaccines. Two participants withdrew due to AEs (urinary tract infection and exacerbation of asthma) and one participant withdrew due to difficulty with attending appointments. This number was well within the 10% drop-out rate accounted for in the study size calculation (Section 2.4.1) allowing adequate statistical power for the analysis of the primary objective. Data

obtained from patients who withdrew from the study was included up to the point of withdrawal, as defined in the study protocol in accordance with the International Conference on Harmonization (ICH) Guidance on General Considerations for Clinical Trials, which states "The protocol should specify procedures for the follow-up of patients who stop treatment prematurely"[238].

Formal reactogenicity data was not collected, as this study was designed primarily to investigate immunological responses to polysaccharide and conjugate vaccines. However, in a US multicentre study of 1359 adults 19-55 years of age[185], 38.4% and 24.9% of MenACWY-CRM recipients reported injection-site pain and headache respectively, in similar proportions to the comparator quadrivalent conjugate vaccine. No SAEs were reported. A South American multicentre study in 2831 adults [233] reported 43-46% injection-site reactions and 39% systemic reactions in MenACWY-CRM recipients (aged 19-65) compared to 40% injection-site or systemic reactions in MenACWY-PS recipients s (aged 55-65 years only). One possibly vaccine related SAE was reported in the MenACWY-CRM group (spontaneous abortion).

There were a total of 11 protocol violations (7 in Group 1 and 4 in Group 2), related to either prior meningococcal vaccination not disclosed at the time of enrolment or to visits being incorrectly timed for B cell assays. As specified in the protocol, participants were not excluded on account of these protocol violations. The primary objective was assessed using both intention to treat (ITT) and per protocol (PP) analyses. All other comparisons were made using ITT in accordance with the ICH Guidance on Statistical Principles for Clinical Trials[239] which states: "The intention-to-treat principle implies that the primary analysis should include all randomized subjects. Compliance with this principle would necessitate complete follow-up of all randomized subjects for study outcomes."

A feedback questionnaire revealed that participants had several reasons for taking part in the study. In common with other vaccine studies [240, 241] involving healthy volunteers, altruism ('to aid medical research'), was the most common reason given for taking part (144/148). Of note, 35/148 respondents decided to participate due to personal knowledge of someone who had developed meningitis. Although bacterial meningitis and associated septicaemia are uncommon conditions, with approximately 3400 cases per year in the UK [242], they are strongly emotive and have a high public profile due to awareness campaigns run by organisations such as Meningitis UK [243] and the Meningitis Research Foundation [242]. Vaccine studies are also perceived as having direct personal benefit ('to protect myself from meningitis'), to the participant, as cited by 22/148 respondents. Although participants in patient-centred studies often have misconceptions about the degree of personal benefit they may receive [244], healthy volunteers in Phase 1 trials do not usually cite personal benefit as a reason for participation in research [245]. Other reasons cited for enrolling in this study included working in the healthcare profession or prior participation in clinical trials. Financial reward was not given as a reason for participation by any of the respondents as no formal payment was made. However, each participant was reimbursed with £20 for travel expenses and time for each visit, to a total of £100 for completion of the whole study.

3.5. Conclusion

The MenACWY-CRM/MenACWY-PS study was successfully planned, initiated, conducted and completed according to the principles of Good Clinical Practice. The next two chapters of this DPhil thesis will present and analyse the data collected from this study.

Chapter 4 – The immunogenicity of MenACWY-CRM and MenACWY-PS in healthy adult volunteers

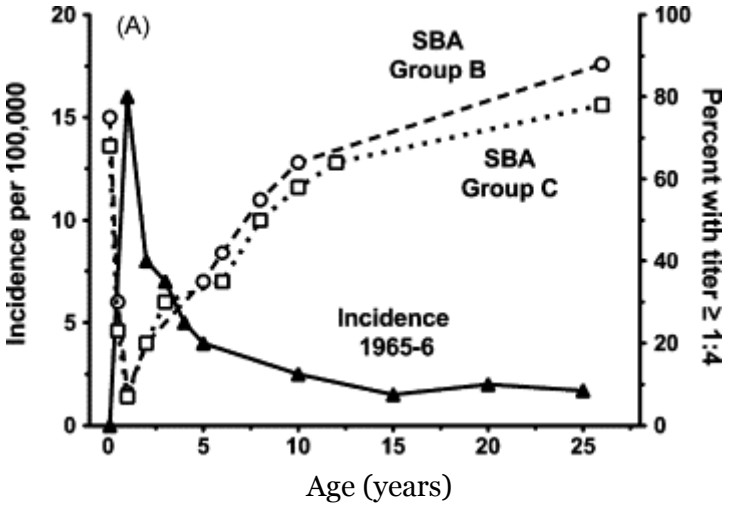
4.1. Introduction

This chapter describes the differences in the ability of quadrivalent meningococcal protein polysaccharide conjugate and plain polysaccharide vaccines to provide short-term protection as measured by serum bactericidal activity. Antigen specific memory B cell responses to each vaccine are also discussed as predictors of long-term protection.

4.1.1. Serum bactericidal activity (SBA) as a correlate of protection

The importance of serum bactericidal activity (SBA) as a correlate of protection from meningococcal disease was first demonstrated by Goldschneider [27] who showed the classic inverse relationship between incidence of meningococcal disease and SBA across a range of ages shown in Figure 17.

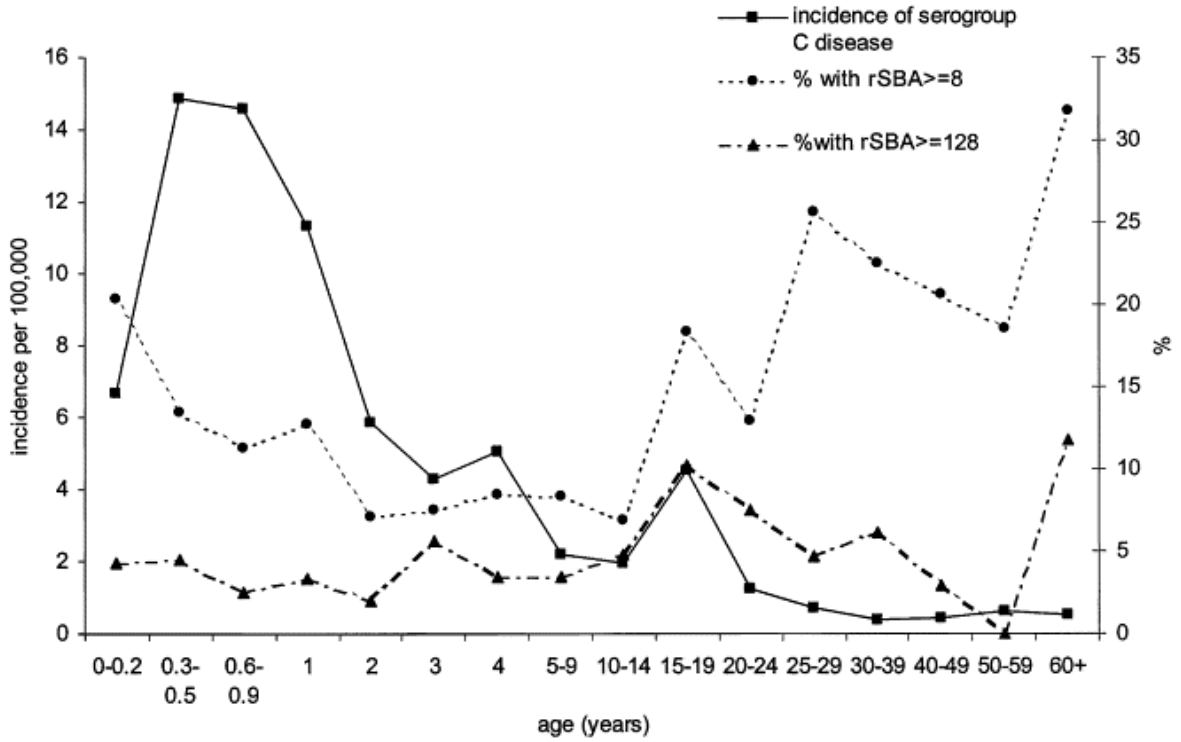
Figure 17 The incidence of all cause meningococcal disease and the percentage of the population with hSBA titres ≥1:4 for Men B (circles) or Men C (squares) against age in years (x axis). Reproduced with permission from Elsevier [76].



To test the hypothesis that a deficiency in SBA resulted in increased susceptibility to meningococcal disease, Goldschneider conducted a prospective study in nearly 15,000 American military recruits. Serum samples were collected on entry to the army and episodes of meningitis recorded over the following 8 weeks of their military training. Only 3/54 individuals who developed meningitis had SBA titres ≥4 compared to 444/540 controls who did not develop meningococcal infection. These observations established an SBA titre of ≥4 as being not only an appropriate individual correlate of short-term protection, but also a valid correlate at population level.

Goldschneider used human serum as an exogenous complement source for the SBA assay (hSBA), which can be difficult to obtain and standardise across laboratories[246]. For this reason, baby rabbit serum is now recommended by the World Health Organisation for use in standardised SBA assays (rSBA) when evaluating the immunogenicity of meningococcal polysaccharide vaccines [247]. However, meningococci are known to be more susceptible to antibody-mediated lysis in the presence of rabbit complement than human complement[92] due to their inability to bind nonhuman Factor H binding protein [248], an inhibitor of the complement pathway. Consequently, hSBA titres and rSBA titres are not directly equivalent. Thus, prior to the introduction of the MenC conjugate vaccine in 1999, the correlates of protection were re-evaluated using rSBA. Trotter et al repeated the populations studies of Goldschneider and showed a similar inverse relationship between a rSBA titre of ≥8 and MenC incidence [249].

Figure 18 Age incidence of MenC disease in relation to per cent of population with rSBA titre >8 (circles) or >128 (triangles). Reproduced with permission[249].



Borrow *et al.* measured hSBA and rSBA titres on individual samples of sera from unvaccinated and vaccinated individuals across a range of ages [250]. Based on these studies, an rSBA of >1:8 was proposed as a population based correlate of protection, which was then extrapolated back to give an individual correlate of protection of >1:8 without further formal prospective studies. Post-licensure vaccine efficacy studies after the introduction of the MenC vaccine in the UK confirmed that an rSBA titre of >1:8 at 8 weeks after immunisation best correlated with previous data on vaccine effectiveness at preventing meningococcal disease [251].

Although the above study by Borrow has shown a correlation between hSBA and rSBA titres in regard to MenC, serogroup A, W and Y specific rSBA titres do not accurately predict seroprotection as defined by a hSBA≥4 [252]. In addition, unlike the situation with human complement, with the use of rabbit complement IgM contributes significantly

to any measured SBA [253]. Since this only produced early on in the immune response and is short-lived, this may give a misleading interpretation of protective efficacy when SBA is measured only a month after immunisation as is typical for many vaccine studies. SBA assays were therefore performed using a human complement source for serogroups A, C, W & Y in the present study. There is no validated correlate of protection against serogroups A, W & Y.

4.1.2. Antibody persistence and immunological priming: independent correlates of short and long term protection?

Immunological priming - the ability to mount a secondary antibody response on re-encounter with antigen - is a characteristic feature of immune responses generated by thymus dependent (TD) antigens such as the MenC conjugate vaccine [254]. The relative importance of antibody persistence versus immunological priming in the protection afforded by conjugate vaccines is uncertain. Although rSBA titres may be indicators of short-term protection, the efficacy of the MenC conjugate vaccine is significantly underestimated by rSBA titres at 7-9 months post vaccination [251]. This indicates that SBA at the time of exposure to *N. meningitidis* may be less important than immunological memory as a long-term predictor of protection. In contrast, the decline in MenC polysaccharide vaccine efficacy over time is associated with a decline in vaccine-induced SBA [255, 256] as immunological memory is not generated by thymus independent (TI) antigens (Section 1.4.9).

4.1.3. The cellular basis of immunological priming by conjugate vaccines

Memory B cells have been shown to persist in humans decades after antigen exposure[135]. By rapidly differentiating into antibody-secreting plasma cells on re-encounter with antigen, MBCs form the basis of immunological priming. Blanchard-

Rohner *et al.* showed that the memory B cell response immediately after priming with MenC conjugate correlate with both functional antibody and the degree of the booster response up to 7 months later[215].

Conjugate vaccines may afford a longer duration of protection as a result of their ability to generate memory B cells through thymus dependent germinal centre formation (Section 1.5.6). In infants, priming with serogroup C conjugate induces persisting MBCs in infants in contrast to priming with serogroup C polysaccharide [189]. Similarly, pneumococcal conjugate vaccines have been demonstrated to induce memory B cell generation in adults in contrast to pneumococcal polysaccharide vaccines [257].

4.2.Methods

This chapter relates to the first month of the MenACWY-CRM/MenACWY-PS clinical trial discussed in Chapter 3. Participants were bled immediately prior to receiving either a single dose of MenACWY-CRM or MenACWY-PS, and again at 7 and 28 days after vaccination as shown in Table 18.

Table 18 Timeline of procedures in the first 28 days of the MenACWY-CRM/MenACWY-PS clinical vaccine trial in healthy adult volunteers.

	V1 Day 0	V2 Day 7	V3 Day 28
Group 1 n=75	**MenACWY-CRM conjugate**		
Group 2 n=75	**MenACWY-PS polysaccharide**		
Assays	SBA Memory B cells	SBA Plasma cells	SBA Memory B cells

hSBA assays were performed at each time point as described in Section 2.2.2 and as previously described [214]. 'Seroresponse' was defined as a composite endpoint that incorporated two categories of pre-vaccination immune status: for initially seronegative subjects, (baseline hSBA titre <1:4), seroresponse was defined as a post-vaccination hSBA titre of ≥1:8; for those initially seropositive, (baseline hSBA titre ≥1:4), seroresponse was defined as at least a 4-fold increase in the pre-vaccination titre [233].

Memory B cells were measured at baseline at day zero and at day 28 as described in Section 2.3.7. Plasma cells were measured at day 7 as described in Section 2.3.6. The time points for quantifying memory B cells and plasma cells were based on prior studies of B cell kinetics after vaccination in adults [134] and infants [258]. The carrier protein used in MenACWY-CRM is CRM197, a naturally occurring mutant diphtheria toxoid. CRM197 is known to elicit diphtheria toxoid specific B cells and antibody [215]. As CRM197 was not available as an antigen to coat the ELISPot plates, diphtheria toxoid was used as a

substitute. MBCs and plasma cells specific for serogroups A, C, W & Y and diphtheria toxoid were measured. No serological assays were performed for diphtheria toxoid or CRM197.

4.3. Results

4.3.1. SBA responses to a single dose of MenACWY-CRM conjugate or MenACWY-PS polysaccharide vaccine

75 participants from Group 1 received a single dose of MenACWY-CRM and 75 participants from Group 2 received a single dose of MenACWY-PS. The hSBA GMTs of these individuals pre-vaccination and at days 7 and 28 post vaccination are shown in Table 19.

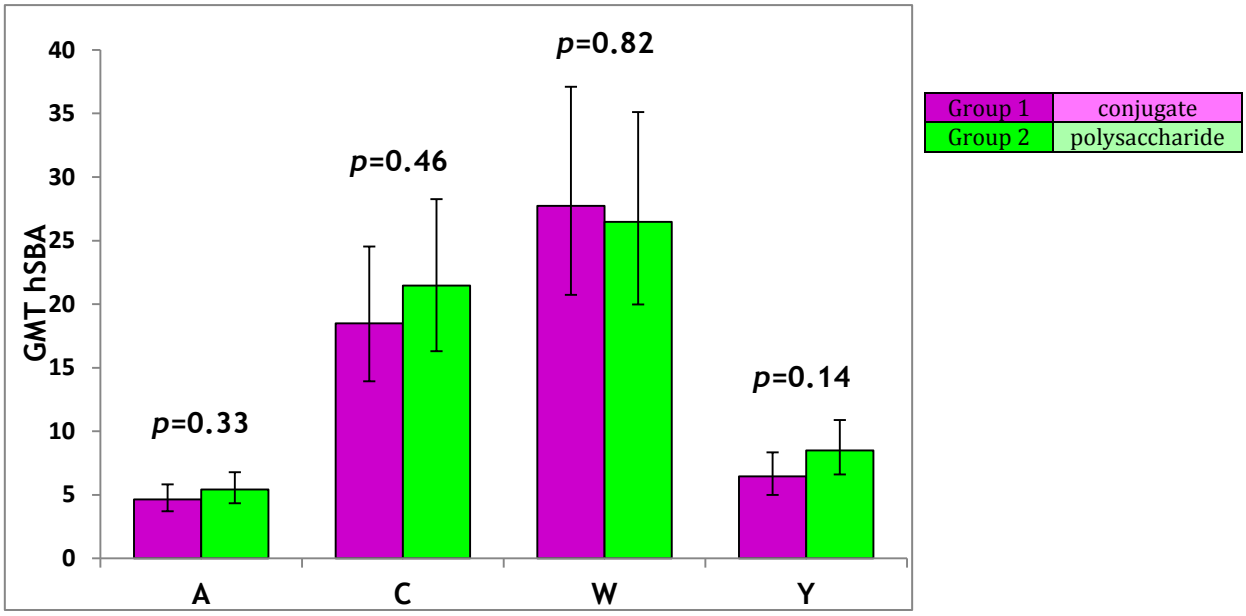
Table 19 Unadjusted hSBA geometric mean titres against serogroups A, C, W & Y at baseline and at 7 and 28 days after vaccination with MenACWY-CRM (Group 1) or MenACWY-PS (Group 2).

Serogroup	Group	V1 (day 0)	V2 (day 7)	V3 (day 28)
A	1	**3.2** (2.6, 4.0) [75]	**4.3** (3.2, 5.7) [72]	**16.7** (10.9, 26.1) [71]
	2	**3.9** (3.0, 5.1) [75]	**5.9** (4.2, 8.3) [73]	**26.5** (16.1, 43.5) [75]
C	1	**6.3** (4.6, 8.5) [73]	**14.3** (9.7, 21.3) [71]	**32.7** (20.06, 53.19) [68]
	2	**11.1** (8.2, 15.0) [75]	**27.0** (18.8, 38.6) [74]	**98.9** (64.9, 150.6) [75]
W	1	**12.0** (8.0, 18.0) [75]	**20.67** (13.4, 31.8) [68]	**64.1** (40.7, 100.8) [66]
	2	**26.2** (17.5, 39.3) [74]	**34.5** (23.4, 50.7) [73]	**53.1** (35.6, 79.0) [74]
Y	1	**3.8** (3.0, 4.7) [75]	**5.5** (4.1, 7.5) [70]	**15.4** (10.1, 23.5) [71]
	2	**5.6** (4.2, 7.5) [75]	**9.8** (7.1, 13.7) [74]	**21.7** (13.9, 33.8) [75]

95% confidence intervals are shown in round brackets and numbers of samples are shown in square brackets. The numbers of samples at each time point varied due to missed visits, participant drop out, insufficient serum for assays or failed assays.

Baseline titres (day 0) differed significantly between the study groups for serogroups C & W, with higher titres in Group 2 (Mann-Whitney U tests p=0.008 and p=0.007, respectively). Subsequent between group analyses were performed using an analysis of covariance (ANCOVA) model with adjustment for baseline (day 0) hSBA titres. The values presented in the between group comparisons therefore differ from the raw GMTs presented in Table 19. There was no overall correlation between age and baseline hSBA titre for any of the four serogroups (data not shown).

Figure 19 hSBA GMTs & 95% CI 7 days after vaccination with a single dose of MenACWY-CRM (n=68-72) or a single dose of MenACWY-PS (n=73-74), ANCOVA with adjustment for baseline hSBA values at day 0.



The primary objective of the study (Section 3.2.2) was to compare serogroup A specific hSBA GMTs seven days after immunisation with either MenACWY-CRM or MenACWY-PS. As shown in Figure 20 , there were no statistically significant differences between the conjugate and the polysaccharide groups in meningococcal serogroup A, C, W or Y specific GMTs measured at day 7 with adjustment for baseline values at day 0.

Figure 20 shows that 28 days after the initial immunisation, MenACWY-PS generates significantly higher serogroup C specific hSBA titres than MenACWY-CRM (p=0.02). However, MenACWY-CRM generates higher serogroup W specific hSBA titres that MenACWY-PS (p<0.01). There are no significant differences observed for serogroup A and Y hSBA responses at 28 days between Group 1 and 2.

Figure 20 hSBA GMTS & 95%CI 28 days after vaccination with a single dose of MenACWY-CRM (n=62-66) or a single dose of MenACWY-PS (n=68-70), ANCOVA with adjustment for baseline hSBA values at day 0. Note the difference in scale of the y axis from Figure 19.

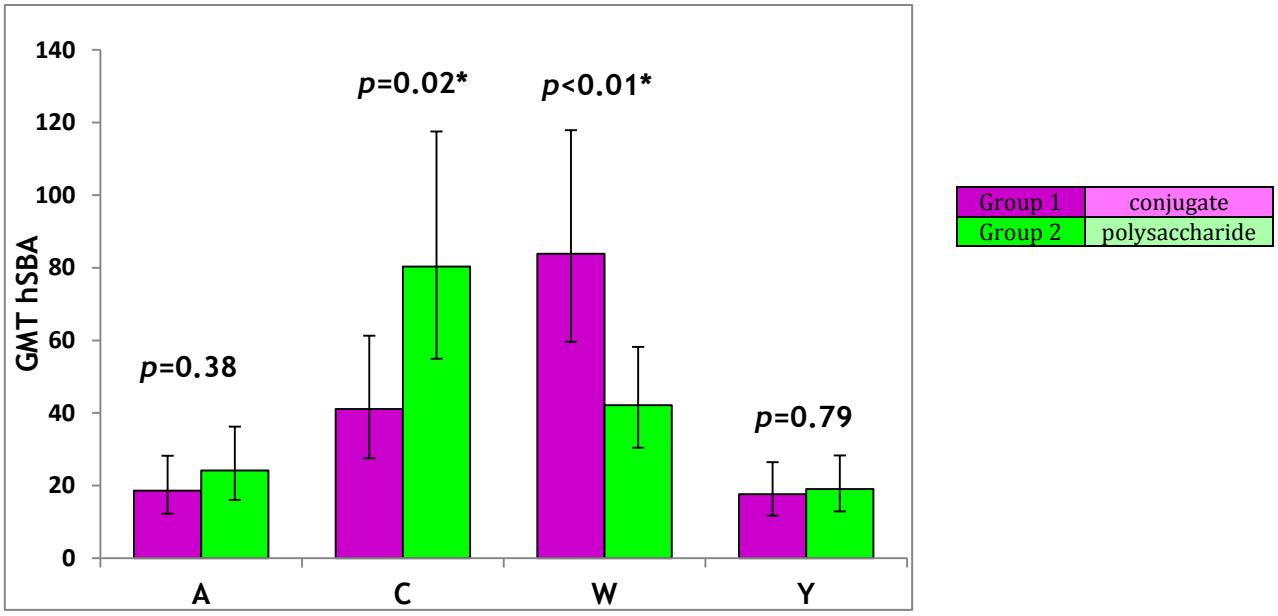
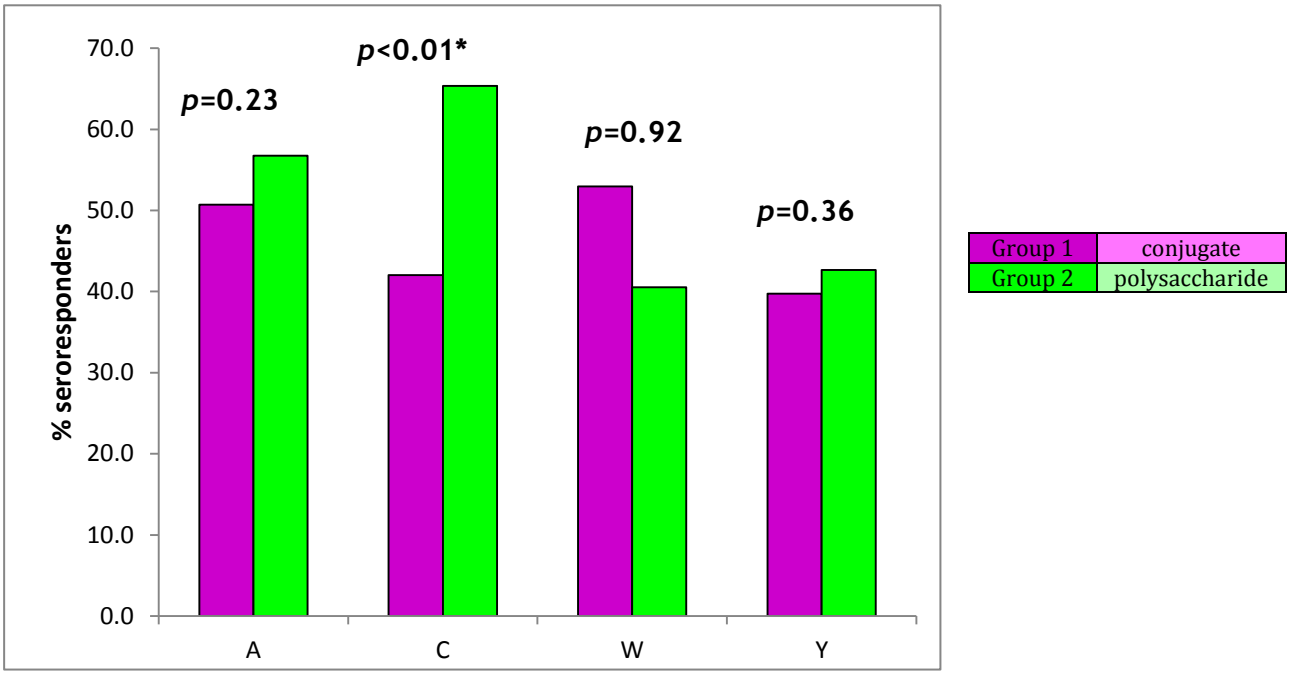


Figure 21 Percentage of seroresponders at 28 days (if baseline hSBA titre <1:4 then post-vaccination hSBA titre of ≥1:8 OR if baseline hSBA titre ≥1:4 then at least a 4-fold increase in the pre-vaccination titre), following a single dose of MenACWY-CRM (n=68-73) or MenACWY-PS (n=74-75), independent 2 sample t tests.



The 'seroresponse' endpoint at 28 days is, by definition (Section 4.2), adjusted for the baseline (day 0) hSBA titre and is an alternative to the ANCOVA analysis. As shown in Figure 21, the polysaccharide vaccine appears to generate a significantly higher proportion of seroresponders to serogroup A than the conjugate vaccine ($p<0.01$), a pattern similar to the ANCOVA analysis at 28 days (Figure 20).

4.3.2. Plasma cell responses to a single dose of MenACWY-CRM conjugate or MenACWY-PS polysaccharide vaccine

Antigen specific plasma cell numbers were determined on 47 participants from Group 1 and 50 participants from Group 2 as shown inTable 20.

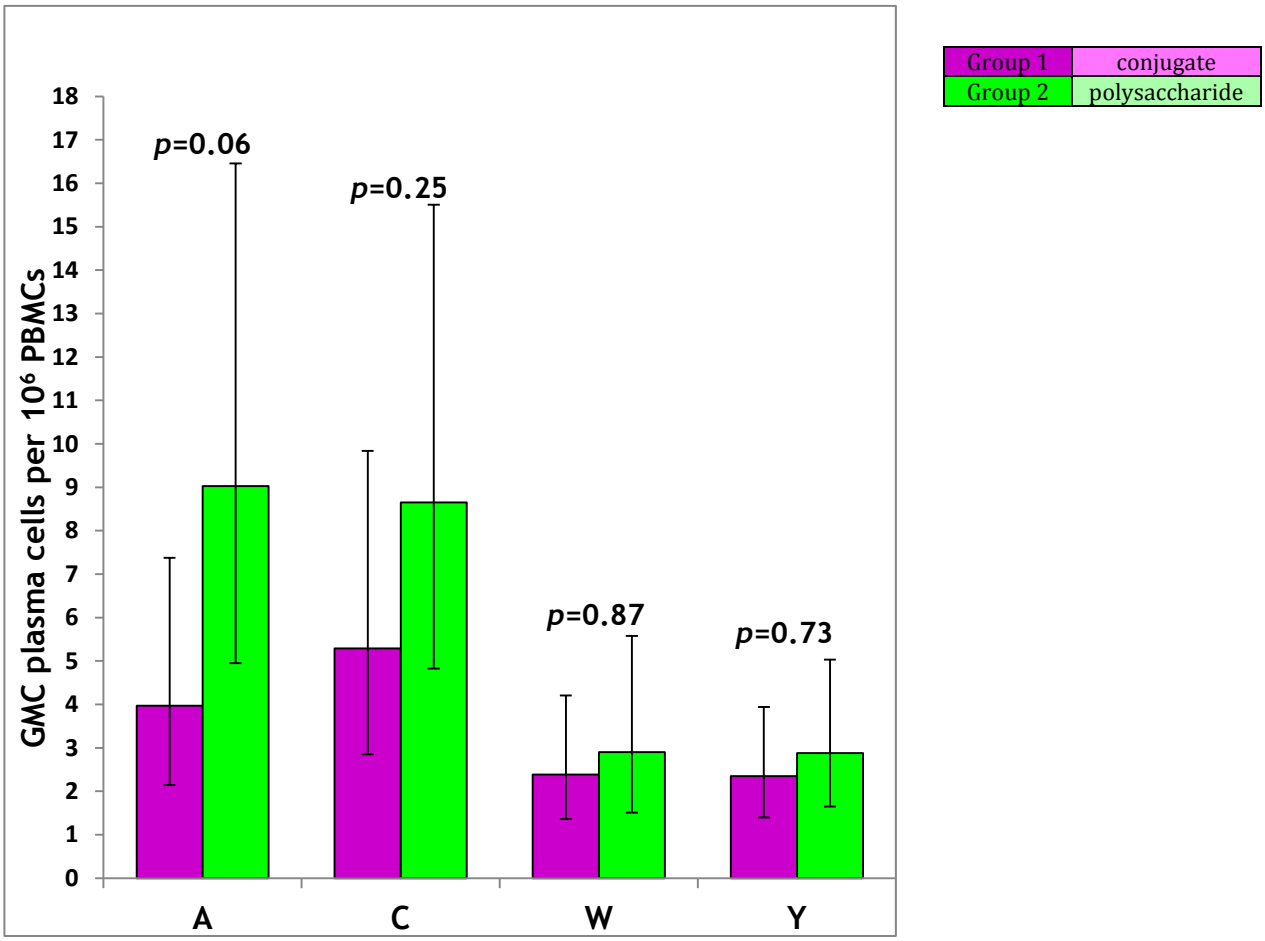
Table 20 Geometric mean counts of plasma cells specific for meningococcal serogroups A, C, W & Y and diphtheria toxoid 7 days after vaccination with MenACWY-CRM (Group 1) or MenACWY-PS (Group 2).

Serogroup	Group	V2 (day 7)
A	1	**3.97** (2.14, 7.38) [47]
	2	**9.03** (4.95, 16.46) [50]
C	1	**5.29** (2.85, 9.84) [47]
	2	**8.65** (4.83, 15.51) [50]
W	1	**2.40** (1.36, 4.21) [47]
	2	**2.90** (1.51, 5.58) [50]
Y	1	**2.35** (1.40, 3.94) [47]
	2	**2.88** (1.65, 5.03) [50]
Diptheria toxoid	1	**19.86** (10.73, 36.77) [47]
	2	**0.44** (0.34, 0.57) [50]

95% confidence intervals are shown in round brackets and numbers of samples are shown in square brackets. The numbers of samples between groups varied due to missed visits, insufficient blood for assays or failed assays.

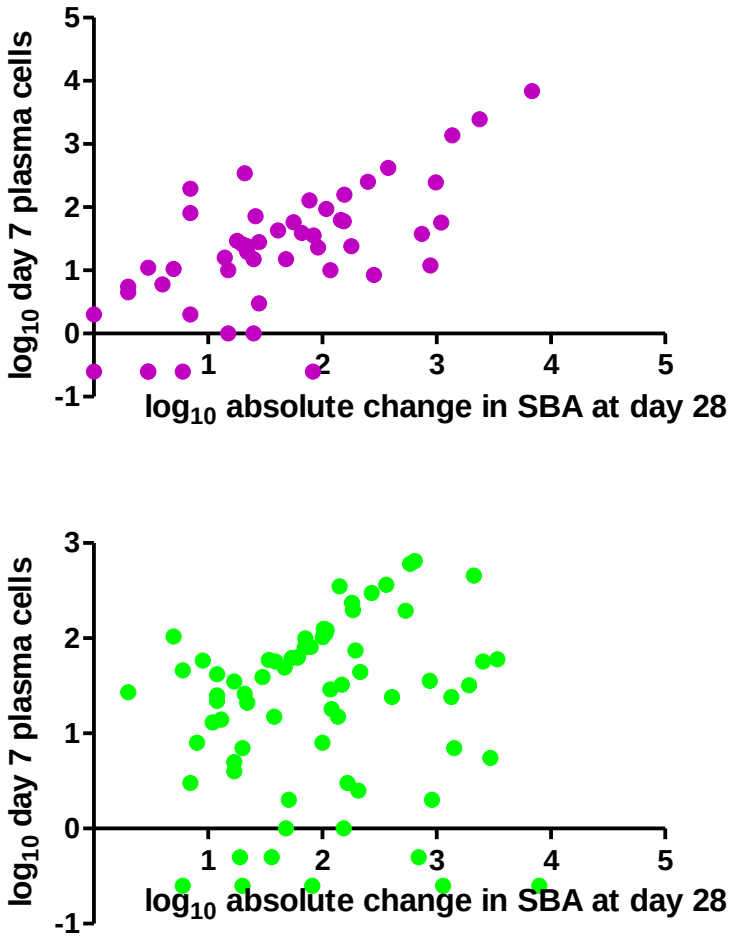
Plasma cell GMCs were compared between Group 1 and 2 at day 7 to assess the response to a single dose of either conjugate or polysaccharide (Table 20 and Figure 22). Overall, there is a trend for MenACWY-PS to generate a greater plasma cell response at 7 days than MenACWY-CRM across all four serogroups, although this is not statistically significant for any individual serogroup. There was no adjustment for baseline plasma cell counts as the frequencies of antigen specific plasma cells prior to vaccination are below the threshold of detection of this *ex vivo* ELISpot method [215].

Figure 22 Absolute plasma cell GMCs 7 days after a single dose of MenACWY-CRM vaccine (n=47) or MenACWY-PS vaccine (n=50), independent 2 sample t tests.



Serogroup C specific plasma cell counts at day 7 correlated with the absolute change in serogroup C specific hSBA GMT's at day 28 for the conjugate group (Pearson *r* coefficient =0.68), but not for the polysaccharide group (Pearson r coefficient =0.09) as shown in Figure 23.

Figure 23 Correlation between log_{10} MenC specific plasma cells at day 7 and log_{10} absolute change in MenC specific hSBA titres from baseline to 28 days after a single dose of MenACWY-CRM (purple) and MenACWY-PS (green), Pearson correlation analysis.



	conjugate	polysaccharide
Number of pairs	50	70
r (95% CI)	0.68 (0.50-0.81)	0.09 (-0.15-0.32)
p value	<0.0001	0.45

4.3.3. Memory B cell responses to a single dose of MenACWY-CRM or MenACWY-PS

Memory B cells frequencies were ascertained at baseline and 28 days on 53-58 participants who received a single dose on MenACWY-CRM (Group 1) and on 55-59 participants who received a single dose of MenACWY-PS (Group 2) as shown in Table 21.

Table 21 Unadjusted geometric mean counts of memory B cells specific for meningococcal serogroups A, C, W & Y and diphtheria toxoid at baseline and 28 days after vaccination with MenACWY-CRM (Group 1) or MenACWY-PS (Group 2).

Serogroup	Group	V1 (day 0)	V3 (day 28)
A	1	**1.62** (1.13, 2.31) [57]	**2.78** (1.86, 4.13) [58]
	2	**1.78** (1.24, 2.55) [57]	**1.99** (1.37, 2.87) [59]
C	1	**1.27** (0.88, 1.82) [58]	**4.61** (3.06, 6.96) [58]
	2	**2.04** (1.36, 3.07) [57]	**2.42** (1.68, 3.48) [59]
W	1	**0.94** (0.67, 1.32) [55]	**1.26** (0.84, 1.89) [54]
	2	**1.25** (0.86, 1.81) [55]	**1.41** (0.97, 2.05) [57]
Y	1	**1.01** (0.70, 1.46) [55]	**1.28** (0.85, 1.91) [53]
	2	**1.37** (0.95, 1.96) [55]	**1.28** (0.90, 1.82) [58]
Diptheria toxoid	1	**4.73** (3.18, 7.06) [58]	**19.92** (13.00, 30.52) [53]
	2	**7.05** (4.89, 10.17) [57]	**5.71** (3.84, 8.49) [59]

95% confidence intervals are shown in round brackets and numbers of samples are shown in square brackets. The numbers of samples at each time point varied due to missed visits, participant drop out, insufficient blood for assays or failed assays.

There were no significant differences in baseline titres (day 0) between Groups 1 & 2 for any of the 4 meningococcal serogroups or diphtheria toxoid (independent 2 sample t tests, data not shown). Subsequent between group analyses were performed using an analysis of covariance (ANCOVA) model with adjustment for baseline (day 0) memory B cell GMCs. The values shown in Figure 24 therefore differ from the raw memory B cell GMCs presented in Table 21.

Figure 24 Memory B cell GMCs and 95% CI 28 days after a single dose of MenACWY-CRM (n=53-58) or MenACWY-PS (n=55-59) vaccine, ANCOVA with adjustment for baseline (day 0) values.

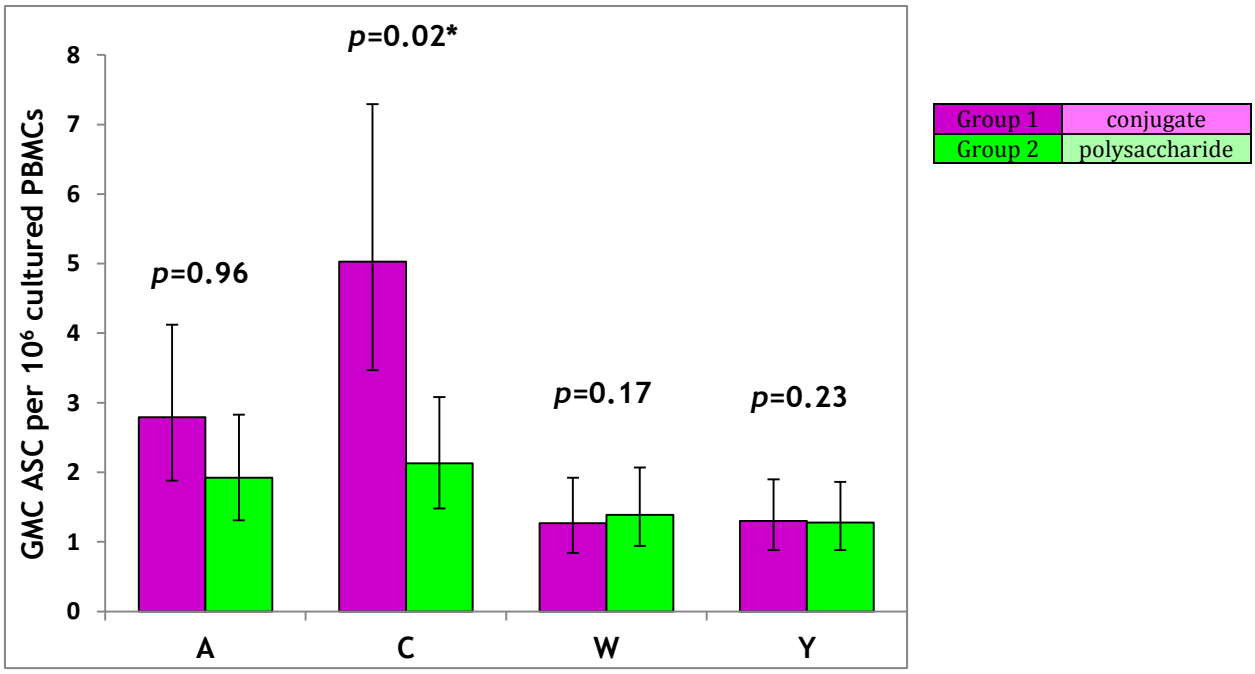
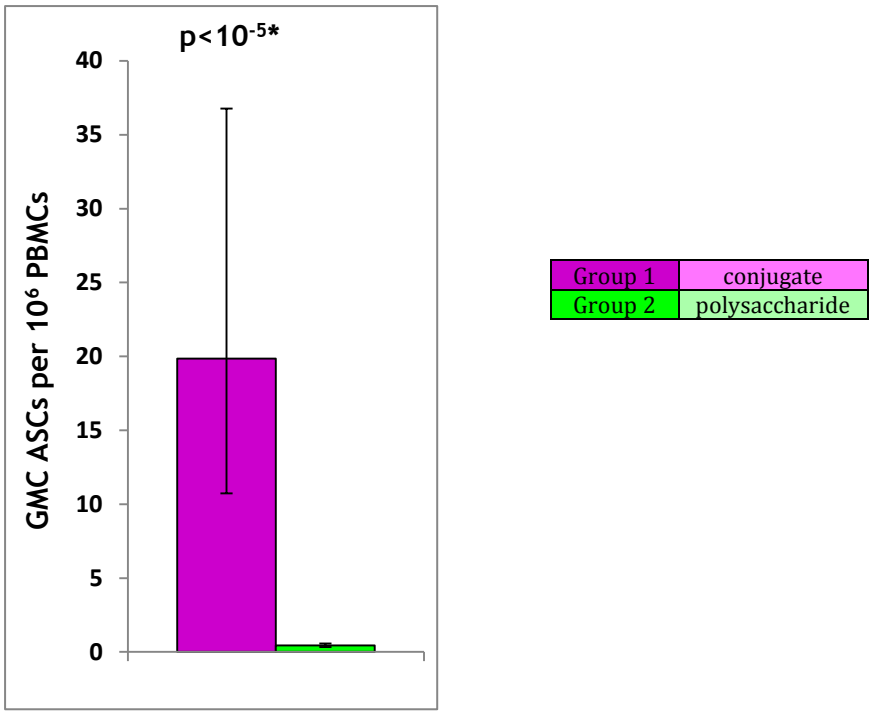


Figure 24 shows that serogroup C specific memory B cell frequencies are significantly higher 28 days after vaccination with MenACWY-CRM than MenACWY-PS (p=0.02). There are no significant differences between the conjugate and the polysaccharide vaccine for serogroups A, W or Y.

4.3.4. B cell response to carrier protein

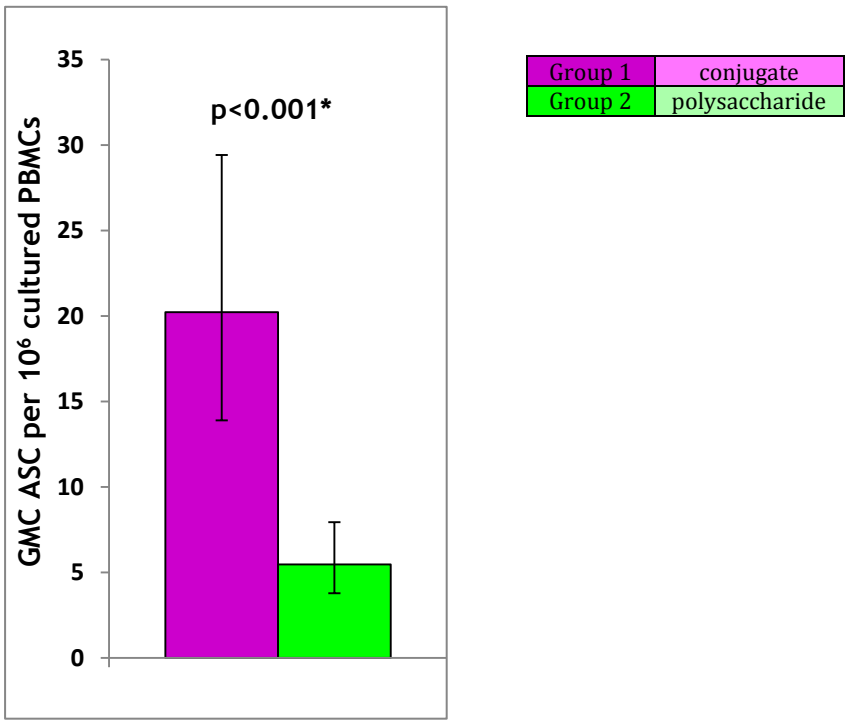
The CRM-containing conjugate vaccine elicited significantly higher frequencies of diphtheria-specific plasma cells than the polysaccharide vaccine ($p<10^{-5}$) as shown in Figure 25.

