IMMUNE RESPONSES TO PROTOTYPE GMMA VACCINE AGAINST *NEISSERIA MENINGITIDIS* IN AFRICA

by

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Abstract

Neisseria meningitidis is a major cause of meningitis epidemics in sub-Saharan Africa. A promising broadly-protective vaccine approach is outer membrane particle-based vaccine comprising GMMA (Generalised Modules for Membrane Antigens). To better understand vaccine potential and immune responses induced by GMMA, we (i) typed outer membrane vaccine antigens fHbp, NadA, NHBA and PorA in African serogroup A, W and X isolates, (ii) produced GMMA from recombinant strains expressing fHbp variant 1 or NadA-3, and (iii) generated and characterised monoclonal antibodies (mAb) against GMMA.

Characterisation of 94 isolates showed limited variability of fHbp, NadA and PorA over time and geographical region. Mice immunised with GMMA developed cross-reactive bactericidal antibody responses against diverse serogroup A, W and X isolates that targeted fHbp, NadA and other unidentified antigens. We obtained 33 hybridoma clones producing GMMA-binding mAbs. Four mAbs of immunoglobulin subclasses IgG1, IgG2a/b and IgG3 bound NadA and four IgG1 mAbs bound fHbp. The other 25 mAbs recognised seven other antigens. In bactericidal assays all anti-NadA mAbs killed the vaccine serogroup A strain. Five mAbs against unknown antigens also showed bactericidal activity against another A isolate. Conclusively, the results provide support for developing a GMMA-based vaccine with broad coverage against meningococcal meningitis for Africa.

Dedication

To my family

Tack för allt ni har gjort för mig.
Jag älskar er.

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Table of contents

List of figures

List of tables

List of abbreviations

aa	Amino acid
ANOVA	Analysis of variance
AP	Alternative pathway
Alum	Aluminium hydroxide
BBB	Blood brain barrier
BCR	B cell receptor
bp	Base pair
BSA	Bovine serum albumin
C1	Complement protein 1
C4bp	Complement 4 binding protein
CC	Clonal complex
CECAM	Carcinoembryonic antigen cell adhesion molecules
Cfu	Colony forming unit
Cm	Chloramphenicol
CMP-NANA	Cytidine 5'-monophospho-N-acetylneuraminic acid
CNS	Central nervous system
CO_2	Carbon dioxide
CP	Classical Pathway
cps	Capsule
CRM_{197}	Diphtheria cross-reactive material 197
CSF	Cerebrospinal fluid
dOMV	Detergent extracted outer membrane vesicle
DPBS	Dulbecco's phosphate buffer
ELISA	Enzyme-linked immunosorbent assay

ermC	Erythromycin
ET	Electrophoretic type
FACS	Fluoresence activated cell sorting
fH	Complement factor H
fHbp	Factor H binding protein
fw	Forward
GBS	Group B *Streptococcus*
GMMA	Generalised modules for membrane antigens
GMT	Geometric mean titre
GNA	Genome-derived neisserial antigen
GPR	Growth phase regulatory region
Hib	*Haemophilus influenzae* b
HGPRT	Hypoxanthine-guanine-phosphoribosyl transferase
4HPA	4-Hydroxyphenylacetic acid
hsp90	Heat-shock protein 90
IFA	Immunofluorescence
Ig	Immunoglubulin
IHF	Integration host factor
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IP	Intraperitoneal
IS	Insertion sequence
IV	Intravenous
kan	Kanamycin
kDA	Kilo-dalton

KND	Kassena-Nankana District
KO	Knock-out
LCCD	Late complement component deficiency
LNT	lacto-*N*-neotetraose
LOS	Lipooligosaccharide
LP	Lectin pathway
mAb	Monoclonal antibody
MAC	Membrane attach complex
MASP-2	Mannose-binding lectin-associated serine protease-2
MCDMI	Meningitis chemically defined medium 1
MH	Mueller Hinton
MHC	Major histocompatability
MBL	Mannose-binding lectin
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
NadA	Neisserial adhesin A
NadR	Neisserial adhesin Regulator
NCAM-1	Neural-cell adhesion binding antigen
NHBA	Neisserial heparin binding antigen
NHD	Nouna Health District
NIPH	Norwegian Institute of Public Health
NOMV	Native outer membrane vesicle
NspA	Neisserial surface protein A
nt	Nucleotide
OCA	Oligomeric coiled-coil adhesin