Figure 25 Diphtheria specific plasma GMCs & 95%CI 7 days after vaccination with a single dose of MenACWY-CRM (n=47) or a single dose of MenACWY-PS (n=50), independent 2 sample t test.



The number of diphtheria-specific MBCs at baseline as a proportion of total IgG secreting memory cells was 0.01%. The memory B cell frequency at day 28 in Group 2 was not significantly different from that at baseline. As shown in Figure 26, MenACWY-CRM elicited significantly higher diphtheria toxoid specific memory B cells at day 28 than MenACWY-PS ($p<0.001$).

Figure 26 Diphtheria specific memory B cell GMCs & 95%CI 28 days after vaccination with a single dose of MenACWY-CRM (n=56) or a single dose of MenACWY-PS (n=57), ANCOVA with adjustment for baseline values at day 0.



4.4. Discussion

4.4.1. MenACWY-PS elicits higher hSBA titres at 28 days to serogroups A,C & Y than MenACWY-CRM

This is the first study in adults to compare MenACWY-CRM with MenACWY-PS. Whilst in the USA, MPVS4 is used as a quadrivalent polysaccharide vaccine, the MenACWY-PS vaccine used in this study is the only quadrivalent polysaccharide vaccine licensed outside of the Americas. The primary endpoint of the study was the meningococcal serogroup A specific hSBA GMT measured at Day 7 (Visit 2) following the initial immunisation with MenACWY-CRM and MenACWY-PS. This time point was chosen as meningococcal specific antibody starts to rise from baseline 4-7 days after immunisation with either a conjugate or polysaccharide vaccine [254, 259, 260]. There are few data comparing the magnitude of the antibody responses to a primary dose of

conjugate and polysaccharide vaccine. Snape *et al.* showed that in adolescents primed with MenC conjugate vaccine, a conjugate booster elicits higher SBA GMTs than a polysaccharide booster at day 5 [254]. However, a study by de Voer *et al.* in adults primed with MenC conjugate showed no difference in the magnitude of the SBA response between individuals boosted with conjugate or polysaccharide [259]. Serogroup A responses were chosen for the primary endpoint due to investigate whether it acts as TD or TI antigen. Both quadrivalent vaccines are currently licensed in the UK as travel vaccines for individuals travelling to areas at risk of A,C W or Y meningococcal disease[230]. SBA responses at 7 days were also chosen as the primary endpoint to determine pragmatically which vaccine would be most useful for travellers who required immunisation at short notice prior to travel. In this study, there were no significant differences in the hSBA GMTs at day 7 between participants who received a single dose of MenACWY-CRM or those who received a single dose of MenACWY-PS, despite an apparent trend towards higher GMTs in the polysaccharide group for A, C & Y (Figure 19).

Antibody titres following meningococcal vaccination plateau by day 28 [254] and this is time point is therefore used as the standard outcome in vaccine immunogenicity trials [247]. At 28 days after the initial vaccination, there is a similar trend towards higher hSBA GMTs in the polysaccharide group to serogroups A & Y, with statistically significant higher titres for serogroup C (Figure 20). However, for serogroup W, MenACWY-CRM generates higher SBA tires than MenACWY-PS.

The percentage of 'seroresponders' at 28 days is an alternative to ANCOVA to compare vaccine responses in populations with elevated baseline antibody titres. In this study, 'seroresponse' was defined as the development of seroprotective antibody levels (ie ≥1:4) in individuals previously seronegative for the specific capsular antigen or a four-fold or

greater increase in antibody in individuals already seropositive to that antigen [233]. A similar pattern to the 28 day ANCOVA was seen (Figure 21), with a trend to higher seroresponse rates in the MenACWY-PS group for A & Y and a significantly higher rate for C. However, for serogroup W, MenACWY-CRM appeared to generate a higher percentage of seroresponders. A similar pattern was seen at day 7, though with lower percentages of overall seroresponse (data not shown).

However, as shown in Table 19, both MenACWY-CRM and MenACWY-PS vaccines induced hSBA GMTs above 8 at 7 days and 28 days for all 4 serogroups. A hSBA titre of ≥4 has been established as the threshold for the correlate of protection for serogroup C conjugate vaccines [246], and although there are no validated correlates of protection for serogroups A,W and Y, a value of ≥1:8 has been used as an immunological endpoint in pre-licensure immunogenicity studies of conjugate quadrivalent meningococcal vaccines in adults [233]. This suggests that there may be no immunological advantage in using the newer, more expensive conjugate vaccine over the polysaccharide vaccine to provide short-term protection to adults travelling to high risk areas.

4.4.2. MenACWY-CRM generates greater serogroup W responses at 28 days than MenACWY-PS

Both ANCOVA analysis and comparison of the seroresponder rate at day 7 and day 28 showed a trend for the polysaccharide to elicit better antibody responses to serogroups A, C & Y and for the conjugate to elicit a better response to serogroup W. The reason for the apparent difference in hSBA trends with serogroup W is unclear. One possibility is a difference in the structure of the MenW polysaccharide in the conjugate and polysaccharide vaccines. Men W polysaccharide is structurally similar to MenC and MenY polysaccharides and consists of 6-Gal*p*-α1-4-Neu*p*NAc-α2 sialic acid [261]. Protective anti-capsular antibodies are generated against the polysaccharide backbone as well as the O acetylated sialic acid residues [262]. The degree of O Acetylation at the O7 and O9

position of the sialic acid residues of MenW polysaccharide influences immunogenicity [263]. The O acetylation status of the bacterial strain used in the SBA assay also influences the absolute titre measured [264]. Thus differences in the degree of the O acetylation of the serogroup W component of the conjugate and polysaccharide vaccines and the meningococcal strains used in the SBA assay may have influenced hSBA titre results. However, no information could be ascertained as to the relative proportions of O acetylation of each vaccine. Furthermore, the same serogroup W strain of bacteria was used for the SBA assay in both groups.

The apparently anomalous behaviour of serogroup W responses could also be due to negative feedback effects of the higher serogroup W baseline hSBA titres seen in Group 2 (MenW specific hSBA GMT was 12.03 and 26.22 at day 0 for Groups 1 and 2 respectively). No individuals in either group were known to have had prior vaccination with a MenW polysaccharide containing vaccine. These high baseline titres may therefore be a result of natural priming in these individuals from nasopharyngeal carriage. However, Men W forms a relatively small proportion of meningococcal isolates from invasive disease in the UK [31] and a recent study in university students suggests that carriage of MenW in the UK is relatively infrequent [225]. MenW specific hSBA titres in 30-70 year olds in the general population are usually below the protective threshold of rSBA >1:8[227], suggesting that carriage may also be unusual in this age group. An alternative explanation may be that the exposure to other bacterial species with cross-reactive epitopes to MenW, may have varied between the study groups, thus giving rise to falsely high SBA titres as has been described for other meningococcal serogroups [44, 45]. A detailed examination of the hSBA assay procedure and sample handling did not reveal any systematic reason for this apparent discrepancy in baseline titres.

High pre-vaccination titres may inhibit further rises in antibody by negative feedback after a threshold level. The inverse relationship between baseline antibody titres and subsequent antibody responses as measured by ELISA has been described in adults to diphtheria toxoid [265] and tetanus toxoid [266]. The mechanism for this may be competition for antigen between pre-existing antibody and membrane bound B cell receptor molecules, thus reducing the stimulus to *de novo* antigen specific B cell proliferation.

4.4.3. Other studies comparing the immunogenicity of quadrivalent meningococcal conjugate and polysaccharide vaccines

Previous studies in adolescents [267] and young children [268] have shown that MenACWY-CRM elicits superior SBAs to MPVS4 28 days after primary vaccination. There are no published data directly comparing MenACWY-CRM with MenACWY-PS in either of these age groups. Only one other study has been published that compares MenACWY-CRM to a quadrivalent polysaccharide vaccine in adults [233]. This study, conducted in Brazilian and Argentine adults aged 55-65 years, showed post-vaccination hSBA GMTs were 1.2- to 5.4-fold higher for MenACWY-CRM than for the polysaccharide comparator for the four serogroups. However, there are important differences between the South American study and the study described in this thesis.

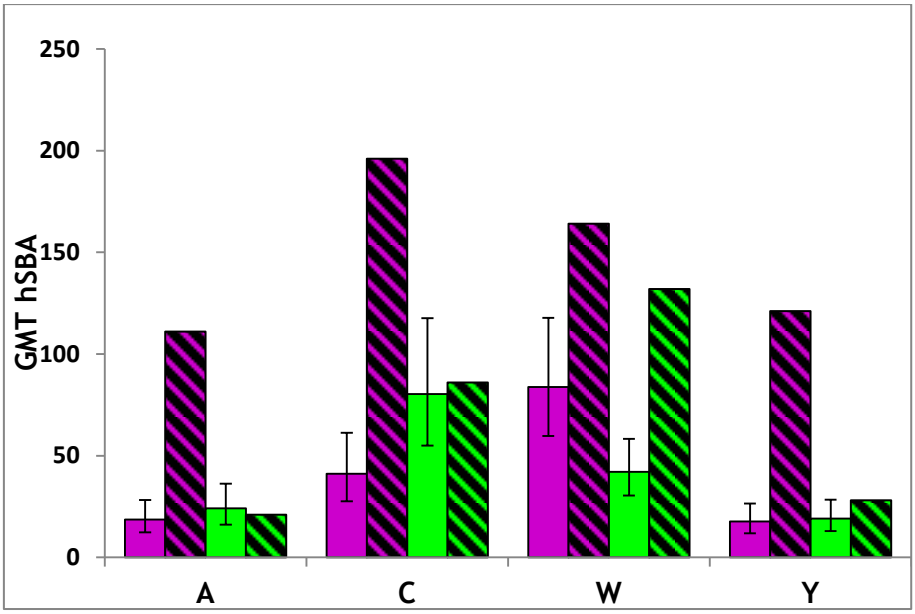
Firstly, the comparator polysaccharide vaccines used were different – the South American study used MPSV4 (Menomune®, Sanofi-Pasteur), as opposed to MenACWY-PS (ACWYVax®, Glaxo-Smith-Kline) in the current study. The Summary of Product Characteristics (SPC) of both vaccines state that they each contain the same amount of capsular polysaccharide (50μg each of serogroups A, C, W & Y). However, independent measurements of the polysaccharide content of both MPVS4 and MenACWY-PS suggest that they can both contain more polysaccharide than specified in the SPC (up to 60 μg of

polysaccharide per serogroup), that the content may vary between batches and that serogroups C, W and Y content are particularly subject to variation [B. Bolgiano, National Institute of Biological Standards and Control, personal communication]. Thus it is possible that absolute differences in the polysaccharide content, and thus the antigen dose delivered by MPVS4 and MenACWY-PS, may explain the differences in relative immunogenicity of each vaccine observed in the two studies. Dose ranging studies in the early development of polysaccharide vaccines suggest that there is no immunological advantage to using a polysaccharide vaccine dose of 100 μg over 50 μg, but there are no published data for smaller increases in dose [269, 270]. Studies of lower dose ranges (5-10 μg polysaccharide) show non-inferiority to full dose polysaccharide [271], but there is no evidence that either MPVS4 or MenACWY-PS contain less than 50 μg of polysaccharide amount [B Bolgiano, National Institute of Biological Standards and Control, personal communication].

A second major difference is that the study described in this thesis was conducted in Oxfordshire, in the United Kingdom, while the study by Stamboulian and colleagues was performed in Buenos Aires, Argentina. The natural priming of adults by nasopharyngeal carriage may differ in two such geographically distinct populations. It is unclear whether nasopharyngeal carriage of meningococci results in TD or TI responses. It is possible that intact organisms present capsular polysaccharide linked to subcapsular proteins, forming a natural conjugate-like TD antigen, with the subsequent generation of memory. Thus in a population previously primed by high rates of carriage, responses to a single vaccination with conjugate or polysaccharide may actually reflect dose-effect of a booster on pre-existing memory cells rather than a true primary TD or TI response. In this situation, MenACWY-PS, with 50μg of each serogroup polysaccharide , may be expected to elicit greater hSBA responses than MenACWY-CRM, which contains 10μg of MenA and 5μg each of MenC, MenW & MenY. Meningococcal carriage rates in adults in Europe are

roughly 7-8% [9], and there are few recent data on carriage in South America. Interestingly, the UK has a higher incidence of invasive disease (2 per 100,000), than Brazil (1.8 per 100,00) or Argentina (0.96 per 100,000)[36], though this may be confounded by differences in diagnosis and reporting rates. The baseline SBA GMTs are not described in the South American study, although the absolute SBA titres achieved after vaccination were higher than for the study described in this thesis [233]. The hSBA GMTs 28 days after vaccination are shown for each of the studies in Figure 27.

Figure 27 Absolute hSBA GMTs 28 days after a single dose of conjugate or polysaccharide vaccine. The study described in this thesis uses MenACWY-CRM or MenACWY-PS (open bars), the study by Stamboulian *et al.* uses MenACWY-CRM or MPVS4 (hatched bars). 95% CI are shown for the Oxford study, but were not available for the Stamboulian *et al.* study.



It is not possible to comment further on the relative differences in hSBA titres post-vaccination without the baseline titres of the South American study. Duplicate SBA assays can give varying results when performed in different laboratories [Reviewed 272]. However, both studies were performed at the laboratories of Novartis Vaccines and Diagnostics, using the same hSBA assay [214]. Thus inter-laboratory variation alone cannot account for the discrepancies between the results from each study. Furthermore, a

quality review of the data from this study did not reveal any systematic errors to explain the observed results.

If the wide variation in baseline titres is taken to represent the heterogeneity of the adult population prior exposure to meningococci, a larger sample cohort may be required to robustly distinguish true primary responses to MenACWY-PS and MenACWY-CRM. The sample size of this study (Section 2.4.1) was calculated assuming similar baseline values between the two groups.

4.4.4. MenACWY-PS drives plasma cell production while MenACWY-CRM drives MBC production

Previous B cell kinetic studies suggest that antigen-specific plasma cells are below the level of detection at baseline and found at their highest frequency in peripheral blood 7 days after primary antigen exposure in adults[134]. A single dose of MenACWY-PS appears to result in higher plasma cell numbers for all four serogroups (Figure 22) than a single dose of MenACWY-CRM, although this difference is not statistically significant. This finding is supported by a recent study which analysed B cell populations produced in response to pneumococcal vaccines by flow cytometry [257] and showed that larger numbers of cells with the phenotype of plasma cells ($CD19^{+}$, $CD20^{-}$, $CD38^{hi}$) were produced in response to a polysaccharide vaccine than to a conjugate.

Memory B cells can be detected by cultured ELISpot assays from 7 days after primary antigen exposure, reaching a plateau around 28 days [134, 189]. It is unclear whether these circulating memory B cells detected at day 7 represent newly generated cells or pre-formed cells from prior natural exposures which have been displaced from bone marrow niches. Unlike hSBA titres, memory B cell GMCs were similar at baseline between the two groups, suggesting similar immunological experience of meningococcal antigens. At day 28, serogroups A & C specific memory B cells showed an increase from baseline in

response to MenACWY-CRM (Figure 24). No significant changes were seen in antigen specific memory B cells after MenACWY-PS.

The memory B cell results described are similar to a study in adolescents by Kelly *et al.* which showed that a MenC conjugate vaccine induced a memory B cell response at 28 days while a MenC polysaccharide vaccine did not [189]. However, the authors of this study demonstrated that the plasma cell response to MenC conjugate at 7 days was three times greater in magnitude than that to MenC polysaccharide. The plasma cell responses at day 7 in both this and the adolescent study correlate with the absolute change in SBA from baseline to day 10 in the conjugate group (Figure 23) but not in the polysaccharide group. The authors suggest that the lack of correlation in the plasma cell group may have been due to low absolute numbers of plasma cells below the threshold of detection. However, the results in Figure 20 and Figure 22 show that in the MenACWY-CRM/MenACWY-PS study, MenC specific plasma cell counts and hSBAs are higher after a polysaccharide vaccine than after a conjugate vaccine, which is contrary to this proposed explanation. A further important difference between the Kelly study and MenACWY-CRM/MenACWY-PS study described here is that both MenC vaccines used in the former contained the same amount of MenC capsular polysaccharide (10 μg). Thus the higher plasma cell counts observed in the MenACWY-PS group may simply reflect a dose effect of greater antigenic load as discussed in Section 4.4.6.

MenW and MenY specific memory B cell and plasma cell frequencies were lower than MenA and MenC specific populations. This reasons for this are not clear, but may be due to MenW and MenY acting as less efficient antigens in the ELISpot assay.

4.4.5. B cell responses to the carrier protein

MenACWY-CRM contains the capsular polysaccharides of serogroups A,C,W&Y individually chemically conjugated to CRM197, a naturally occurring mutant diphtheria toxoid known to elicit diphtheria toxoid specific B cells and antibody [215]. Diphtheria toxoid specific B cells were therefore measured as a marker of the cellular immune response to CRM197. The number of diphtheria specific MBCs at baseline as a proportion of total IgG producing cells was 0.01%, which is similar to previous published data [273]. There was an increase in the number of diphtheria-specific plasma cells and memory cells in response to MenACWY-CRM (Group 1) but not to MenACWY-PS (Group 2). This is consistent with a TD type response to the CRM197 protein antigen in Group 1.

There was no change in diphtheria toxoid–specific memory B cell frequencies from baseline in Group 2 as MenACWY-PS does not contain CRM197. A prior study with protein vaccines (in this case, diphtheria) [273] has also demonstrated a lack of memory B cell generation to an antigen not present in the vaccine (tetanus toxoid). However, another study using a pneumococcal protein-conjugate vaccine [216] did show a memory B cell response to a tetanus toxoid antigen not present in the vaccine. This is postulated to be due to 'bystander activation' due to an increased availability of activated helper T cells during an antigen specific response (Section 1.4.9) [139].

In Group 1, the plasma cell and MBC responses to the CRM197 carrier protein was of greater magnitude than to any of the meningococcal serogroup polysaccharides of the same vaccine. This may be due to the adults in the study having received multiple diphtheria toxoid containing vaccines in the course of their lives and thus already have pre-existing immunological memory to diphtheria. However, as similar results have also been observed in infants[215], who have not had prior diphtheria vaccines this suggests that diphtheria toxoid may in fact act as a more efficient antigenic stimulus in the *in vitro* ELISpot assay than meningococcal polysaccharides.

4.4.6. Differences in antigen dose and route of administration are confounding factors

An alternative explanation for the differences in plasma cell and memory B cell responses between the two groups may be the disparity in antigen load provided by each vaccine. MenACWY-CRM contains 5 µg of serogroups C, W and Y capsular polysaccharide and 10µg of serogroups A polysaccharide. MenACWY-PS, however, contains 50µg each of serogroups A, C, W and Y polysaccharide. It is possible that the higher antigenic dose contained in the polysaccharide vaccine drives the observed higher initial plasma cell production. Polysaccharide vaccines generally contain longer chains of several hundred repeating monosaccharide units, whilst the polysaccharide moiety of conjugate vaccines consists of shorter oligosaccharide units [160, 274]. The strength of the extra-follicular B cell response to TI type 2 antigens such as bacterial capsular polysaccharides has been shown to be directly related to both the number and density of repetitive epitopes as well as the molecular mass of the antigen[142, 275]. Thus to induce an antibody response, a TI antigen requires a valency of at least 20 repeating units and a molecular mass of at least 100,000 kDA[276]. This would imply that MenACWY-PS would generate a greater extra-follicular response than MenACWY-CRM. A lower antigen density, as might be expected in a conjugate vaccine may preferentially drive a germinal centre pathway response [142]. The relative importance of the extra-follicular as compared to germinal centre responses to MenACWY-PS and MenACWY-CRM cannot be clearly defined in this study without further antibody avidity data or somatic hypermutation analysis. Evidence of affinity maturation by means of avidity ELISAs or immunoglobulin sequence analysis would suggest germinal centre involvement during the immune response.

The equivalence of the absolute measured polysaccharide content of plain polysaccharide vaccines and conjugate vaccines is uncertain – *i.e.* does MenACWY-PS actually contain 5-10 times more biologically available polysaccharide than MenACWY-CRM? Using equivalent antigen dosages (*i.e.* a 1/5th dose of MenACWY-PS vs. a full dose of

MenACWY-CRM) would allow this issue to be addressed. A follow-on clinical study (OVG 2012/02) is planned to investigate this issue and will be discussed in more detail later in this thesis.

A further confounding factor between the 2 vaccine groups is the route of administration of each vaccine. The conjugate vaccine was given intramuscularly, while the polysaccharide was administered subcutaneously as specified in the SPC for each vaccine. The type of antigen presenting cell and the route taken by antigen to the draining lymph node will vary according to the location of the antigen depot, whether in the subcutaneous adipose tissue or in the muscle, which could theoretically alter the magnitude or nature of the immune response. However, Ruben *et al.* administered the same dose of MPVS4 polysaccharide vaccine to adults either subcutaneously or intramuscularly and showed no difference in rSBA GMTs or the proportion of individuals with a 4-fold rise at 28 days [277]. The differences in route may lead to more subtle variations in the immune response. For example, intramuscular vaccination produces higher IgG2 titres than subcutaneous vaccination but has no effect on IgG1 titres [278]. TD antigens such as conjugate vaccines produce predominantly antibody of the IgG1 subtype while TI antigens produce IgG2 [279, 280]. IgG1 is a more potent activator of the classical complement pathway than IgG2, thus a shift in the IgG1/IgG2 ratio may alter the hSBA titres observed. There are no published data on route of administration and B cell responses.

4.5. Conclusions

The clinical study described in this chapter demonstrates that both MenACWY-CRM and MenACWY-PS are immunogenic in adults. Furthermore, both vaccines offer similar short-term protection as measured by the clinical correlate of protection of a hSBA titre of ≥1:4, although there are individual serogroup differences between the vaccine groups.

Striking differences are observed in the B cell responses to each vaccine. A single dose of MenACWY-PS tends to generate plasma cells at day 7, while MenACWY-CRM generates memory B cells for serogroups A and C. Differences in the antigen content and route of administration of MenACWY-CRM and MenACWY-PS may be confounding factors in interpreting these results.

Chapter 5 – MenACWY-PS causes hyporesponsiveness

5.1. Introduction

Vaccine-induced hyporesponsiveness is the inability to mount a booster response of at least the same magnitude as that produced to the priming dose. Hyporesponsiveness has been described after vaccination of newborns [281, 282] with protein toxoid vaccines. However, hyporesponsiveness has been more extensively studied in the context of polysaccharide vaccination, in particular with pneumococcal and meningococcal plain polysaccharide vaccines. This chapter describes the phenomenon of polysaccharide induced hyporesponsiveness in quadrivalent meningococcal vaccines at the level of functional antibody, memory B cell and plasma cell responses.

5.1.1. Repeated doses of polysaccharide vaccine induce hyporesponsiveness

Gold *et al.* first observed that repeated doses of a serogroup C polysaccharide vaccine resulted in lower antigen specific antibody responses as early as 1975 [172]. Infants were primed at 3 months and boosted at 7 or 12 month of age with MenC plain polysaccharide (MenC-PS). No anamnestic response was seen after to booster doses. Instead, the MenC specific geometric mean antibody titres as measured by radioimmunassay were significantly lower after the booster at either 7 or 12 months than after the priming dose at 3 months. The phenomenon was dose-dependent: infants receiving 25-100 μg of polysaccharide demonstrated depressed responses while those receiving a smaller dose of 10 μg did not.

A series of studies in Gambian infants performed in the late 1990's investigated this further. Infants who had received 2 doses of MenC-PS under the age of 6 months were

shown to mount a lower MenC-specific IgG response (measured by ELISA) to a MenC polysaccharide booster at 18 months than previously polysaccharide naïve age-matched controls [174]. These children were boosted with a further dose of MenC-PS at 5 years of age [283] which elicited lower serum bactericidal activity (SBA) titres than in unvaccinated controls. However, at 5 years of age, there were no differences in MenC-specific IgG between previously vaccinated and unvaccinated children. This may be explained by ELISA measuring antibodies of varying affinities, while SBA assays measure functional activity of antibodies of all isotypes and are thus more likely to represent the response to a clinical infection. Other studies in infants [170, 179], adolescents [169] and adults [191, 192, 284], have also demonstrated MenC-PS induced impaired immune responses at SBA level to subsequent polysaccharide which can last for up to 5 years . These are summarised in Table 22. The repeated observations of lower antibody responses after preceding polysaccharide vaccine has led to the concept of hyporesponsiveness, the mechanisms for which remain a matter for speculation.

Hyporesponsiveness has also been observed in individuals given a MenC polysaccharide vaccine as a booster following priming with MenC conjugate where interference with responses to a subsequent dose of MenC conjugate vaccines was observed [180]. Children primed with 2 doses of MenAC conjugate in infancy and boosted at 2 years with MenAC-PS had reduced SBA titres to a MenAC-PS booster at 5 years compared with children who received MenAC conjugate or a polio vaccine at 2 years [285].

Hyporesponsiveness after multiple doses of polysaccharide vaccine has also been described for other serogroups. Al-Mazrou *et al.* showed that children who have received 2 doses of quadrivalent polysaccharide vaccine (MenACWY-PS) have lower antibody responses to serogroups C, W and Y than children who had 1 dose of the same vaccine

[286]. However, in this study children who received 2 doses of vaccine were 6-24 months of age while those who received only one dose were 24-48 months of age. Thus the poor response to multiple doses of vaccine may simply relate to poor TI responses in children under the age of 2 (Section 1.4.2).

5.1.2. Can conjugate vaccines overcome hyporesponsiveness induced by polysaccharide vaccines?

Several studies have examined whether polysaccharide induced hyporesponsiveness can be remedied by conjugate vaccination. University students primed with MenAC-PS were boosted with MenC-CRM conjugate or MenAC-PS 6 months later. Although individuals with a conjugate booster had higher SBA titres than those boosted with polysaccharide, their SBA titres were lower than previously vaccine naïve controls who received only a single dose of MenC-CRM [284]. This was not carrier specific and MenC-PS induced hyporesponsiveness has also been demonstrated to a MenC-TT booster [287]. Similar impaired responses to a quadrivalent MenACWY-TT conjugate 3 years after prior polysaccharide vaccine have been reported in adolescents [171] to serogroups A, C, W & Y. Children who received 2 doses of a MenAC polysaccharide vaccine in infancy followed by a MenAC conjugate vaccine at 2 years of age, mounted higher SBA responses to a further booster dose of MenAC polysaccharide at 5 years of age than previously vaccine naïve controls[288]. This indirectly suggests the induction of memory by the conjugate vaccine despite prior polysaccharide vaccination although no formal antibody measurements were made after the conjugate vaccine at 2 years. Subsequently, another study in children 2-3.5 years of age primed with MenC-PS and boosted 6 months later with a MenC-CRM conjugate showed no difference in either the SBA response or the development of a memory response with avidity matured antibody compared with polysaccharide naïve controls who received only a single dose of conjugate [190], suggesting that in children, conjugate may abrogate the effects of polysaccharide induced hyporesponsiveness.

Table 22 Key studies demonstrating hyporesponsiveness to meningococcal polysaccharide vaccines

Study	Vaccine(s)	Regimen	Finding	Assay
Borrow, 2000[232]	MenA/C PS	Prime adults Boost 6m	Prior PS impaired MenA Ab response to PS booster	ELISA
Borrow, 2001[190]	Men A/C PS + MenC-CRM	Prime infants, boost 7m later	<1yr age prior PS impairs MenC SBA to MenC-CRM boost	SBA, ELISA, avidity
Borrow, 2001[289]	MenA/C PS + MenC-CRM	Boost adults	Prior PS impairs MenAc Ab response to MenC-CRM booster	SBA, ELISA
Findlow, 2011[290]	MenACWY PS	Prime 12-23m toddlers Boost 10m later	Prior PS impairs MenC & W Ab response to low dose PS booster	SBA
Gold, 1975[172]	Men C PS + Men A PS	Prime 3m infants, boost 7 or 12 months	Impaired Ab response to booster doses MenC, no difference to MenA	RIA
Granoff, 1998[191]	MenACWY PS	Prime adults Boost 4yr	Prior PS impairs MenC ab response to low dose PS booster	SBA
Jokhdar, 2004 [169]	MenA/C PS	Boost adolescents	Increasing numbers of doses of PS associated with reduced MenC response to PS booster	SBA
Lakshman, 2002[192]	MenA/C PS	Prime adults boost 12m	Prior PS impairs MenC SBA and IgG response to PS booster and MenA SBA to PS booster	SBA, ELISA
Leach, 1997 [174]	Men A/C PS	Prime 3,6m, boost 18-24m	2 prior doses of PS impaired MenC response to PS booster, no difference in MenA	ELISA
MacDonald, 1998 [179]	MenACWY PS	Prime 15-24 m (2 doses 2 m apart),Boost 3 yrs	Prior PS impaired MenC Ab response to PS booster	SBA, ELISA
MacDonald, 2000[288]	MenACWY PS + MenC-CRM	Prime 18, 20 m, Boost 3 yrs Boost 4 yrs (con)	3 doses of prior PS impaired MenC Ab response to MenC-CRM booster more than 1 dose	SBA, ELISA
Maclennan, 1999[283]	MenA/C PS	Prime 3,6m, boost 18-24m, boost 5 yrs	3 prev doses of PS impairs response to Men A & Men C	SBA
Richmond, 2000 [284]	MenA/C PS + MenA/C PS or MenC-CRM	Prime adults Boost 6m	Prior PS impairs MenC Ab response to PS booster and conjugate booster	SBA, ELISA
Southern, 2004[287]	Men A/C PS	Boost adults	Magnitude and persistence of MenC ab response to MenC-TT conjugate impaired by ≥1 doses prior PS	SBA, ELISA

5.1.3. Multiple doses of serogroup A may not lead to hyporesponsiveness

Although hyporesponsiveness has been well described with serogroup C, evidence of the effect of repeated doses of MenA polysaccharide is conflicting. The same study by Gold *et al.* (Section 5.1.1) that demonstrated hyporesponsiveness to multiple doses of MenC polysaccharide in infants, also showed that the antibody response to MenA polysaccharide increases with each successive dose [172]. Käyhty *et al.* immunised children under the age of 17 months with 2 doses of MenA polysaccharide vaccine 2 months apart during a MenA epidemic in Finland and showed that the antibody responses to the second dose were significantly higher than those to the first dose [291]. Both these studies used radioimmunoasays to quantify antibody and did not assess antibody functional activity. A more recent study by Jokhdar *et al.* showed that MenA specific SBA responses in Saudi individuals aged 10-29 years of age to a MenAC polysaccharide vaccine were higher in those who had received 2 or more previous doses of a MenA polysaccharide containing vaccine.

In contrast, 2 studies have shown impairment of MenA specific SBA responses after multiple doses of MenA polysaccharide vaccine. Borrow *et al.* immunised 18-25 years with 2 doses of a MenAC polysaccharide vaccine 6 months apart and showed reduced MenA specific IgG titres by ELISA and MenA specific SBA after the second dose when compared to after the first dose [232]. An important difference between this and the Saudi was that serogroup A carriage rates are much lower in the UK, so the study populations may have differed in the extent of immunological priming and natural boosting. The Gambian study described in Section 5.1.1 also demonstrated immunological hyporesponsiveness to repeated vaccination with MenA polysaccharide using MenA specific IgG ELISA and SBA assays. However although the rates of natural exposure may have been similar to that of the Saudi population, the Gambian study population was conducted in children who received the MenAC polysaccharide vaccine at 3, 6, 18-24 months and 5 years of age.

5.1.4. Hyporesponsiveness to vaccines against other encapsulated bacteria

Hyporesponsiveness has also been described after multiple doses of pneumococcal polysaccharide vaccine (PPV) in children [292, 293] and in adults re- immunised at intervals of to 18 months to 6 years [294, 295]. In adults, polysaccharide-induced hyporesponsiveness appears to wane with a 10 year interval between doses [296], suggesting recovery of immune function with time. Several studies have examined the effect of prior PPV on the response to subsequent pneumococcal conjugate vaccine (PCV) in children. These show no clear pattern of hyporesponsiveness to PCV in children who have previously received PPV [reviewed 297]. However, studies in healthy adults clearly demonstrate that prior PPV impairs the subsequent response to a PCV booster [257, 298]. The investigation of hyporesponsiveness to pneumococcal polysaccharides is complicated by the large number of disease causing serotypes (>90), and the varying immunogenicity of their capsular polysaccharides in different age groups [reviewed 299].

The other widely studied bacterial capsular polysaccharide vaccine is the polyribositol phosphate (PRP) containing *Haemophilus influenzae* type b (Hib) vaccine. The immune response to a second dose of Hib polysaccharide vaccine given after 3.5 years is no different from that to the first dose [300]. Although this demonstrates lack of priming for memory, this is not true hyporesponsiveness. The children who made no immune response to PRP were those who had higher initial levels of anti-PRP antibodies at baseline, suggesting that high antibody may exert negative feedback on further responses.

5.2.Methods

The chapter relates to the responses of participants in the MenACWY-CRM/MenACWY-PS clinical trial previously described in Chapter 3 to a MenACWY-CRM booster. Participants were primed with either MenACWY-CRM (Group 1) or MenACWY-PS (Group 2) as shown in Table 23.

Table 23 Timeline of procedures in the MenACWY-CRM/MenACWY-PS clinical vaccine trial in healthy adult volunteers.

	V1 Day 0	V2 Day 7	V3 Day 28	V4 Day 35	V5 Day 56
Group 1 n=75	**MenACWY-CRM conjugate**		**MenACWY-CRM conjugate**		
Group 2 n=75	**MenACWY-PS polysaccharide**		**MenACWY-CRM conjugate**		
Assays	SBA Memory B cells	SBA Plasma cells	SBA Memory B cells	SBA Plasma cells	SBA Memory B cells

hSBA assays were performed on serum at each time point using methods described in Section 2.3.2 [214]. Antigen specific plasma cell and memory B cell geometric mean counts (GMCs) were determined 7 and 28 days after each vaccination respectively as described in Section 2.3.6 and 2.3.7.

5.3. Results

5.3.1. SBA responses to a MenACWY-CRM booster

73 participants from Group 1 (MenACWY-CRM primed) received a MenACWY-CRM booster, while all 75 participants from Group 2 (MenACWY-PS primed) received a MenACWY-CRM booster. The absolute hSBA GMTs for these individuals at baseline and 7 and 28 days after each priming and boosting vaccine is shown in Table 24.

Table 24 Unadjusted hSBA geometric mean titres against serogroups A, C, W & Y at baseline and at 7 and 28 days after each vaccination for Group 1 (MenACWY-CRM + MenACWY-CRM) or Group 2 (MenACWY-PS + MenACWY-CRM).

Serogroup	Group	V1 (day 0)	V2 (day 7)	V3 (day 28)	V4 (day 35)	V5 (day 56)
A	1	**3.24** (2.60, 4.03) [75]	**4.26** (3.17, 5.73) [72]	**16.87** (10.91, 26.09) [71]	**29.89** (19.68, 45.41) [72]	**30.30** (19.62, 46.78) [73]
	2	**3.88** (2.98, 5.05) [75]	**5.90** (4.20, 8.28) [73]	**26.46** (16.11, 43.46) [75]	**29.76** (18.50, 47.87) [73]	**38.17** (23.99, 60.71) [72]
C	1	**6.26** (4.61, 8.48) [73]	**14.34** (9.65, 21.30) [71]	**32.67** (20.06, 53.19) [68]	**53.21** (34.62, 81.79) [72]	**56.20** (37.70, 83.78) [72]
	2	**11.06** (8.18, 14.96) [75]	**26.97** (18.84, 38.59) [74]	**98.89** (64.94, 150.58) [75]	**92.74** (60.65, 141.79) [74]	**119.55** (77.97, 183.30) [73]
W	1	**12.03** (8.03, 18.02) [75]	**20.66** (13.44, 31.75) [68]	**64.06** (40.72, 100.77) [66]	**74.21** (48.95, 112.50) [71]	**77.44** (50.74, 118.20) [68]
	2	**26.22** (17.51, 39.27) [74]	**34.46** (23.43, 50.69) [73]	**53.06** (35.64, 78.99) [74]	**57.11** (39.54, 82.48) [73]	**61.47** (39.78, 94.99) [69]
Y	1	**3.76** (3.03, 4.65) [75]	**5.53** (4.08, 7.49) [70]	**15.44** (10.12, 23.54) [71]	**21.79** (14.32, 33.17) [72]	**20.68** (13.98, 30.58) [73]
	2	**5.59** (4.17, 7.48) [75]	**9.83** (7.06, 13.69) [74]	**21.66** (13.88, 33.80) [75]	**22.44** (14.40, 34.96) [71]	**27.14** (17.48, 42.12) [71]

95% confidence intervals are shown in round brackets and numbers of samples are shown in square brackets. The numbers of samples at each time point varied due to missed visits, participant drop out, insufficient serum for assays or failed assays.

5.3.2. Plasma cell frequencies after a MenACWY-CRM booster

Antigen specific plasma cell frequencies were determined at day 35 (*i.e.* 7 days after booster vaccination), on 44 participants from Group 1 (MenACWY-CRM primed), and 47 participants from Group 2 (MenACWY-PS primed), as shown in Table 25.

Table 25 Geometric mean counts of plasma cells specific for meningococcal serogroups A, C, W & Y and diphtheria toxoid 7 days after vaccination with a MenACWY-CRM priming and MenACWY-CRM booster vaccine (Group 1) or 7 days after vaccination with a MenACWY-PS priming and MenACWY-CRM booster vaccine (Group 2).

Serogroup	Group	V2 (day 7)	V4 (day 35)
A	1	**3.97** (2.14, 7.38) [47]	**3.75** (2.37, 5.93) [44]
	2	**9.03** (4.95, 16.46) [50]	**1.41** (0.85, 2.34) [47]
C	1	**5.29** (2.85, 9.84) [47]	**4.26** (2.72, 6.67) [44]
	2	**8.65** (4.83, 15.51) [50]	**1.32** (0.84, 2.05) [47]
W	1	**2.40** (1.36, 4.21) [47]	**1.53** (0.97, 2.43) [44]
	2	**2.90** (1.51, 5.58) [50]	**0.84** (0.57, 1.23) [47]
Y	1	**2.35** (1.40, 3.94) [47]	**2.01** (1.33, 3.02) [44]
	2	**2.88** (1.65, 5.03) [50]	**0.83** (0.58, 1.20) [47]
Diphtheria toxoid	1	**19.86** (10.73, 36.77) [47]	**9.26** (5.62, 15.26) [44]
	2	**0.44** (0.34, 0.57) [50]	**28.03** (14.63, 53.70) [47]

95% confidence intervals are shown in round brackets and numbers of samples are shown in square brackets. The numbers of samples between groups varied due to missed visits, insufficient blood for assays or failed assays.

5.3.3. Memory B cell frequencies after a MenACWY-CRM booster

Memory B cells frequencies were ascertained at pre-booster (i.e. day 28), and 28 days after a MenACWY-CRM booster dose (i.e. day 56), on 51-58 participants who were primed with MenACWY-CRM (Group 1) and on 56-59 participants who were primed with MenACWY-PS (Group 2) as shown in Table 26.

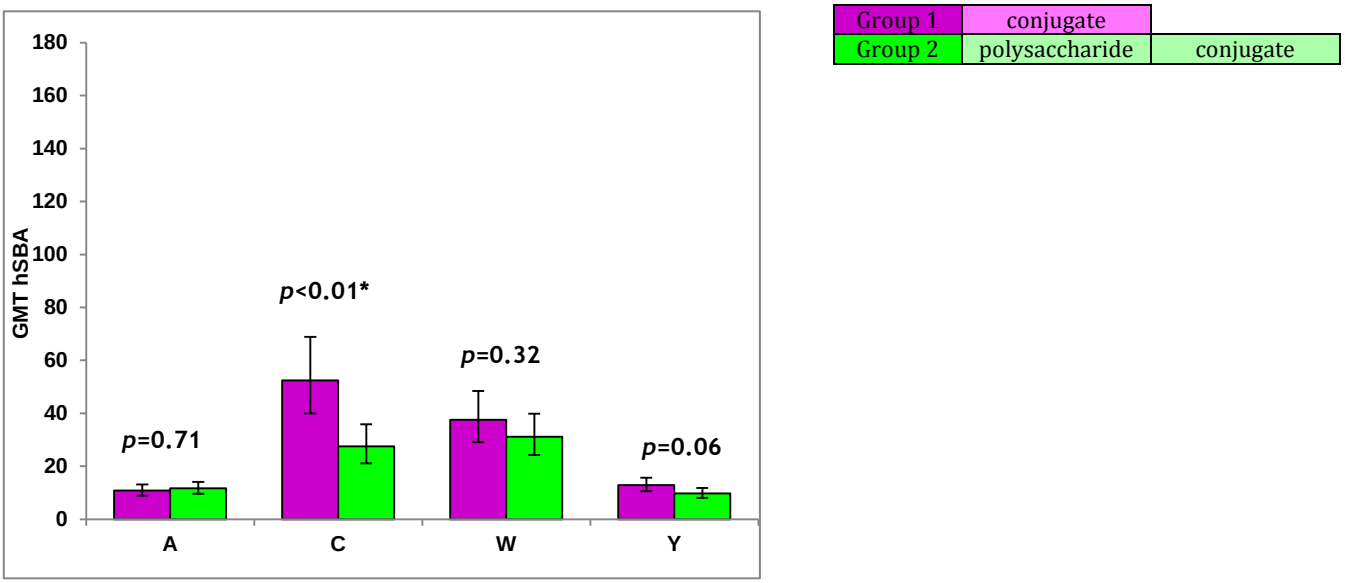
Table 26 Geometric mean counts of memory B cells specific for meningococcal serogroups A, C, W & Y and diphtheria toxoid 7 days after vaccination with a MenACWY-CRM priming and MenACWY-CRM booster vaccine (Group 1) or 7 days after vaccination with a MenACWY-PS priming and MenACWY-CRM booster vaccine (Group 2).

Serogroup	Group	V1(day 0)	V3 (day 28)	V5 (day 56)
A	1	**1.62** (1.13, 2.31) [57]	**2.78** (1.86, 4.13) [58]	**5.29** (3.74, 7.48) [57]
	2	**1.78** (1.24, 2.55) [57]	**1.99** (1.37, 2.87) [59]	**2.85** (2.02, 4.02) [58]
C	1	**1.27** (0.88, 1.82) [58]	**4.61** (3.06, 6.96) [58]	**6.92** (4.82, 9.94) [57]
	2	**2.04** (1.36, 3.07) [57]	**2.42** (1.68, 3.48) [59]	**3.00** (2.06, 4.38) [58]
W	1	**0.94** (0.67, 1.32) [55]	**1.26** (0.84, 1.89) [54]	**2.04** (1.40, 2.98) [53]
	2	**1.25** (0.86, 1.81) [55]	**1.41** (0.97, 2.05) [57]	**1.65** (1.16, 2.35) [56]
Y	1	**1.01** (0.70, 1.46) [55]	**1.28** (0.85, 1.91) [53]	**2.33** (1.65, 3.30) [51]
	2	**1.37** (0.95, 1.96) [55]	**1.28** (0.90, 1.82) [56]	**1.55** (1.04, 2.31) [56]
Diphtheria toxoid	1	**4.73** (3.18, 7.06) [58]	**19.92** (13.00, 30.52) [58]	**25.72** (16.45, 40.20) [57]
	2	**7.05** (4.89, 10.17) [57]	**5.71** (3.84, 8.49) [59]	**26.08** (17.63, 38.47) [58]

5.3.4. Antibody responses to MenACWY-CRM with or without prior MenACWY-PS

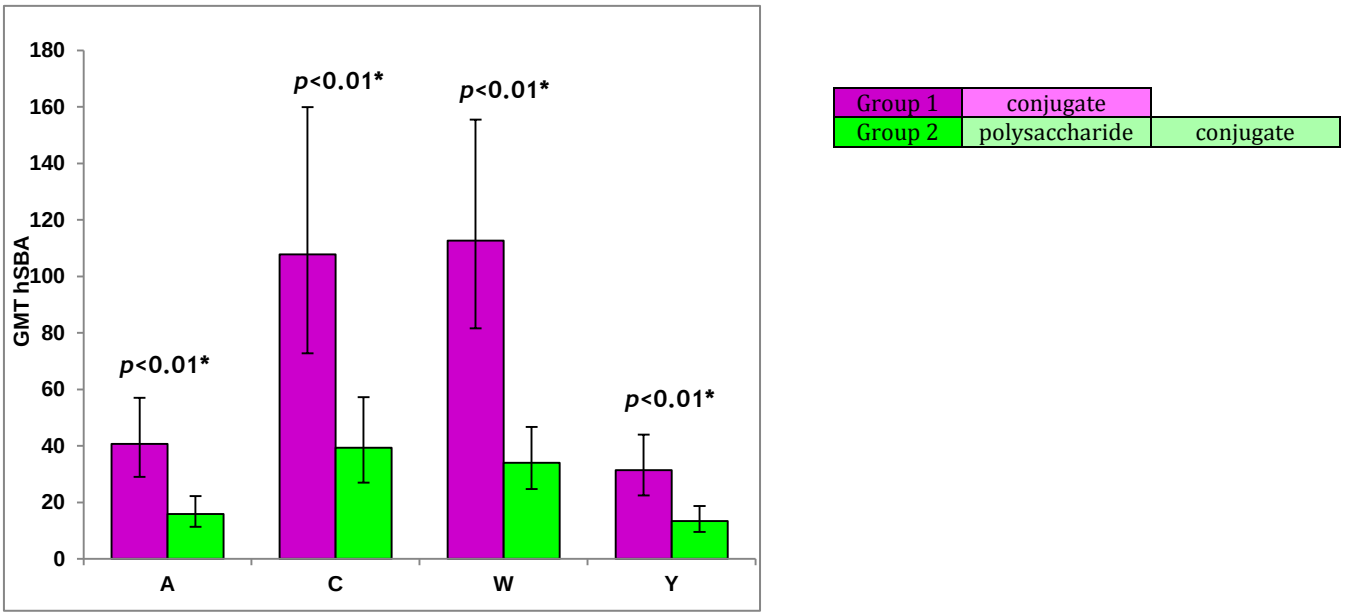
Between group analyses were performed using an analysis of covariance (ANCOVA) model with adjustment for hSBA titres prior to the first dose of conjugate (day 0 in Group 1 and day 28 in Group 2 respectively). The values presented in Figure 28 and Figure 29 therefore differ from the raw hSBA GMTs presented in Table 24.

Figure 28 hSBA GMTs & 95% CI 7 days after a dose of MenACWY-CRM conjugate, with (n=71-74) or without (n=68-72) a prior dose of Men ACWY-PS polysaccharide, ANCOVA with adjustment for baseline pre-conjugate.



As shown in Figure 28, 7 days after conjugate vaccination, individuals who have received a prior dose of MenACWY-PS have significantly lower MenC specific SBA titres than previously polysaccharide naïve individuals ($p<0.01$). By 28 days after conjugate vaccination, this effect has attained significance across all 4 serogroups, as shown in Figure 28 ($p<0.01$ for all comparisons) .

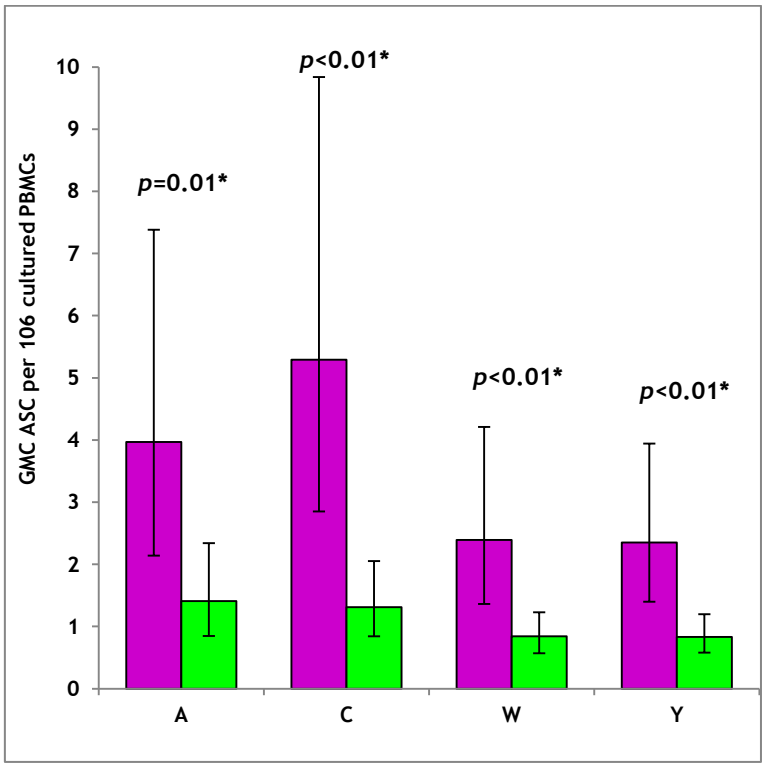
Figure 29 hSBA GMTs & 95% CI 28 days after a dose of MenACWY-CRM conjugate, with (n=68-73) or without (n=66-71) a prior dose of Men ACWY-PS polysaccharide, ANCOVA with adjustment for baseline pre-conjugate.



5.3.5. Antigen specific B cell responses to MenACWY-CRM with or without prior MenACWY-PS

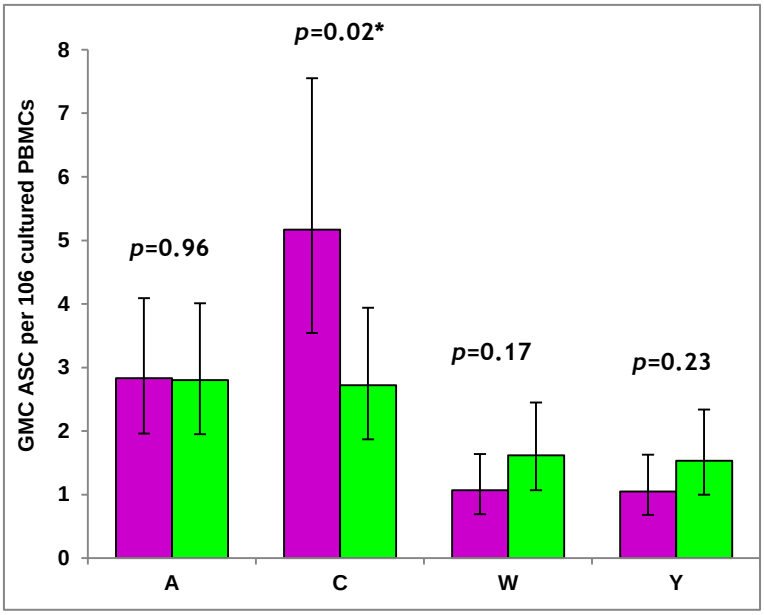
The descriptive analyses of the antigen specific plasma cell responses are shown in Table 25. Plasma cell counts at baseline (day 0, day 28) were below the level of detection (data not shown), so no adjustment of the values for baseline were made in the case of the plasma cell data. Figure 30 shows that antigen specific plasma cell responses to conjugate vaccine are significantly attenuated across all 4 serogroups in individuals who have received prior polysaccharide, compared to polysaccharide naïve individuals ($p \leq 0.01$ for all 4 serogroups). At this comparison point, prior polysaccharide impairs the MenC specific memory B cell response 28 days after a conjugate as shown in Figure 31 (p=0.02).

Figure 30 Plasma cell GMC and 95% CI, 7 days after a single dose of conjugate MenACWY-CRM with (N=47) or without (N=47) a prior dose of MenACWY-PS polysaccharide, independent 2 sample t tests.



Group 1	conjugate	
Group 2	polysaccharide	conjugate

Figure 31 Memory B cell GMC & 95% CI 28 days after a dose of MenACWY-CRM conjugate, with (n=56-58) or without (n=57-58) a prior dose of Men ACWY-PS polysaccharide, ANCOVA with adjustment for baseline pre-conjugate

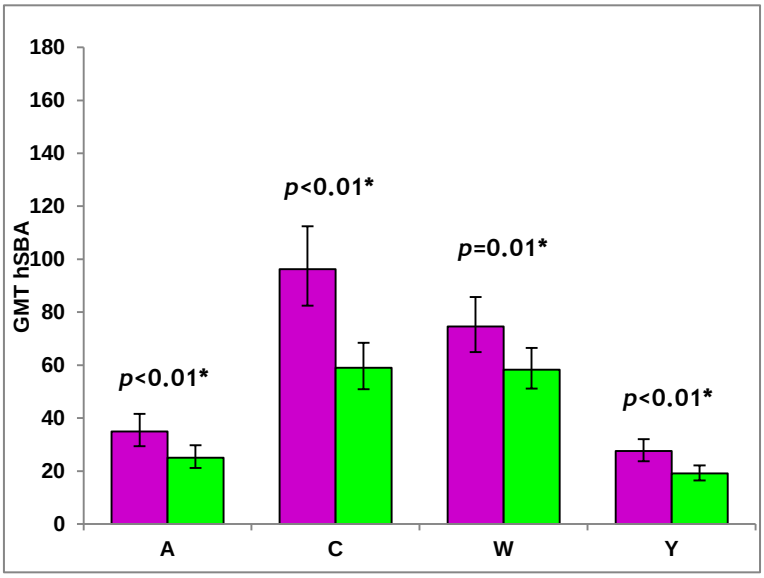


Group 1	conjugate	
Group 2	polysaccharide	conjugate

5.3.6. Antibody responses to a conjugate booster with either conjugate or polysaccharide priming

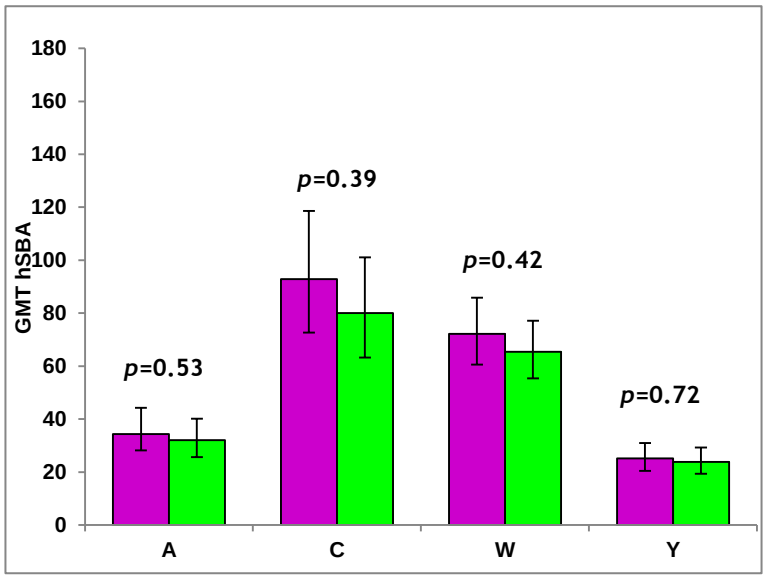
Figure 32 shows that SBA responses to all 4 serogroups measured 7 days after a conjugate booster are lower in polysaccharide primed individuals than in conjugate primed individuals ($p \leq 0.01$ across all 4 serogroups), though these differences have lost significance by day 28 after a conjugate booster (Figure 33).

Figure 32 hSBA GMTs & 95% CI 7 days after a dose of MenACWY-CRM conjugate, with MenACWY-CRM conjugate priming (n=71-72) or Men ACWY-PS polysaccharide priming (n=71-74), ANCOVA with adjustment for baseline pre-booster



Group 1	conjugate	conjugate
Group 2	polysaccharide	conjugate

Figure 33 hSBA GMTs & 95% CI 28 days after a dose of MenACWY-CRM conjugate, with MenACWY-CRM conjugate priming (n=68-73) or Men ACWY-PS polysaccharide priming (n=69-73), ANCOVA with adjustment for baseline pre-booster



Group 1	conjugate	conjugate
Group 2	polysaccharide	conjugate

5.3.7. Antigen specific B cell responses to a conjugate booster with either conjugate or polysaccharide priming

Figure 34 demonstrates strong evidence of impaired plasma cell responses across all serogroups at day 7 after a conjugate booster in polysaccharide primed individuals as compared to conjugate primed individuals (all $p<0.01$).

Figure 34 Plasma cell GMCs & 95%CI 7 days after a MenACWY-CRM booster, with MenACWY-CRM conjugate priming (n=44) or MenACWY-PS polysaccharide priming (n=47), independent 2 sample t tests

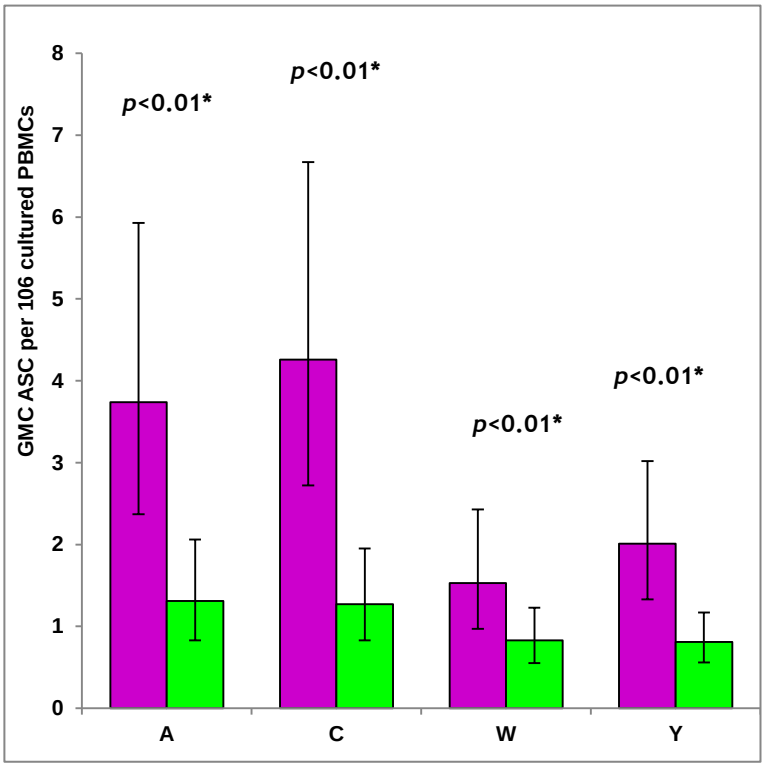
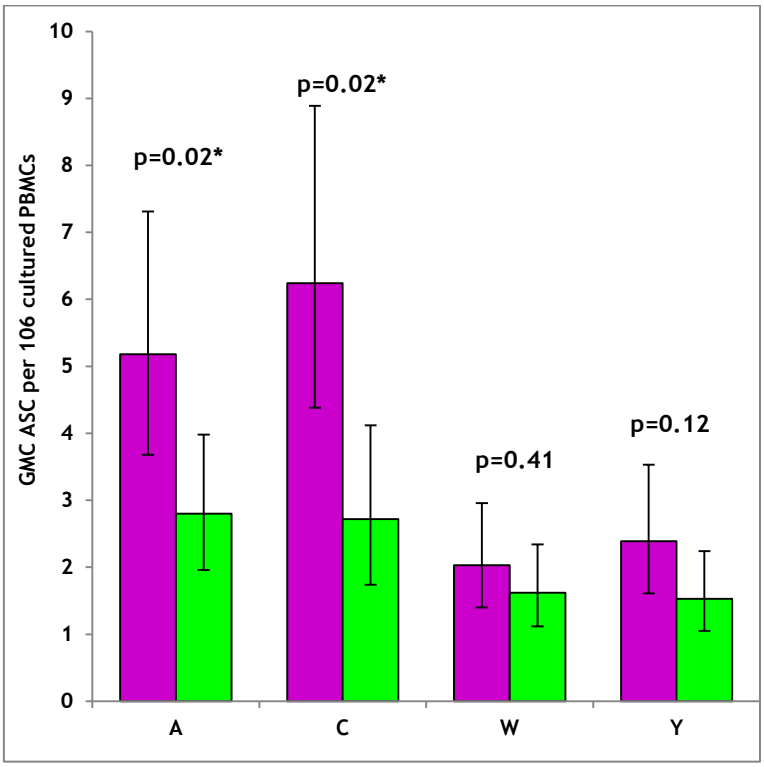


Figure 35 shows a significantly impaired MenA and MenC specific memory B cell responses 28 days after a MenACWY-CRM booster in individuals who have received priming with MenACWY-PS compared with those primed with MenACWY-CRM ($p=0.02$ for both MenA and MenC)

Figure 35 Memory B cell GMC & 95%CI 28 days after MenACWY-CRM conjugate booster, with either MenACWY-CRM conjugate (n=51-57) or MenACWY-PS polysaccharide (n=56-58) priming, ANCOVA with adjustment for baseline pre-booster.

Group 1	conjugate	conjugate
Group 2	polysaccharide	conjugate



5.3.8. Temporal trends in antigen specific B cell responses

The plasma cell response to in Group 2 is greatest to the priming dose rather than the booster as shown in Figure 36. The temporal patterns of memory B cell production in each group suggest a trend towards higher cell counts over the course of the trial in Group 1 than Group 2 (Figure 37).

Figure 36 MenA, MenC, MenW and MenY specific plasma cell GMCs and 95%CI at days 7 and 35 of the MenACWY-CRM/MenACWY-PS clinical study.

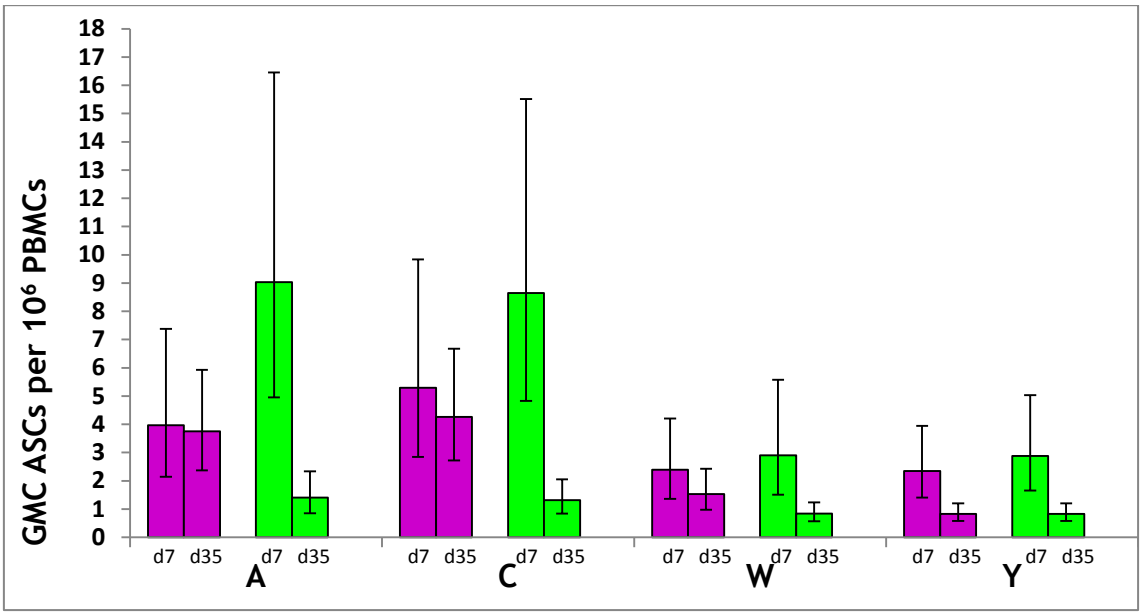
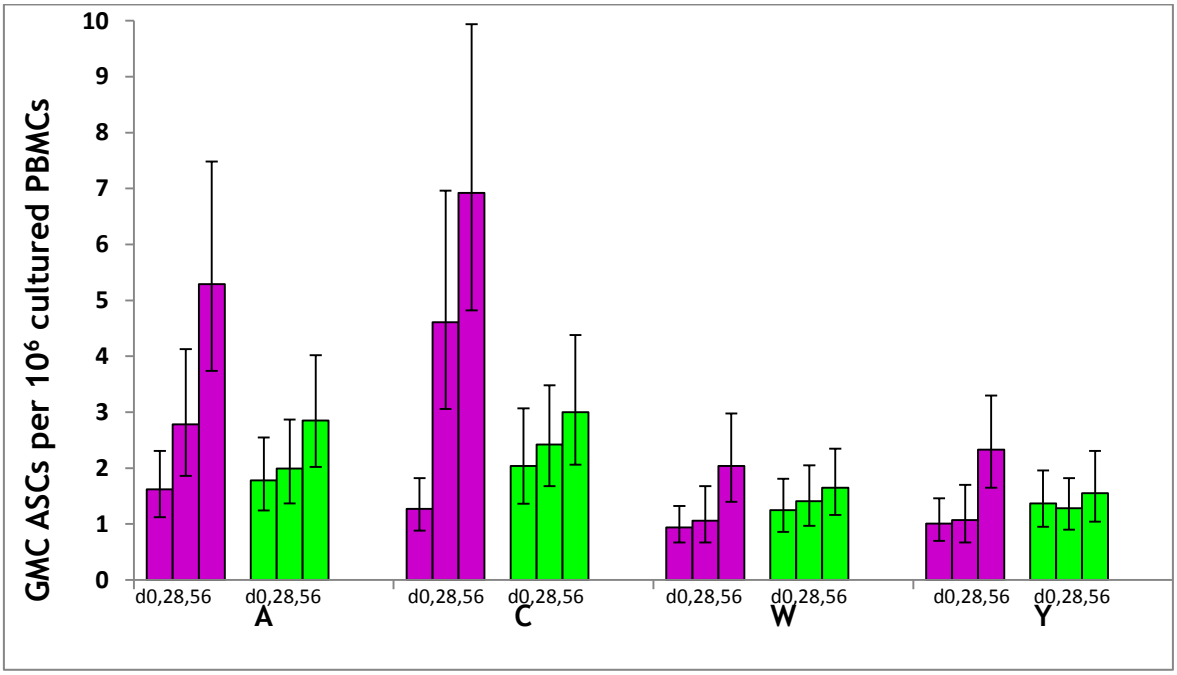


Figure 37 MenA, MenC, MenW and MenY specific memory B cell GMCs and 95%CI at days 0, 28 and 56 of the MenACWY-CRM/MenACWY-PS clinical study.



5.4. Discussion

5.4.1. Prior MenACWY-PS impairs the antibody and plasma cell response to subsequent MenACWY-CRM

Meningococcal serogroup C polysaccharide induced hyporesponsiveness has been well demonstrated at the antibody level using radioimmunoassay, ELISA and SBA assays (summarised Table 22). The data presented in Figure 29 show that prior MenACWY-PS impairs the functional antibody response to all 4 serogroups 28 days after a dose of MenACWY-CRM and confirms the previously demonstrated phenomenon of polysaccharide vaccine induced hyporesponsiveness at antibody level.

However, Figure 30 shows that MenACWY-PS also impairs the plasma cell response to a dose of MenACWY-CRM at 7 days for all 4 serogroups. Thus in Group 2, the plasma cell response to the conjugate booster is less than response to the priming polysaccharide (Figure 36). This is the first time that hyporesponsiveness has been demonstrated to meningococcal polysaccharide at the antigen specific B cell level. This is consistent with the observation in the previous chapter that day 7 plasma cells correlate with day 28 SBA titres.

5.4.2. MenACWY-PS fails to prime for immunological memory

Memory B cells underlie the anamnestic response to subsequent antigenic challenge and do not constitutively produce antibody, but rapidly differentiate into plasma cells and further memory B cells on stimulation by antigen [100]. Thus memory B cell responses have classically been inferred by a potentiated antibody response after booster vaccination. The SBA GMT 7 days after a conjugate booster is significantly higher in individuals primed with MenACWY-CRM than MenACWY-PS (Figure 32). This suggests that priming with conjugate elicits bactericidal antibodies faster than priming with polysaccharide. The higher SBA titre at day 7 elicited in Group 1 by a conjugate booster

may reflect higher avidity antibody with greater bactericidal activity generated through the germinal centre by the priming dose of conjugate. It has been suggested that the response to a conjugate vaccine closely parallels natural infection by meningococci, where the capsular polysaccharide of the invading bacteria are presented as TD antigens linked to subcapsular proteins[301]. Thus the slower response to a conjugate booster after priming with polysaccharide implies that individuals who have received a prior polysaccharide may be less able to mount a fast protective immune response in the event of invasive disease. The difference in SBA titres has lost significance by 28 days after the conjugate booster but is still higher in the MenACWY-CRM primed Group 1(Figure 33). It can be speculated that the slower response to conjugate in Group 2 may be more similar to the kinetics of a primary response than the secondary-type response seen in Group 1. However, given the short interval between the first and second vaccines (1 month) and the consequent high SBA titres at the time of boosting, true memory responses in individuals with already high antibody titres may be difficult to ascertain [180]. Avidity maturation indices and demonstration of antibody somatic hypermutation may provide better indicators of memory at the antibody level [194]. Priming with conjugate also elicits higher frequencies of plasma cells against all 4 serogroups 7 days after a conjugate booster than priming with polysaccharide (Figure 34).

Antigen specific memory B cell responses are of greater magnitude and peak earlier in secondary responses compared to primary responses [134]. Figure 31 shows lower MenC specific memory B cell frequencies 28 days after a conjugate vaccine in those who have received prior polysaccharide compared to polysaccharide naïve individuals. However, it may be that memory B cells should have been measured at a shorter interval after the conjugate vaccination to elucidate newly generated memory B cells from a faster secondary response. A more accurate indication of the direct effect of priming vaccine on memory B cell generation may actually be to compare the memory B cell response to a

booster conjugate vaccine between the 2 groups. Figure 35 shows that MenA and MenC specific memory B cells 28 days after a conjugate booster are higher in conjugate primed individuals, compared to polysaccharide primed individuals. A similar trend is seen for MenW and MenY specific memory B cells, but these differences are not significant, perhaps due to the small sample size. The temporal patterns of memory cell production in each group suggest a trend towards higher cell counts over the course of the trial in Group 1 than Group 2. Taken together, these findings suggest that MenACWY-CRM is better able to prime for immunological memory than MenACWY-PS.

5.4.3. Possible mechanisms for the hyporesponsiveness observed in this study

As discussed in the introduction to thesis, hyporesponsiveness caused by polysaccharide is antigen specific [302, 303] and dose dependent [304]. This argues against a non-specific mechanism of immune suppression by polysaccharide for example via T suppressor cells. Four possible explanations for the phenomenon of hyporesponsiveness are considered below.

5.4.3.1. Persistent antigen

Murine studies with radiolabelled pneumococcal polysaccharide suggest that polysaccharides can persist in the circulation for considerable time after vaccination[305]. On injecting polysaccharide specific antibody, the amount of detectable polysaccharide decreases, then rises again as the antibody levels wane. The authors postulate that this continuously released free antigen constantly binds to and neutralises specific antibody, thereby reducing the functional antibody detected in response to subsequent vaccination. The persistence of the polysaccharide may be related to its size. Polysaccharide vaccines generally contain higher molecular weight saccharides than conjugate vaccines (Section 4.4.6). This would explain the apparent dose dependency observed in relation to

polysaccharide-induced hyporesponsiveness [172, 306]. However, this theory does not explain the effects seen in this study at cellular level.

5.4.3.2. Inhibitory effect of high serum antibody

A high antibody titre elicited by a polysaccharide vaccine with its high antigen load may exert a negative feedback on further antibody production – perhaps by binding to free antigen and limiting the amount available to stimulate specific B cells. This mechanism has been used to explain the observed temporary drop in anti-Hib antibody titres immediately after a dose of PRP polysaccharide vaccine, the magnitude of which correlates with pre-immunisation anti-PRP titres [307]. This would also be consistent with the apparent reversal of hyporesponsiveness over time [296] once antibody has waned. However, other studies with dose intervals of 6 months to 1 year have shown that despite antibody titres pre-boost returning to pre-priming levels, hyporesponsiveness persists [288, 308].

In the study described in this thesis, the interval between doses was 1 month and antibody levels had not returned to baseline levels before the second dose of vaccine. At day 28 after the first vaccination, MenC SBA titres were higher in the MenACWY-CRM group while MenW titres were higher in the MenACWY-PS group (Section 4.4.1). However, hyporesponsiveness was demonstrated across all 4 serogroups, which suggests that absolute antibody levels alone do not mediate hyporesponsiveness.

A strong immune response to a primary vaccine may exert a ceiling on subsequent responses to a booster vaccine. In Table 24, it may be seen that the magnitude of the absolute increase in SBA titres at day 28 is smaller after a second dose of conjugate than after a single dose of conjugate across all 4 serogroups. A similar pattern of a smaller rise in absolute MenC-specific memory B cells after a MenACWY-CRM booster, as compared

to a primary dose of MenACWY-CRM, is shown in Table 26. Serogroup C induces the largest increase in memory B cell numbers from day 0 to day 28, thus the threshold of response may not have been reached by the other serogroups. This effect is not restricted to the oligosaccharide component of the conjugate vaccine but is also seen for the carrier protein at memory B cell level. There was no significant difference between plasma cell responses after a first or second dose of conjugate (data not shown). Using longer intervals between priming and boosting that allow time for germinal centres to involute and thus SBA titres and memory counts to return to baseline values may provide a clearer picture of hyporesponsiveness without the confounder of a potential ceiling on the magnitude of the immune response.

5.4.3.3. Reduction in specific antibody isotypes

Polysaccharide vaccines may selectively impair the production of an immunoglobulin isotype. TI antigens such as polysaccharide vaccines induce IgG2 subclass responses in humans and IgG3 responses in mice [280, 309]. Conjugate vaccines, however, invoke IgG1 and to a lesser extent, IgG2 [290]. Murine studies suggest that polysaccharide may deplete the IgG2a and IgG2b subclasses [301, 306]. These subclasses in mice are responsible for binding complement with high affinity through their Fc receptor, thereby activating the classical complement pathway [310]. Thus hyporesponsiveness may be due to an impairment of a specific complement binding isotype. The resulting reduced complement binding capacity of serum would be detected as a lower SBA titre or overall lower levels of IgG would be detected by ELISA. Antibody isotypes were not measured in this study. Again, this mechanism does not explain the lower number of plasma cells or memory B cell s seen after MenACWY-PS vaccination.

5.4.3.4. Depletion of the memory B cell pool

The final theory to explain hyporesponsiveness is that polysaccharide vaccines drive terminal differentiation of antigen specific cells into plasma cells, without regeneration of the memory B cell pool, so fewer B cells are available to respond to subsequent antigenic challenge. [168]. The antigen specific B cell responses described in Chapter 4 of this thesis support this theory, with a single dose of MenACWY-PS generating higher plasma cell titres than MenACWY-CRM which in turn appears to generate memory B cells. On administering a booster dose of MenACWY-CRM to MenACWY-PS, fewer plasma cells are produced at 7 days in comparison to individuals receiving a single dose of MenACWY-CRM (Figure 30). This is reflected in the discrepancy in SBA titres at 28 days after conjugate (Figure 29). In contrast, on administering a MenACWY-CRM booster to the MenACWY-CRM primed group, more plasma cells and memory B cells are generated (Figure 34 and Figure 35) suggesting that there has been no corresponding depletion in antigen specific cells in this group?

A recent study in elderly adults by Clutterbuck *et al.* comparing varying regimes of PPV and PCV also supports terminal differentiation into plasma cells and memory B cell depletion as the mechanism underlying hyporesponsiveness [257]. In a subset of participants, a booster dose of PPV elicited higher numbers of cells with the phenotype of plasma cells ($CD19^{+}CD20^{-}CD38^{hi}$) than a PCV booster. However, the antigen specificity of these plasma cells was not determined and different priming regimes were used in these participants. Comparison of a single dose of PCV to priming with PPV followed by PCV demonstrated an attenuation of antigen-specific memory B cell responses that persisted for up to 6 months after polysaccharide vaccination. Immunophenotyping confirmed a similar pattern of fewer pneumococcal antigen specific switched memory cells ($CD19^{+}CD20^{+}CD27^{hi}IgM^{-}$) in participants who had received prior PPV as part of their vaccine regimen. Interestingly, the authors also showed a reduction in pneumococcal

specific B1b cells ($CD19^{+}CD5^{neg}CD27^{-}IgM^{hi}$), a population of B cells thought to be involved in TI-2 responses [122]. This suggests that polysaccharides selectively bind the BCR of antigen specific B cell subsets, causing differentiation into antibody secreting plasma cells, resulting in depletion of that subset. Thus the partial recovery of MenC specific antibody responses observed in MenAC -PS primed adults by administration of MenC-CRM may relate to the stimulation of different subsets of antigen-specific B cells by the conjugate vaccine [284].

A confounding factor in interpreting whether the B cell depletion suggested by the findings described in this chapter is a true feature of a polysaccharide versus conjugate responses is that MenACWY-PS contains 5-10 times more polysaccharide than MenACWY-CRM. It may simply be that higher doses of antigen drive plasma cell production and exhaustion of the B cell pool. A study which investigated the MenC specific B cell response in infants also showed that a MenC conjugate vaccine induced memory B cells whereas a MenC polysaccharide vaccine did not [189]. However, this study showed no difference in plasma cell responses between groups. This may have been because both the MenC polysaccharide and conjugate vaccine used each contained 10 μg of polysaccharide. The studies by Gold *et al.* in infants [172] did not demonstrate hyporesponsiveness with 10 μg of MenC polysaccharide. Using equivalent dosages of polysaccharide e.g. by comparing a $1/5^{th}$ dose MenACWY-PS (containing 10 μg each of MenA, MenC, MenW & MenY) to MenACWY-CRM (containing 10 μg of MenA and 5 μg each of MenC, MenW & MenY) will clarify whether the hyporesponsiveness observed in this study is confounded by antigen dosage.

This antigen-specific B cell depletion may be brought about by targeted apoptosis of B cell populations by polysaccharide. Recent murine work shows that in MenC-CRM primed mice, a MenC-PS booster drives apoptosis of MenC-specific switched IgG^{+} memory cells in

the spleen in the first 24 hours after vaccination [311]. This subsequently manifests as less proliferation of antigen specific plasma cells ($B220^{-}CD138^{+}$) and memory B cells ($B220^{+}CD138^{+}$) in the spleen 5 days after vaccination. Fewer MenC specific plasma cells are also found in the bone marrow 5 days after MenC-PS, which the authors hypothesize is due to a failure of MenC-specific plasmablasts to differentiate and migrate to the bone marrow. Of note, the MenC-PS booster used in this study contained 4 times more polysaccharide that the conjugate.

5.4.4. Serogroup A polysaccharide also causes hyporesponsiveness

MenC polysaccharide has been well established as a TD antigen, with poor immunogenicity in young children [172], hyporesponsiveness to subsequent boosters ([168, 284, 287, 288, 312] and the inability to prime for memory [179, 190]. In contrast to MenC, antibody responses to MenA polysaccharide have been demonstrated in infancy[172, 313] and MenA appears to prime for anamnestic booster responses [169]. MenA polysaccharide is structurally distinct from that of the other serogroups and this may underlie these observed differences. Alternatively, the early infant studies of the 1960's where immunogenicity was demonstrated, may have used preparations of MenA polysaccharide which were contained meningococcal protein impurities, thus these vaccines may not have acted as pure TI antigens.

The results of this chapter show that MenA polysaccharide acts similarly to MenC, by causing hyporesponsiveness of antibody and plasma cell responses (Figure 29 and Figure 30) and also fails to prime for memory responses (Figure 32, Figure 34 and Figure 35). This is the first definitive evidence that, contrary to previous observations, MenA polysaccharide acts as a TI antigen. These observations support the current strategy of the African Meningitis Vaccine Project (http://www.path.org/menafrivac/index.php) to

vaccinate populations of the African meningitis belt with a MenA conjugate vaccine over a MenA polysaccharide vaccine.

A future study is planned to investigate the phenotypes of antigen specific B cells elicited by MenACWY-PS and MenACWY-CRM. This will test the hypothesis that marginal zone or B1b cells are elicited in response to both the MenA and MenC components of MenACWY-PS while follicular B cells are elicited in response to the MenA and MenC components of MenACWY-CRM.

5.4.5. What is the clinical significance of meningococcal polysaccharide vaccine induced hyporesponsiveness?

Nasopharyngeal carriage of a particular pneumococcal serotype has been shown to impair the response to the same serotype in a subsequent conjugate vaccine [302, 314]. If conjugate vaccines present a TD response in much the same way as infection with an intact organism, then this suggests that exposure to polysaccharide, whether by carriage or vaccination may impair the immune response to subsequent invasive disease. This been shown in a study of PPV versus PCV in HIV positive Ugandan adults, in which a significant increase in pneumonic events occurred in the PPV group within the first 6 months after vaccination [315]. Although individual cases of MenC-PS vaccine failure have been reported [316, 317], the overall incidences of carriage and invasive disease are lower for meningococci than pneumococci, and similar increase in disease susceptibility has not been observed with meningococcal polysaccharide vaccines.

The absolute Men C-specific SBA GMT of participants in Group 2 of this study remains well above the putative correlate of protection defined as a hSBA of ≥4 [27] both 7 and 28 days after the conjugate booster (titres of 53.2 and 56.2 respectively). A similar correlate of protection has not been established for serogroups A, W & Y. Both

MenACWY-PS and MenACWY-CRM are recommended by the Departments of Health of England and Wales for individuals over 5 years of age who are at risk from meningococcal disease [230]. The current guidance suggests that those who are at on-going risk should have booster doses of MenACWY-PS every 5 years. However, given the ability of MenACWY-PS to impair subsequent responses to conjugate boosters and thus potentially to invasive disease, the advice to administer multiple doses of polysaccharide to these individuals may need to be reconsidered.

5.4.6. Polysaccharide vaccine induced hyporesponsiveness may have evolved as a bacterial strategy to evade immune recognition.

TI-2 antigens such as bacterial capsular polysaccharides are repetitive structures that require only a high molecular weight and an optimum density of epitopes to elicit an antibody response [276]. Many mammalian cells have potentially TI-2 type molecules associated with their cell surfaces and intracellular matrix including glycosaminoglycans such as chondroitin and glycolipids such as gangliosides. These molecules do not normally elicit B cell responses despite cross-linking B cell surface receptors (BCRs). This tolerance is thought to be mediated by 'self-markers' on the self-antigen simultaneously cross-linking BCRs and inhibitory receptors on the B cell surface. Duong et al demonstrated that sialic acid residues on a potentially TI-2 antigen interact with sialic acid-binding Ig-like lectins (siglecs) such as CD22 on B cells, inducing tolerance *in vivo* [318]. Crosslinking of the BCR and engagement of CD22 causes arrest of the cell cycle and drives B cells into apoptosis in vitro [319]. The capsules of serogroups B,C,W & Y are sialic acid based and this sialylation is critical for virulence [13]. It may be hypothesised from these findings that meningococci have evolved polysaccharide capsules with the ability to engage siglecs as a form of molecular mimicry to evade the immune system – thus polysaccharide

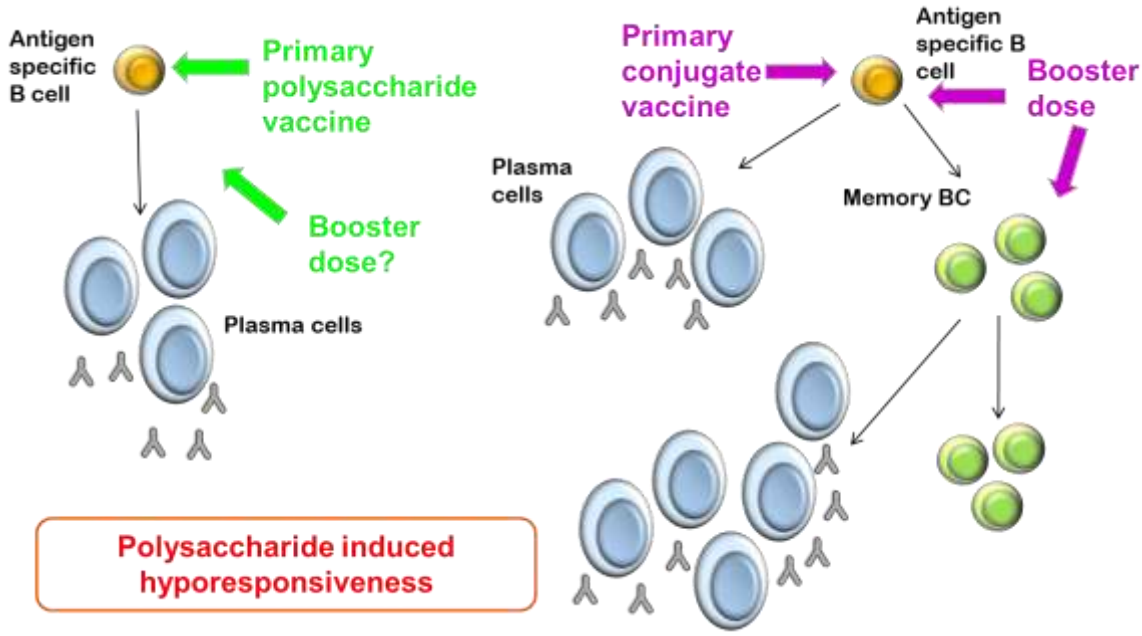
induced hyporesponsiveness may be due to encapsulated bacteria manipulating mechanisms to provide tolerance to self-antigens.

Conversely, it has been suggested that downregulation of B cell responses to polysaccharides may have evolved as a protective mechanism by the host [320]. TI-2 antigens do not require a second signal for the development of an immune response [321] and are slow to degrade *in vivo* [305]. Thus hyporesponsiveness may prevent stimulation of polysaccharide specific B cells and the resultant overproduction of antibody.

5.5. Conclusion

MenACWY-PS causes impaired antibody and plasma cell responses to subsequent vaccination with MenACWY-CRM and fails to prime for immunological memory. Taken together with the results from Chapter 4, MenACWY-PS induced hyporesponsiveness may be due to the polysaccharide driving terminal differentiation of antigen specific B cells into plasma cells without replenishment of the memory B cell pool.

Figure 38 Differences in B cell responses to primary and secondary polysaccharide and conjugate vaccines



In this study, both the MenA and MenC components of the polysaccharide vaccine cause hyporesponsiveness and fail to prime for memory, suggesting that MenA may act as a TI antigen and not as a TD antigen. Further work is needed to ascertain if there are differences in B cell subsets responding to quadrivalent meningococcal conjugate and polysaccharide vaccines which explain the distinctions in immunological responses observed in this study.