OD	Optical density
OE	Over-expressed
OM	Outer membrane
OMP	Outer membrane protein
OMV	Outer membrane vesicle
OpI/II/III	Operator I/II/III
ORF	Open reading frame
P	Probability
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PNP	P-Nitrophenyl phosphate
PorA/B	Porin A/B
Ps	Polysaccharide
rHis-fHbp	Recombinant Hexa-Histidine tagged fHbp
rHis-NadA	Recombinant Hexa-Histidine tagged NadA
RNAP	RNA polymerase
Rv	Reverse
SBA	Serum bactericidal assay
spec	Spectinomycin
ST	Sequence type
STPHI	Swiss Tropical and Public Health Institute
T4P	Type IV pili
TAE	Tris base, acetic acid and EDTA
TbpAB	Transferrin binding protein AB
TCR	T cell receptor

tet	Tetracycline
Th1/2	T helper 1/2 cells
TLR-4	Toll like receptor 4
TNFα	Tumor necrosis factor α
TT	Tetanus toxoid
v.1/2/3	Variant 1/2/3
VR	Variable region
WHO	World Health Organisation
WT	Wild type

CHAPTER 1

GENERAL INTRODUCTION

1.1 Meningococcal meningitis

Meningitis is an inflammation of the membrane surrounding the human brain and spinal cord, also known as the meninges. The inflammation is usually caused by fungal, protozoan, viral or bacterial infection, of which bacterial infection causes the most severe life-threatening symptoms (1). Medical records that document bacterial meningitis date back to the early 1800s (2). In the United States, Europe and most developed countries, *Streptococcus pneumoniae, Haemophilus influenzae* type b (Hib), *Neisseria meningitidis,* Group B *Streptococcus* (GBS) and *Listeria monocytogenes* are the most common causes of bacterial meningitis, while in Africa *S. pneumoniae*, *N. meningitidis* and Hib are the leading causative agents (3). The first outbreaks of *N. meningitidis* were reported in Geneva, Switzerland and New Bedford, Massachusetts U.S. in 1805 and 1806, respectively (4). Description of the intracellular oval *N. meningitidis* in a sample of cerebrospinal fluid (CSF) was firstly made by Marchiafava and Celli in 1884, and Anton Weichselbaum isolated the meningococcus in 1887 distinguishing it from pneumococci. *N. meningitidis* is a Gram-negative β-proteobacterium of the family *Neisseriaceae* that colonise the epithelia of the nasopharynx of humans. The anaerobic diplococcus is a strictly human pathogen (1,5). Since the initial clinical descriptions, meningococci represent a major global public health problem whereby approximately 135,000 are killed yearly due to invasive disease (6). Morbidity includes limb loss, cognitive dysfunction, visual impairment and hearing loss, developmental delays, seizure disorders and behavioural problems.

1.2 Clinical presentations

The severity of infection associated with *N. meningitidis* is the rapid onset and progression from first symptoms to severe disease (7). This depends on the amount of viable bacteria that have reached the blood stream. The incubation period of meningococcal disease is usually 3 to 4 days. During low degree of bacteraemia, bacteria are cleared and patients present with febrile flu-like symptoms. When bacteria are not cleared, overt disease develop at the start of the bacteraemia phase with initial symptoms of fever, vomiting, drowsiness, stiff neck and severe muscle pain (8). Occasionally, patients develop meningitis associated with acute inflammation of the meninges (**Figure 1.1**). Inflammation develops when a higher concentration of bacteria is present in the CSF than in the plasma. This leads to a large compartmentalised inflammatory response in the subarachnoid space with an increase in concentrations of cytokines tumour necrosis factor (TNF-α) and interleukins (IL-1β, IL-6, IL-8 and IL-10), different chemokines and other mediators (2). Patients can also develop fulminant meningococcal septicaemia, which is characterised by high levels of bacteria in the blood circulation. Large bacterial growth causes a destructive intravascular inflammatory response leading to circulatory collapse. Such vascular complications can cause loss of limbs and other symptoms including impaired adrenal, renal, pulmonary functions and disseminated intravascular coagulation with thrombotic lesions in the skin, limbs and occasionally the lungs. (9). Some of these patients may develop transient meningococcaemia with fever and a non-specific rash. On rare occasions, chronic meningococcaemia can develop that lasts from weeks to months with intermittent symptoms such as fever. However, ~30% of patients may also present without distinct signs of meningitis or fulminant septicaemia, but instead have persistent hypotension. Patients are classified