Chapter 6 – Next generation sequencing as a tool to explore antibody repertoire

6.1. Introduction

This chapter discusses the development of laboratory methods to prepare B cell VDJ gene sequences for 454 sequencing, with the aims to:

- Compare the normal B cell repertoire of 2 different individuals
- Investigate the *Haemophilus influenzae* type b polysaccharide-specific antigen responses after vaccination
- Investigate differences in B cell subset repertoire in responses to conjugate and polysaccharide vaccination

6.1.1. Antigen specific antibody repertoires are characterised by specific VDJ sequences

Several studies have examined the antibody repertoire generated in response to specific antigens such as bacterial polysaccharides [322-325], viral glycoproteins [326-328] and autoimmune antigens [329]. These suggest that genetically diverse individuals utilise similar combinations of VDJ combinations for a given antigen. One of the best studied examples of this phenomenon is the antibody repertoire produced following *Haemophilus influenzae* type b (Hib) polysaccharide. This was first investigated by Insel, who demonstrated by isoelectric focussing 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 [330]. Affinity chromatography purification of Hib-specific antibody and subsequent amino acid sequencing revealed a V_H3 family dominance of the response to Hib polysaccharide vaccination [331]. More recently, B cells enriched in specificity for Hib polysaccharide from vaccinated infants were used to construct phage display antibody libraries [332]. Nucleotide sequencing of

the antibodies expressed revealed usage of a single V_H gene segment (V3-23) and only two J_H and two V_L and J_L segments, consistent with findings in older studies using Hib specific-antibody secreting hybridomas [333].

Arnaout performed a detailed analysis of the antibody repertoires against known antigens, so called 'public repertoires' [334]. He searched the reference database for immunogenetics (International ImMunoGeneTics Information System, or IMGT) for sequences linked to specific antigens. The sequences were obtained, for example, from immortalised B cell lines from vaccinated individuals, and identified nearly 300 such sequences against 16 specific antigens. He showed that there was little overlap in VDJ usage between antibodies of different specificity and that this occurred less often than would be predicted by chance. This suggests that VDJ segment recombination is not random and that the antibody repertoire reflects the exposure to antigen. An intriguing possibility arising from this observation is that of developing a diagnostic fingerprint of VDJ repertoire in order to reveal prior exposure to antigen. This might be used, for example, in investigating effective immune responses to a vaccine. However, the studies discussed in this section have relied on small samples sizes, and a detailed genetic analysis of the antigen specific repertoire has been limited by the capabilities of traditional Sanger sequencing. There are no published studies using next-generation sequencing methods to investigate antigen-specific antibody repertoire.

6.1.2. Analysis of antibody repertoires using 454 sequencing

454 sequencing (Section 1.7) was first used to study the breadth of the antibody repertoire in zebrafish [335]. Zebrafish are a useful experimental model for studying adaptive immune responsessince immunoglobulin diversity is generated through similar mechanisms to humans, with a multiplicity of V, D and J segments, DNA recombinase

enzymes and mechanisms for junctional diversity. However, the potential repertoire of zebrafish is several orders of magnitude smaller than that of humans as they posess fewer individual gene segments. In a proof-of-concept study, Weinstein *et al.* obtained approximately 1 million sequences in total from 14 individual fish, and developed a 'capture-recapture' statistical method for estimating the total size of the antibody repertoire based on the variation in abundance of the observed sequences. This suggested that individual zebrafish used between 50-86% of potential VDJ combinations. By comparing VDJ usage between individual fish, the investigators were able to show that the most common sequences were relatively preserved between individual fish, perhaps due to a similar pattern of antigenic exposure in their fish tanks.

Several studies have since used 454 sequencing to investigate the antibody repertoire in humans, summarised in Table 27. Boyd *et al.* showed that clonal expansions of B cells could be identified on the basis of their VDJ sequences and that the largest clones formed between 0.15%-1.5% of all circulating B cells in healthy adults[336]. These clones persisted for up to 14 months. The investigators suggest that the ability to detect and track small clonal expansions means that 454 is a potential tool for monitoring residual malignant clones post chemotherapy for leukaemia.

A study by Wu *et al.* looked at the VDJ usage between B cell subsets – this showed that the repertoire of naïve B cells is more diverse and less oligoclonal than the repertoire of antigen-experienced IgM or switched memory B cells [128]. In addition, the repertoire and CDR3 characteristics of the switched memory cells differed from the IgM memory cells, which supports previous studies which suggest that the two populations are derived from immunologically distinct pathways [104, 129, 337].

Table 27 Key studies using 454 sequencing to analyse immunoglobulin repertoire in humans

Study	Cell pop	Substrate	Finding
Arnaout 2011	PBMCs	DNA	Preferential pairing of certain D with certain J Over representation of CDR3s ? selection of BC that express these
Boyd 2009	PBMCs	DNA	Investigation of clonality in BC pops. Largest clones 0.15-1.5% of circ BC. Clones persist over time. Ability to use as a detection for residual disease without multiparameter flow cytometry
Boyd 2010	PBMCs	DNA	Allelic variation in IgH locus between individuals
Briney 2012	Naïve,IgM memory, IgG memory	mRNA	Jh4 used in memory pop. Diff in IgG/IgM memory. Increased oligoclonality in memory pop. Intrasubset variability greater than intradonor variability.
Glanville 2009	PBMCS phage display combinatorial library	mRNA	Non-random pairing of heavy and light chains, estimates of diversity
Ippolito 2012	PBMCs	Total RNA	Preferential pairing of VJ and DJ combinations
Prabakharan 2012	Cord blood	mRNA	IgM populations – IgHv1 most common family, differences between adult and foetal repertoire
Wu 2010	Naïve, transitional, switched memory, IgM memory	Total RNA	Ag experienced cells more oligoclonal, shorter CDR3. Differences in IgM/IgG memory repertoire and CDR3 characteristics.
Wu 2011	Naïve, IgM memory, switched memory, CD27-	Total RNA	CD27- and switched memory cells have similar VH usage, suggesting a single developmental origin

6.1.3. Conjugate and polysaccharide vaccines may evoke different antibody repertoires

Licensed vaccines against *N. meningitidis* utilise the polysaccharide capsule of the organisms as a vaccine antigen. Both plain polysaccharide and protein-polysaccharide conjugate vaccines are available for use in the prevention of meningococcal disease. Multiple studies have shown a difference in the quality of the antibody response induced by conjugate and polysaccharide vaccinations. T cell dependent (TD) antigens such as conjugate vaccines elicit T cell help and stimulate germinal centre formation with associated antibody class-switching and somatic hypermutation, forming high affinity antibody. In contrast, T cell independent (TI) antigens like polysaccharide vaccines are associated with limited hypermutation of the variable domain, although this may be due to the subset of B cells responding to TI antigens [104].

Harris *et al.* showed that despite similar serum bactericidal activity (SBA) geometric mean titres (GMTs) in adults who received a MenAC-CRM conjugate and MenAC-PS polysaccharide, antibodies induced by conjugate had a higher avidity index, suggestive of an affinity maturation process. The conjugate induced antibodies, but not the polysaccharide induced antibodies, also transferred protection in a mouse experimental model [197]. A similar increase in MenC specific IgG avidity in response to MenC-CRM but not MenAC-PS has been demonstrated in children [190]. Subtle changes in the polysaccharide component of a vaccine may also directly influence the expressed antibody repertoire. Adults immunised with a Hib vaccine containing high molecular weight polysaccharide were compared with those receiving a vaccine with an oligomeric form of polysaccharide [338]. The oligomeric vaccine induced antibodies that were of higher avidity index than those induced by the high molecular weight vaccine. In addition, the two antibody repertoires were distinct as defined by anti-idiotype antibodies (monocolonal antibodies generated against the antigen binding site). Thus the length and molecular weight of a polysaccharide antigen, as presented by a polymeric plain

polysaccharide vaccine or an oligomeric protein-polysaccharide conjugate vaccine may favour the emergence of B cell clones with distinct patterns of VDJ usage. This may partly explain the functional differences observed in the antibody response to these two types of vaccine.

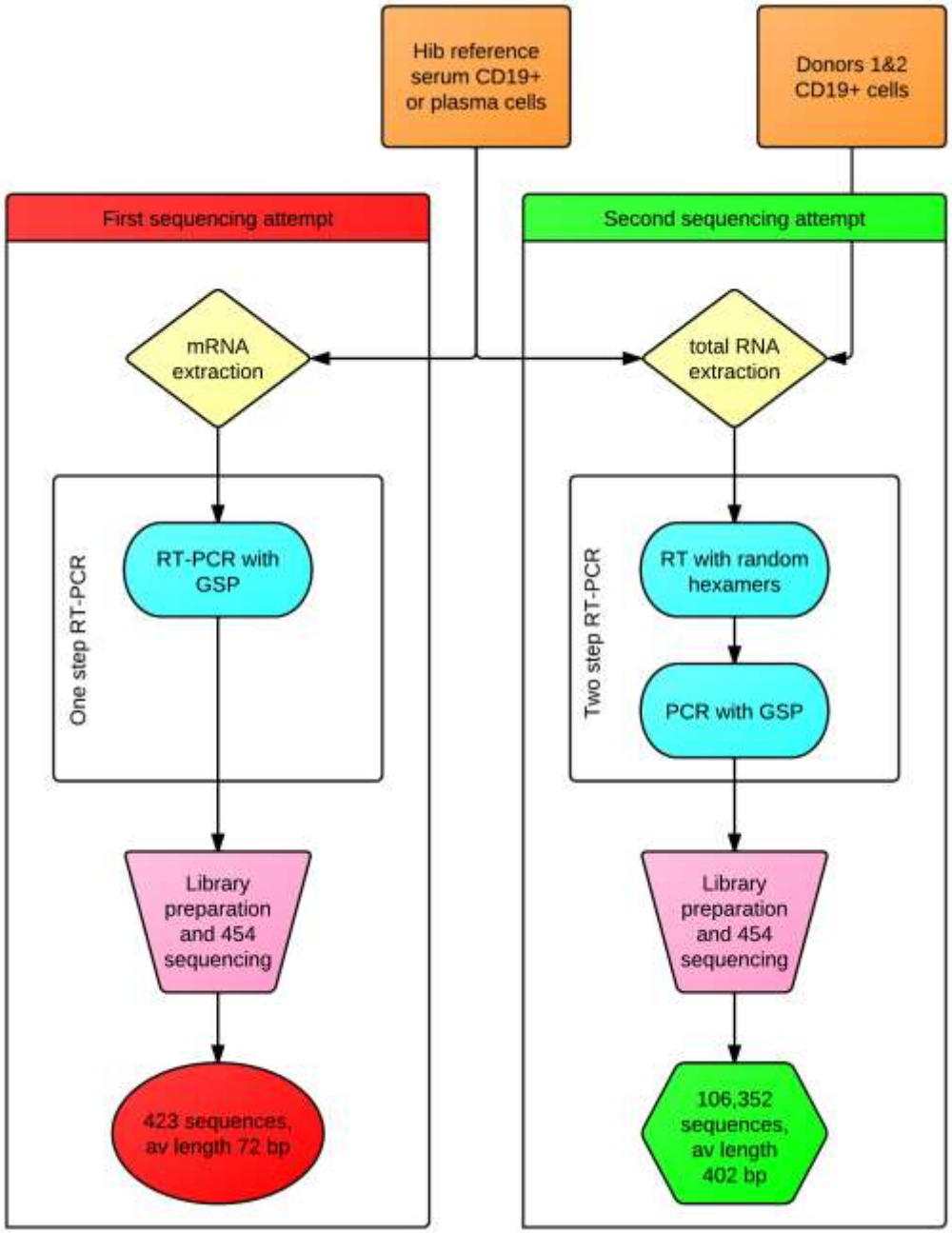
This DPhil thesis aimed to use 454 sequencing to further investigate these observed differences in antibody quality between polysaccharide and conjugate responses using B cell subsets derived from the MenACWY-CRM/MenACWY-PS study described in Chapter 3. The overall repertoire in response to each type of vaccine and the repertoires of naïve, IgM memory and IgG memory populations between the two vaccine groups were to be compared. As a pilot study, 454 sequencing methods were to be applied to analyse the repertoire of 2 healthy adults at baseline and also to analyse antigen specific V_H sequences before and after receiving a Hib conjugate vaccine.

6.2.Methods

6.2.1. Overview

The strategies used to develop the methods for analysing immunoglobulin heavy chain variable domain genes are shown in Figure 39.

Figure 39 Overview of methods used to analyse V_H gene sequences in this chapter.



RT = reverse transcription, PCR = polymerase chain reaction, GSP = gene specific primers, bp = base pairs. Note that the MenACWY-CRM/MenACWY-PS study samples did not yield sufficient RNA for downstream 454 sequencing attempts.

6.2.2. CD19^{+} B cell separation for comparison of repertoire between two individuals

20 mL of heparinised blood were obtained from two healthy adult volunteers, termed Donors 1 and 2 in accordance with University of Oxford policies [339]. CD19+ B cells were separated from PBMCs using a magnetic cell sorter as described in Section 2.3.9 and immediately followed by RNA extraction and two step RT-PCR.

6.2.3. CD19+ B cells and plasma cells to study Hib specific antibody repertoire

Hib polysaccharide specific antibody responses were studied as part of a single centre, open-label clinical study in 20 healthy adult volunteers aged 18-65 years of age in Oxford, UK (Hib reference study[2]). Participants were recruited by poster advertisement. Exclusion criteria were as follows: systemic hypersensitivity to any component of the vaccine; febrile illness (oral temperature >37.5°C) on the day of inclusion; congenital or acquired immunodeficiency or receipt of immunosuppressive therapy; chronic illness that could interfere with trial conduct or completion (e.g. cardiac, renal, diabetes, or auto-immune disorders); receipt of blood or blood-derived products within the previous 3 months; and thrombocytopenia or bleeding disorder contraindicating intramuscular vaccination. Written informed consent was obtained from the participants before enrolment. Ethical approval was obtained from the Oxfordshire Research Ethics Committee B (OxREC Reference: 08/H0605/74).

Participants were vaccinated with a Hib-MenC vaccine (Menitorix®, GlaxoSmithKline Vaccines, Brentford, UK; Lot number A76CA058A), containing *Haemophilus influenzae* type b (Hib) polyribosyl ribitol phosphate (PRP) polysaccharide and meningococcal serogroup C polysaccharide individually conjugated to a tetanus toxoid carrier (Table 28).

[2] Full study title 'Creation of a human anti-*Haemophilus influenzae* type b Reference Serum'

One 0.5 mL dose of the vaccine was injected into the deltoid muscle using a 23G 25 mm needle.

Table 28 Contents of a 0.5ml dose of Menitorix®

Haemophilus influenzae type b polysaccharide (polyribosyl ribitol phosphate)	5 µg
conjugated to tetanus toxoid as carrier protein	12.5 µg
Neisseria meningitidis serogroup C (strain C11) polysaccharide	5 µg
conjugated to tetanus toxoid as carrier protein	5 µg

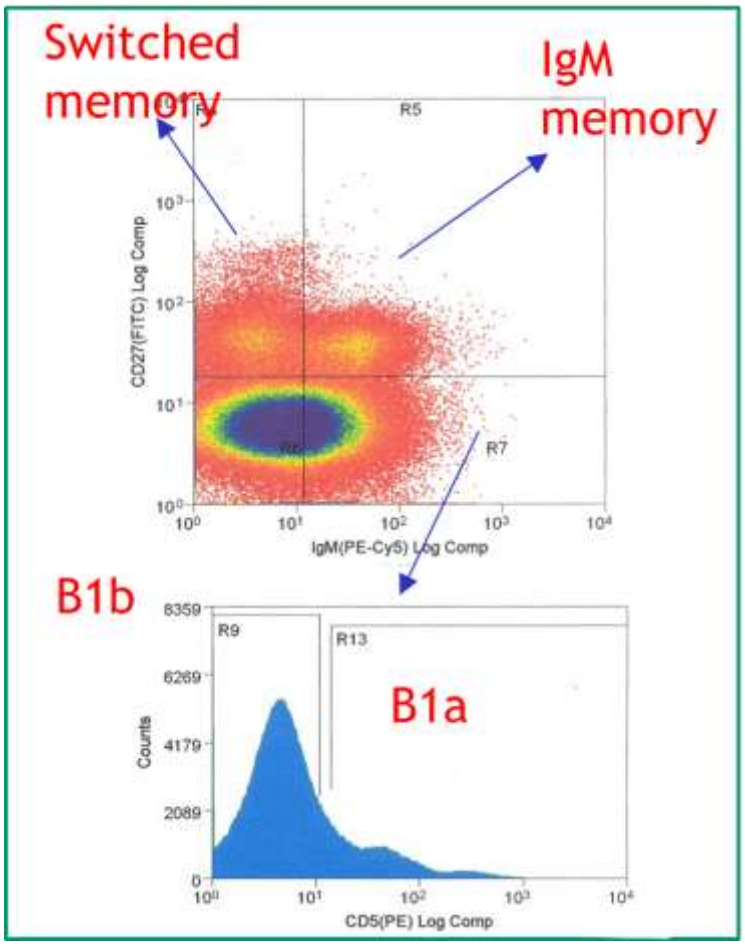
Blood was obtained from participants immediately prior to vaccination(20 mL) and 7 days after vaccination (50 mL) as described in Section. 2.3.1. Desired cell populations were separated using a magnetic cell sorter as described in Section 2.3.9. $CD19^+$ cells were separated at day 0 from all participant samples. At day 7, plasma cells were obtained from 11 participants while CD19+ cells were obtained from 9 participants. CD19+ B cells and plasma cells were separated from peripheral blood mononuclear cells (PBMCs) as described in section 2.3.3 and frozen at -80°C until RNA extraction. Previous studies have shown that antigen specific plasma cells peak in the peripheral circulation at day 7 after primary vaccination in adults [134]. This time point was therefore chosen to maximise the likelihood of detecting Hib-specific plasma cells. Cell population purities were determined by fluorescent activated cell sorting (FACS) using a 4 colour flow cytometer (FACSCalibur™) as described in Section 2.3.11 using anti-CD19 and anti-CD38 flurochrome labelled monoclonal antibodies as described in Section 2.2.3.

6.2.4. B cell subset separation for conjugate and polysaccharide vaccine induced repertoire analysis

PBMCs from 6 participants of MenACWY-CRM/MenACWY-PS clinical study (Chapter 3) were sorted into B cell subsets for antibody repertoire analysis. 3 of these individuals were from Group 1 and received 2 doses of MenACWY-CRM one month apart and the remaining 3 individuals were from Group 2 and received a dose of MenACWY-PS followed

by a dose of MenACWY-CRM one month later. CD19+ B cells were separated from PBMCs as described in Section 2.3.3. Sorted cells were incubated with IgM-PECy5, CD5-PE, CD19-APC and CD27-FITC as described in Section 2.2.3 to ascertain IgM memory populations ($CD29^{+}IgM^{+}$), switched memory populations ($CD27^{+}IgM^{-}$) and B1b populations ($CD27^{-}IgM^{+}CD5^{+}$). The cell populations were sorted on the basis of these cell surface markers using a high speed cell sorter (MoFlo™) as described in Section 2.3.11. An example of the gating strategy used is shown in Figure 40.

Figure 40 Example gating strategy for B cell subset separation in conjugate or polysaccharide vaccinated individuals using the MoFlo™ high speed cell sorter.



To each separated subset was added RNAlater RNA stabilisation solution (Applied Biosystems, Foster City, USA; AM7020). The manufacturer's instructions suggested that the cells be pelleted prior to the addition of RNAlater. However, given the low cell numbers obtained by sorting (Table 33), the cells could not be successfully pelleted from the cytometer sorting buffer. Therefore, 1 mL of RNAlater was added to each sorted population, allowed to equilibrate overnight at 4 °C, then stored at -80 °C until RNA extraction.

6.2.5. mRNA extraction

The initial run of parallel sequencing used mRNA as a template for cDNA synthesis. mRNA was extracted from cells, using the Oligotex Direct mRNA Mini Kit (Qiagen, Crawley, UK; 72022). Briefly, frozen cell pellets were thawed, followed by the addition of 600 µL OL1 lysis buffer (to which 30 µL β-mercaptoethanol (Sigma-Aldrich, Dorset, UK ; M7522) had been added per 1 mL OL1 buffer), and the sample mixed well by vortexing. The lysate was homogenised by pipetting onto a QIAshredder spin column (Qiagen; 79656) placed in a 2 ml collection tube and centrifuging for 2 minutes (min). 1.2 mL of ODB dilution buffer was added to the lysate, mixed by pipetting and centrifuged for 3 min. The supernatant was transferred to a new RNAse free tube. 35 µL of Oligotex suspension (containing latex microspheres with adherent polydT oligonucleotides which bind to the polyA tails of mRNA molecules) was added to the sample, mixed by vortex and incubated at 30°C for 10 min. The Oligotex:mRNA complex was pelleted by centrifuging for 5 min and the supernatant pipetted off. The pellet was resuspended in 100 µL OL1 lysis buffer and mixed by vortex. A further 400 µL of ODB dilution buffer was added, the sample incubated at 70°C for 10 min and then re-pelleted by centrifuging for 5 min. The pellet was resuspended in 350 µL OW1 wash buffer, pipetted onto a spin column and centrifuged for 1 min. The flow-through was discarded and the column transferred to a

clean collection tube. The column was washed with 350 µL OW2 wash buffer for 1 min and the flow through discarded. This was repeated once using the same collection tube. The spin column was transferred to a new 2 mL collection tube and 50 µL hot (70°C) OEB elution buffer used to resuspend the Oligotex:mRNA beads in the spin column. This was centrifuged for a further 1 min. The mRNA so obtained was immediately used in cDNA synthesis or frozen at -80°C until required. All centrifugation steps were performed at 13,000 revolutions per minute (rpm).

6.2.6. Total RNA extraction

Total RNA was extracted using a RNeasy Mini Kit (Qiagen; 74106). Briefly, frozen cell pellets were thawed followed by the addition of 600 µL RLT lysis buffer (to which 10 µL β-mercaptoethanol had been added per 1 mL RLT buffer) and the sample mixed well by pipetting. The sample was homogenised by passing the lysate 5-10 times through a 20G needle attached to an RNAse free syringe. An equal volume of ice-cold 70% ethanol was added and the sample mixed well by pipetting. 700 µL of the sample was transferred to a 1 mL RNA binding column placed in a 2 mL collection tube and centrifuged at 15 seconds (s) and the flow through discarded. The remaining aliquot was centrifuged for 15 s in the same spin column and the flow through discarded. 500 µL RW1 wash buffer added to the column and centrifuged for a further 15 s and the flow through discarded. 500 µL RPE wash buffer was added to the column, washed for 15 s and the flow through discarded. This was repeated but the column washed for 2 min. The column was spun for a further minute to eliminate RPE carryover, then the lid opened and allowed to air dry for 2 ½ min. The spin column was inverted onto filter paper and allowed to air dry for a further 2 ½ min. 30 µL of RNAse free water was added to the column and the RNA was eluted by centrifuging for 2 min. The eluted RNA was immediately used for cDNA synthesis or frozen at -80 °C. Fresh 2 mL collection tubes were used after each spin step to minimise

reagent carryover. All centrifugation steps were performed at 13,000 rpm. This method was adapted for B cell subsets stored in RNAlater by adding a further 2 volumes of RLT lysis buffer to the sample prior to the addition of ethanol.

6.2.7. First strand cDNA synthesis

Direct cell lysis and cDNA synthesis without RNA extraction was attempted using the Cells-to-cDNA II kit (Life Technologies, Paisley, UK; AM1723), but this proved unsuccessful. Subsequent experiments used cDNA synthesised from total RNA using random hexamer priming. Each 20 µL reverse transcription reaction contained 1 µL RNAsin RNAse inhibitor (Promega, Maddison, USA; N251B), 1 µL 50 µM random hexamers (Applied Biosciences, Warrington, UK; N808-0127), 1 µL 10mM dNTP mix (see section 2.X), 4 µL 5x buffer, 2 µL DTT and 1 µL SuperScript III reverse transcriptase (Invitrogen, Paisley, UK; 18080-093). Reverse transcription was performed in thin-walled PCR tubes with a thermocycler (DNA Engine PTC-200; MJ Research, Waltham, USA). A variety of reaction temperatures and times were trialled. Optimal cDNA yields were obtained with reverse transcription at 42 °C for 60 min, followed by inactivation at 95 °C for 10 min. Each experiment included a no reverse transcription (NRT) negative control to identify possible DNA contamination of the starting RNA. This included all the reagents necessary for the reaction apart from the reverse transcriptase enzyme.

6.2.8. Polymerase chain reaction (PCR)

The first and second rounds of PCR amplification used cDNA and first round PCR product as a template respectively. Each 50 µL reaction contained 0.25 µL (1.25 units) Taq DNA polymerase (Qiagen; 201205), 10 µL Q solution, 5 µL 10x buffer, 1 µL 10mM dNTP mix, 1 µL each 10 mM sense and anti-sense primers (Section 6.2.10), 4 µL template DNA

and 27.75 µL distilled water. 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 they were placed in a thermocycler preheated to 94 °C . PCR conditions were as follows: an initial denaturation a 94 °C for 3 min; followed by 30 (first round) or 15 (second round)cycles of denaturation ay 94 °C for 30 s, annealing at 58 °C for 30 s, 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 the second round PCR, and the products pooled. Each experiment included a no template (NT) negative control to check for exogenous DNA contamination of the reagents. This included all the reagents necessary for the reaction apart from the DNA template.

6.2.9. One step reverse transcription polymerase chain reaction (RT-PCR)

One-step RT-PCR combines first-strand cDNA synthesis (reverse transcription) and the polymerase chain reaction (PCR) in the same reaction tube, theoretically reducing the possibility of contamination and simplifying the reaction set-up. One step RT-PCR utilises the same gene specific primers for both cDNA synthesis and subsequent PCR amplification. The Titan One Tube RT-PCR Kit (Roche Applied Science, Mannheim, Germany; 11939823001) was used for the first run or parallel sequencing. Various annealing temperatures and starting mRNA template volumes were trialled prior to determining the final protocol. Briefly, each 50 µL reaction contained 4 µL dNTP mix (Section 2.2.2), 2.5 µL DTT solution, 1 µL RNAse inhibitor, 1 µL each 0.4 µM sense and antisense primers, 10 µL 5xRT-PCR buffer, 1 µL Titan enzyme mix (containing AMV reverse transcriptase and Tth DNA polymerase), 5 µL mRNA template and 24.5 µL distilled water. RT-PCR was carried out in a thermocycler (DNA Engine PTC-200; MJ Research, Waltham, USA) in thin-walled PCR tubes. RT-PCR conditions were typically as

follows: reverse transcription at 50 °C for 30 min; followed by an initial denaturation at 94 °C for 10 s; followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for for 30 s, elongation at 68 °C for 45 s; followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for for 30 s, elongation at 68 °C for 45 s, with cycle elongation of 5 s for each cycle (e.g. cycle 11 has additional 5 s, cycle 12 has additional 10 s etc); final elongation was at 68 °C for 7 min. Each RT-PCR experiment also had NRT and NT negative controls. Subsequent library preparation used 2 step RT-PCR with separate reactions for cDNA synthesis and subsequent PCR amplification.

6.2.10. Oligonucleotide primers

Several published primer sets were trialled; their details are shown in Table 29 below. The Sblattero primers [340] did not yield consistent results and were not used in further sequencing. The Van Dongen primers [341] were used in one-step RT-PCR to amplify Ig genes for the first attempt at 454 sequencing. These primers are designed to detect clonally rearranged VDJ genes in lymphoid malignancies. The forward primers are consensus sequences located in FR1 and are potentially able to amplify all the variants of a given VH family. The reverse primer is a consensus primer able to amplify all 6 JH genes. Thus while the whole of the VDJ sequence is amplified, no information is obtained about the isotyope of antibody. The Van Dongen primers are designed to be used in a multiplex PCR reaction, with the 6 forward primers used in equimolar amounts in each reaction. However, attempts at multiplex RT-PCR using V_H3, V_H4 and V_H1 family forward primers (the three most common V_H families in a normal repertoire[204]) did not yield discrete products, therefore these primers were only used in separate reactions. The Wu primers [128] were used for all subsequent sequencing. This primer set has six consensus forward primers for each of the families V_H 1-6, located in the FR1 region. The V_H7 family is amplified by the VH1 family primer. The reverse primers are located in the C_H1 constant

domain for IgM, IgA and IgG, enabling identification of the isotype of the antibody. The forward primers are designed to be used in a multiplex reaction, allowing inference of the relative proportions of each family in a given sample, subject to the bias that different primers may amplify targets with differing efficiency. A pair of primers that amplified a 600 bp sequence of the β actin structural protein gene were used in each PCR reaction as a positive control. All supplied primers were purified and desalted (Sigma-Aldrich; Custom DNA Oligos) and made into 10 μM working stocks using distilled water which were stored at -20 °C until required.

6.2.11. 454 library preparation and sequencing

The PCR amplicons were prepared for sequencing (Section 1.7.2) using the Roche GS FLX Titanium Rapid Library Preparation Protocol [211] and Multiplex Identifier (MID) tags 1-8. Briefly, amplicons were purified from the pooled PCR reactions and quantified using the fluorescent dyes ribogreen (first attempt at parallel sequencing) or picogreen (second attempt at parallel sequencing). A and B sequencing adaptors incorporating a fluorescence activated molecule (FAM) and MID tags were blunt-end ligated on to the amplicon sequences. The library was then quantified against a standard fluorescent curve, using qPCR and by electropherogram. Emulsion PCR and pyrosequencing (see Section 1.7.3) were performed on up to 2 x 10^6 molecules from each library using the Roche GS FLX Titanium XL+ sequencing platform [212, 213] on one quarter of a sequencing plate with the potential to yield up to 200,000 reads in total. Single read accuracy on this platform is quoted as greater than 99.5% over 600 bases [213].

6.2.12. Analysis of sequences

The raw sequences were processed using standard Roche GS FLX Titanium XL+ software algorithms which included filtering and trimming of low quality reads [342]. Individual library sequence data were obtained as FASTA files of the base-called reads. These were uploaded to the IMGT high throughput portal, IMGT/HighV-QUEST [343]. The resulting IMGT annotated output files were interrogated using the Microsoft Excel based programme, Immunoglobulin Analysis Tool (IgAT) version 1.02 [344]. All subsequent comparisons between libraries on normally distributed data (CDR3 length, somatic hypermutation rate) used unpaired t tests. Comparisons of V_H family usage used χ^2 tests.

Table 29 Primers used to amplify antibody VH domains

Primer set	Primer name	Sequence (5'-3')	Annealing temperature	Method of use
Positive control	ßactin for	CCAAGGCCAACCGCGAGAAGATGAC	66˚C	All PCR amplifications as a positive control
	ßactin rev	AGGGTACATGGTGGTGCCGCCAGAC	66˚C	
Van Dongen, 2003 [341]	Vh1-FR1 for	GGCCTCAGTGAAGGTCTCCTGCAAG	50˚C	Used in separate reactions in first sequencing run
	Vh3-FR1 for	CTGGGGGGTCCCTGAGACTCTCCTG	50˚C	
	Vh4-FR1 for	CTTCGGAGACCCTGTCCCTCACCTG	50˚C	
	JH cons rev	CTTACCTGAGGAGACGGTGACC	50˚C	
Sblattero, 1998 [340]	Degenerate VH3 rev	GAGGTTGCAGCTGKTGGAGWCY	50˚C	Not used for final library prep
	CH1µ for	GGTTGGGGCGGATGCACTCC	50˚C	
	CH1γfor	SGATGGGCCCTTGGTGGARGC	50˚C	
Wu, 2010 [128]	IGHV1 for	CCTCAGTGAAGGTCTCCTGCAAGG	58˚C	Used as a V_H family multiplex forward primers with either IgA, IgG or IgM reverse primers in second sequencing run
	IGHV2 for	TCCTGCGCTGGTGAAACCCACACA	58˚C	
	IGHV3 for	GGTCCCTGAGACTCTCCTGTGCA	58˚C	
	IGHV4 for	TCGGAGACCCTGTCCCTCACCTGC	58˚C	
	IGHV5 for	CAGTCTGGAGCAGAGGTGAAA	58˚C	
	IGHV6 for	CCTGTGCCATCTCCGGGGACAGTG	58˚C	
	CHA rev	GGCTCCTGGGGGAAGAAGCC	58˚C	
	CHG rev	GAGTTCCACGACACCGTCAC	58˚C	
	CHM rev	GGGGAATTCTCACAGGAGAC	58˚C	

6.3. Results

6.3.1. CD19+ B cells obtained from 2 healthy volunteers

PBMC and CD19+ counts, RNA amounts obtained from 2 healthy volunteers are shown in Table 30 below.

Table 30 PBMC and CD19+ B cell counts; extracted RNA quantity and quality; and V_H libraries prepared from 2 healthy volunteers for antibody repertoire analysis

Volunteer	PBMC count ($x10^6$)	$CD19^+$ count ($x10^6$)	Total RNA (ng)	RNA A_{260}/A_{280}	Ig gene	MID
01	22.5	0.8	920	2.04	VH multiplex/IgA	MID 1
					VH multiplex/IgG	MID 2
					VH multiplex/IgM	MID3
02	21.9	1.2	882	1.85	VH multiplex/IgA	MID 8
					VH multiplex/IgG	MID 8
					VH multiplex/IgM	MID 8

6.3.2. $CD19^+$ B cell and plasma cells from Hib reference serum study

20 participants were enrolled in the Hib reference study. The cell counts and purities of the separated cells (CD19+ at V1 and plasma cells or CD19+ at V2) are shown in Table 31 below.

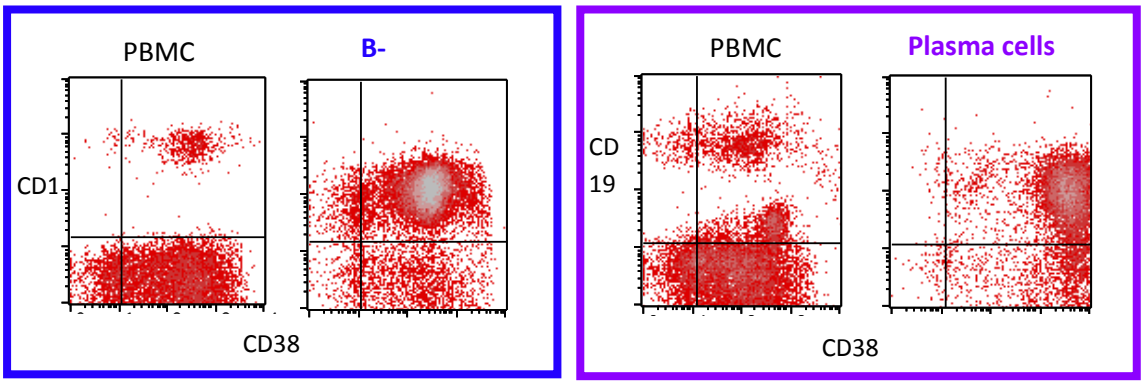
Table 31 PBMC count, CD19$^+$ B cell or plasma cell count and separated population purity before (day 0) and 7 days after vaccination with a Hib-MenC conjugate vaccination in the Hib reference study.

Participant	V1 (day 0) 20 mLs			V2 (day 7) 50 mLs		
	PBMC count (x10^6)	CD19$^+$ count (x10^6)	Purity (%)	PBMC count (x10^6)	CD19$^+$ or plasma cell count (x10^6)	Purity (%)
001	17.1	0.8	82	52.5	*	95
002	21.3	1.2	95	60.6	*	99
003	24.3	1.2	94	46.8	*	99
004	21.3	0.8	93	37.8	*	99
005	18.0	0.8	94	54.6	*	99
006	38.7	2.2	94	68.1	*	99
007	3.2	*	N/A	N/A	N/A	N/A
008	29.4	*	93	34.5	*	99
009	18.6	*	97	29.1	*	<15
010	22.2	*	98	93.3	*	<15
011	24.0	*	96	34.2	*	<15
012	26.4	*	96	48.9	1.4	86
013	18.3	*	97	64.2	2.8	94
014	20.4	*	90	60.3	*	87
015	30.9	*	96	45	*	92
016	17.4	*	92	66.3	*	77
017	21.9	*	91	48	*	90
018	26.4	*	90	56.1	*	97
019	20.4	*	93	56.7	*	97
020	41.7	*	95	74.7	*	96

* = total cell count not measured. Participant 007 had insufficient blood obtained at V1 to process PBMCs and did not have further blood taken at V2. Participants 001-011 had plasma cells separated at day 7 (results underlined), while participants 012-020 had CD19$^+$ B cells separated at day 7. Samples from participants 002, 005 and 006 were used for library preparation for the first 454 sequencing run did not yield any V_H sequences (results in red). Samples from participants 001 and 004 were used in the second sequencing run (results in green).

Purity estimates were made by flow cytometryic analysis using anti-CD38 and anti-CD19 fluorchrome labelled monoclonal antibodies. Example purity data from participant 009 is shown in Figure 41 .

Figure 41 Example purity data comparing PBMCs and sorted populations using anti-CD19 for B cells at day 0 and anti-CD38 for plasma cells at day 7.



mRNA yields from Hib reference study samples used for the first run of parallel sequencing were not formally measured.

Due to difficulties with obtaining sufficient quantities of RNA from the frozen Hib reference study samples for downstream PCR, extracted total RNA quality was assessed on a subset of samples. This was done by performing RT and using quantitative PCR (qPCR) on the resultant cDNA with primers for the Abl tyrosine kinase gene. These results are shown in Table 32. The Abl tyrosine kinase gene is a housekeeping gene which is normally expressed at a frequency of 50,000 copies/µL in this protocol. The Abl gene copy number derived from the frozen Hib CD19+ B cell pellet RNA (*Hib*) is an order of magnitude less that of freshly extracted RNA from a similar starting number of B cells (*MNR1* and *MNR2*).

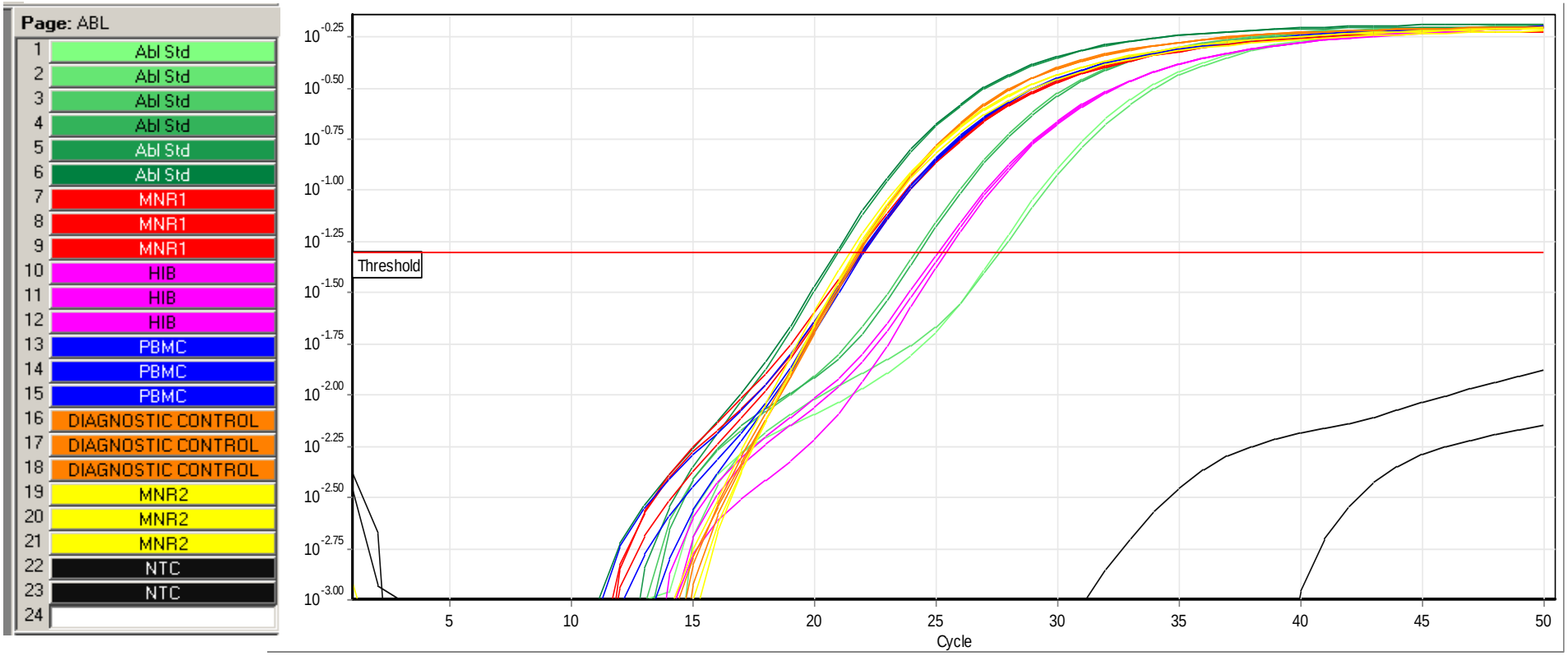
Table 32 qPCR of Abl tyrosine kinase gene copy number derived from RNA of CD19+ cells from Hib study participant 009 V1 (*Hib*), fresh CD19+ cells (*MNR1 & MNR2*) and *PBMC*s as compared to Abl standard constructs (*Abl Std 1-3*)

Name	Type	Given Conc (copies/µL)	Calc Conc (copies/µL)
Abl Std1	Standard	1,000	1,049
Abl Std1	Standard	1,000	953
Abl Std2	Standard	10,000	10,555
Abl Std2	Standard	10,000	9,488
Abl Std3	Standard	100,000	96,456
Abl Std3	Standard	100,000	103,598
MNR1	Fresh BC		49,073
MNR1	Fresh BC		55,387
MNR1	Fresh BC		47,247
Hib	Frozen BC		5,550
Hib	Frozen BC		4,912
Hib	Frozen BC		4,400
PBMC	Fresh PBMC		49,213
PBMC	Fresh PBMC		49,921
PBMC	Fresh PBMC		45,560
MNR2	Fresh BC		69,536
MNR2	Fresh BC		61,259
MNR2	Fresh BC		57,199
NTC	No template		
NTC	No template		

NTC= no template negative control. Abl std = recombinant Abl tyrosine kinase construct for calibration. All samples were run in triplicate apart from the Abl standard and NTC which were run in duplicate.

The qPCR quantification plots are shown below. Nanodrop quantification of the Hib sample gave a total RNA of 420ng per 10^6 cells with an A_{260}/A_{280} ratio of 2.25, as compared to the MNR1 and MNR2 samples which 1140 ng and 1280 ng of total RNA per 10^6 cells respectively and an A_{260}/A_{280} ratios of 2.04 and 2.04 respectively.

Figure 42 qPCR standardised curves of fluorescence (y axis) versus cycle number (x axis) for frozen CD19+ cells from Hib reference study participant 009 V1 (*Hib*), fresh CD19+ cells (*MNR1 & MNR2*) and *PBMC*s as compared to Abl standard constructs (*Abl Std 1-3*)



NTC= no template negative control. Abl std = recombinant Abl tyrosine kinase gene construct for calibration. All samples were run in triplicate apart from the Abl standards and NTC which were run in duplicate.

6.3.3. B cell subset numbers for repertoire to conjugate versus polysaccharide vaccine responses

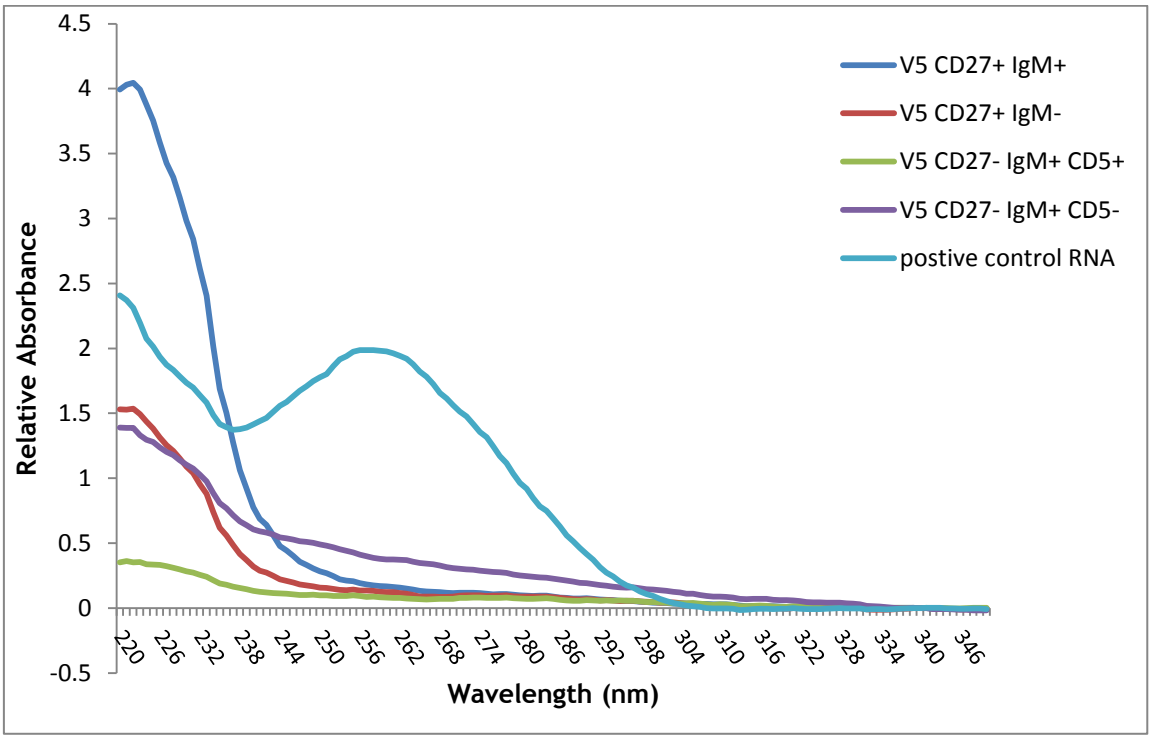
An average of 10^4-10^5 cells in each subset were obtained from the participants of the MenACWY-CRM/MenACWY-PS clinical study as shown in Table 33. Total RNA was extracted from separated subsets as described in Section 6.2.6, but with two extra volumes of RLT buffer. Unfortunately RNA yields were very low, of the order of 3-9 ng/μL. The absorption spectrum measured on a spectrophotometer revealed a ratio of absorbance at 260nm to 280 nm (A260/A280 ratio) of 1.1-1.7. An A260/A280 of 1.8-2.0 is generally considered indicative of RNA of sufficient quality for further applications [345]. The RNA from the MenACWY-CRM/MenACWY-PS study was not therefore used for downstream sequencing.

Table 33 B cell subset numbers by participant number and visit for Group 1 (MenACWY-CRM + MenACWY-CRM) and Group 2 (MenACWY-PS + MenACWY-CRM).

			Cell count ($x10^4$)				
Participant number	Group	Subset	V1 day 0	V2 day 7	V3 day 28	V4 day 35	V5 day 56
137	2	CD27+IgM+	2.1	2.4	0.28	1.3	2.0
		CD27+IgM-	2.6	3.5	0.78	2.2	2.5
		CD27-IgM+CD5+	0.75	0.82	0.42 §	1.3 §	1.1
		CD27-IgM+CD5-	3.3	3.5			5.4
139	1	CD27+IgM+	1.0	1.5	1.1	*	0.9
		CD27+IgM-	2.2	3.2	2.4	*	2.1
		CD27-IgM+CD5+	0.3	3.2	1.3 §	*	0.7
		CD27-IgM+CD5-	2.0	16.1		*	2.9
140	2	CD27+IgM+	3.7	1.1	1.4	*	2.4
		CD27+IgM-	6.7	1.4	4.6	*	4.3
		CD27-IgM+CD5+	1.4	1.3	1.5 §	*	1.3
		CD27-IgM+CD5-	6.5	2.7		*	6.6
141	1	CD27+IgM+	1.1	1.1	*	0.4	1.2
		CD27+IgM-	3.0	3.7	*	1.3	3.6
		CD27-IgM+CD5+	3.9	2.6	*	1.0	5.3
		CD27-IgM+CD5-	5.9	3.7	*	1.7	7.2
142	2	CD27+IgM+	2.0	1.0	*	2.7	1.1
		CD27+IgM-	2.1	1.4	*	3.0	1.0
		CD27-IgM+CD5+	2.1	1.3	*	2.8	1.8
		CD27-IgM+CD5-	5.7	2.7	*	10.2	4.0
147	1	CD27+IgM+	1.0	0.3	2.3	1.7	3.8
		CD27+IgM-	3.8	1.7	8.0	5.9	11.4
		CD27-IgM+CD5+	1.6	0.6 §	5.0	5.2	10.2
		CD27-IgM+CD5-	2.6		5.4	5.6	11.5

* = sample not obtained. § = CD5 labelling not performed. CD27+IgM+=IgM memory; CD27+IgM-=switched memory; CD27-IgM+CD5+=B1a; CD27-IgM+CD5-=B1b.

Figure 43 Example absorbance spectrum of total RNA extracted from 4 different B cell subsets of a single participant (137) at V5 with A260/A280 ratios of 1.1-1.7. Note the positive control RNA has an A260/A280 ratio of 2.05.



Attempts at two step RT-PCR using random hexamers for reverse transcription and the Wu variable domain primer set or βactin primers (Table 29) for PCR did not yield any products (data not shown).

6.3.4. First attempt at 454 sequencing

One-step RT-PCR with the Van Dongen primers listed in Table 29 were used to generate amplicons for the first run of parallel sequencing. The amplicons were gel purified but were not formally quantified using a spectrophotometer prior to library preparation.

Initial quantification of amplicon DNA generated after RT-PCR by ribogreen at the Wellcome Trust Sanger Institute suggested that adequate double stranded DNA was

available for 454 library preparation. Blunt end ligation of the A & B sequencing adaptors and the MIDs required for parallel sequencing were then performed as part of the library preparation. However, subsequent qPCR based on the adaptor sequences showed that the library preparation was weak, with far fewer DNA molecules than initially estimated (Table 34) . This was thought to be due to inefficient blunt-end ligation of the adaptors to the original amplicons. A HIV protein sequence was used as a control for qPCR. A minimum of 2.5 x 10^5 DNA molecules/µl is required for emulsion PCR [212], and for this reason, the samples highlighted in red were excluded from further sequencing. The samples with the strongest samples (highlighted in green) were used for the initial run of emulsion PCR.

Table 34 First attempt at 454 sequencing. Quantification of amplicons from the Hib reference serum study by picogreen and quantification of adaptor ligated libraries by qPCR. An HIV protein is used as a control.

Participant/ Visit number	Ig gene primers used to amplify V_H region (forward/reverse)	MID	Average fragment size	Estimated molecules $x10^8$/µL by ribogreen	Estimated molecules/µL by qPCR
005/ V1	V_H3/J_H	1	699	10.4	Assay not run
005/ V2	V_H3/J_H	2	514	12.0	Assay not run
002/ V1	V_H3/J_H	3	590	5.7	Assay not run
002/ V2	V_H3/J_H	4	350	4.5	Assay not run
006/ V1A§	V_H3/J_H	5	609	6.0	Assay not run
006/ V1B§	V_H3/J_H	6	783	0.7	Assay not run
006/ V2	V_H3/J_H	7	344	11.4	8.0×10^5
005/ V1	V_H4/J_H	8	591	6.6	3.4×10^5
005/ V2	V_H4/J_H	9	408	6.7	3.9×10^6
002/ V1	V_H4/J_H	10	648	5.9	5.3×10^5
002/ V2	V_H4/J_H	11	350	4.1	1.7×10^4
006/ V1A*	V_H4/J_H	12	545	5.3	1.6×10^4
006 /V1B*	V_H4/J_H	1	571	4.2	9.8×10^4
006/ V2	V_H4/J_H	2	348	5.5	1.6×10^6
002/ V2	$V_H3/J_H + V_H4/J_H$†	3	347	4.5	5.1×10^5
006/ V2	$V_H3/J_H + V_H4/J_H$†	4	342	6.1	2.1×10^6
HIV control protein	-	1	460	2.0	3.7×10^7

All primers were based on Van Dongen *et al.* [341] and are listed in **Table 29**. Libraries highlighted in red had insufficient DNA for further sequencing. Libraries highlighted in green were used for the initial trial of 454 sequencing. §,* = duplicate samples. † = equal volumes of separate PCR reactions using the 2 different primer sets were pooled prior to library preparation.

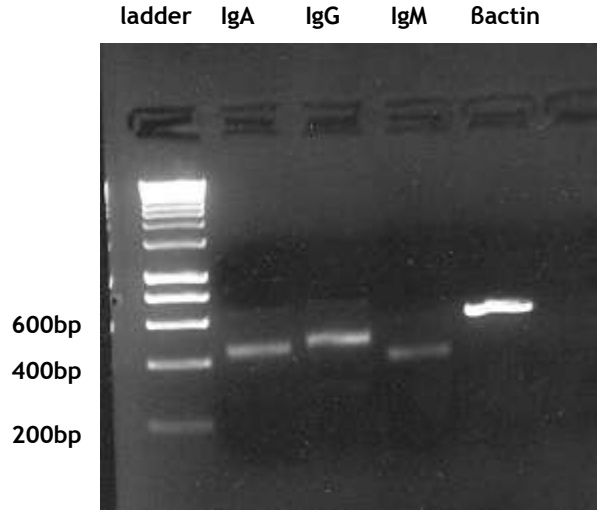
Only 423 sequences were generated after the initial trial of parallel sequencing. These were an average length of 72 bp, considerably shorter than the expected 400-450 bp

sequence expected with a variable domain sequence, but suggestive of an adaptor dimer (each adaptor is 30 bp long and the MID sequence is 10 bp long). The sequences could not be aligned to the IMGT variable gene database to allocate gene identity.

6.3.5. Second attempt at 454 sequencing

In view of the poor yield of sequences at the first 454 sequencing attempt, an alternative strategy of amplicon generation was pursued for the second attempt at 454 sequencing. This used total RNA as a template, with 2 step RT-PCR using random hexamers for reverse transcription and Wu primers for PCR. 2 successive rounds of PCR amplification were performed to increase amplicon yield as discussed in Section 6.2.8. An example of first round PCR products is shown in Figure 44.

Figure 44 Example agarose gel electrophoresis of first round PCR products using multiplex VH forward primers and IgA/IgG/IgM back primers yielding a ~400bp product or a βactin control primer set yielding a ~600bp product..



The pooled second round PCR products for each sample were quantified at the Wellcome Trust Sanger Institute using picogreen. Sufficient DNA was present in all samples to proceed to library preparation. Following adaptor ligation, the amplicon libraries were quantified by qPCR and all libraries contained sufficient amplicons to proceed to emulsion PCR as shown in Table 35 below.

Table 35 Second attempt at 454 sequencing. Quantification of amplicons by picogreen, quantification of adaptor ligated libraries by qPCR and number of sequences obtained per library post 454 sequencing.

Sample ID	Subject	MID	Ig gene primers used to amplify V_H region (forward/reverse)	Estimated molecules $x10^8$/µL by picogreen	Estimated molecules $x10^7$/µL by qPCR	Number of sequences obtained post 454 pyrosequencing
BC 1	Donor 1	6	V_H multiplex/IgA	16.2	10.6	5691
BC1	Donor 1	7	V_H multiplex/IgG	18.3	11.6	5033
BC1	Donor 1	8	V_H multiplex/IgM	18.7	25.0	5322
BC 2	Donor 2	1	V_H multiplex/IgA + V_H multiplex/IgG + V_H multiplex/IgM	9.0	8.9	15369
BC3 pre Hib	Hib ref study 001/V1	9	V_H multiplex/IgA + V_H multiplex/IgG + V_H multiplex/IgM	15.9	12.4	18203
PC 3 post Hib	Hib ref study 001/V2	10	V_H multiplex/IgA + V_H multiplex/IgG + V_H multiplex/IgM	17.7	15.2	18483
BC4 pre Hib	Hib ref study 004/V1	11	V_H multiplex/IgA + V_H multiplex/IgM	10.5	11.7	22142
PC 4 post Hib	Hib ref study 004/V2	12	V_H multiplex/IgA + V_H multiplex/IgG + V_H multiplex/IgM	10.8	9.8	16109

BC = sample derived from sorted CD19+ B cells. PC = sample derived from sorted plasma cells. Pre/post Hib = pre and 7 days post Hib conjugate vaccination, V_H multiplex = $V_H1/V_H2/V_H3/V_H4/V_H5/V_H6$ forward primers used in equimolar amounts. All primers were based on Wu *et al.* [128] and are listed in **Table 29**. Donor 1 IgA, IgG and IgM amplicons were individually tagged using separate 3 MIDs while for the rest of the subjects, the Ig class amplicons were pooled and tagged with a single MID per sample. For subsequent analyses, the sequence results of all Ig classes of Donor 1 were considered together as BC1. No V_H multiplex/IgG amplicon was observed on gel electrophoresis after 2 rounds of PCR amplification for Hib ref study 004/V1 therefore a library was not made with this sample. The average fragment size of all libraries post adaptor ligation was 450 bp.

6.3.6. Overview of sequence data

Between 15,000-22,000 sequences were derived from each sample as shown in Table 36. Of the sequences that were matched to the IMGT database, >99% were functional, as would be expected for mRNA derived sequences. The overall sequence diversity (defined in this case as the number of unique sequences expressed as a percentage of the total functional sequences), of the CD19+ B cell samples at baseline (BC1, BC2, BC3 pre-Hib and BC4 pre-Hib) was 85-89%. However, after Hib vaccination (BC3 post-Hib and BC4 post-Hib), a greater number of functional sequences were clonally related leading to a fall in overall diversity (54-59%). For individuals 3 and 4, the mean CDR3 lengths in pre-vaccination B cells are significantly different from the mean CDR3 lengths in post-vaccination plasma cells ($p<0.0001$ in both cases, unpaired t test). There was no change in hydrophobicity of the CDR3 for individual 3 after vaccination. There was a trend to increased hydrophilicity of the plasma cell CDR3 regions of individual 4 after vaccination when compared to the CDR3 hydrophobicity in pre-vaccination B cells, although this difference was not significant. For individuals 3 and 4, the mean CDR3 SMH rate in pre-vaccination B cells are significantly different from the mean CDR3 SMH rate in post-vaccination plasma cells ($p<0.0001$ in both cases, unpaired t test).

Table 36 Summary of sequence data

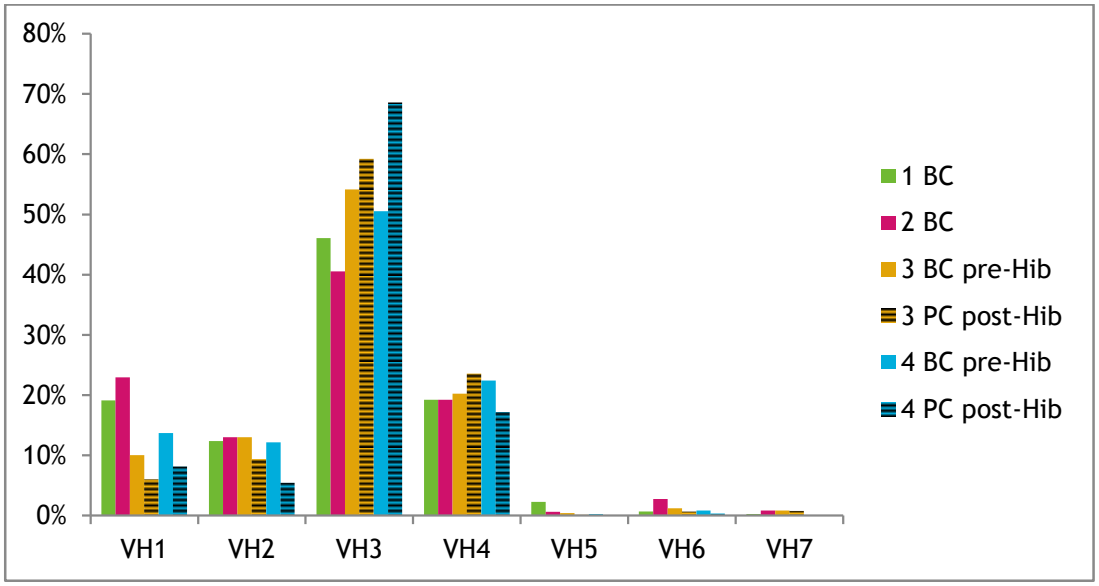
Sample ID	Total sequences post 454	Number of sequences mapped to IMGT database (functional, non-functional)	Sequence diversity (unique sequences/functional sequences %)	Mean CDR3 length (bp)	Mean CDR3 hydrophobicity (Kyte-Doolittle)	Mean CDR3 SMH rate (per 1000 bp)
1 BC	16046	9713, 2	85	46.2	-0.06	61.3
2 BC	15369	9478, 3	89	44.9	-0.06	47.1
3 BC Pre-Hib	18203	10985, 7	89	42.2	-0.04	46.7
3 PC Post-Hib	18483	12525, 1	54	39.6	-0.04	66.0
4 BC Pre-Hib	22142	15484, 9	87	43.8	-0.02	30.8
4 PC Post-Hib	16109	11192, 1	59	33.0	-0.06	56.1

CDR3 = complementarity determining region 3, SMH= somatic hypermutation. Sequences were defined as functional if they had an open reading frame throughout the sequence. Sequences were considered clonally related if they (i) used the same V and J segment genes, (ii) had an identical CDR3 length, and (iii) had a highly homologous CDR3 region, defined as ≤10% difference in CDR3 nucleotide sequence. All non-clonally related sequences were defined as unique. The diversity of a given repertoire was expressed as the percentage of functional sequences that were unique. As a default for the IgH chain, the CDR3 was defined as amino acids 105–117, according to the IMGT unique numbering system, and the corresponding nucleotide length expressed in bp. Hydrophobicity was determined according to the normalized Kyte–Doolittle scale, which assigns an arbitrary value to each amino acid [346]. Negative numbers represent polar/hydrophilic amino acids and positive values represent hydrophobic amino acids. The somatic hypermutation rate was expressed as the number of mutations per 1000 bp.

6.3.7. V_H, D_H and J_H family usage between samples

As shown in Figure 45, the VH3 family comprised the majority of V gene segments across all samples. VH family usage differed significantly between all four B cell samples at baseline (all comparisons $p<0.0001$, $\chi 2$ test). For individuals 3 and 4, the V_H family usage in pre-vaccination B cells are significantly different from the V_H family usage in post-vaccination plasma cells ($p<0.0001$ in both cases, $\chi 2$ test). This difference appears to be largely due to the increased use of V_H3 post vaccination.

Figure 45 V_H family usage between samples expressed as a percentage of the total functional repertoire (y axis).

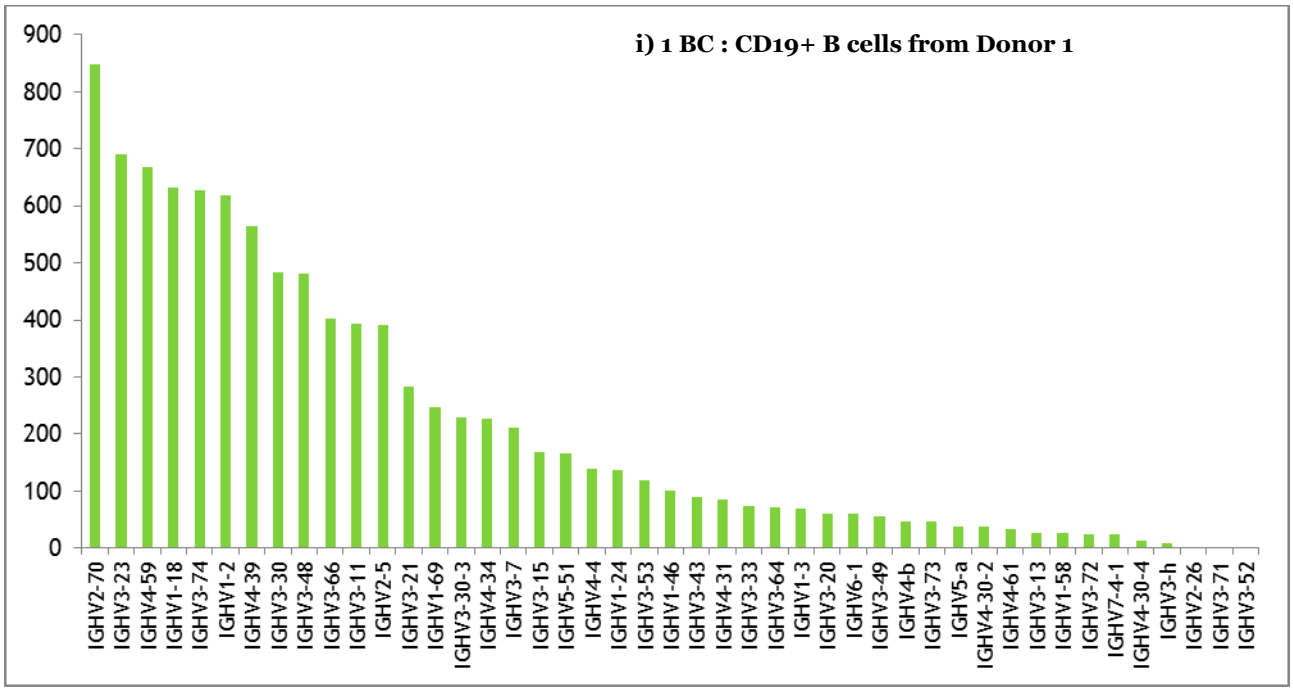


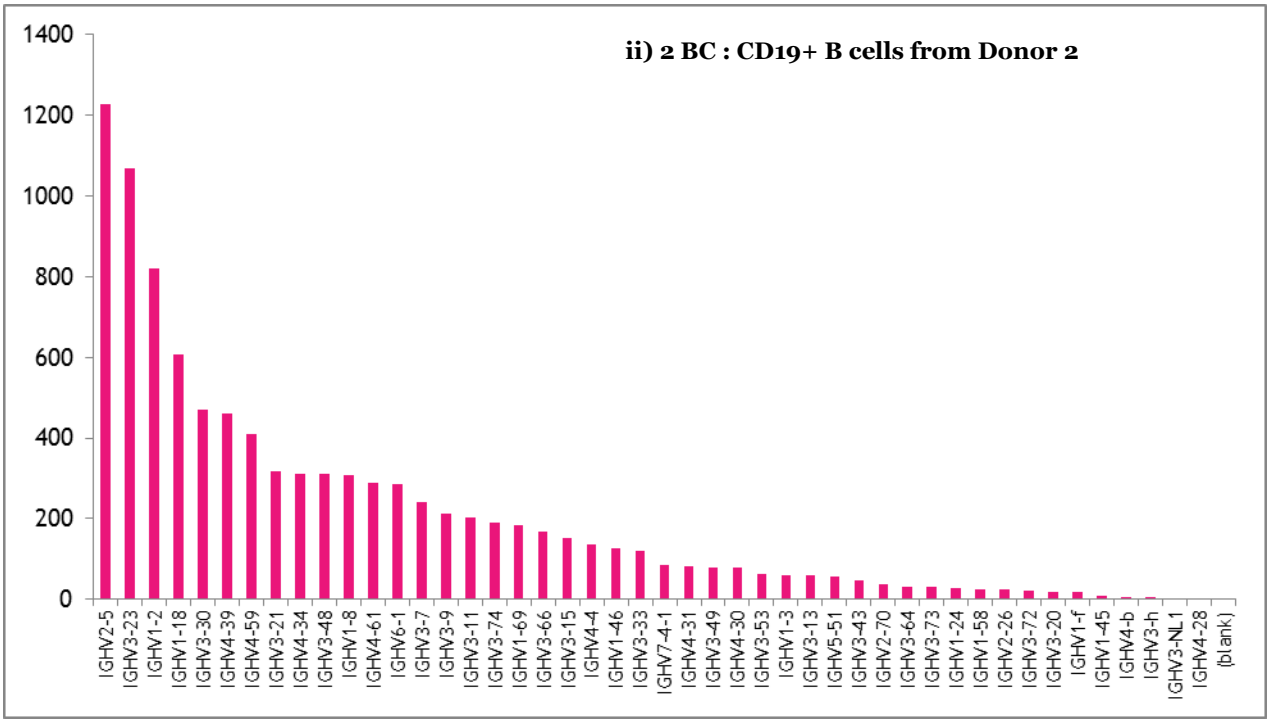
1 BC/2BC = CD19+ B cells from donors 1 and 2 respectively; 3 BC pre-Hib/4 BC pre-Hib = CD19+ B cells from Hib reference study participants 001 and 004 respectively prior to receiving a Hib conjugate vaccine; 3 PC post-Hib/4 PC post-Hib = plasma cells from Hib reference study participants 001 and 004 respectively, 7 days after receiving a Hib conjugate vaccine.

There were uneven frequencies of V gene usage across the samples, with a few genes being highly represented and the majority being used rarely, resulting in a highly skewed distribution as shown in Figure 46. The most frequent V_H gene segments in B cells at baseline varied between individuals, although V3-23 was the most common segment used by individuals 2, 3 and 4 and the second most common segment used by individual 1, in

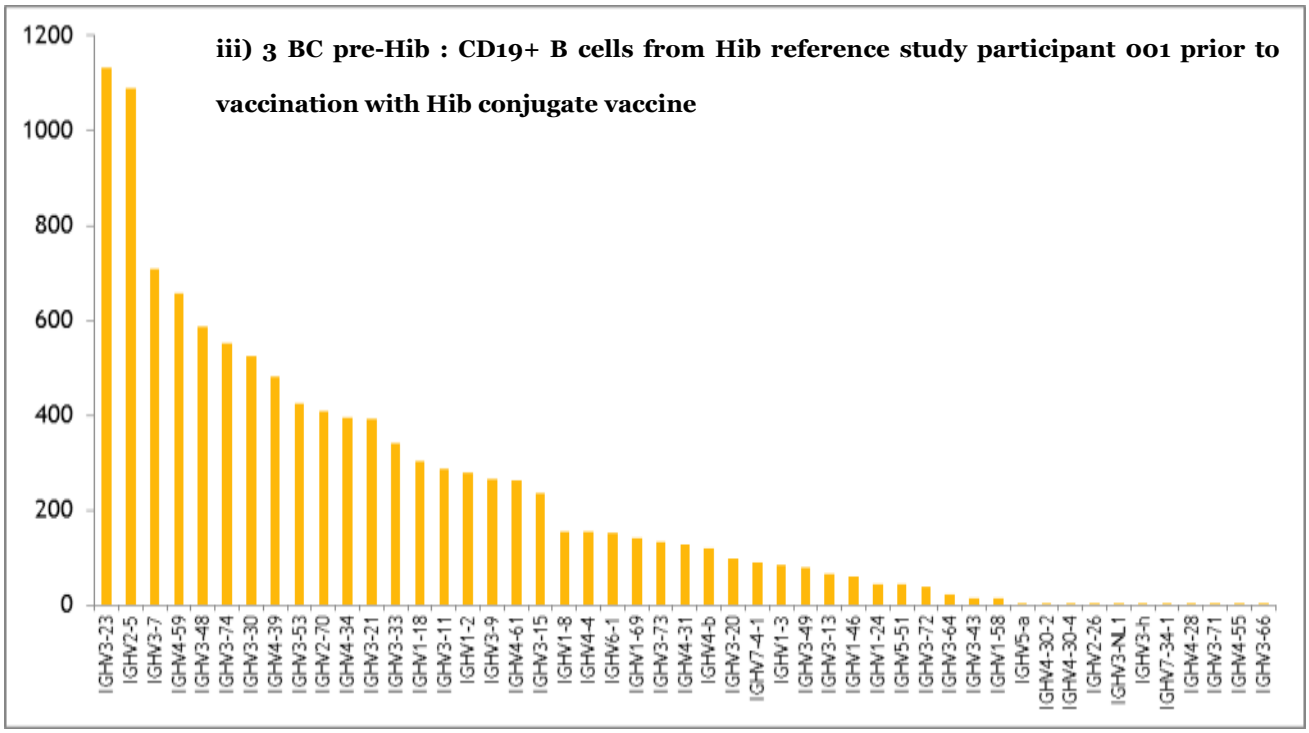
common with previous published data [347, 348]. Post vaccination plasma cells of individual 3 used predominantly V3-23 segments, while those of individual 4 used V3-74 followed by V3-23.

Figure 46 Individual V_H gene segment usage between samples i) 1 BC, ii) 2 BC, iii) 3 BC pre-Hib, iv) 3 PC post-Hib, v) 4 BC pre-Hib, vi) 4 PC post-Hib, expressed as the number of copies (y axis).

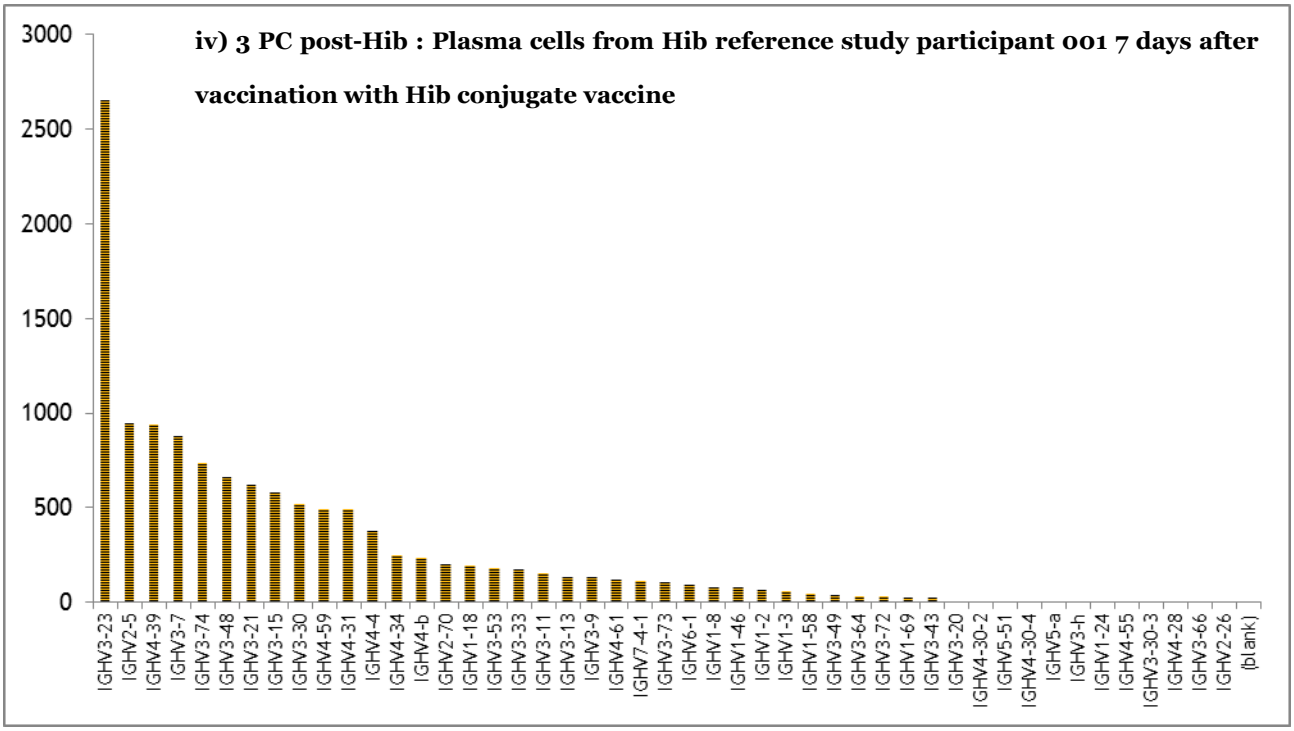


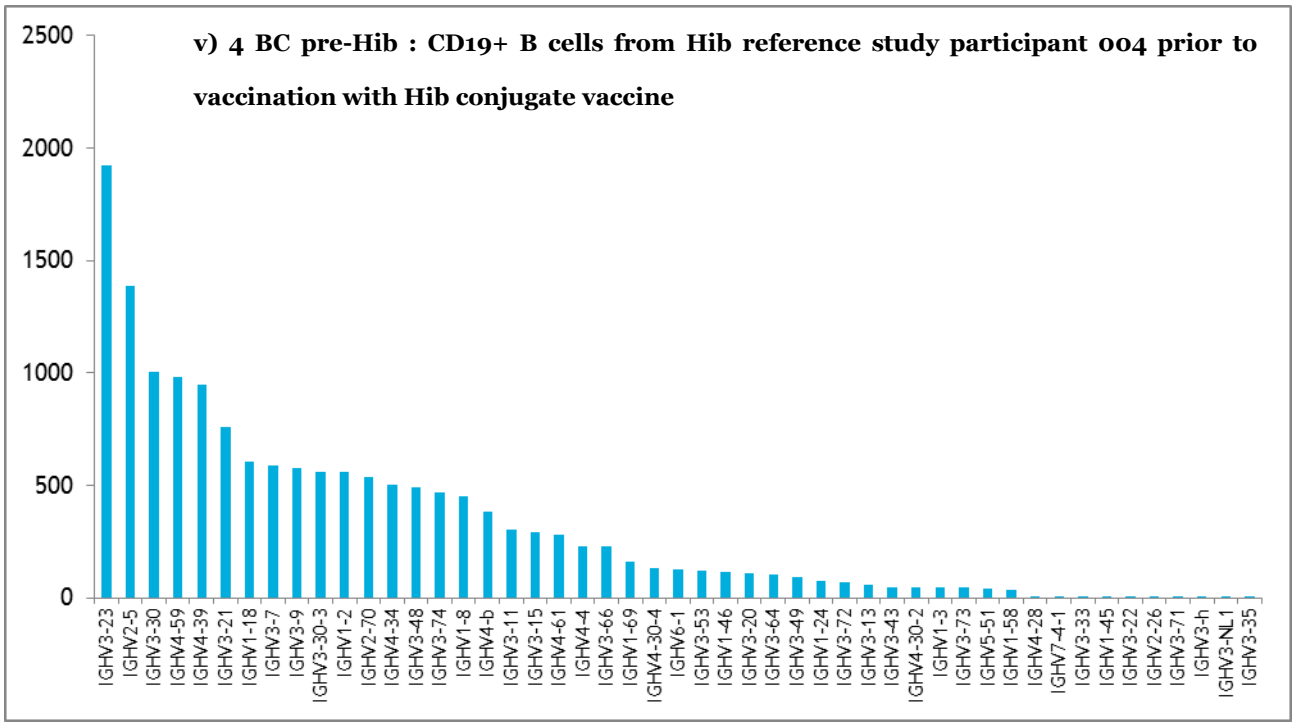


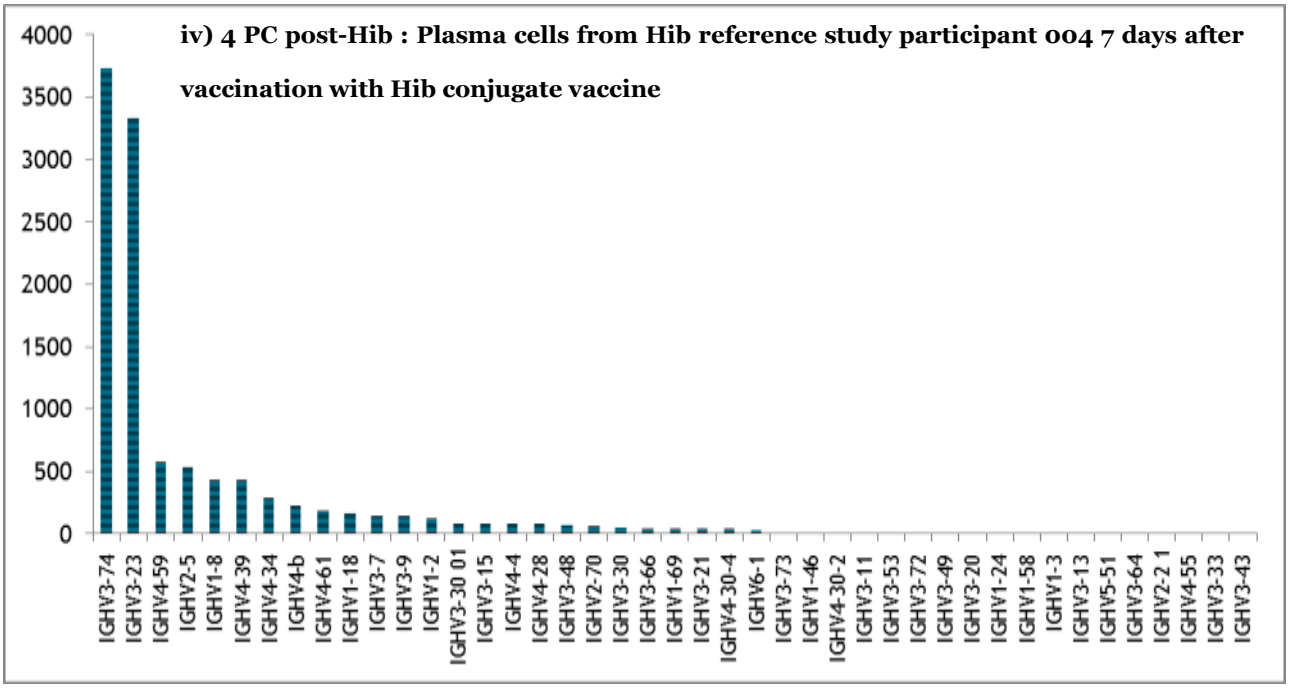
iii) 3 BC pre-Hib : CD19+ B cells from Hib reference study participant 001 prior to vaccination with Hib conjugate vaccine



iv) 3 PC post-Hib : Plasma cells from Hib reference study participant 001 7 days after vaccination with Hib conjugate vaccine

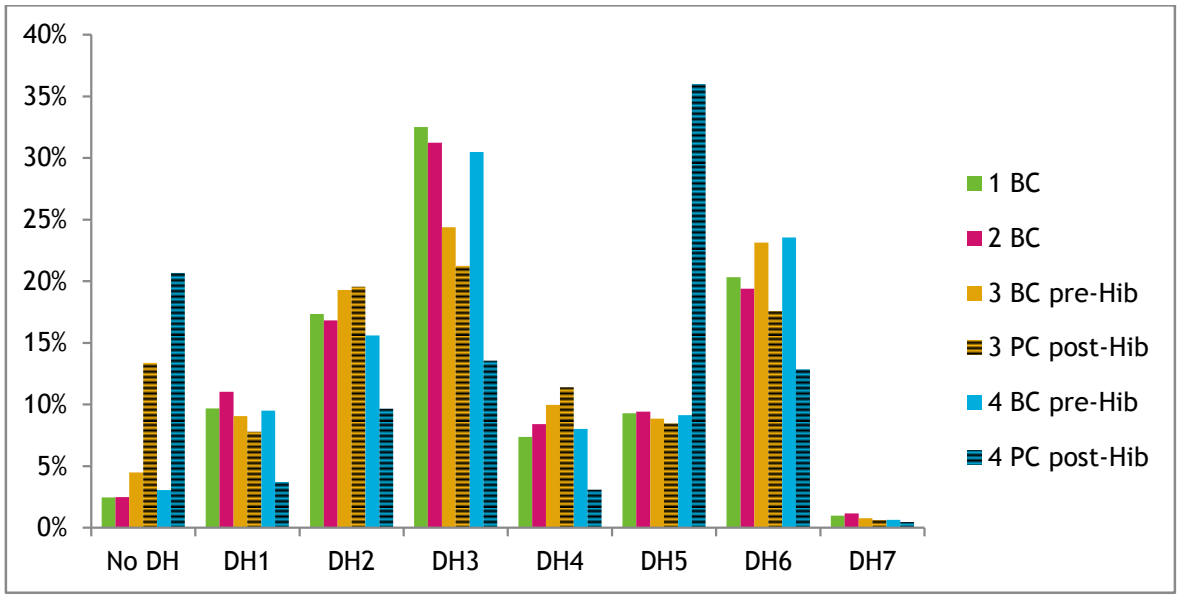



v) 4 BC pre-Hib : CD19+ B cells from Hib reference study participant 004 prior to vaccination with Hib conjugate vaccine
2500
2000
1500
1000
500
0
IGHV3-23
IGHV2-5
IGHV3-30
IGHV4-59
IGHV4-39
IGHV3-21
IGHV1-18
IGHV3-7
IGHV3-9
IGHV3-30-3
IGHV1-2
IGHV2-70
IGHV4-34
IGHV3-48
IGHV3-74
IGHV1-8
IGHV4-b
IGHV3-11
IGHV3-15
IGHV4-61
IGHV4-4
IGHV3-66
IGHV1-69
IGHV4-30-4
IGHV6-1
IGHV3-53
IGHV1-46
IGHV3-20
IGHV3-64
IGHV3-49
IGHV1-24
IGHV3-72
IGHV3-13
IGHV3-43
IGHV4-30-2
IGHV1-3
IGHV3-73
IGHV5-51
IGHV1-58
IGHV4-28
IGHV7-4-1
IGHV3-33
IGHV1-45
IGHV3-22
IGHV2-26
IGHV3-71
IGHV3-h
IGHV3-NL1
IGHV3-35


iv) 4 PC post-Hib : Plasma cells from Hib reference study participant 004 7 days after vaccination with Hib conjugate vaccine
4000
3500
3000
2500
2000
1500
1000
500
0
IGHV3-74
IGHV3-23
IGHV4-59
IGHV2-5
IGHV1-8
IGHV4-39
IGHV4-34
IGHV4-b
IGHV4-61
IGHV1-18
IGHV3-7
IGHV3-9
IGHV1-2
IGHV3-30 01
IGHV3-15
IGHV4-4
IGHV4-28
IGHV3-48
IGHV2-70
IGHV3-30
IGHV3-66
IGHV1-69
IGHV3-21
IGHV4-30-4
IGHV6-1
IGHV3-73
IGHV1-46
IGHV4-30-2
IGHV3-11
IGHV3-53
IGHV3-72
IGHV3-49
IGHV3-20
IGHV1-24
IGHV1-58
IGHV1-3
IGHV3-13
IGHV5-51
IGHV3-64
IGHV2-2 1
IGHV4-55
IGHV3-33
IGHV3-43

Frequencies of D_H family usage were similar across the samples (Figure 47) with the notable exception of an increased usage of D_H5 in BC4 post-Hib ($p<0.0001$, unpaired t test). Sequences where the identity of the D_H gene segment could not be reliably ascertained (No D_H) were more common after vaccination ($p<0.0001$, unpaired t test).

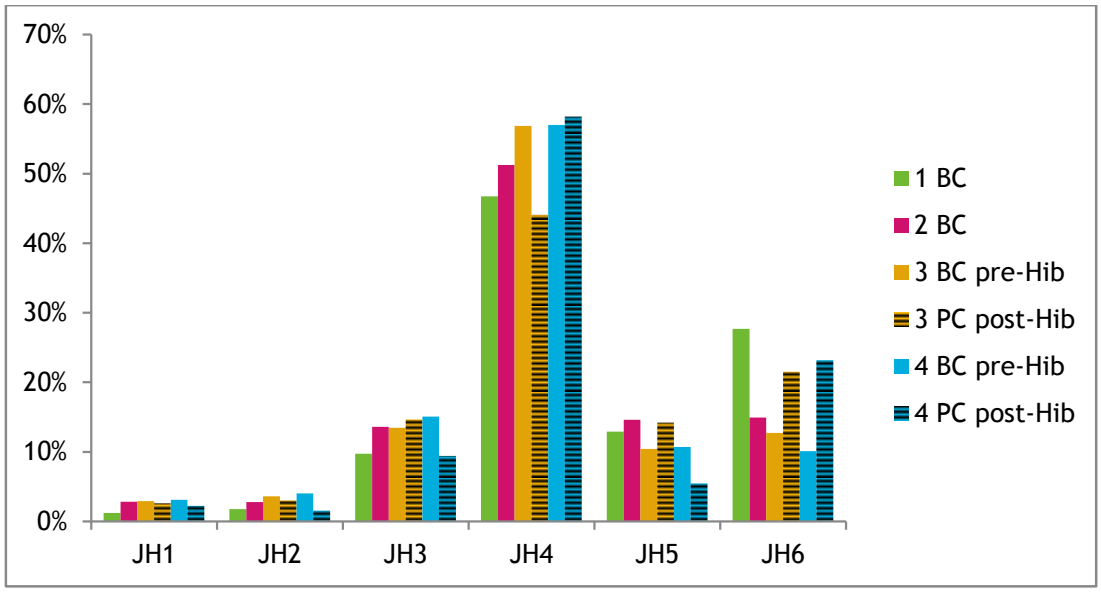
Figure 47 D_H family usage between samples, expressed as a percentage of the total functional repertoire (y axis).