in four groups based on the sequence of events and prognosis: (i) patients with bacteraemia and no shock, (ii) bacteraemia and shock, (iii) shock and meningitis, and patients with meningitis alone (iv) (8).

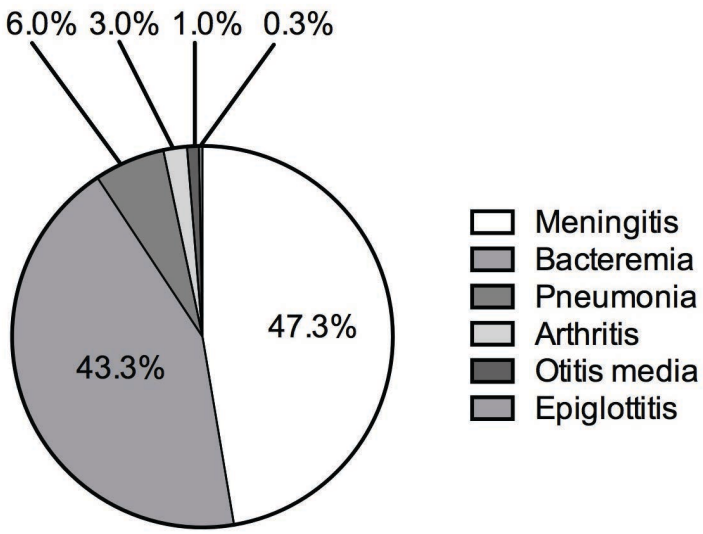

Figure 1.1. Clinical manifestations due to infection by *Neisseria meningitidis*.

The most common clinical presentation due to invasive meningococcal disease is meningitis and bacteraemia. Bacteraemia can occur without meningitis in 5-20% of invasive infections. Other less common presentations are pneumonia (6%), arthritis (2%), otitis media (1%) and epiglottitis (0.3%). Figure adapted and altered from Center for Disease Control and Prevention (10).

In industrialised countries, more than 60% that get invasive meningococcal disease develop meningitis without shock, while in developing countries the proportion is higher. Mortality due to meningitis ranges from 8%-12.5% in developed countries and is roughly half in developing countries. In epidemic situations, there is a general clinical pattern of meningococcal disease. However, for reasons not fully understood, the number of patients that present with specific clinical features vary from outbreak to outbreak (5). Therefore, rapid and proper diagnosis is pivotal.