1 BC/2BC = CD19+ B cells from donors 1 and 2 respectively; 3 BC pre-Hib/4 BC pre-Hib = CD19+ B cells from Hib reference study participants 001 and 004 respectively prior to receiving a Hib conjugate vaccine; 3 PC post-Hib/4 PC post-Hib = plasma cells from Hib reference study participants 001 and 004 respectively, 7 days after receiving a Hib conjugate vaccine.

Figure 48 shows that J_H4 was the most utilised J_H gene segment family across all samples. However for individuals 3 and 4, there was a significantly increased usage of the J_H6 family in post-vaccination plasma cells compared to pre-vaccination B cells ($p<0.0001$ in both cases, unpaired t test).

Figure 48 J_H family usage between samples, expressed as a percentage of the total functional repertoire (y axis).

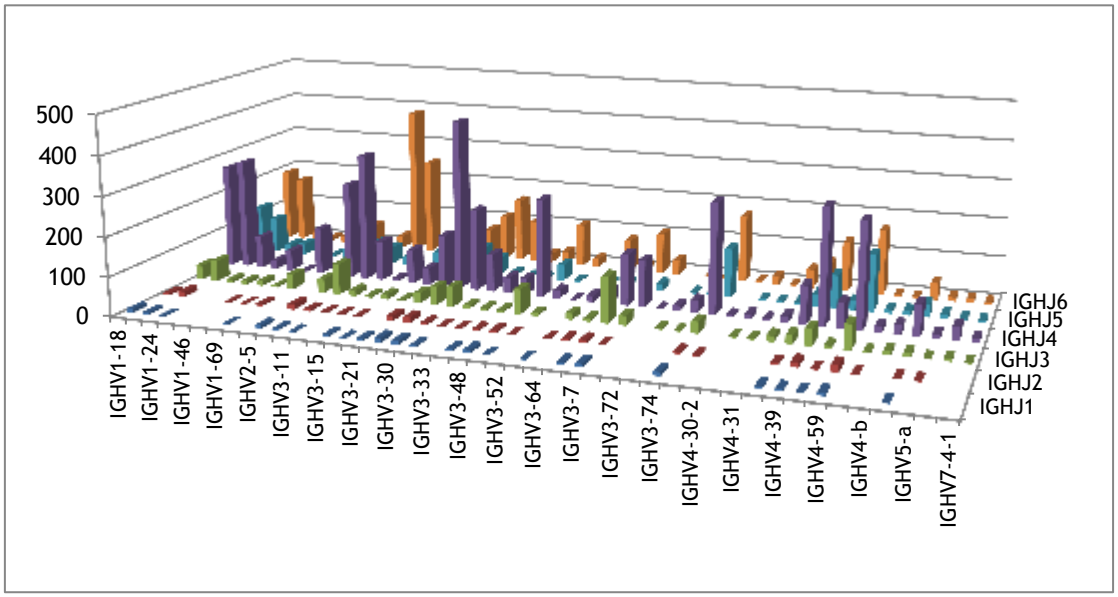


1 BC/2BC = CD19+ B cells from donors 1 and 2 respectively; 3 BC pre-Hib/4 BC pre-Hib = CD19+ B cells from Hib reference study participants 001 and 004 respectively prior to receiving a Hib conjugate vaccine; 3 PC post-Hib/4 PC post-Hib = plasma cells from Hib reference study participants 001 and 004 respectively, 7 days after receiving a Hib conjugate vaccine.

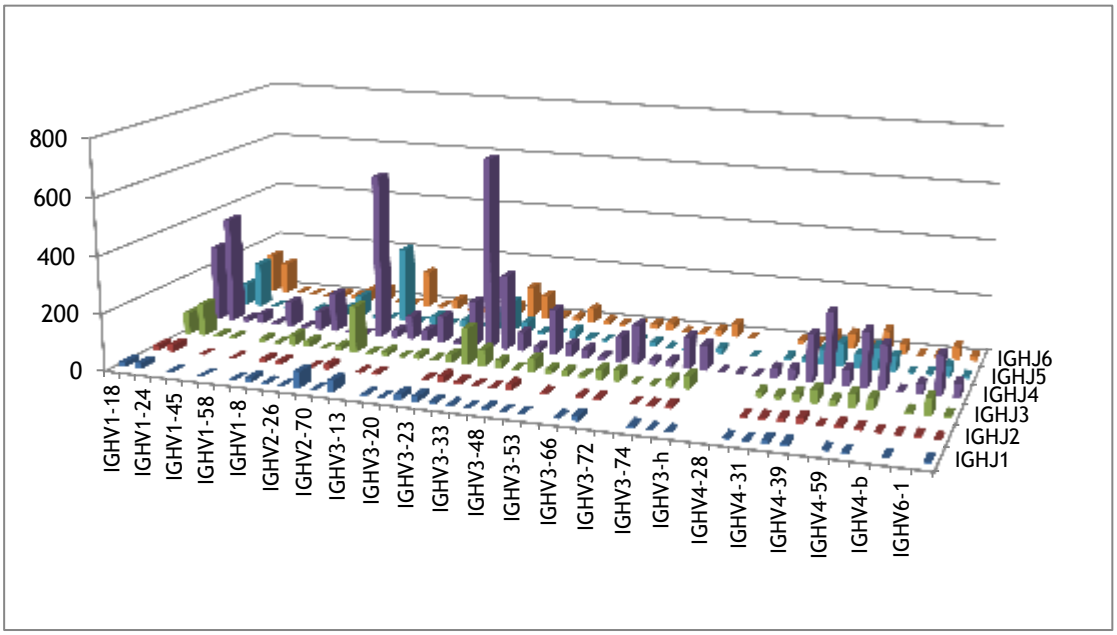
6.3.8. VJ Pairing between samples

Figure 49 Three dimensional representation of V_H gene usage in conjunction with J_H gene usage for i) 1 BC, ii) 2 BC, iii) 3 BC pre-Hib, iv) 3 PC post-Hib, v) 4 BC pre-Hib, vi) 4 PC post-Hib. Note differences in scale of the number of sequences (y axis) between graphs.

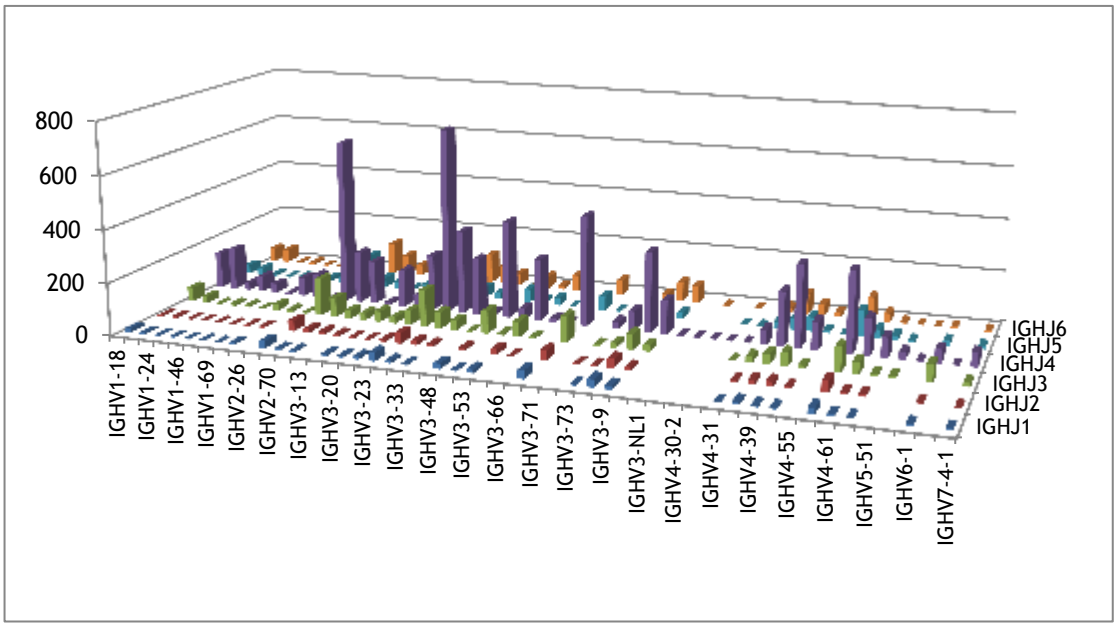
i) 1 BC : CD19+ B cells from Donor 1



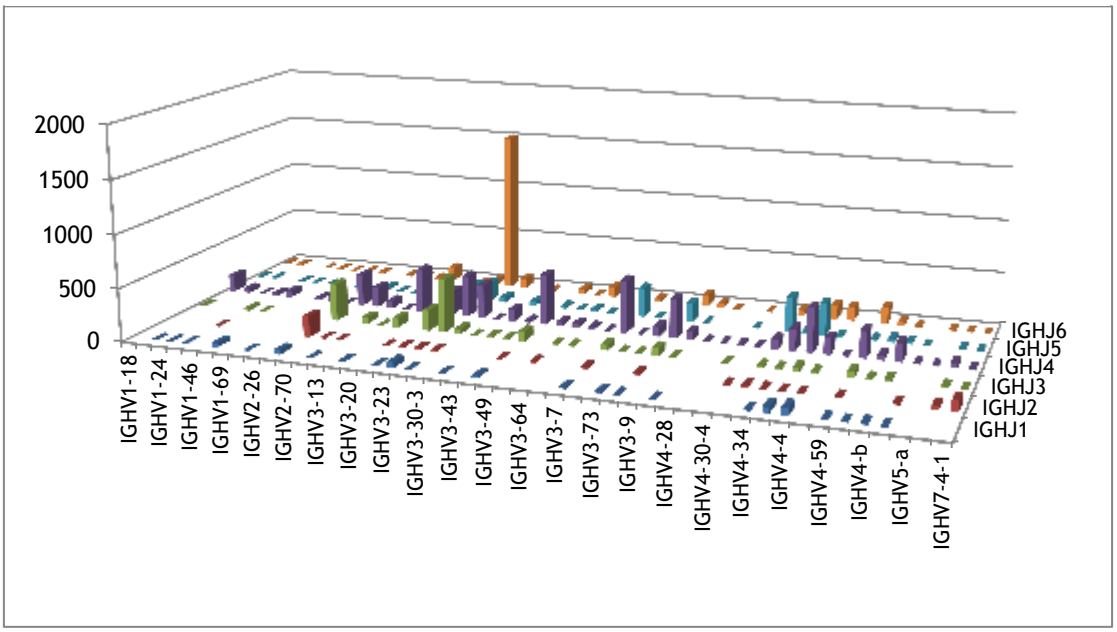
ii) 2 BC : CD19+ B cells from Donor 2



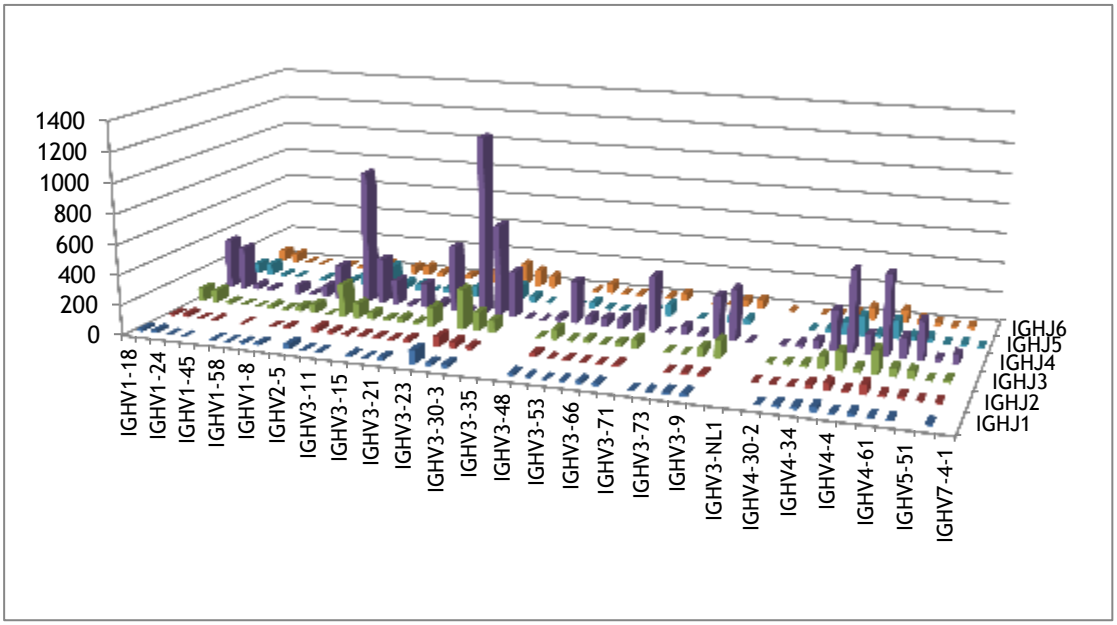
iii) 3 BC pre-Hib : CD19+ B cells from Hib reference study participant 001 prior to vaccination with Hib conjugate vaccine



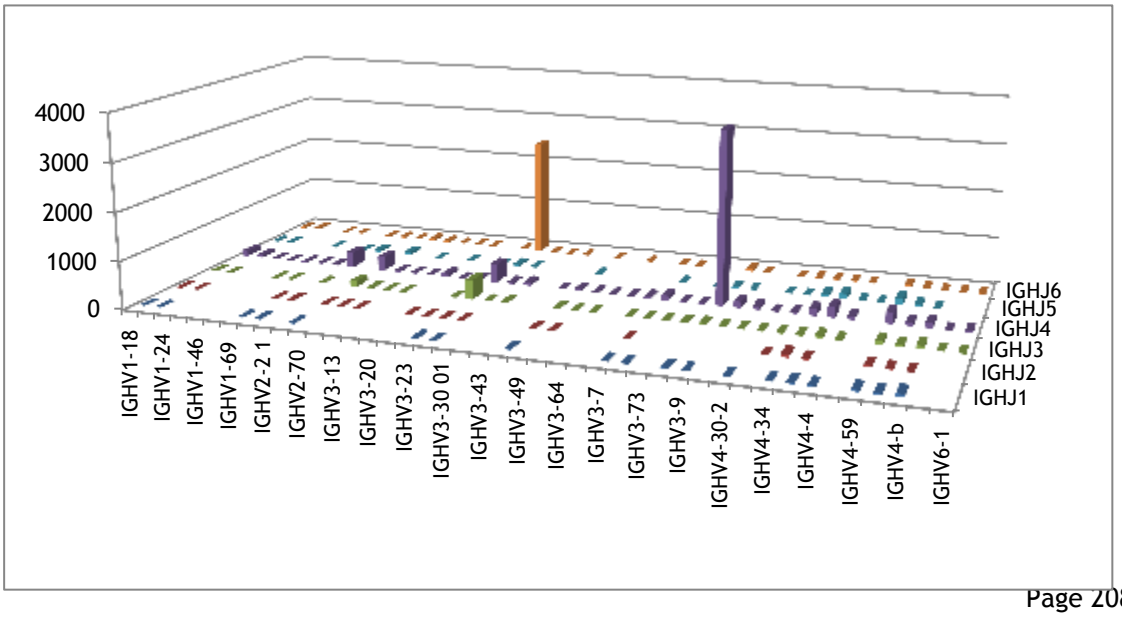
iv) 3 PC post-Hib : Plasma cells from Hib reference study participant 001 7 days after vaccination with Hib conjugate vaccine



v) 4 BC pre-Hib : CD19+ B cells from Hib reference study participant 004 prior to vaccination with Hib conjugate vaccine



vi) 3 PC post-Hib : Plasma cells from Hib reference study participant 004 7 days after vaccination with Hib conjugate vaccine



The V3-23/J4 combination was the most common pairing in all four B cell samples at baseline (Figure 49). Hib vaccine specific sequences post vaccination are known to be highly restricted to V3-23/J4 or V3-23/J6 combinations [332]. V3-23/J6 was the most frequent and second most frequent V_H/J_H pairing in plasma cells from individuals 3 and 4 post vaccination respectively. The frequency of V3-23/J6 usage increased from 1% to 12% and from 1% to 26% in individuals 3 and 4 respectively after vaccination. Although V3-23/J4 was the 7th and 4th most common pairing after vaccination in individuals 3 and 4 respectively, the frequency of expression halved after vaccination (6% to 3% in individual 3 and 7% to 4% in individual 4).

Overall, the three dimensional representation of V_H/J_H pairing shown in Figure 49 suggests that post-vaccination plasma cell repertoires tends to be more oligoclonal than the pre-vaccination B cell repertoire within the same individual.

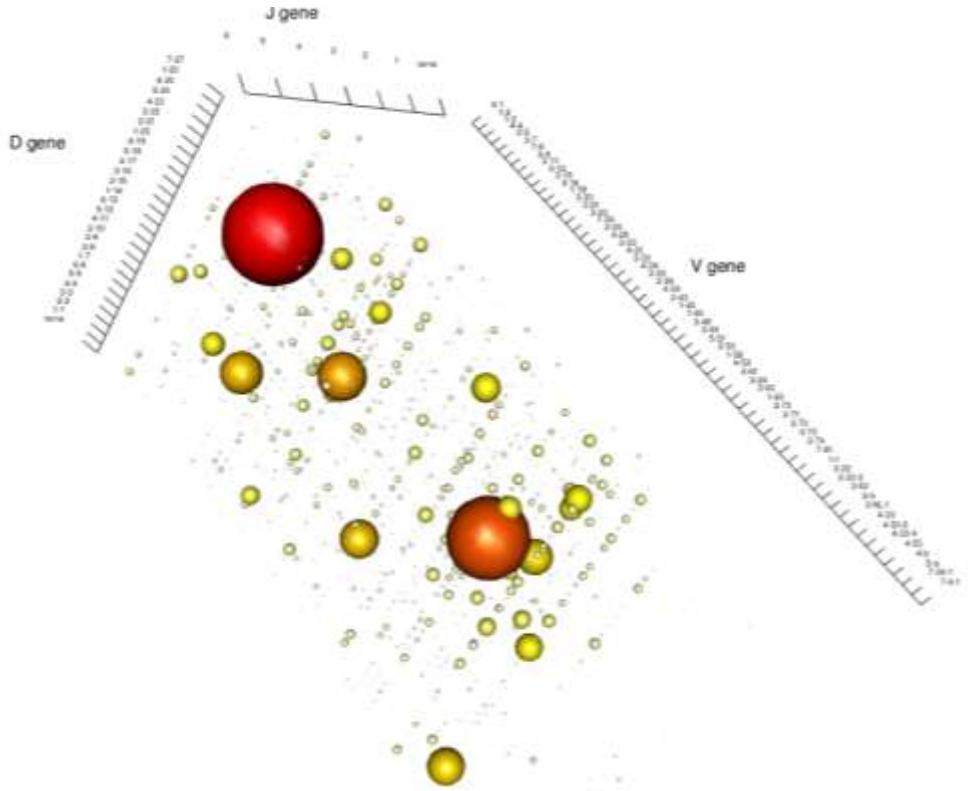
6.3.9. Unique VDJ combinations between samples

Comparison of the combinations of VDJ gene segments used in the 4 baseline B cell samples suggests considerable inter-individual variation in the antibody repertoire. This data is summarised in Figure 50. There was no overlap between the 20 most frequent clones of each sample. The mean clone size based on unique VDJ combinations for 1 BC, 2 BC, 3 BC pre-Hib and 4 BC pre-Hib were 4.1, 4.6, 4.3 and 5.5 copies respectively.

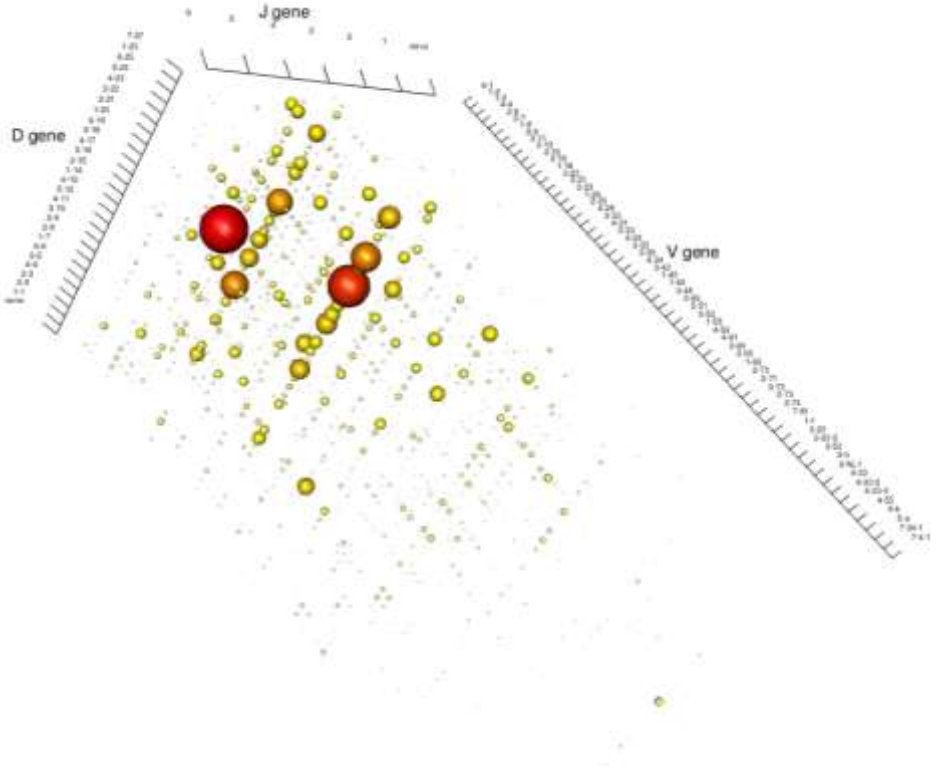
The largest group of sequences in the post vaccination plasma cells of individual 3 (8% of functional sequences) have no assignable D segment, but consist of V3-23/J6 pairings. The 3 next most common combinations are: V3-23/D2-21/J6, V4-31/D4-17/J5 and V3-23/D2-8/J3 with frequencies of 2.7%, 2.5% and 2.1% respectively. The post vaccination plasma cell repertoire of individual 4 is dominated by the clone V3-74/D5-18/J4 which represents 37.1% of functional sequences. A set of sequences with V3-23/J6 pairing without a defined D segment identity comprises 24.7% of all sequences and the 2 next most common clones are V1-8/D6-13/J4 and V3-23/D2-8/J3 with frequencies of 3.1% and 2.7% respectively. The mean clone size post vaccination in individuals 3 and 4 were 12.9 and 16.8 copies respectively.

Figure 50 Four dimensional representation of VH, DH and JH usage for samples i) 1 BC, ii) 2 BC, iii) 3 BC pre-Hib, iv) 3 PC post-Hib, v) 4 BC pre-Hib and vi) 4BC post-Hib

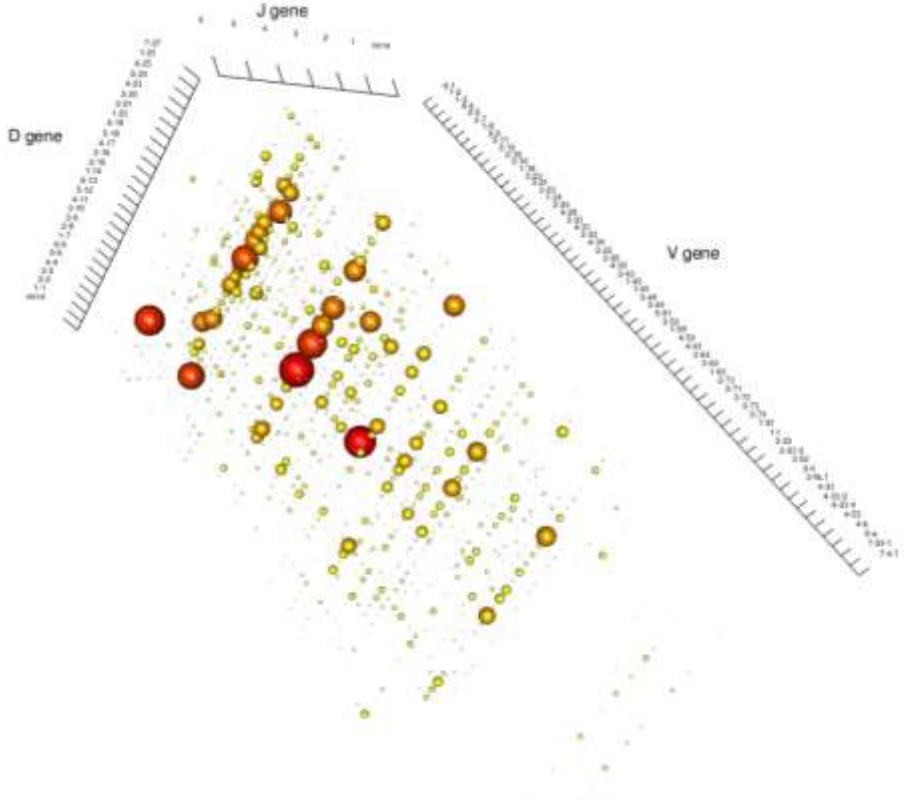
i) 1 BC : CD19+ B cells from Donor 1



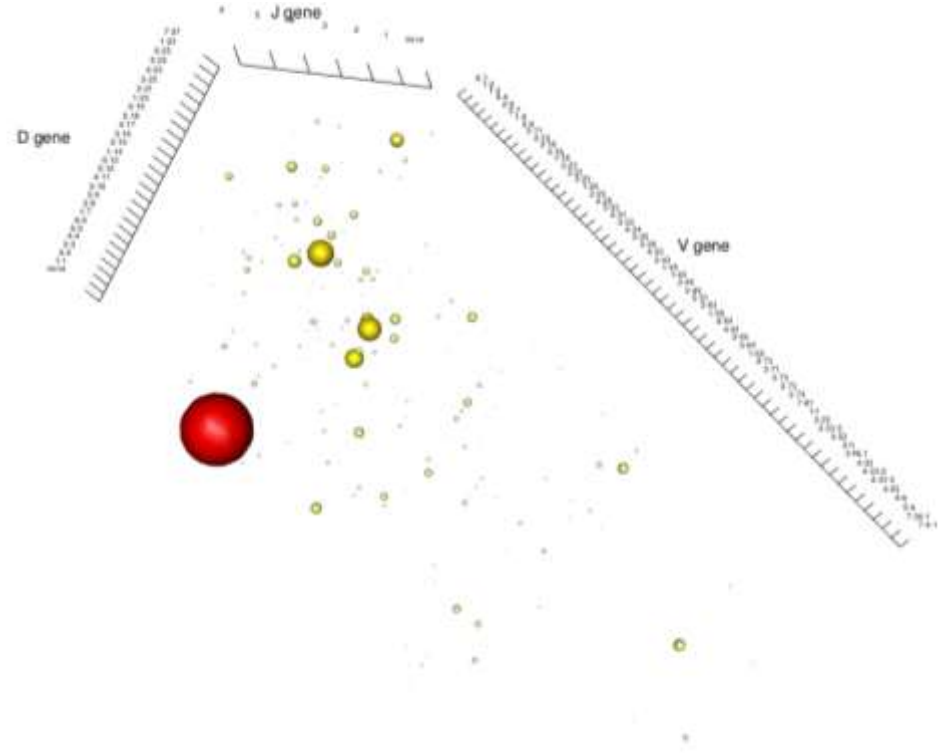
ii) 2 BC : CD19+ B cells from Donor 2



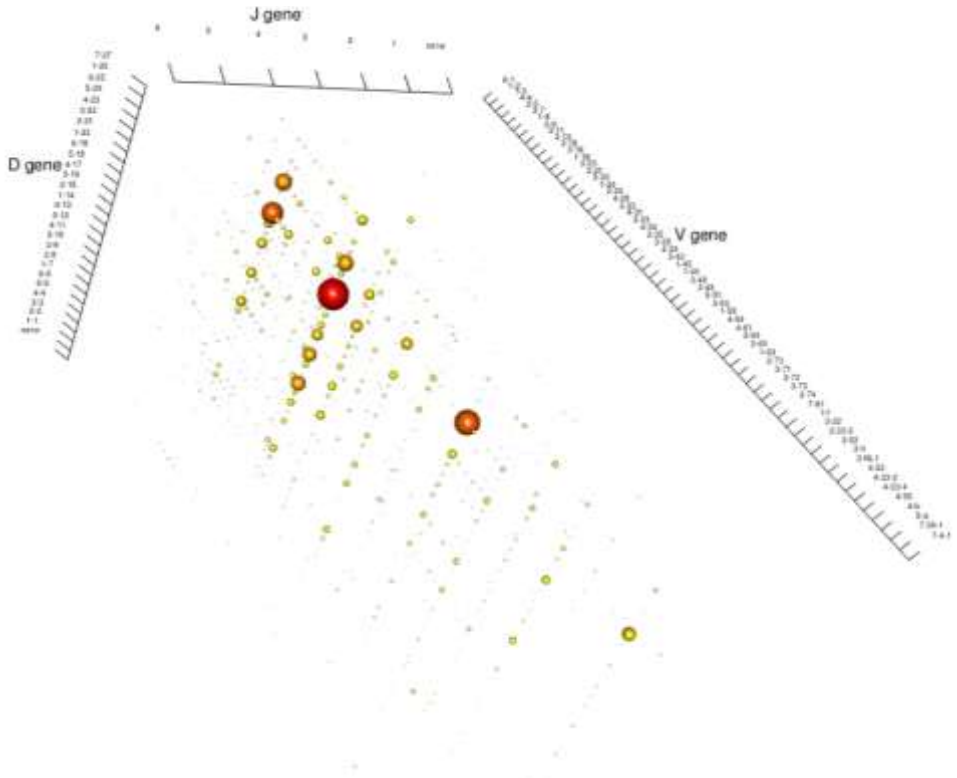
iii) 3 BC pre-Hib : CD19+ B cells from Hib reference study participant 001 prior to vaccination with Hib conjugate vaccine



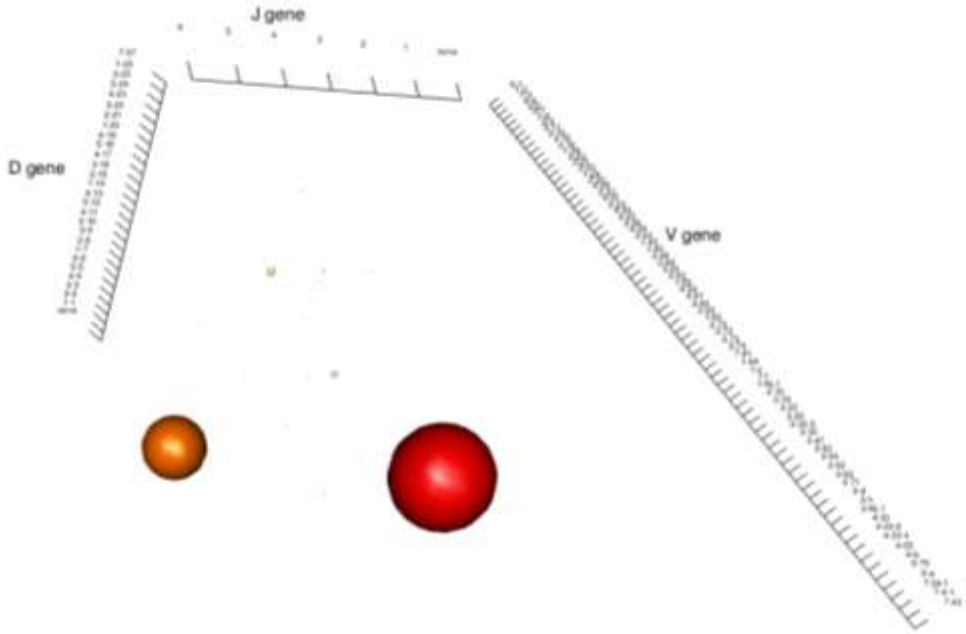
iv) 3 PC post-Hib : Plasma cells from Hib reference study participant 001 7 days after vaccination with Hib conjugate vaccine



v) 4 BC pre-Hib : CD19+ B cells from Hib reference study participant 004 prior to vaccination with Hib conjugate vaccine



vi) 4 PC post-Hib : Plasma cells from Hib reference study participant 004 7 days after vaccination with Hib conjugate vaccine



6.4. Discussion

6.4.1. Method development – RNA extraction and stabilisation

Investigation of the antibody repertoire requires complex sample handling, with the potential to lose material and thus introduce bias at many stages. Obtaining discrete B cell populations containing small numbers cells from heparinised blood requires multiple steps, including PBMC separation, antibody labelling and MACS (and additional FACS in the case of subset sorting). Cells lose viability with repeated handling and thus a proportion of B cells are inevitably lost during cell separation. This was minimised by processing samples within 4 hours of bleeding participants and keeping samples on ice where possible.

RNA is particularly vulnerable to enzymatic degradation by RNAses and must be handled and stored under RNAse-free conditions [345]. PBMC RNA expression levels are particularly sensitive to *ex vivo* handling [349]. The total RNA yield from the CD19+ cells freshly sorted by MACS as described in Section 6.2.1 is approximately 900 ng per 10^6 cells (Table 30) when estimated with a spectrophotometer. This is in accordance with published estimates of B cell RNA content [350]. This RNA was also of sufficient quality for downstream sequencing applications as suggested by the A260/A280 ratio [345].

The Hib reference study B cells (Section 6.2.3) were magnetically sorted, pelleted and snap-frozen at -80°C for subsequent RNA extraction which in some instances took place up to 2 years after the initial separation. The first run of sequencing utilised mRNA extracted from the Hib reference study samples (6.2.5). mRNA forms about 2-5% of total RNA in a cell [350], thus yields were expected to be low and therefore not directly quantified. Instead, the ability to obtain products after RT-PCR were used as a way of indirectly assessing mRNA content.

The Hib samples used in the second run of sequencing used total RNA as a template for cDNA synthesis (section 6.2.6). A subset of these samples had the extracted total RNA assessed for quality by qPCR and spectrophotometer (Section 6.3.2). These results showed that the total RNA content of the frozen cells was an order of magnitude less than that extracted on the same day from a similar number of freshly prepared cells (Figure 42). This suggests that snap-freezing cell pellets may not be the optimal way of long-term storage of samples for subsequent RNA extraction.

An alternative method of stabilising RNA for subsequent extraction was therefore planned for the B cell subsets from the MenACWY vaccine study. Subset separation involved obtaining small numbers of B cells (mean 3.2 x 10^4 cells, Table 33), by MACS followed by FACS. Given the multiple handling steps required for subset separation, the cell viability and quantity of extractable RNA was likely to be less than that of the Hib reference study samples. A previous study has examined the effect of an RNA stabilisation reagent, RNAlater, on RNA expression profiles of FACS sorted T cells [351]. This showed that RNA degradation was minimal in FACS sorted samples treated with RNAlater and stored for 1 month at 4°C. The study used larger sorted cell numbers (3 x 10^5 cells) and reported a 30% cell loss on sample recovery from RNAlater. The MenACWY vaccine study B cell subsets were thus stabilised with RNAlater, with the aim that this would provide more efficient RNA recovery than pelleting and snap-freezing the cells. Unfortunately, RNA yields from the sorted subsets proved disappointing and thus these samples could not be processed further for 454 sequencing. The failure to obtain usable RNA from the ACWY vaccine trial sorted B cell samples may have been due to low starting cell numbers and inadequate extraction of RNA from the large volumes of RNAlater required to stabilise the samples. Future sorting studies using similar small populations of cells may benefit from alternative strategies to preserve RNA e.g. sorting cells directly into an RNA

stabilising cell lysis buffer such as RLT buffer or Trizol reagent for storage at -80 °C prior to subsequent RNA extraction.

6.4.2. Method development – amplicon preparation and parallel sequencing

The DNA content of the amplicon pools post-PCR for the first attempt at 454 sequencing at the Wellcome Trust Sanger Institute using ribogreen showed that the individual amplicon pools contained between 0.7-12 x 10^8 DNA molecules/μL. However, following adaptor ligation, qPCR showed that the amplicon libraries contained considerably less DNA with between 1.6 x 10^4 - 3.9 x 10^5 DNA molecules/μL. The expected efficiency of adaptor ligation to amplicons is 10-30% [211]. It was not clear if the discrepancy between the DNA content of the amplicons and the ligated libraries was due to inaccurate quantification with ribogreen or inefficiency of adaptor ligation to one step RT-PCR products. Ribogreen measures both RNA and double stranded DNA, and the amplicons may have been contaminated with residual RNA from the one step RT-PCR reaction, giving a falsely high value. As the amplicons were not quantified by spectroscopy prior to being sent to the Wellcome Trust Sanger Institute, it is not possible to ascertain whether there was an error in the ribogreen quantification.

454 sequencing is a relatively new technology and the Wellcome Trust Sanger Institute had not previously used one-step RT-PCR products for amplicon library 454 sequencing (Richard Rance, Senior Scientific Manager, Wellcome Trust Sanger Institute, personal communication). It is possible one step RT-PCR amplicons are not suitable for 454 library preparation for mechanistic reasons. For example, the 5' and 3' ends of amplicons produced may differ between one-step RT-PCR and two-step RT-PCR which affects the efficiency of blunt end ligation of adaptors. However, 454 library preparation involves an end repair step using T4 DNA polymerase and E. coli DNA polymerase I Klenow fragment

[211]. The 3' to 5' exonuclease activity of these enzymes removes 3'overhangs and the polymerase activity fills in the 5' overhangs. Thus amplicons produced by either method should be suitable for blunt end ligation. Alternatively, a residual reagent from the one-step RT-PCR may have interfered with downstream ligation. However, the 454 library preparation protocol involves a purification step which should remove residual buffers and salts from the amplicon pool. Amplicons derived from one step RT-PCR have recently been used by a group to investigate antibody repertoire [347]. Thus the reason for the inefficient adaptor ligation in the first attempt at 454 sequencing remains uncertain.

The first attempt at 454 sequencing yielded only 423 sequences with an average length of 78 bp, none of which could be identified as immunoglobulin gene sequences against the IMGT database. Each A and B sequencing adaptor is 30 bp long and each MID tag is 10 bp long. These short sequences are this likely to represent products of adaptor dimerisation as a result of insufficient amplicon products and inefficient adaptor ligation.

To prepare sufficient DNA for the second attempt at 454 sequencing, 2 rounds of PCR were performed and the products of the second round were pooled (Section 6.2.8). Amplicons were quantified by spectroscopy before library preparation. Quantification of the amplicons at the Wellcome Trust Sanger Institue was performed using picogreen, which measures only double stranded DNA. This showed between 9-19 x 10^8 DNA molecules/μL, which was similar to the spectroscopy results. The second attempt at 454 sequencing used two-step RT-PCR to prepare amplicons as has been described previously by other groups [128, 207] and successfully resulted in the generation of 106, 352 sequences with an average length of 402 bb.

6.4.3. Differences in repertoire between individuals at baseline

The overall degree of B cell sequence diversity, expressed as the proportion of functional sequences that were unique, was similar between the 4 individuals at baseline (85-89%). Analysis of individual V_H, D_H or J_H segment usage appears to suggest similarities between individuals. For example, V3-23 is the most or second most commonly used V_H gene segment in all four individuals (Figure 46) in accordance with other published work [128, 347, 348]. D gene segments are short (10-15 bp), and are affected by *p* and *n* nucleotide addition and exonuclease activity at both 5' and 3' ends. For this reason, D_H identity cannot be reliably assigned, particularly in those sequences which also have high rates of somatic hypermutation [348]. Nevertheless, the D3-10, D3-22, D6-13 and D6-19 segments comprised 4 out of the 5 of the most commonly used D_H segments across all 4 baseline B cell samples. Both D3-10 and D3-22 have previously been described to be commonly used DH segments [348]. J4 and J6 are the most commonly used J_H gene segments, consistent with prior reports [128, 208, 348].

Due to the difficulties in ascertaining D_H identity, previous studies have analysed V_H and J_H pairings alone using 454 sequencing and have shown a dominance of V3-23/J4 pairings [128, 336, 352]. Given the frequencies of individual V_H and J_H usage found in this study, it is not surprising that the most common V_H/J_H pairing in the 4 B cell samples was also V3-23/J4 as shown in Figure 49.

However, the immunoglobulin heavy chain consists of recombined VDJ gene segments, and consideration of individual segments or pairings alone may not provide a true picture of the repertoire diversity. As shown in Figure 50, the overall pattern of B cell recombined VDJ gene usage differs considerably between individuals, with no overlap between the 50 most frequent clones of each sequence set. The average clone size within individuals ranged from 4.1-5.5 copies. This suggests that although the repertoire landscape may be similar between individuals, with 20-30 clones appearing at modest

frequencies and the rest of the clones occurring in very low copy numbers, the individual clones differ between individuals.

Weinstein *et al.* compared the frequencies of recombined VDJ gene expression in zebrafish and showed similar patterns of expression between individual fish[335]. However, there are only 975 potential VDJ combinations in zebrafish as compared to approximately 6000 in humans (Section 1.6.1). Additionally zebrafish are inbred experimental animals reared under similar conditions. The four individuals who donated B cells for this study varied in age, gender and ethnicity and consequently may have different antibody repertoires skewed by their genetic backgrounds and prior antigenic exposure. Recent work by Briney *et al.* showed differential VDJ usage across naïve, IgM memory and IgG memory subsets [347]. The baseline sequences were obtained from B cells sorted on surface CD19+ expression alone. Thus variations in proportions of circulating B cell subsets may also have contributed to the observed differences between individuals.

6.4.4. The response to Hib conjugate vaccine

The characteristics of the post-vaccination plasma cell CDR3 segments differed from those of the pre-vaccination B cells. The mean CDR3 lengths were shorter post vaccination, in keeping with increased exonuclease activity and somatic hypermutation as has been previously described [207]. The calculated rate of somatic hypermutation was also higher in post-vaccination CDR3 regions. There was an overall shift to a more oligoclonal response, as evidenced by the reduced sequence diversity and increased clone size. Although reduced CDR3 hydrophobicity has been described in antigen experienced cells, [128], this was not observed consistently post-vaccination in this study.

Following vaccination with the Hib conjugate, V3-23 was the most common or second most common V segment found in plasma cells in individuals 3 and 4 respectively, although the significance of this alone is uncertain as V3-23 was also the most common segment used in these individuals pre-vaccination. A high proportion of D_H segments could not be accurately identified in either individual after vaccination, in keeping with an extensively mutated CDR3. J4 and J6 were the most commonly used J_H segments as has previously been described in antigen experienced B cell subsets [128] .

The sequences of Hib specific antibody have been described as highly restricted to V3-23/J4 or V3-23/J6 combinations [332]. Within the two individuals who were vaccinated in this study, V3-23/J6 appeared to play a greater role in Hib responses than V3-23/J4.

In contrast to the differences in unique VDJ recombinations between individuals at baseline, the post-vaccination samples between individuals 3 and 4 showed some similarities. Both individuals had large numbers of sequences with no assignable DH segment but with V3-23/J6 segments. These sequences are likely to comprise multiple large clones with highly mutated CDR3 regions rather than a single large clone. Further detailed analysis of the actual nucleotide sequences (rather than VDJ identity), of this group may reveal homology between the transcripts of the 2 individuals and thus convergence of antibody gene sequence elicited by the vaccine. The unique VDJ combination (V3-23/D2-8/J3) was expressed in both individuals 3 and 4 at a frequency of 2.1% and 2.7% respectively. There was no overlap in VDJ usage between clones of this size in the 4 baseline samples, which suggests that this may be a vaccine induced response common to both individuals.

As part of the Hib reference study, the Hib polysaccharide specific IgG antibody concentrations were determined at day 28 after vaccination on individuals 3 and 4 using

an ELISA and were found to be 49 and 343 μg/mL respectively. Avidity indices were expressed as the percentage mean reduction in IgG concentration with chaotrope compared to without chaotrope and were 45.9 and 59.8 for individuals 3 and 4 respectively. Thus both individuals had high affinity antibody after vaccination, consistent with the expression of V3-23/J6.

6.4.5. The identification of a clonal expansions

It is not possible to ascertain whether identical transcripts derived from 454 sequencing reflect the presence of *bona fide* identical clones derived from clonal expansion, varying levels of mRNA transcription antibody secreting cells, or biases in cDNA generation during reverse transcription or PCR amplification. For this reason all comparisons of clone size made in this thesis are relative rather than absolute.

6.4.6. Plasma cells and CD19+ B cell repertoires may not be directly comparable

Pre and post vaccination sequences were derived from CD19+ B cells and plasma cells respectively. Previous work has demonstrated that antigen specific plasma cells peak in the peripheral circulation peak 7 days after vaccination [134]. In the absence of a method to isolate Hib polysaccharide-specific B cells, the post-vaccination repertoire was analysed on sequences obtained from plasma cells at day 7 to enrich for Hib-specific cells. Antigen specific plasma cells are not detectable in the absence of antigenic stimulation [134, 216] and were therefore not used for the baseline comparison. The burst of plasma cells detected at day 7 are newly emigrated from germinal centres in lymph nodes and are the result of antigenic stimulation and clonal expansion. There are no published data on 454 sequencing of sorted plasma cells, but the expressed repertoire of plasma cells at seven days after vaccination likely to be highly oligoclonal. Furthermore, the levels of mRNA

transcripts may be higher in actively secreting plasma cells. Consideration of the plasma cell repertoire alone in the absence of the other circulating B cells involved in an immune response (such as memory B cells) may skew the observed sequences disproportionately. However, for the purposes of this thesis, the analysis of plasma cells at day 7 showed that it is possible to identify the canonical Hib polysaccharide specific sequences using 454 sequencing.

6.4.7. The response to vaccination is directed against multiple components

The Hib conjugate vaccine used in this study contains Hib PRP polysaccharide and MenC polysaccharide individually conjugated to tetanus toxoid (Table 28). The antibody repertoire observed in the post-vaccination plasma cells is thus a composite of the responses to all 3 antigens. Although Hib-specific V3-23/J6 sequences are highly over represented in both individuals post vaccination, the V3-74/D5-18/J4 clone which represents 37.1% of functional sequences in individual 4, may be a response to either the MenC or tetanus toxoid.compnents of the vaccine. Monovalent polysaccharide vaccines, including the plain Hib polysaccharide vaccine, are no longer widely available in the UK. Identification of specific antigen response may require using monovalent protein antigen vaccines (*e.g*. hepatitis B) or developing methods for sorting antigen specific cells [353].

6.4.8. Future work

The analysis of the large body of data generated by the second attempt at 454 sequencing is ongoing and is beyond the scope of this thesis. Of particular interest is the investigation of the antibody class usage after vaccination, using the constant domain sequences at the 3' ends of the amplicons. This will confirm if the high avidity antibody seen at day 28 post vaccination is indeed due IgG encoded by the V3-23/J6 sequences

dominating the repertoire at day 7. The canonical Hib specific antibody has been shown have a conserved CDR3 amino acid sequence of Gly–Tyr–Gly–Phe/Met–Asp [332]. A more detailed analysis of the amino acid sequence of the CDR3 regions post vaccination may reveal underlying patterns of functional convergence which are not observed at the VDJ identity level. Further samples from the Hib reference study are also currently being prepared for 454 sequencing. In particular, CD19+ B cell samples at baseline will be compared with CD19+ B cell samples at day 7 post vaccination to ascertain if oligoclonal Hib specific responses can also be detected in this population.

6.5. Conclusions

Successful 454 sequencing is reliant on high quality amplicon preparation. Minimal handling of cells, the use of RNA stabilisation agents and accurate quantification of amplicons are important steps in generating a library suitable for downstream sequencing. The pilot work in this chapter shows that the large datasets generated by 454 sequencing can be used to identify differences between the normal antibody repertoires of individuals, while demonstrating convergence in the their antigen specific repertoires after vaccination.

The methods established in this chapter are will form the basis of future studies of antibody repertoire using 454 sequencing. In particular, these will be used, as initially planned, to characterise VDJ sequences of different B cell subsets in response to conjugate and polysaccharide meningococcal vaccination to ascertain the relative contributions of follicular B cells, marginal zone B cells and naïve B cells in the response to each of these vaccines.

Chapter 7 – Discussion

This thesis has investigated the immune responses of adults to quadrivalent conjugate and polysaccharide meningococcal vaccines, and developed methods to analyse the antibody repertoire of B cells in response to vaccination.

7.1. A comparison of the immunogenicity of MenACWY-CRM and MenACWY-PS

Previous studies in young children and adolescents have shown that MenACWY-CRM elicits significantly higher hSBA GMTs against all 4 serogroups 28 days after vaccination than an alternative quadrivalent polysaccharide vaccine, MPVS4 [267, 268]. The poor immunogenicity of the MPVS4 in the young may be explained by a relative immaturity of the marginal zone of the spleen, and thus diminished immune responses to thymus independent (TI) antigens such as polysaccharide vaccines [104, 113]. However a study comparing MenACWY-CRM with MPVS4 in adults aged 54-65 also demonstrated superior immunogenicity of the conjugate over the polysaccharide 28 days after vaccination [233].

In the MenACWY-CRM/MenACWY-PS clinical study, there was no difference in the primary endpoint of day 7 hSBA GMTs for serogroup A, or for the serogroups C, W and Y after either MenACWY-CRM or MenACWY-PS vaccination. By day 28, participants who received MenACWY-CRM had higher hSBA GMTs against serogroup W, while participants who received MenACWY-PS had higher titres against serogroup C. The differences observed in this study compared to the previously described MenACWY-CRM/MPVS4 adult study may reflect variations in nasopharyngeal meningococcal carriage rates and natural immunological priming between the 2 study populations.

Both MenACWY-CRM and MenACWY-PS vaccines induced hSBA GMTs above 8 at 7 days and 28 days for all 4 serogroups. A hSBA titre of ≥4 has been established as the threshold for the correlate of protection for serogroup C conjugate vaccines [246], and although there are no validated correlates of protection for serogroups A,W and Y, a value of ≥1:8 has been used as an immunological endpoint in pre-licensure immunogenicity studies of conjugate quadrivalent meningococcal vaccines in adults [233]. Thus, there does not seem to be any short-term immunological advantage in using MenACWY-CRM over MenACWY-PS.

7.2. MenACWY-CRM and MenACWY-PS elicit distinct antigen specific B cell responses

In this study, there was a trend to higher frequencies of serogroup A, C, W and Y specific plasma cells 7 days after a single dose of MenACWY-PS compared to after a dose of MenACWY-CRM. Furthermore, MenACWY-CRM generated significantly more serogroup A and C specific memory B cells after 28 days than MenACWY-PS. These observations are consistent with plasma cells being rapidly generated by the TI polysaccharide vaccine via the extra-follicular pathway, while both memory B cells and plasma cells are generated through a germinal centre pathway by the thymus dependent (TD) conjugate vaccine. Further characterisation of these distinct responses may be possible by examining the phenotype of the antigen specific B cells and the degree of somatic hypermutation of the B cell receptors elicited by each vaccine.

Antibody persistence was not measured beyond 28 days in this study. However, memory B cells frequencies in infants after a priming MenC vaccine have been shown to correlate with both the persistence of antibody and the magnitude of antibody responses to a booster 7 months later [215]. Persistent levels of protective antibody are important in

rapidly invasive meningococcal disease. Thus, the higher memory B cell frequencies elicited by MenACWY-CRM may reflect a greater potential for persistent antibody and thus longer-term protection than MenACWY-PS.

Differences in capsular polysaccharide content between MenACWY-PS and MenACWY-CRM confound the results described in Chapter 4. It is possible that the distinct pattern of plasma cell and memory B cell responses observed reflect the higher antigenic dose presented by MenACWY-PS compared to MenACWY-CRM. Furthermore, MenACWY-PS was administered subcutaneously, while MenACWY-CRM was administered intramuscularly. Thus, there may also have been differences in the antigen processing and presentation pathways for each vaccine which have contributed to the observed results.

7.3.MenACWY-PS impairs immune responses to subsequent MenACWY-CRM

Chapter 5 demonstrates that for all 4 serogroups, both the day 28 hSBA responses and the day 7 plasma cell responses to MenACWY-CRM are lower in individuals who have received a prior dose of MenACWY-PS, compared to those who are polysaccharide naïve. This is the first study to show clear evidence of hyporesponsiveness in antigen specific plasma cell frequencies after meningococcal polysaccharide vaccination.

In contrast to MenACWY-CRM, MenACWY-PS does not appear to prime for immunological memory. Thus individuals primed with MenACWY-PS exhibit lower A,C W and Y hSBA GMTs and plasma cell frequencies 7 days after a MenACWY-CRM booster than those with MenACWY-CRM. Furthermore, the serogroup A and C specific memory B cell response to a MenACWY-CRM booster is higher in MenACWY-CRM primed individuals than in MenACWY-PS primed individuals.

Taken together, the results of Chapters 4 and 5 support the theory that polysaccharide vaccines induce hyporesponsiveness by driving the terminal differentiation of antigen specific B cells into plasma cells without replenishment of the memory B cell pool [168]. Again, it is difficult to ascertain if the subsequent impaired responses to MenACWY-CRM are related to the higher antigenic dose of the priming MenACWY-PS or an inherent property of the polysaccharide antigen.

7.4. Serogroup A acts as a thymus independent antigen

Despite prior evidence suggesting that serogroup A polysaccharide may act as a TD antigen, Chapters 4 and 5 show that both the MenA and MenC components of MenACWY-PS have similar effects on antigen specific B cell frequencies and hSBA GMTs. This thesis presents the first clear evidence that serogroup A polysaccharide acts as TI antigen and elicits impaired immune responses to subsequent conjugate vaccination. This work supports the shift in strategy by the World Health Organisation from mass vaccination with MenA polysaccharide vaccines during cyclical serogroup A meningitis outbreaks, to using MenA conjugate vaccines instead [43].

7.5. The development of techniques for RNA extraction and 'next generation' sequencing

'Next generation' sequencing technologies such as 454 sequencing allow the simultaneous sequencing of several million individual DNA sequences in a short period of time [354] and permit the investigation of the breadth of the antibody repertoire sequence. The initial aim of this thesis was to identify the heavy chain variable domain

(V_H) sequences of low frequency B cell subset populations. B cells from 2 normal individuals and B cells and plasma cells from the Hib reference study were to be used as pilot samples during the method development. Samples from the MenACWY-PS/MenACWY-CRM study were to be used for subsequent B cell subset sequence analysis.

High quality RNA is an essential prerequisite for 454 library preparation. Minimal handling of cells and prompt RNA extraction and stabilisation were found to be important steps in ensuring high quality RNA. Unfortunately, it was not possible to obtain adequate RNA from the B cell subsets from the MenACWY-CRM/MenACWY-PS study, and these samples were not analysed further. Therefore only RNA from the B cells and/or plasma cells of normal donors or the Hib reference study were used for subsequent library preparation. The optimal method of obtaining RNA from small B cell populations may involve sorting cells by flow cytometry into a cell lysis/RNA stabilising buffer.

Multiple primer sets and reverse transcription (RT) and polymerase chain reaction (PCR) conditions were trialled, before the optimum conditions were determined. Use of the Wu primer set [128] and the two step RT-PCR conditions discussed in Chapter 6 gave the best yield of the V_H amplicons. Following successful amplicon library generation and 454 sequencing, over 106,000 functional sequences were obtained. The methods established in this thesis will be applied to future studies of antibody gene repertoire.

7.6.454 sequencing reveals differences in antibody repertoire between individuals and after vaccination

Despite differences in age, sex and ethnicity, there appeared to be broad similarities between individuals in their usage of specific V_H and J_H segments. For example, V3-23/J4 was the most common pairing seen in the B cells of all 4 individuals at baseline. However, more detailed analysis of the unique VDJ combination revealed distinct antibody repertoires, with little overlap between the 50 largest clones of the each of the 4 individuals. The overall pattern of clonality (as defined by identical VDJ usage) was similar between individuals, with approximately 20 moderately sized clones and multiple smaller clones.

The plasma cell sequences obtained 7 days after a *Haemophilus influenzae* type b (Hib) conjugate vaccine, had shorter complementarity determining regions (CDR3) and higher rates of somatic hypermutation as has previously been described for antigen experienced cells [207]. Assignment of identity to the D_H segment was always not possible due to high levels of junctional diversity and somatic hypermutation, but there appeared to be a trend towards multiple large clones using V3-23/J6 gene segments, a combination which has been identified with Hib polysaccharide specific antibody [332]. Both individuals also mounted a high avidity anti-Hib antibody response, which suggests that the observed dominance of V3-23/J6 represents Hib specific sequences.

Analysis of the data from the 454 sequencing is ongoing. Comparisons of the antibody isotype distribution and further detailed analyses of the nucleotide and amino acid sequences before and after vaccination are planned. In particular, clonal lineages based on actual CDR3 amino acid sequences, rather than VDJ identity, may reveal convergent evolution of the repertoire.

7.7. Future work

A follow on vaccine study (ACWY2) has been planned and will start recruiting participants in December 2012. This study will address issues raised by ACWY1 and an overview of the study design is shown in Table 37 below.

Table 37 Overview of the design of the 'ACWY2' follow-on study

	V1 Day 0	V2 Day 7	V3 Day 28	V4 Day 35	V5 Day 56
Group 1 n=5	MenACWY-CRM conjugate (i.m.)		MenACWY-CRM conjugate (i.m.)		
Group 2a n=5	MenACWY-PS polysaccharide (full dose s.c.)		MenACWY-CRM conjugate (i.m.)		
Group 2b n=5	MenACWY-PS polysaccharide ($1/5^{th}$ dose s.c.)		MenACWY-CRM conjugate (i.m.)		
Group 2c n=5	MenACWY-PS polysaccharide ($1/5^{th}$ dose i.m.)		MenACWY-CRM conjugate (i.m.)		
Assays	SBA, memory B cells, phenotyping, antibody repertoire	SBA, plasma cells, phenotyping, antibody repertoire	SBA, memory B cells, phenotyping, antibody repertoire	SBA, plasma cells, phenotyping, antibody repertoire	SBA, memory B cells, phenotyping, antibody repertoire

s.c. = subcutaneously; i.m. = intramuscularly. MenACWY-CRM contains 10 μg of MenA polysaccharide and 5 μg each of MenC, MenW and MenY polysaccharides. MenACWY-PS contains 50 μg of polysaccharide from each of the 4 serogroups. Full dose MenACWY-CRM will be used throughout the study.

The follow-on study will be conducted in adults aged 30-70, with similar inclusion and exclusion criteria to those of the original study described in Chapter 3. Participants will receive a priming dose of MenACWY-CRM or MenACWY-PS, followed by a MenACWY-CRM booster 1 month later. hSBA assays and B cell ELISpot assays to enumerate plasma

cells and memory B cells will be performed as described in Chapter 2. However, there are a few key distinctions between the original study and the follow-on study.

The issue of the differences in antigenic dose contained in MenACWY-CRM and MenACWY-PS will be addressed by the inclusion of a group (Group 2b) which uses a 1/5th dose of MenACWY-PS. The 1/5th dose of MenACWY-PS contains the same amount of MenA polysaccharide as MenACWY-CRM and only twice (rather than 10 times) the amount of MenC, MenW and MenY polysaccharides. A further group (Group 2c) will administer both the 1/5th dose of MenACWY-PS and the MenACWY-CRM by the intramuscular route.

Fluorescent antibody panels are currently being developed to identify discrete B cell subsets and antigen specific B cells by flow cytometry to compare responses to conjugate and polysaccharide vaccines. For example, it will be possible to investigate if MenACWY-CRM preferentially elicits follicular B cells and switched memory B cells, while MenACWY-PS generates marginal zone and B1 B cells. This thesis found that MenA polysaccharide appears to act in an immunologically similar manner to MenC polysaccharide. Thus, the comparison of the phenotypes of the MenA and MenC specific cells elicited by MenACWY-PS will be of particular interest.

The RNA extraction and stabilisation techniques, RT, multiplex PCR and library preparation methods developed in Chapter 6 will be applied to sorted B cells subsets from the ACWY2 study. 454 sequencing of these samples will allow the investigation of the fine detail of somatic hypermutation and variable gene usage in the antibody repertoires elicited by MenACWY-PS and MenACWY-CRM and thus the nature of TD and TI antibody responses in adults.

In conclusion, the functional antibody, antigen specific B cell responses form the basis for novel insights into the mechanisms of action of TD and TI antigens. The molecular techniques described in this thesis will eventually be applied to sequencing the variable domains of antigen specific B cells generated by vaccination. Correlating this information with functional antibody responses will enable the identification of vaccine-specific repertoire signatures. This will allow the development of sequence based approaches to assessing vaccine immunogenicity and an in-depth understanding of B-cell immunology through an ability to track B-cell clones.

Bibliography

1. Herpin, *Meningitis*. These de Paris, 1803. **391**.
2. Vieusseux, G., *Mémoire sur la maladie qui a regné à Genève auprintemps de1805*. . Journal de Médecine, Chirurgie et Pharmacie, 1805. **11**: p. 186-193.
3. Danielson, L. and E. Mann, *A history of a singular and very noted disease, which has lately made its appearance in Medfield*. Medical and Agricultural Register, 1806. **1**: p. 65-69.
4. Koch, R., *Die aetiologie der Tuberkulose*. Mitt Kaiser Gesundh., 1884. **2**: p. 1-88.
5. Weichselbaum, A., *Ueber die Aetiologie der akuten Meningitis cerebro-spinalis*. Fortschritte der Medicin., 1887. **5**: p. 573-583.
6. Flexner, S., *Experimental cerebrospinal meningitis and its serum treatment*. Journal of the American Medical Association, 1906. **47**: p. 560-6.
7. Flexner, S., *The results of the serum treatment in thirteen hundred cases of epidemic meningitis*. . J Exp Med, 1913. **17**: p. 553-576.
8. Apicella, M.A., *Neisseria meningitidis*, in *Principles and Practise of Infectious Diseases*, G. Mandell, J. Bennett, and R. Dolin, Editors. 2009, Philadelphia, 2498.: Elsevier Churchill Livingstone Publishers.
9. Christensen, H., et al., *Meningococcal carriage by age: a systematic review and meta-analysis*. The Lancet Infectious Diseases, 2010. **10**(12): p. 853-861.
10. Aycock, W.L., J.H. Mueller, and F.B. Carroll, *Meningococcus carrier rates and meningitis incidence*. Bacteriological Reviews, 1950. **14**(2): p. 115-160.
11. Sadarangani, M. and A.J. Pollard, *Serogroup B meningococcal vaccines—an unfinished story*. The Lancet Infectious Diseases, 2010. **10**(2): p. 112-124.
12. Hill, D.J., et al., *Cellular and molecular biology of Neisseria meningitidis colonization and invasive disease*. Clinical Science, 2010. **118**(9): p. 547-564.
13. Vogel, U., S. Hammerschmidt, and M. Frosch, *Sialic acids of both the capsule and the sialylated lipooligosaccharide of Neisseria meningitis serogroup B are prerequisites for virulence of meningococci in the infant rat*. Med Microbiol Immunol, 1996. **185**(2): p. 81-7.
14. Mackinnon, F.G., et al., *Demonstration of lipooligosaccharide immunotype and capsule as virulence factors for Neisseria meningitidis using an infant mouse intranasal infection model*. Microbial Pathogenesis, 1993. **15**(5): p. 359-366.
15. Jarvis, G.A. and N.A. Vedros, *Sialic acid of group B Neisseria meningitidis regulates alternative complement pathway activation*. Infection and Immunity, 1987. **55**(1): p. 174-180.
16. Hoang, L.M.N., et al., *Rapid and fatal meningococcal disease due to a strain of Neisseria meningitidis containing the capsule null locus*. Clinical Infectious Diseases, 2005. **40**(5): p. e38-e42.
17. Johswich, K.O., et al., *Invasive potential of nonencapsulated disease isolates of Neisseria meningitidis*. Infection and Immunity, 2012. **80**(7): p. 2346-2353.
18. Goldschneider, I., E.C. Gotschlich, and M.S. Artenstein, *Human immunity to the meningococcus. II. Development of natural immunity*. J Exp Med, 1969. **129**(6): p. 1327-48.
19. Swartley, J.S., et al., *Capsule switching of Neisseria meningitidis*. Proceedings of the National Academy of Sciences, 1997. **94**(1): p. 271-276.
20. Harrison, L.H., C.L. Trotter, and M.E. Ramsay, *Global epidemiology of meningococcal disease*. Vaccine, 2009. **27**(Supplement 2): p. B51-B63.
21. Maiden, M.C.J., et al., *Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms*. Proceedings of the National Academy of Sciences, 1998. **95**(6): p. 3140-3145.
22. Jolley, K.A., *Primary Neisseria MLST website*, 2012: University of Oxford.
23. Yazdankhah, S.P., et al., *Distribution of serogroups and genotypes among disease-associated and carried isolates of Neisseria meningitidis from the Czech*

Republic, Greece, and Norway. Journal of Clinical Microbiology, 2004. **42**(11): p. 5146-5153.
24. Jolley, K.A., et al., *Ribosomal multilocus sequence typing: universal characterization of bacteria from domain to strain*. Microbiology, 2012. **158**(Pt 4): p. 1005-1015.
25. Hill, D., et al., *The Meningitis Reserach Foundation Meningococal Genome Library*, in *XIIIth International Pathogenic Neisseria Conference*2012: Wurzburg, Germany.
26. Health Protection Authority. *Epidemiological data - meningococcal disease*. 2012 [cited 2012 07/02/2012]; Available from: http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/MeningococcalDisease/EpidemiologicalData/.
27. Goldschneider, I., E.C. Gotschlich, and M.S. Artenstein, *Human immunity to the meningococcus. I. The role of humoral antibodies*. J Exp Med, 1969. **129**(6): p. 1307-26.
28. Imrey, P.B., et al., *Outbreak of serogroup C meningococcal disease associated with campus bar patronage*. American Journal of Epidemiology, 1996. **143**(6): p. 624-630.
29. Bruce, M.G., et al., *Risk factors for meningococcal disease in college students*. JAMA, 2001. **286**(6): p. 688-93.
30. MacLennan, J., et al. *Social behavior and meningococcal carriage in British teenagers*. Emerg Infect Dis, 2006 **12**.
31. Authority, H.P. *Epidemiological data - meningococcal disease*. 2011; Available from: http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/MeningococcalDisease/EpidemiologicalData/.
32. Stephens, D.S., *Uncloaking the meningococcus: dynamics of carriage and disease*. The Lancet, 1999. **353**(9157): p. 941-942.
33. Andersen, J., et al., *Dynamics of the meningococcal carrier state and characteristics of the carrier strains: a longitudinal study within three cohorts of military recruits*. Epidemiol Infect, 1998. **121**(1): p. 85-94.
34. Ala'Aldeen, D.A.A., et al., *Dynamics of meningococcal long-term carriage among university students and their implications for mass vaccination*. Journal of Clinical Microbiology, 2000. **38**(6): p. 2311-2316.
35. Edwards, E.A., et al., *Immunological investigations of meningococcal disease. III. Brevity of group C acquisition prior to disease occurrence*. Scand J Infect Dis, 1977. **9**(2): p. 105-10.
36. Halperin, S.A., et al., *The changing and dynamic epidemiology of meningococcal disease*. Vaccine, 2012. **30 Suppl 2**(0): p. B26-36.
37. World Health Organisation, *Outbreak news. Meningococcal disease, African meningitis belt*. Wkly Epidemiol Rec, 2009. **84**(15): p. 117-8.
38. Horn, A.E., *Report on an investigation of cerebrospinal fever in the northern territories of the Gold Coast in 1908*. Journal of Tropical Medicine and Hygiene, 1908. **11**: p. 358-365.
39. Lapeyssonie, L., *La méningite cérébro-spinale en Afrique*. Bulletin of the World Health Organization, 1963(28): p. 3-114.
40. Greenwood, B., *Meningococcal meningitis in Africa*. Transactions of the Royal Society of Tropical Medicine and Hygiene, 1999. **93**(4): p. 341-353.
41. Organisation, W.H. *Meningococcal Disease Fact Sheet*. 2012 [cited 2012; Available from: http://www.who.int/mediacentre/factsheets/fs141/en/index.html.
42. Olyhoek, T., B.A. Crowe, and M. Achtman, *Clonal population structure of Neisseria meningitidis serogroup A isolated from epidemics and pandemics between 1915 and 1983*. Rev Infect Dis, 1987. **9**(4): p. 665-92.

43. World Health Organisation. *Meningococcal Disease: situation in the African Meningitis Belt*. 2012 23/03/2012; Available from: http://www.who.int/csr/don/2012_03_23/en/index.html.
44. Guirguis, N., et al., *Escherichia coli K51 and K93 capsular polysaccharides are crossreactive with the group A capsular polysaccharide of Neisseria meningitidis. Immunochemical, biological, and epidemiological studies*. The Journal of Experimental Medicine, 1985. **162**(6): p. 1837-1851.
45. Robbins, J.B., et al., *Enteric bacteria cross-reactive with Neisseria meningitidis groups A and C and Diplococcus pneumoniae types I and III*. Infection and Immunity, 1972. **6**(5): p. 651-656.
46. Pollard, A.J. and C. Frasch, *Development of natural immunity to Neisseria meningitidis*. Vaccine, 2001. **19**(11–12): p. 1327-1346.
47. Kasper DL, et al., *Harrison's Principles of Internal Medicine*. 18 ed. Vol. 8. 2011, New York: McGraw-Hill Medical Publishing Division.
48. Cookson, S.T., et al., *Disco fever: Epidemic meningococcal disease in northeastern Argentina associated with disco patronage*. Journal of Infectious Diseases, 1998. **178**(1): p. 266-269.
49. Krasinski, K., et al., *Possible association of mycoplasma and viral respiratory infections with bacterial meningitis*. Am J Epidemiol, 1987. **125**(3): p. 499-508.
50. Medzhitov, R. and J.C. Janeway, *The Toll receptor family and microbial recognition*. Trends in Microbiology, 2000. **8**(10): p. 452-456.
51. Schmitt, C., A. Villwock, and O. Kurzai, *Recognition of meningococcal molecular patterns by innate immune receptors*. International Journal of Medical Microbiology, 2009. **299**(1): p. 9-20.
52. Mogensen, T.H., et al., *Live Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns*. Journal of Leukocyte Biology, 2006. **80**(2): p. 267-277.
53. Miller, S.I., R.K. Ernst, and M.W. Bader, *LPS, TLR4 and infectious disease diversity*. Nat Rev Micro, 2005. **3**(1): p. 36-46.
54. Zughaier, S.M., et al., *Neisseria meningitidis lipooligosaccharide structure-dependent activation of the macrophage CD14/Toll-like receptor 4 pathway*. Infection and Immunity, 2004. **72**(1): p. 371-380.
55. van der Ley, P. and L. Steeghs, *Lessons from an LPS-deficient Neisseria meningitidis mutant*. Journal of Endotoxin Research, 2003. **9**(2): p. 124-128.
56. Pridmore, A.C., et al., *A lipopolysaccharide-deficient mutant of Neisseria meningitidis elicits attenuated cytokine release by human macrophages and signals via Toll-like receptor (TLR) 2 but not via TLR4/MD2*. Journal of Infectious Diseases, 2001. **183**(1): p. 89-96.
57. Massari, P., et al., *Meningococcal porin PorB binds to TLR2 and requires TLR1 for signaling*. The Journal of Immunology, 2006. **176**(4): p. 2373-2380.
58. Hemmi, H., et al., *A Toll-like receptor recognizes bacterial DNA*. Nature, 2000. **408**(6813): p. 740-745.
59. Gazi, U. and L. Martinez-Pomares, *Influence of the mannose receptor in host immune responses*. Immunobiology, 2009. **214**(7): p. 554-561.
60. Gough, P.J. and S. Gordon, *The role of scavenger receptors in the innate immune system*. Microbes and Infection, 2000. **2**(3): p. 305-311.
61. Woof, J.M. and D.R. Burton, *Human antibody-Fc receptor interactions illuminated by crystal structures*. Nat Rev Immunol, 2004. **4**(2): p. 89-99.
62. Peters, M.J., et al., *Severe meningococcal disease is characterized by early neutrophil but not platelet activation and increased formation and consumption of platelet–neutrophil complexes*. Journal of Leukocyte Biology, 2003. **73**(6): p. 722-730.

63. Pérez, O., et al., *Immune response induction and new effector mechanisms possibly involved in protection conferred by the Cuban anti-meningococcal BC vaccine.* Infection and Immunity, 2001. **69**(7): p. 4502-4508.
64. Mayer-Scholl, A., P. Averhoff, and A. Zychlinsky, *How do neutrophils and pathogens interact?* Current Opinion in Microbiology, 2004. **7**(1): p. 62-66.
65. Casey, R., et al., *The acute-phase reactant C-reactive protein binds to phosphorylcholine-expressing Neisseria meningitidis and increases uptake by human phagocytes.* Infection and Immunity, 2008. **76**(3): p. 1298-1304.
66. Estabrook, M.M., D. Zhou, and M.A. Apicella, *Nonopsonic phagocytosis of group C Neisseria meningitidis by human neutrophils.* Infection and Immunity, 1998. **66**(3): p. 1028-1036.
67. Criss, A.K. and H.S. Seifert, *A bacterial siren song: intimate interactions between Neisseria and neutrophils.* Nat Rev Micro, 2012. **10**(3): p. 178-190.
68. Palucka, K. and J. Banchereau, *Dendritic cells: A link between innate and adaptive immunity.* Journal of Clinical Immunology, 1999. **19**(1): p. 12-25.
69. Kolb-Mäurer, A., et al., *The role of human dendritic cells in meningococcal and listerial meningitis.* International Journal of Medical Microbiology, 2003. **293**(4): p. 241-249.
70. van Deuren, M., et al., *Correlation between proinflammatory cytokines and antiinflammatory mediators and the severity of disease in meningococcal infections.* Journal of Infectious Diseases, 1995. **172**(2): p. 433-439.
71. D'Amelio, R., et al., *Complement deficiency and antibody profile in survivors of meningococcal meningitis due to common serogroups in Italy.* Scand J Immunol, 1992. **35**(5): p. 589-95.
72. Figueroa, J., J. Andreoni, and P. Densen, *Complement deficiency states and meningococcal disease.* Immunol Res, 1993. **12**(3): p. 295-311.
73. Ram, S., L.A. Lewis, and P.A. Rice, *Infections of people with complement deficiencies and patients who Have undergone splenectomy.* Clinical Microbiology Reviews, 2010. **23**(4): p. 740-780.
74. Lassiter, H.A., et al., *Complement factor 9 deficiency in serum of human neonates.* Journal of Infectious Diseases, 1992. **166**(1): p. 53-57.
75. Ross, S.C., et al., *Killing of Neisseria meningitidis by human neutrophils: Implications for normal and complement-deficient individuals.* Journal of Infectious Diseases, 1987. **155**(6): p. 1266-1275.
76. Granoff, D.M., *Relative importance of complement-mediated bactericidal and opsonic activity for protection against meningococcal disease.* Vaccine, 2009. **27, Supplement 2**(0): p. B117-B125.
77. Platonov, A.E., et al., *Long term effects of vaccination of patients deficient in a late complement component with a tetravalent meningococcal polysaccharide vaccine.* Vaccine, 2003. **21**(27–30): p. 4437-4447.
78. Matsunami, T. and J.A. Kolmer, *The relation of the meningococcidal activity of the blood to resistance to virulent meningococci.* The Journal of Immunology, 1918. **3**(3): p. 201-212.
79. Kasper, D.L., et al., *Antigenic specificity of bactericidal antibodies in antisera to Neisseria meningitidis.* Journal of Infectious Diseases, 1973. **127**(4): p. 378-387.
80. Cartwright, K.A., et al., *The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica.* Epidemiol Infect, 1987. **99**(3): p. 591-601.
81. Gold, R., et al., *Carriage of Neisseria meningitidis and Neisseria lactamica in infants and children.* Journal of Infectious Diseases, 1978. **137**(2): p. 112-121.
82. Kim, J.J., R.E. Mandrell, and J.M. Griffiss, *Neisseria lactamica and Neisseria meningitidis share lipooligosaccharide epitopes but lack common capsular and class 1, 2, and 3 protein epitopes.* Infection and Immunity, 1989. **57**(2): p. 602-608.

83. Oliver, K.J., et al., *Neisseria lactamica Protects against Experimental Meningococcal Infection*. Infection and Immunity, 2002. **70**(7): p. 3621-3626.
84. Janeway, C.A., *Immunobiology*. 8th ed2011, Abindon, UK: Garlan Science.
85. Flaegstad, T., T. Gutteberg, and B.E. Kristiansen, *Antibodies to meningococci in children with meningococcal disease*. Scand J Infect Dis, 1990. **22**(5): p. 547-51.
86. Granoff, D.M., et al., *Antibody responses to the capsular polysaccharide of Neisseria meningitidis serogroup B in patients with meningococcal disease*. Clinical and Diagnostic Laboratory Immunology, 1995. **2**(5): p. 574-82.
87. Rosenqvist, E., et al., *Antibody responses to serogroup B meningococcal outer membrane antigens after vaccination and infection*. Journal of Clinical Microbiology, 1988. **26**(8): p. 1543-1548.
88. Lowell, G.H., et al., *Antibody-dependent mononuclear cell-mediated antimeningococcal activity: Comparison of the effects of convalescent and postimmunization immunoglobulins G, M, and A*. The Journal of Clinical Investigation, 1980. **66**(2): p. 260-267.
89. Pollard, A.J., et al., *Humoral immune responses to Neisseria meningitidis in children*. Infection and Immunity, 1999. **67**(5): p. 2441-2451.
90. Sjursen, H., et al., *IgG subclass antibodies to serogroup B meningococcal outer membrane antigens following infection and vaccination*. APMIS, 1990. **98**(12): p. 1061-9.
91. Vidarsson, G., et al., *Activity of human IgG and IgA subclasses in immune defense against Neisseria meningitidis serogroup B*. The Journal of Immunology, 2001. **166**(10): p. 6250-6256.
92. Griffiss, J. and D. Goroff, *IgA blocks IgM and IgG-initiated immune lysis by separate molecular mechanisms*. The Journal of Immunology, 1983. **130**(6): p. 2882-2885.
93. Hamadeh, R.M., et al., *Anti-alpha-galactosyl immunoglobulin A (IgA), IgG, and IgM in human secretions*. Clinical and Diagnostic Laboratory Immunology, 1995. **2**(2): p. 125-31.
94. Nurkka, A., et al., *Salivary antibody response to vaccination with meningococcal A/C polysaccharide vaccine in previously vaccinated and unvaccinated Gambian children*. Vaccine, 2000. **19**(4-5): p. 547-56.
95. Zhang, Q., et al., *Mucosal immune responses to meningococcal group C conjugate and group A and C polysaccharide vaccines in adolescents*. Infection and Immunity, 2000. **68**(5): p. 2692-2697.
96. Allman, D. and S. Pillai, *Peripheral B cell subsets*. Current Opinion in Immunology, 2008. **20**(2): p. 149-157.
97. Vos, Q., et al., *B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms*. Immunological Reviews, 2000. **176**(1): p. 154-170.
98. Rijkers, G.T., et al., *Infant B cell responses to polysaccharide determinants*. Vaccine, 1998. **16**(14–15): p. 1396-1400.
99. Avci, F.Y. and D.L. Kasper, *How bacterial carbohydrates influence the adaptive immune system*. Annu Rev Immunol, 2010. **28**: p. 107-30.
100. McHeyzer-Williams, L.J. and M.G. McHeyzer-Williams, *Antigen-specific memory B cell development*. Annual Review of Immunology, 2005. **23**(1): p. 487-513.
101. Ho, F., et al., *Distinct short-lived and long-lived antibody-producing cell populations*. European Journal of Immunology, 1986. **16**(10): p. 1297-1301.
102. MacLennan, I.C.M., et al., *Extrafollicular antibody responses*. Immunological Reviews, 2003. **194**(1): p. 8-18.
103. Shaffer, A.L., et al., *Blimp-1 orchestrates plasma cell differentiation by extinguishing the mature B cell gene expression program*. Immunity, 2002. **17**(1): p. 51-62.

104. Weller, S., et al., *Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire.* Blood, 2004. **104**(12): p. 3647-3654.
105. Oliver, A.M., F. Martin, and J.F. Kearney, *IgM high/CD21 high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells.* The Journal of Immunology, 1999. **162**(12): p. 7198-7207.
106. Weill, J.-C., S. Weller, and C.-A. Reynaud, *Human marginal zone B cells.* Annual Review of Immunology, 2009. **27**(1): p. 267-285.
107. Martin, F., A.M. Oliver, and J.F. Kearney, *Marginal zone and B1b cells unite in the early response against T-independent blood-borne particulate antigens.* Immunity, 2001. **14**(5): p. 617-629.
108. Dunn-Walters, D.K., P.G. Isaacson, and J. Spencer, *Analysis of mutations in immunoglobulin heavy chain variable region genes of microdissected marginal zone (MGZ) B cells suggests that the MGZ of human spleen is a reservoir of memory B cells.* J Exp Med, 1995. **182**(2): p. 559-66.
109. Tian, C., et al., *Evidence for preferential Ig gene usage and differential TdT and exonuclease activities in human naive and memory B cells.* Mol Immunol, 2007. **44**(9): p. 2173-83.
110. White, H.N. and Q.-H. Meng, *Recruitment of a distinct but related set of VH sequences into the murine CD21hi/CD23– marginal zone B cell repertoire to that seen in the class-switched antibody response.* The Journal of Immunology, 2012. **188**(1): p. 287-293.
111. Weller, S., et al., *CD40-CD40L independent Ig gene hypermutation suggests a second B cell diversification pathway in humans.* Proceedings of the National Academy of Sciences, 2001. **98**(3): p. 1166-1170.
112. Weller, S., et al., *Somatic diversification in the absence of antigen-driven responses is the hallmark of the IgM+IgD+CD27+ B cell repertoire in infants.* The Journal of Experimental Medicine, 2008. **205**(6): p. 1331-1342.
113. Kruschinski, C., et al., *Age-dependent development of the splenic marginal zone in human infants is associated with different causes of death.* Human Pathology, 2004. **35**(1): p. 113-121.
114. Kruetzmann, S., et al., *Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen.* The Journal of Experimental Medicine, 2003. **197**(7): p. 939-945.
115. Song, H. and J. Cerny, *Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen.* The Journal of Experimental Medicine, 2003. **198**(12): p. 1923-1935.
116. Bankoti, R., et al., *Marginal zone B cells regulate antigen-specific T cell responses during Infection.* The Journal of Immunology, 2012. **188**(8): p. 3961-3971.
117. Berland, R. and H.H. Wortis, *Origins and functions of B-1 cells with notes on the role of CD5.* Annual Review of Immunology, 2002. **20**(1): p. 253-300.
118. Baumgarth, N., J.W. Tung, and L.A. Herzenberg, *Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion.* Springer Semin Immunopathol, 2005. **26**(4): p. 347-62.
119. Schwartz, J.T., et al., *Natural IgM mediates complement-dependent uptake of Francisella tularensis by human neutrophils via complement receptors 1 and 3 in nonimmune serum.* The Journal of Immunology, 2012.
120. Montecino-Rodriguez, E., H. Leathers, and K. Dorshkind, *Identification of a B-1 B cell-specified progenitor.* Nat Immunol, 2006. **7**(3): p. 293-301.
121. Haas, K.M., et al., *B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae.* Immunity, 2005. **23**(1): p. 7-18.

122. Alugupalli, K.R., *A distinct role for B1b lymphocytes in T cell-independent immunity*. Curr Top Microbiol Immunol, 2008. **319**: p. 105-30.
123. Roy, B., et al., *Somatic hypermutation in peritoneal B1b cells.* Molecular Immunology, 2009. **46**(8–9): p. 1613-1619.
124. Gao, J., et al., *Novel functions of murine B1 cells: Active phagocytic and microbicidal abilities.* European Journal of Immunology, 2012. **42**(4): p. 982-992.
125. Griffin, D. and T.L. Rothstein, *Human B1 cell frequency: Isolation and analysis of human B1 cells.* Frontiers in Immunology, 2012. **3**.
126. Griffin, D.O., N.E. Holodick, and T.L. Rothstein, *Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+CD27+CD43+CD70−*. The Journal of Experimental Medicine, 2011. **208**(1): p. 67-80.
127. Descatoire, M., et al., *A human equivalent of mouse B-1 cells?* The Journal of Experimental Medicine, 2011. **208**(13): p. 2563-2564.
128. Wu, Y.C., et al., *High-throughput immunoglobulin repertoire analysis distinguishes between human IgM memory and switched memory B-cell populations.* Blood, 2010. **116**(7): p. 1070-8.
129. Dogan, I., et al., *Multiple layers of B cell memory with different effector functions.* Nat Immunol, 2009. **10**(12): p. 1292-1299.
130. Tangye, S.G. and K.L. Good, *Human IgM+CD27+ B cells: Memory B cells or "memory" B cells?* The Journal of Immunology, 2007. **179**(1): p. 13-19.
131. Mamani-Matsuda, M., et al., *The human spleen is a major reservoir for long-lived vaccinia virusâ€"specific memory B cells.* Blood, 2008. **111**(9): p. 4653-4659.
132. Good, K.L., D.T. Avery, and S.G. Tangye, *Resting human memory B cells are intrinsically programmed for enhanced survival and responsiveness to diverse stimuli compared to naive B cells.* J Immunol, 2009. **182**(2): p. 890-901.
133. Liu, Y.J., et al., *Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2.* Immunity, 1995. **2**(3): p. 239-48.
134. Blanchard-Rohner, G., et al., *Appearance of peripheral blood plasma cells and memory B cells in a primary and secondary immune response in humans.* Blood, 2009. **114**(24): p. 4998-5002.
135. Crotty, S., et al., *Cutting edge: Long-term B cell memory in humans after smallpox vaccination.* J Immunol, 2003. **171**(10): p. 4969-4973.
136. Macallan, D.C., et al., *B-cell kinetics in humans: rapid turnover of peripheral blood memory cells.* Blood, 2005. **105**(9): p. 3633-3640.
137. Vieira, P. and K. Rajewsky, *Persistence of memory B cells in mice deprived of T cell help*. International Immunology, 1990. **2**(6): p. 487-494.
138. Maruyama, M., K.-P. Lam, and K. Rajewsky, *Memory B-cell persistence is independent of persisting immunizing antigen.* Nature, 2000. **407**(6804): p. 636-642.
139. Bernasconi, N.L., E. Traggiai, and A. Lanzavecchia, *Maintenance of serological memory by polyclonal activation of human memory B cells.* Science, 2002. **298**(5601): p. 2199-2202.
140. Clutterbuck, E.A., et al., *Serotype-specific and age-dependent generation of pneumococcal polysaccharide-specific memory B-cell and antibody responses to immunization with a pneumococcal conjugate vaccine.* Clinical and Vaccine Immunology, 2008. **15**(2): p. 182-193.
141. Fahey, J.L. and S. Sell, *The immunoglobulins of mice. V: The metabolic (catabolic) properties of five immunoglobulin classes.* J Exp Med, 1965. **122**: p. 41-58.
142. Paus, D., et al., *Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation.* The Journal of Experimental Medicine, 2006. **203**(4): p. 1081-1091.