1.3 Diagnosis

The first initial signs of meningitis are based on the recognition of symptoms of stiff neck, fever, rash and altered mental status. However, diagnosis based on identification of clinical presentations is a challenge as symptoms are variable, not specific or might not be present. Therefore, in conjunction with clinical symptoms, gold-standard detection and confirmation of infection by meningococci is done by blood and CSF culture. CSF is taken by lumbar puncture, and an initial sign of inflammation is a raised opening pressure. Culturing of CSF and blood are done on chocolate or blood agar (11). Colonies of *N. meningitidis* on blood agar appear grey, smooth, convex, glistening and with a defined edge while colonies on chocolate agar are large, colourless and opaque. Following characterisation of the colonies, non-culture methods such as Gram staining, Kovac's oxidase test and carbohydrate utilisation testing (both positive) are recommended to confirm morphological identity of cultures. CSF samples are further analysed for indicators such as presence of bacteria, polymorphonuclear pleocytosis, decreased glucose content and raised protein levels (11,12). However, culture-based tests take 48-72 hours to complete and are not very sensitive (40-80%), especially if prior antibiotic therapy is initiated (8). Alternative diagnostic methods such as antigen detection using latex agglutination assays and polymerase chain reaction (PCR) has become an increasingly used form of diagnosis for meningococcal meningitis. Latex agglutination tests provides quick results in less than 15 minutes, and the test uses serum containing bacterial antibodies or commercial antisera against the capsular polysaccharide. However, this test has a variable sensitivity between 22-93% (12). Polymerase chain reaction (PCR) can be performed on CSF, blood and serum samples with an overall sensitivity of 88% for all serogroups (13). In the United Kingdom, patients are often diagnosed by PCR without

culture. Improved antigen and nucleic acid detection systems has also allowed for the enhanced accuracy of epidemiological studies, disease surveillance control measures and rapid augmentation of course of treatment for each patient (14).

1.4 Treatment

Successful treatment depends on the speed and severity of clinical deterioration of the patient and the speed of diagnosis. The type of treatment given is based on the clinical manifestations; however, prompt start of antibiotic therapy is pivotal after obtaining blood and CSF (15). Before the 1920s meningococcal disease was fatal in up to 70-90% of cases (9). With the discovery of sulphonamides and other antimicrobial agents, case fatality rate of systemic meningococcal disease has decreased to about 10%. Resistance to sulphonamides prompted the switch to penicillin or chloramphenicol in the 1950s and 1960s. β-lactam antibiotics such as benzylpenicillin are, when used early, effective in stopping the proliferation of *N. meningitidis* in the CSF by killing the bacteria within 3-4 hours after intravenous treatment. Chloramphenicol is bactericidal for *N. meningitidis* as it can penetrate the blood brain barrier more effectively than β-lactam antibiotics (9). However, resistance to these antibiotics have also been reported, and patients infected with penicillin-resistant strains are given third-generation cephalosporin derivatives such as ceftriaxone (8). Antibiotics can also be given as a preventative measure whereby contacts of cases can be given ceftriaxone, azithromycin and quinolones to aid control of localised outbreaks. However, the most effective preventative measures are vaccines.

Depending on the clinical manifestations there are additional forms of treatment. Although controversial due to lack of good clinical trials, transfusions have been used for treating sepsis, especially in neonates (8,16). The transfusions reduce the TNF in plasma and replenish immunomodulatig compounds such as anticoagulant factors protein C and protein S. Patients with sepsis are given infusion of protein C, which is important for regulating blood clotting, to restore the plasma concentration. If adrenal haemorrhage develops, low doses of steroids is given to maintain adrenal functions (9). Despite the availability of effective antibiotics and intensive care, due to the high morbidity and mortality, 7-15% of individuals who develop meningococcal meningitis and about 40% that develop sepsis die (17). A step towards reduction of mortality is the development and improved detection, classification and typing systems for enhanced disease surveillance.

1.5 Classification and typing

Characterisation of the invasive strain is important for informed public health responses, epidemiologic monitoring, vaccine development and the management and control of meningococcal disease (18). The first routine classification and typing systems were immunological based using monoclonal antibody (mAb) panels that identified variants in the capsule (serogroup), outer membrane proteins (OMP; serotype [Porin A (PorA)] and serosubtype [PorB]) and lipooligosaccharide (immunotype [LOS]) (9). However, limitations including reproducibility between labs and incomplete coverage as a result of recombination and phase variability, which are common in *N. meningitidis*, have resulted in an increase of non-typable isolates by these means. Genetic applications to classification and typing such as multilocus

sequence typing (MLST) and multilocus enzyme eletrophoresis (MLEE), which determines sequence or electrophoretic types (ST, ET), and sequencing of OMP PorB, PorA and FetA have increased the understanding of epidemiology of meningococcal disease (19).