143. Amanna, I.J. and M.K. Slifka, *Mechanisms that determine plasma cell lifespan and the duration of humoral immunity*. Immunological Reviews, 2010. **236**(1): p. 125-138.
144. Bortnick, A., et al., *Long-lived bone marrow plasma cells are induced early in response to T cell-independent or T cell-dependent antigens*. The Journal of Immunology, 2012. **188**(11): p. 5389-5396.
145. Manz, R.A., A. Thiel, and A. Radbruch, *Lifetime of plasma cells in the bone marrow*. Nature, 1997. **388**(6638): p. 133-134.
146. Slifka, M.K., et al., *Humoral immunity due to long-lived plasma cells*. Immunity, 1998. **8**(3): p. 363-372.
147. Wols, H.A.M., et al., *The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity*. The Journal of Immunology, 2002. **169**(8): p. 4213-4221.
148. Belnoue, E., et al., *APRIL is critical for plasmablast survival in the bone marrow and poorly expressed by early-life bone marrow stromal cells*. Blood, 2008. **111**(5): p. 2755-2764.
149. Smith, K.G., et al., *The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response*. EMBO J, 1997. **16**(11): p. 2996-3006.
150. Cambridge, G., et al., *Serologic changes following B lymphocyte depletion therapy for rheumatoid arthritis*. Arthritis & Rheumatism, 2003. **48**(8): p. 2146-2154.
151. Mesin, L., et al., *Long-lived plasma cells from human small intestine biopsies secrete immunoglobulins for many weeks in vitro*. The Journal of Immunology, 2011. **187**(6): p. 2867-2874.
152. van Laar, J.M., et al., *Sustained secretion of immunoglobulin by long-lived human tonsil Plasma cells*. The American Journal of Pathology, 2007. **171**(3): p. 917-927.
153. Pollard, A.J., et al., *Cellular immune responses to Neisseria meningitidis in children*. Infection and Immunity, 1999. **67**(5): p. 2452-2463.
154. Robinson, K., et al., *Characterization of humoral and cellular immune responses elicited by meningococcal carriage*. Infection and Immunity, 2002. **70**(3): p. 1301-1309.
155. Davenport, V., et al., *Evidence for naturally acquired T cell-mediated mucosal immunity to Neisseria meningitidis*. The Journal of Immunology, 2003. **171**(8): p. 4263-4270.
156. Dochez, A.R. and O.T. Avery, *The elaboration of specific soluble substance by pneumococcus during growth*. J Exp Med, 1917. **26**(4): p. 477-93.
157. Heidelberger, M. and O.T. Avery, *The soluble specific substance of pneumococcus*. J Exp Med, 1923. **38**(1): p. 73-9.
158. Macleod, C.M., et al., *Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides*. J Exp Med, 1945. **82**(6): p. 445-65.
159. Kabat, E.A., H. Kaiser, and H. Sikorski, *Preparation of the type-specific polysaccharide of the type I meningococcus and a study of its effectiveness as an antigen in human beings*. J Exp Med, 1944. **80**(4): p. 299-307.
160. Gotschlich, E.C., T.Y. Liu, and M.S. Artenstein, *Human immunity to the meningococcus. 3. Preparation and immunochemical properties of the group A, group B, and group C meningococcal polysaccharides*. J Exp Med, 1969. **129**(6): p. 1349-65.
161. Gotschlich, E.C., I. Goldschneider, and M.S. Artenstein, *Human immunity to the meningococcus. V. The effect of immunization with meningococcal group C polysaccharide on the carrier state*. J Exp Med, 1969. **129**(6): p. 1385-95.

162. Artenstein, M.S., et al., *Prevention of meningococcal disease by group C polysaccharide vaccine.* New England Journal of Medicine, 1970. **282**(8): p. 417-420.
163. Gold, R. and M.S. Artenstein, *Meningococcal infections. 2. Field trial of group C meningococcal polysaccharide vaccine in 1969-70.* Bull World Health Organ, 1971. **45**(3): p. 279-82.
164. Mäkelä, P.H., et al., *Effect of group-A meningococcal vaccine in army recruits in Finland.* The Lancet, 1975. **306**(7941): p. 883-886.
165. Department of Health, *Immunisation against infectious diseases*, 2011. p. 235-252.
166. Zangwill, K.M., et al., *Duration of antibody response after meningococcal polysaccharide vaccination in US air force personnel.* Journal of Infectious Diseases, 1994. **169**(4): p. 847-852.
167. Dellicour, S. and B. Greenwood, *Systematic review: Impact of meningococcal vaccination on pharyngeal carriage of meningococci.* Tropical Medicine & International Health, 2007. **12**(12): p. 1409-1421.
168. Granoff, D.M. and A.J. Pollard, *Reconsideration of the use of meningococcal polysaccharide vaccine.* Pediatr Infect Dis J, 2007. **26**(8): p. 716-22.
169. Jokhdar, H., et al., *Immunologic hyporesponsiveness to serogroup C but not serogroup A following repeated meningococcal A/C polysaccharide vaccination in Saudi Arabia.* Clin. Diagn. Lab. Immunol., 2004. **11**(1): p. 83-88.
170. Findlow, H., et al., *Meningococcal group C and W135 immunological hyporesponsiveness in African toddlers.* Clin Vaccine Immunol, 2011. **18**(9): p. 1492-6.
171. Keyserling, H., et al., *Safety, immunogenicity, and immune memory of a novel meningococcal (Groups A, C, Y, and W-135) polysaccharide diphtheria toxoid conjugate vaccine (MCV-4) in healthy adolescents.* Arch Pediatr Adolesc Med, 2005. **159**(10): p. 907-913.
172. Gold, R., et al., *Clinical evaluation of group A and group C meningococcal polysaccharide vaccines in infants.* J Clin Invest, 1975. **56**(6): p. 1536-47.
173. Lepow, M.L., et al., *Persistence of antibody following immunization of children with groups A and C meningococcal polysaccharide vaccines.* Pediatrics, 1977. **60**(5): p. 673-80.
174. Leach, A., et al., *Induction of immunologic memory in Gambian children by vaccination in infancy with a group A plus group C meningococcal polysaccharide-protein conjugate vaccine.* J Infect Dis, 1997. **175**(1): p. 200-4.
175. Wyle, F.A., et al., *Immunologic response of man to group B meningococcal polysaccharide vaccines.* Journal of Infectious Diseases, 1972. **126**(5): p. 514-522.
176. Finne, J., M. Leinonen, and P.H. Mäkelä, *Antigenic similarities between brain components and bacteria causing meningitis: Implications for vaccine development and pathogenesis.* The Lancet, 1983. **322**(8346): p. 355-357.
177. Avery, O.T. and W.F. Goebel, *Chemo-immunological studies on conjugated carbohydrate-proteins : V. The immunological specifity of an antigen prepared by combining the capsular polysaccharide of type III pneumococcus with foreign protein.* J Exp Med, 1931. **54**(3): p. 437-47.
178. Miller, E., D. Salisbury, and M. Ramsay, *Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story.* Vaccine, 2001. **20, Supplement 1**(0): p. S58-S67.
179. MacDonald, N.E., et al., *Induction of immunologic memory by conjugated vs plain meningococcal C polysaccharide vaccine in toddlers: a randomized controlled trial.* JAMA, 1998. **280**(19): p. 1685-9.

180. Vu, D.M., et al., *Priming for immunologic memory in adults by meningococcal group C conjugate vaccination.* Clinical and Vaccine Immunology, 2006. **13**(6): p. 605-610.
181. MacLennan Jm, S.F.H.P.T. and et al., *Safety, immunogenicity, and induction of immunologic memory by a serogroup c meningococcal conjugate vaccine in infants: A randomized controlled trial.* JAMA: The Journal of the American Medical Association, 2000. **283**(21): p. 2795-2801.
182. Ramsay, M.E., et al., *Efficacy of meningococcal serogroup C conjugate vaccine in teenagers and toddlers in England.* The Lancet, 2001. **357**(9251): p. 195-196.
183. Borrow, R., P. Balmer, and E. Miller, *Meningococcal surrogates of protection—serum bactericidal antibody activity.* Vaccine, 2005. **23**(17–18): p. 2222-2227.
184. Zhang, Q., et al., *Primary and booster mucosal immune responses to meningococcal group A and C conjugate and polysaccharide vaccines administered to university students in the United Kingdom.* Infection and Immunity, 2001. **69**(7): p. 4337-4341.
185. Reisinger, K.S., et al., *Quadrivalent meningococcal vaccination of adults: Phase III comparison of an investigational conjugate vaccine, MenACWY-CRM, with the licensed vaccine, Menactra.* Clinical and Vaccine Immunology, 2009. **16**(12): p. 1810-1815.
186. Baxter, R., et al., *Immunogenicity and safety of an investigational quadrivalent meningococcal ACWY tetanus toxoid conjugate vaccine in healthy adolescents and young adults 10 to 25 years of age.* Pediatr Infect Dis J, 2011. **30**(3): p. e41-8.
187. Pichichero, M.E., et al., *Serum antibody responses of weanling mice and two-year-old children to pneumococcal-type 6A–protein conjugate vaccines of differing saccharide chain lengths.* Vaccine, 1998. **16**(1): p. 83-91.
188. Decker, M.D., et al., *Comparative trial in infants of four conjugate Haemophilus influenzae type b vaccines.* The Journal of Pediatrics, 1992. **120**(2, Part 1): p. 184-189.
189. Kelly, D.F., et al., *CRM197-conjugated serogroup C meningococcal capsular polysaccharide, but not the native polysaccharide, induces persistent antigen-specific memory B cells.* Blood, 2006. **108**(8): p. 2642-2647.
190. Borrow, R., et al., *Influence of prior meningococcal C polysaccharide vaccination on the response and generation of memory after meningococcal C conjugate vaccination in young children.* Journal of Infectious Diseases, 2001. **184**(3): p. 377-380.
191. Granoff, D.M., et al., *Induction of immunologic refractoriness in adults by meningococcal C polysaccharide vaccination.* J Infect Dis, 1998. **178**(3): p. 870-4.
192. Lakshman, R., et al., *Prior meningococcal A/C polysaccharide vaccine does not reduce immune responses to conjugate vaccine in young adults.* Vaccine, 2002. **20**(31–32): p. 3778-3782.
193. Anttila, M., et al., *Avidity of IgG for Streptococcus pneumoniae Type 6B and 23F polysaccharides in infants primed with pneumococcal conjugates and boosted with polysaccharide or conjugate vaccines.* Journal of Infectious Diseases, 1998. **177**(6): p. 1614-1621.
194. Goldblatt, D., A.R.J.P.M. Pinto Vaz, and E. Miller, *Antibody avidity as a surrogate marker of successful priming by Haemophilus influenzae type b conjugate vaccines following infant immunization.* Journal of Infectious Diseases, 1998. **177**(4): p. 1112-1115.
195. Richmond, P., et al., *Ability of 3 Different Meningococcal C Conjugate Vaccines to Induce Immunologic Memory after a Single Dose in UK Toddlers.* Journal of Infectious Diseases, 2001. **183**(1): p. 160-163.
196. Goldblatt, D., R. Borrow, and E. Miller, *Natural and vaccine-induced immunity and immunologic memory to Neisseria meningitidis serogroup C in young adults.* Journal of Infectious Diseases, 2002. **185**(3): p. 397-400.

197. Harris, S.L., A. Finn, and D.M. Granoff, *Disparity in functional activity between serum anticapsular antibodies induced in adults by immunization with an investigational group A and C Neisseria meningitidis-diphtheria toxoid conjugate vaccine and by a polysaccharide vaccine.* Infection and Immunity, 2003. **71**(6): p. 3402-3408.
198. Baxendale, H.E., et al., *Immunogenetic analysis of the immune response to pneumococcal polysaccharide.* European Journal of Immunology, 2000. **30**(4): p. 1214-1223.
199. Tonegawa, S., *Somatic generation of antibody diversity.* Nature, 1983. **302**(5909): p. 575-81.
200. Lefranc, M.P., *Nomenclature of the human immunoglobulin heavy (IGH) genes.* Exp Clin Immunogenet, 2001. **18**(2): p. 100-16.
201. Lefranc, M.P., *Nomenclature of the human immunoglobulin lambda (IGL) genes.* Exp Clin Immunogenet, 2001. **18**(4): p. 242-54.
202. Lefranc, M.P., *Nomenclature of the human immunoglobulin kappa (IGK) genes.* Exp Clin Immunogenet, 2001. **18**(3): p. 161-74.
203. Hougs, L., et al., *The first dose of a Haemophilus influenzae type b conjugate vaccine reactivates memory B cells: Evidence for extensive clonal selection, intraclonal affinity maturation, and multiple isotype switches to IgA2.* The Journal of Immunology, 1999. **162**(1): p. 224-237.
204. Brezinschek, H., R. Brezinschek, and P. Lipsky, *Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction.* J Immunol, 1995. **155**(1): p. 190-202.
205. Ohno, S., N. Mori, and T. Matsunaga, *Antigen-binding specificities of antibodies are primarily determined by seven residues of VH.* Proc Natl Acad Sci U S A, 1985. **82**(9): p. 2945-9.
206. Rosner, K., et al., *Third complementarity-determining region of mutated VH immunoglobulin genes contains shorter V, D, J, P, and N components than non-mutated genes.* Immunology, 2001. **103**(2): p. 179-87.
207. Ademokun, A., et al., *Vaccination-induced changes in human B-cell repertoire and pneumococcal IgM and IgA antibody at different ages.* Aging Cell, 2011: p. no-no.
208. Brezinschek, H., et al., *Analysis of the human VH gene repertoire. Differential effects of selection and somatic hypermutation on human peripheral CD5(+)/IgM+ and CD5(-)/IgM+ B cells.* J Clin Invest, 1997. **99**(10): p. 2488 - 501.
209. Margulies, M., et al., *Genome sequencing in microfabricated high-density picolitre reactors.* Nature, 2005. **437**(7057): p. 376-80.
210. Ravn, U., et al., *By-passing in vitro screening--next generation sequencing technologies applied to antibody display and in silico candidate selection.* Nucleic Acids Res, 2010. **38**(21): p. e193.
211. Diagnostics, R., *Rapid Library Preparation Method Manual*2010, Mannheim, Germany: Roche Applied Sciences.
212. Diagnostics, R., *emPCR Amplification Method Manual*2011, Mannheim, Germany: Roche Applied Science.
213. Diagnostics, R., *Sequencing Method Manual*2011, Mannheim, Germany: Roche Applied Science.
214. Snape MD, et al., *Immunogenicity of a tetravalent meningococcal glycoconjugate vaccine in infants: a randomized controlled trial.* JAMA, 2008. **299**(2): p. 173-84.
215. Blanchard-Rohner, G., et al., *The magnitude of the antibody and memory B cell responses during priming with a protein-polysaccharide conjugate vaccine in human infants is associated with the persistence of antibody and the intensity of booster response.* The Journal of Immunology, 2008. **180**(4): p. 2165-2173.

216. Clutterbuck, E.A., et al., *The kinetics and phenotype of the human B-cell response following immunization with a heptavalent pneumococcal-CRM197 conjugate vaccine.* Immunology, 2006. **119**(3): p. 328-337.
217. Novartis Vaccines, *MenACWY Investigator's Brochure* 2010.
218. Trotter, C.L., et al., *Effectiveness of meningococcal serogroup C conjugate vaccine 4 years after introduction.* The Lancet, 2004. **364**(9431): p. 365-367.
219. Taha, M.-K., et al., *Serogroup W135 meningococcal disease in Hajj pilgrims.* The Lancet, 2000. **356**(9248): p. 2159.
220. Hahné, S.J.M., et al., *W135 meningococcal disease in England and Wales associated with Hajj 2000 and 2001.* The Lancet, 2002. **359**(9306): p. 582-583.
221. Ladhani SN, L.J., Newbold LS, Gray SJ, Carr AD, Findlow J, et al. *Invasive meningococcal capsular group Y disease, England and Wales, 2007–2009.* **18**, DOI: 10.3201/eid1801.110901.
222. Harrison, L.H., et al., *Antigenic shift and increased incidence of meningococcal disease.* Journal of Infectious Diseases, 2006. **193**(9): p. 1266-1274.
223. Jounio, U., et al., *Genotypic and phenotypic characterization of carriage and invasive disease isolates of Neisseria meningitidis in Finland.* Journal of Clinical Microbiology, 2012. **50**(2): p. 264-273.
224. Trotter, C.L., et al., *A surveillance network for meningococcal disease in Europe.* FEMS Microbiology Reviews, 2007. **31**(1): p. 27-36.
225. Bidmos, F.A., et al., *Persistence, replacement, and rapid clonal expansion of meningococcal carriage isolates in a 2008 university student cohort.* Journal of Clinical Microbiology, 2011. **49**(2): p. 506-512.
226. Maiden, M.C.J., et al., *Impact of meningococcal serogroup C conjugate vaccines on carriage and herd immunity.* Journal of Infectious Diseases, 2008. **197**(5): p. 737-743.
227. Trotter, C.L., H. Findlow, and R. Borrow, *Seroprevalence of serum bactericidal antibodies against group W135 and Y meningococci in England in 2009.* Clinical and Vaccine Immunology, 2012. **19**(2): p. 219-222.
228. Jackson LA and W. JD, *Laboratory-based surveillance for meningococcal disease in selected areas, United States, 1989-1991.* MMWR CDC Surveillance Summaries, 1993. **42**: p. 21-30.
229. Rosenstein, N.E., et al., *The changing epidemiology of meningococcal disease in the United States, 1992–1996.* Journal of Infectious Diseases, 1999. **180**(6): p. 1894-1901.
230. Joint Committee on Vaccination and Immunisation, *Immunisation Against Infectious Disease ("The Green Book")*, 2011, Stationery Office.: Edinburgh. p. 235-253.
231. Borrow, R., et al., *Influence of prior meningococcal C polysaccharide vaccination on the response and generation of memory after meningococcal C conjugate vaccination in young children.* J Infect Dis, 2001. **184**(3): p. 377-80.
232. Borrow, R., et al., *Reduced antibody response to revaccination with meningococcal serogroup A polysaccharide vaccine in adults.* Vaccine, 2000. **19**(9–10): p. 1129-1132.
233. Stamboulian, D., et al., *Safety and immunogenicity of an investigational quadrivalent meningococcal CRM197 conjugate vaccine, MenACWY-CRM, compared with licensed vaccines in adults in Latin America.* International Journal of Infectious Diseases, 2010. **14**(10): p. e868-e875.
234. European Medicines Agency, *International Conference on Harmonization: Good Clinical Practice*, in *CPMP/ICH/135/95/Step5*1996.
235. Cook, I.F., et al., *Reactogenicity and immunogenicity of an inactivated influenza vaccine administered by intramuscular or subcutaneous injection in elderly adults.* Vaccine, 2006. **24**(13): p. 2395-2402.

236. Morris, C.A., et al., *Intradermal hepatitis B immunization with yeast-derived vaccine: serological response by sex and age.* Epidemiol Infect, 1989. **103**(2): p. 387-94.
237. Mohammed, I. and M.M. Damisah, *The immunological response to polyvalent meningococcal vaccine in Bauchi State, Nigeria.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 1982. **76**(3): p. 351-353.
238. European Medicines Agency, *International Conference on Harmonization: Guidance on general considerations for clinical trials*, in *CPMP/ICH/291/95*1997.
239. European Medicines Agency, *International Conference on Harmonization: Statistical principles of clinical trials*, in *CPMP/ICH/363/96*1998.
240. Dhalla, S. and G. Poole, *Motivators of enrolment in HIV vaccine trials: a review of HIV vaccine preparedness studies.* AIDS Care, 2011. **23**(11): p. 1430-1447.
241. Gray, K., et al., *Participation in two phase II prophylactic HIV vaccine trials in the UK.* Vaccine, 2008. **26**(23): p. 2919-2924.
242. Meningitis Research Foundation. *Meningitis and Septicaemia in the UK: Facts and Figures.* 2012 [cited 2012 28/02/2012]; Available from: http://www.meningitis.org/facts.
243. Meningitis UK. [cited 2012 29/02/2012]; Available from: http://www.meningitisuk.org/.
244. Pentz, R.D., et al., *Therapeutic misconception, misestimation, and optimism in participants enrolled in phase 1 trials.* Cancer, 2012: p. n/a-n/a.
245. Stunkel, L. and C. Grady, *More than the money: A review of the literature examining healthy volunteer motivations.* Contemporary Clinical Trials, 2011. **32**(3): p. 342-352.
246. Balmer, P. and R. Borrow, *Serologic correlates of protection for evaluating the response to meningococcal vaccines.* Expert Review of Vaccines, 2004. **3**(1): p. 77-87.
247. World Health Organisation, *Requirements for meningococcal polysaccharide vaccine.*, in *World Health Organization technical report series*1976.
248. Granoff, D.M., J.A. Welsch, and S. Ram, *Binding of complement factor H (fH) to Neisseria meningitidis is specific for human fH and inhibits complement activation by rat and rabbit sera.* Infection and Immunity, 2009. **77**(2): p. 764-769.
249. Trotter, C., et al., *Seroprevalence of meningococcal serogroup C bactericidal antibody in England and Wales in the pre-vaccination era.* Vaccine, 2003. **21**(11–12): p. 1094-1098.
250. Borrow, R., et al., *Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: reevaluation of correlates of protection.* Infection and Immunity, 2001. **69**(3): p. 1568-1573.
251. Andrews, N., R. Borrow, and E. Miller, *Validation of serological correlate of protection for meningococcal C conjugate vaccine by using efficacy estimates from postlicensure surveillance in England.* Clinical and Diagnostic Laboratory Immunology, 2003. **10**(5): p. 780-786.
252. Gill, C.J., et al., *Correlation between serum bactericidal activity against Neisseria meningitidis serogroups A, C, W-135 and Y measured using human versus rabbit serum as the complement source.* Vaccine, 2011. **30**(1): p. 29-34.
253. Santos, G.F., et al., *Importance of complement source in measuring meningococcal bactericidal titers.* Clinical and Diagnostic Laboratory Immunology, 2001. **8**(3): p. 616-623.
254. Snape, M.D., et al., *Serogroup C meningococcal glycoconjugate vaccine in adolescents: Persistence of bactericidal antibodies and kinetics of the immune response to a booster vaccine more than 3 years after immunization.* Clinical Infectious Diseases, 2006. **43**(11): p. 1387-1394.

255. De Wals, P., G. De Serres, and T. Niyonsenga, *Effectiveness of a mass immunization campaign against serogroup C meningococcal disease in Quebec.* JAMA: The Journal of the American Medical Association, 2001. **285**(2): p. 177-181.
256. Espı´n Rı´os, I., et al., *Seroconversion and duration of immunity after vaccination against group C meningococcal infection in young children.* Vaccine, 2000. **18**(24): p. 2656-2660.
257. Clutterbuck, E.A., et al., *Pneumococcal conjugate and plain polysaccharide vaccines have divergent effects on antigen-specific B cells.* Journal of Infectious Diseases, 2012. **205**(9): p. 1408-1416.
258. Kelly, D.F., et al., *Plasma and memory B-cell kinetics in infants following a primary schedule of CRM197-conjugated serogroup C meningococcal polysaccharide vaccine.* Immunology, 2009. **127**(1): p. 134-143.
259. de Voer, R.M., et al., *Kinetics of antibody responses after primary immunization with meningococcal serogroup C conjugate vaccine or secondary immunization with either conjugate or polysaccharide vaccine in adults.* Vaccine, 2009. **27**(50): p. 6974-6982.
260. Findlow, H., et al., *Serum antibody kinetics following nasal or parenteral challenge with meningococcal polysaccharide in healthy adults.* Clinical and Vaccine Immunology, 2011. **18**(3): p. 424-429.
261. Giardina, P.C., et al., *Analysis of human serum immunoglobulin G against O-acetyl-positive and O-acetyl-negative serogroup W135 meningococcal capsular polysaccharide.* Clinical and Diagnostic Laboratory Immunology, 2005. **12**(5): p. 586-592.
262. Fusco, P.C., et al., *Protective meningococcal capsular polysaccharide epitopes and the role of O acetylation.* Clinical and Vaccine Immunology, 2007. **14**(5): p. 577-584.
263. Varki, A. and P. Gagneux, *Multifarious roles of sialic acids in immunity.* Annals of the New York Academy of Sciences, 2012. **1253**(1): p. 16-36.
264. Balmer, P. and R. Borrow, *Issues surrounding standardization of meningococcal group W135 serology.* Vaccine, 2007. **25, Supplement 1**(0): p. A63-A68.
265. Nicolay, U., et al., *Diphtheria booster vaccination: one or two injections?* Vaccine, 1999. **17**(18): p. 2223-2228.
266. Danilova, E., et al., *Attenuated immune response to tetanus toxoid in young healthy men protected against tetanus.* Vaccine, 2005. **23**(42): p. 4980-4983.
267. Jackson, L.A., et al., *A randomized trial to determine the tolerability and immunogenicity of a quadrivalent meningococcal glycoconjugate vaccine in healthy adolescents.* Pediatr Infect Dis J, 2009. **28**(2): p. 86-91.
268. Black, S., et al., *Immunogenicity and tolerability of a quadrivalent meningococcal glycoconjugate vaccine in children 2–10 years of age.* Vaccine, 2010. **28**(3): p. 657-663.
269. Griffiss, J.M., B.L. Brandt, and D.D. Broud, *Human immune response to various doses of group Y and W135 meningococcal polysaccharide vaccines.* Infection and Immunity, 1982. **37**(1): p. 205-208.
270. Artenstein, M.S., et al., *Cutaneous reactions and antibody response to meningococcal group C polysaccharide vaccines in man.* Journal of Infectious Diseases, 1970. **121**(4): p. 372-377.
271. Guerin, P.J., et al., *Immunogenicity of fractional doses of tetravalent A/C/Y/W135 meningococcal polysaccharide vaccine: Results from a randomized non-inferiority controlled trial in Uganda.* PLoS Negl Trop Dis, 2008. **2**(12): p. e342.
272. Maslanka, S.E., et al., *Standardization and a multilaboratory comparison of Neisseria meningitidis serogroup A and C serum bactericidal assays. The*

Multilaboratory Study Group. Clinical and Diagnostic Laboratory Immunology, 1997. **4**(2): p. 156-67.
273. Nanan, R., et al., *Acute and long-term effects of booster immunisation on frequencies of antigen-specific memory B-lymphocytes*. Vaccine, 2001. **20**(3–4): p. 498-504.
274. Bröker, M., et al., *Chemistry of a new investigational quadrivalent meningococcal conjugate vaccine that is immunogenic at all ages*. Vaccine, 2009. **27**(41): p. 5574-5580.
275. Dintzis, H.M., R.Z. Dintzis, and B. Vogelstein, *Molecular determinants of immunogenicity: the immunon model of immune response*. Proceedings of the National Academy of Sciences, 1976. **73**(10): p. 3671-3675.
276. Dintzis, R., et al., *The immunogenicity of soluble haptenated polymers is determined by molecular mass and hapten valence*. The Journal of Immunology, 1989. **143**(4): p. 1239-1244.
277. Ruben, F.L., et al., *Choosing a route of administration for quadrivalent meningococcal polysaccharide vaccine: intramuscular versus subcutaneous*. Clinical Infectious Diseases, 2001. **32**(1): p. 170-172.
278. Mohanan, D., et al., *Administration routes affect the quality of immune responses: A cross-sectional evaluation of particulate antigen-delivery systems*. Journal of Controlled Release, 2010. **147**(3): p. 342-349.
279. Ambrosino, D.M., et al., *IgG1, IgG2 and IgM responses to two Haemophilus influenzae type b conjugate vaccines in young infants*. Pediatr Infect Dis J, 1992. **11**(10): p. 855-9.
280. Mond, J.J., A. Lees, and C.M. Snapper, *T cell-independent antigens type 2*. Annual Review of Immunology, 1995. **13**(1): p. 655-692.
281. Halasa, N.B., et al., *Poor immune responses to a birth dose of diphtheria, tetanus, and acellular pertussis vaccine*. The Journal of Pediatrics, 2008. **153**(3): p. 327-332.e1.
282. Lieberman, J.M., et al., *Effect of neonatal immunization with diphtheria and tetanus toxoids on antibody responses to Haemophilus influenzae type b conjugate vaccines*. The Journal of Pediatrics, 1995. **126**(2): p. 198-205.
283. MacLennan, J., et al., *Immune response to revaccination with meningococcal A and C polysaccharides in Gambian children following repeated immunisation during early childhood*. Vaccine, 1999. **17**(23-24): p. 3086-93.
284. Richmond, P., et al., *Meningococcal C polysaccharide vaccine induces immunologic hyporesponsiveness in adults that is overcome by meningococcal C conjugate vaccine*. J Infect Dis, 2000. **181**(2): p. 761-4.
285. MacLennan, J., et al., *Immunologic memory 5 years after meningococcal A/C conjugate vaccination in infancy*. Journal of Infectious Diseases, 2001. **183**(1): p. 97-104.
286. Al-Mazrou, Y., et al., *Serologic responses to ACYW135 polysaccharide meningococcal vaccine in Saudi children under 5 years of age*. Infection and Immunity, 2005. **73**(5): p. 2932-2939.
287. Southern, J., et al., *Effects of prior polysaccharide vaccination on magnitude, duration, and quality of immune responses to and safety profile of a meningococcal serogroup C tetanus toxoid conjugate vaccination in adults*. Clin Diagn Lab Immunol, 2004. **11**(6): p. 1100-4.
288. MacDonald, N.E., et al., *Can meningococcal C conjugate vaccine overcome immune hyporesponsiveness induced by previous administration of plain polysaccharide vaccine?* JAMA, 2000. **283**(14): p. 1826-7.
289. Borrow, R., et al., *Comparison of antibody kinetics following meningococcal serogroup C conjugate vaccine between healthy adults previously vaccinated with meningococcal A/C polysaccharide vaccine and vaccine-naïve controls*. Vaccine, 2001. **19**(23–24): p. 3043-3050.

290. Findlow, H., et al., *Immunoglobulin G subclass response to a meningococcal quadrivalent polysaccharide-diphtheria toxoid conjugate vaccine.* Clinical and Vaccine Immunology, 2006. **13**(4): p. 507-510.
291. Käyhty, H., et al., *Serum antibodies to capsular polysaccharide vaccine of group A Neisseria meningitidis followed for three years in infants and children.* Journal of Infectious Diseases, 1980. **142**(6): p. 861-868.
292. Koskela, M., et al., *First and second dose antibody responses to pneumococcal polysaccharide vaccine in infants.* Pediatr Infect Dis, 1986. **5**(1): p. 45-50.
293. Russell, F.M., et al., *Hyporesponsiveness to re-challenge dose following pneumococcal polysaccharide vaccine at 12 months of age, a randomized controlled trial.* Vaccine, 2010. **28**(19): p. 3341-3349.
294. Musher, D.M., et al., *Antibody to capsular polysaccharides of streptococcus pneumoniae: prevalence, persistence, and response to revaccination.* Clinical Infectious Diseases, 1993. **17**(1): p. 66-73.
295. Linnemann, C.C., Jr, M.R. First, and G. Schiffman, *Revaccination of renal transplant and hemodialysis recipients with pneumococcal vaccine.* Arch Intern Med, 1986. **146**(8): p. 1554-1556.
296. Musher, D.M., et al., *Antibody persistence ten years after first and second doses of 23-valent pneumococcal polysaccharide vaccine, and immunogenicity and safety of second and third doses in older adults.* Hum Vaccin, 2011. **7**(9): p. 919-28.
297. O'Brien, K.L., M. Hochman, and D. Goldblatt, *Combined schedules of pneumococcal conjugate and polysaccharide vaccines: is hyporesponsiveness an issue?* Lancet Infect Dis, 2007. **7**(9): p. 597-606.
298. de Roux, A., et al., *Comparison of pneumococcal conjugate polysaccharide and free polysaccharide vaccines in elderly adults: Conjugate vaccine elicits improved antibacterial immune responses and immunological memory.* Clinical Infectious Diseases, 2008. **46**(7): p. 1015-1023.
299. Borrow, R., P.T. Heath, and C.A. Siegrist, *Use of pneumococcal polysaccharide vaccine in children: what is the evidence?* Curr Opin Infect Dis, 2012. **25**(3): p. 292-303.
300. Kayhty, H., et al., *Serum antibodies after vaccination with Haemophilus influenzae type b capsular polysaccharide and responses to reimmunization: no evidence of immunologic tolerance or memory.* Pediatrics, 1984. **74**(5): p. 857-65.
301. Arjunaraja, S., et al., *The nature of an in vivo anti-capsular polysaccharide response is markedly Influenced by the composition and/or architecture of the bacterial subcapsular domain.* The Journal of Immunology, 2012. **188**(2): p. 569-577.
302. Dagan, R., et al., *Nasopharyngeal Carriage of Streptococcus pneumoniae Shortly before Vaccination with a Pneumococcal Conjugate Vaccine Causes Serotype-Specific Hyporesponsiveness in Early Infancy.* Journal of Infectious Diseases, 2010. **201**(10): p. 1570-1579.
303. Borrow, R., et al., *Serotype-specific immune unresponsiveness to pneumococcal conjugate vaccine following invasive pneumococcal disease.* Infection and Immunity, 2008. **76**(11): p. 5305-5309.
304. Felton, L.D., et al., *Studies on the mechanism of the immunological paralysis induced in mice by pneumococcal polysaccharides.* The Journal of Immunology, 1955. **74**(1): p. 17-26.
305. Howard, J.G., et al., *Studies on immunological paralysis. 3. Recirculation and antibody-neutralizing activity of 14C-labelled type 3 pneumococcal polysaccharide in paralysed mice.* Clin Exp Immunol, 1970. **7**(4): p. 583-96.
306. Rodriguez, M.E., et al., *Immunogenicity of Streptococcus pneumoniae type 6B and 14 polysaccharide-tetanus toxoid conjugates and the effect of uncoupled*

polysaccharide on the antigen-specific immune response. Vaccine, 1998. **16**(20): p. 1941-1949.
307. Daum, R.S., et al., *Decline in serum antibody to the capsule of Haemophilus influenzae type b in the immediate postimmunization period.* J Pediatr, 1989. **114**(5): p. 742-7.
308. Lazarus, R., et al., *A randomized study comparing combined pneumococcal conjugate and polysaccharide vaccination schedules in adults.* Clinical Infectious Diseases. **52**(6): p. 736-742.
309. Hutchins, W.A., G.M. Carlone, and M.A.J. Westerink, *Elderly immune response to a TI-2 antigen: Heavy and light chain use and bactericidal activity to Neisseria meningitidis serogroup C polysaccharide.* Journal of Infectious Diseases, 1999. **179**(6): p. 1433-1440.
310. Unkeless, J.C., E. Scigliano, and V.H. Freedman, *Structure and function of human and murine receptors for IgG.* Annual Review of Immunology, 1988. **6**(1): p. 251-281.
311. Brynjolfsson, S.F., et al., *Hyporesponsiveness following booster immunization with bacterial polysaccharides is caused by apoptosis of memory B cells.* Journal of Infectious Diseases, 2012. **205**(3): p. 422-430.
312. Poolman, J. and R. Borrow, *Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines.* Expert Review of Vaccines, 2011. **10**(3): p. 307-322.
313. Peltola, H., et al., *Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age.* N Engl J Med, 1977. **297**(13): p. 686-91.
314. Väkeväinen, M., et al., *Serotype-specific hyporesponsiveness to pneumococcal conjugate vaccine in infants carrying pneumococcus at the time of vaccination.* The Journal of Pediatrics, 2010. **157**(5): p. 778-783.e1.
315. French, N., et al., *23–valent pneumococcal polysaccharide vaccine in HIV-1-infected Ugandan adults: double-blind, randomised and placebo controlled trial.* The Lancet, 2000. **355**(9221): p. 2106-2111.
316. Koh, Y.M., et al., *Outbreak of meningococcal disease linked to a sports club.* The Lancet, 1998. **352**(9129): p. 706-707.
317. Irwin, D.J., E.B. Kaczmarski, and J.M. Miller, *A case of invasive serogroup C meningococcal disease after a community vaccination programme in Rotherham, South Yorkshire.* Journal of Public Health, 1998. **20**(1): p. 112-114.
318. Duong, B.H., et al., *Decoration of T-independent antigen with ligands for CD22 and Siglec-G can suppress immunity and induce B cell tolerance in vivo.* The Journal of Experimental Medicine, 2010. **207**(1): p. 173-187.
319. Séïté, J.-F., et al., *IVIg modulates BCR signaling through CD22 and promotes apoptosis in mature human B lymphocytes.* Blood, 2010. **116**(10): p. 1698-1704.
320. Defrance, T., M. Taillardet, and L. Genestier, *T cell-independent B cell memory.* Current Opinion in Immunology, 2011. **23**(3): p. 330-336.
321. Bretscher, P. and M. Cohn, *A theory of self-nonself discrimination.* Science, 1970. **169**(3950): p. 1042-9.
322. Fernández-Sánchez, A., M. García-Ocaña, and J.R. de los Toyos, *Mouse monoclonal antibodies to pneumococcal C-polysaccharide backbone show restricted usage of VH-DH-JH gene segments and share the same kappa chain.* Immunology Letters, 2009. **123**(2): p. 125-131.
323. Kolibab, K., et al., *Immune Response to Pneumococcal Polysaccharides 4 and 14 in Elderly and Young Adults: Analysis of the Variable Heavy Chain Repertoire.* Infect. Immun., 2005. **73**(11): p. 7465-7476.
324. Zhou, J., et al., *Recurrent variable region gene usage and somatic mutation in the human antibody response to the capsular polysaccharide of Streptococcus pneumoniae type 23F.* Infect Immun, 2002. **70**(8): p. 4083-91.

325. Zhou, J., et al., *Somatic hypermutation and diverse immunoglobulin gene usage in the human antibody response to the capsular polysaccharide of Streptococcus pneumoniae Type 6B*. Infect Immun, 2004. **72**(6): p. 3505-14.
326. Wisnewski, A., L. Cavacini, and M. Posner, *Human antibody variable region gene usage in HIV-1 infection*. Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology, 1996. **11**(1): p. 31-38.
327. Binley, J.M., et al., *Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C*. J Virol, 2008. **82**(23): p. 11651-68.
328. Tian, C., et al., *Immunodominance of the VH1-46 antibody gene segment in the primary repertoire of human rotavirus-specific B cells is reduced in the memory compartment through somatic mutation of nondominant clones*. J Immunol, 2008. **180**(5): p. 3279-88.
329. Chardes, T., et al., *The human anti-thyroid peroxidase autoantibody repertoire in Graves' and Hashimoto's autoimmune thyroid diseases*. Immunogenetics, 2002. **54**(3): p. 141-57.
330. Insel, R.A., A. Kittelberger, and P. Anderson, *Isoelectric focusing of human antibody to the Haemophilus influenzae b capsular polysaccharide: restricted and identical spectrotypes in adults*. The Journal of Immunology, 1985. **135**(4): p. 2810-6.
331. Silverman, G.J. and A.H. Lucas, *Variable region diversity in human circulating antibodies specific for the capsular polysaccharide of Haemophilus influenzae type b. Preferential usage of two types of VH3 heavy chains*. J Clin Invest, 1991. **88**(3): p. 911-20.
332. Lucas, A.H., et al., *Molecular ontogeny of the human antibody repertoire to the Haemophilus influenzae type b polysaccharide: expression of canonical variable regions and their variants in vaccinated infants*. Clinical Immunology, 2003. **108**(2): p. 119-127.
333. Adderson, E., et al., *Restricted immunoglobulin VH usage and VDJ combinations in the human response to Haemophilus influenzae type b capsular polysaccharide. Nucleotide sequences of monospecific anti-Haemophilus antibodies and polyspecific antibodies cross-reacting with self antigens*. J Clin Invest, 1993. **91**(6): p. 2734 - 43.
334. Arnaout, R., *Specificity and overlap in gene segment-defined antibody repertoires*. BMC Genomics, 2005. **6**(1): p. 148.
335. Weinstein, J.A., et al., *High-throughput sequencing of the zebrafish antibody repertoire*. Science, 2009. **324**(5928): p. 807-10.
336. Boyd, S.D., et al., *Measurement and Clinical Monitoring of Human Lymphocyte Clonality by Massively Parallel V-D-J Pyrosequencing*. Science Translational Medicine, 2009. **1**(12).
337. Taillardet, M., et al., *The thymus-independent immunity conferred by a pneumococcal polysaccharide is mediated by long-lived plasma cells*. Blood, 2009. **114**(20): p. 4432-4440.
338. Lucas, A.H. and D.M. Granoff, *Functional differences in idiotypically defined IgG1 anti-polysaccharide antibodies elicited by vaccination with Haemophilus influenzae type B polysaccharide-protein conjugates*. J Immunol, 1995. **154**(8): p. 4195-202.
339. University of Oxford, *Taking Blood Samples from Colleagues or Students for Research and Teaching* 2012, University of Oxford.
340. Sblattero, D. and A. Bradbury, *A definitive set of oligonucleotide primers for amplifying human V regions*. Immunotechnology, 1998. **3**(4): p. 271-8.
341. van Dongen, J.J., et al., *Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene*

recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia, 2003. **17**(12): p. 2257-317.
342. Diagnostics., R., *454 Sequencing system Software Manual*2011, Mannheim, Germany: Roche Applied Science.
343. Alamyar, E., et al., *IMGT/HighV-QUEST: the IMGT(R) web portal for immunoglobulin (IG) or antibody and T cell receptor (TR) analysis from NGS high throughput and deep sequencing*. Immunome Res, 2012. **8**(1): p. 26.
344. Rogosch, T., et al., *IgAT (Immunoglobulin Analysis Tool): A novel tool for the analysis of human and mouse heavy and light chain transcripts*. Frontiers in Immunology, 2012. **3**.
345. Sambrook, J. and D.W. Russel, *Molecular Cloning: A Laboratory Manual*. 3rd ed. Vol. 1. 2001: Cold Spring Harbor Laboratory Press.
346. Kyte, J. and R.F. Doolittle, *A simple method for displaying the hydropathic character of a protein*. Journal of Molecular Biology, 1982. **157**(1): p. 105-132.
347. Briney, B.S., et al., *High-throughput antibody sequencing reveals genetic evidence of global regulation of the naive and memory repertoires that extends across individuals*. Genes Immun, 2012.
348. Arnaout, R., et al., *High-resolution description of antibody heavy-chain repertoires in humans*. PLoS One, 2011. **6**(8): p. e22365.
349. Baechler, E.C., et al., *Expression levels for many genes in human peripheral blood cells are highly sensitive to ex vivo incubation*. Genes Immun, 2004. **5**(5): p. 347-353.
350. Miltenyi Biotec GmbH. *Average RNA yields*. MACS Molecular 2012 05/07/2012]; Available from: http://www.miltenyibiotec.com/download/further_information_en/836/Average_RNA_yields.pdf.
351. Nishimoto, K.P., et al., *Fluorescence activated cell sorting (FACS) using RNAlater to minimize RNA degradation and perturbation of mRNA expression from cells involved in initial host microbe interactions*. Journal of Microbiological Methods, 2007. **70**(1): p. 205-208.
352. Ippolito, G.C., et al., *Antibody Repertoires in Humanized NOD-scid-IL2Rγ^{null} Mice and Human B Cells Reveals Human-Like Diversification and Tolerance Checkpoints in the Mouse*. PLoS ONE, 2012. **7**(4): p. e35497.
353. Frölich, D., et al., *Secondary Immunization Generates Clonally Related Antigen-Specific Plasma Cells and Memory B Cells*. The Journal of Immunology, 2010. **185**(5): p. 3103-3110.
354. Rizzo, J.M. and M.J. Buck, *Key Principles and Clinical Applications of "Next-Generation" DNA Sequencing*. Cancer Prevention Research, 2012.