Invasive and non-invasive meningococcal strains express a surface polysaccharide capsule of several types. The capsule is a major virulence factor and plays a crucial role that aids transmission and colonisation, protects the bacterium from desiccation, phagocytic killing, opsonisation and complement-mediated bactericidal killing (20). The capsule forms the basis of classification into specific serogroups (1). *N. meningitidis* can be divided into 13 serogroups based on the chemical composition of the bacterial capsular polysaccharide designated A, B, C, H, I, K, L, M, X, Y, Z, 29E and W (21,22). The vast majority of invasive disease is caused by serogroups A, B, C, X, Y and W (>90%) (23). The capsular polysaccharide can be either a homopolymer or a heteropolymer consisting of monosaccharide, disaccharide, or trisaccharide repeating units (24). The capsular genes are clustered within a single chromosomal locus, *cps*, divided into six regions A-D, D' and E. Genes in region A encode for biosynthesis and polymerisation enzymes of the polysaccharide. Genes in region B and C are responsible for translocation of the polysaccharide from the cytoplasm to the cell surface, respectively (24). Region D is involved in lipooligosaccharide (LOS) synthesis, genes in region D' are methyltransferases and region E function is unknown (23). Region A of serogroups B, C, W and Y harbour the conserved genes *cssA-C* responsible for synthesis of sialic acid in the form of CMP-NANA (24). In the same region, genes *synD-G* encode for a serogroup-specific polysialyltransferase involved in capsule polymerisation, which determines the functional and nucleotide

specificity for the 4 serogroups and also forms the basis of serogroup–specific PCR for diagnosis (24). In contrast, serogroup A contains repeating units of (α1-6)-linked *N*-acetyl-mannosamine-1-phosphate encoded by *mynA-D*, and serogroup X expresses (α1-4)-linked *N*-acetyl-D-glucosamine-1-phosphate (**Table 1.1**) (25).

Table 1.1. Meningococcal serogroups that commonly cause disease and their capsular structure and operon composition. Adapted and modified from Hill *et al.* (2010) (26).

Serogroup	Capsule structure	Operon
A	Non-sialic acid capsule. Homopolymers of (α1-6)-linked-*N*-acetyl-D-mannosamine-1-phosphate	*mynA-mynD*
B	Sialic acid capsule. Homopolymers of (α2-8)-linked-*N*-acetyl-neuraminic acid	*cssA-cssD*
C	Sialic acid capsule. Homopolymers of (α2-9)-linked-*N*-acetyl-neuraminic acid	*cssA-cssD*
W	Sialic acid capsule. Heteropolymers of (α2-6)-linked-6-D-Gal(α1-4)-*N*-acetyl-neuraminic acid	*cssA-cssD*
Y	Sialic acid capsule. Heteropolymers of (α2-6)-linked-6-D-Glc(α1-4)-*N*-acetyl-neuraminic acid	*cssA-cssD*
X	Non-sialic acid capsule. Homopolymers of (α1-4)-linked-*N*-acetyl-D-glucosamine-1-phosphate	*csxA-C*

During the last 20 years, numerous methods have been developed for epidemiological studies of meningococci and for the identification of infectious strains that cause disease, which are central to epidemiological surveillance. Among these methods, MLEE and MLST are considered as 'gold standards' (19). MLEE is a powerful technique to distinguish various meningococcal clones, which involves characterisation of the alleles of 10-20 housekeeping genes indirectly by the

electrophoretic mobility of the enzymes (20). High levels of discrimination are achieved by analysing many loci, and the unique combination of alleles is called electrophoretic type (ET) (27). This technique has been used for the identification of clusters of closely related strains (clonal complexes (CC)) that are particular liable to cause disease, so called hyper-virulent lineages, because it uses variation that is accumulating very slowly in the population (28). However, the problem with MLEE is that the results are difficult to compare between different laboratories (29). Instead, during the last 10 years, MLEE has been replaced by MLST. MLST characterises the alleles of 7 housekeeping genes (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*) by nucleotide sequencing of 450-500bp from PCR fragments of the genes (28). The unique combination of alleles is termed sequence type (ST), and nucleotide sequence data of the different alleles is found on the MLST website (http://pubmlst.org/neisseria). MLST has also been employed to determine the CC of invasive and carried meningococci for enhanced analysis of clonal diversification and the identification of currently circulating hyper-virulent lineages. STs are grouped together into CC or ST complex based on their similarity to a central allelic profile or genotype. A number given heuristically identifies the ancestral genotype or CC. Different STs that are identical or share at least four loci with the ancestral genotype are grouped into that CC (30). This information is important for both national and international management of meningococcal disease and for studies on population biology and evolution (27). Despite the high diversity of the meningococcal population, only a few CCs are associated with invasive disease. For example, ST-11 complex is predominantly associated with serogroup C and W and has caused disease in Europe, Australia, the U.S., Canada and Africa. However, MLST does not provide

fine discrimination of clonal groups compared to typing utilising antigenic diversity of OMP on the cell envelope (29).

Classical characterisation of *N. meningitidis* OMPs is based on serological methods. All meningococci express either class 2 or 3 OMPs, and most also express class 1. PorA is termed as class 1 OMP while class 2 and 3 have been named PorB (PorB2 and PorB3). PorA is assigned a prefix "P1" followed by numbers, separated by commas that correspond to the subtype designation. PorA has 8 surface exposed loops (loops I-VIII), and loops I and IV being the most variable (31). A panel of mAbs used for serosubtyping react with the peptide epitopes located in the two variable regions VR1 and VR2 that confer the subtype, for example P1.20,9. The variable regions are highly immunogenic and elicit bactericidal antibodies in humans; therefore, serosubtyping has been used to identify PorA epitopes for establishing outer membrane vesicle (OMV) vaccine composition (32). However, it has been established that serological analysis of PorA using a panel of mAbs is not comprehensive. An increasing number of isolates become non-serosubtypable due to PorA not being expressed or the lack of recognition of the mAb to a variant. Therefore, the mAbs scheme has been replaced by nucleotide sequencing that allows for a more detailed analysis. The VR families and variants are now assigned based on amino acid sequence relationship rather than reactivity with specific mAbs (31,33).

The lipopolysaccharide of *N. meningitidis* is a major virulence factor that induces production of pro-inflammatory mediators, which can lead to septic shock. Since meningococcal lipopolysaccharide lack the repeating O-antigen units it is referred to as LOS. The LOS is comprised of three short oligosaccharides termed α, β and γ

chains that are used to divide *N. meningitidis* into 13 immunotypes (L1-L13) based on specific antibody reactions (34). The biosynthesis of the LOS chains is performed by glycoacyltransferases encoded by three genetic loci *lgt*-1, 2 and 3, and both commensal and pathogenic strains share a common *lgt* gene pool whereby horizontal gene transfer contributes to the genetic diversity of this locus. Nowadays, like with PorA serosubtyping, serological immunotyping has been replaced by nucleotide sequencing. However, genotypic characterisation by nucleotide sequencing has become less suitable for modern epidemiologic purposes as typing schemes usually involve variation of a few genes, which in all likelihood are under selection pressure. In the future, whole genome sequencing will provide a measure of comparing the overall relatedness of the chromosomal genome to obtain a more comprehensive understanding of meningococcal pathogenicity.

1.6 Epidemiology and pathogenesis

The epidemiology of *N. meningitidis* varies both geographically and temporally for unclear reasons (**Figure 1.2**). The worldwide annual case incidence is estimated to be around 1.2 million with 135000 deaths and a case fatality rate of at least 10% (6). However, the incidence of meningococcal disease in industrialised countries have predominantly lower annual rates of approximately 0.5-1.1/100000 of population per year while countries in the "meningitis belt" of sub-Saharan Africa have high rates between 10-100/100000 (endemic) and up to 1000/100000 during epidemics (6). The rate of meningococcal disease is also age dependent, and in general the highest peak of disease incidence is seen in children under 2 years of age and a second peak in young adolescents and adults between ages 14-25 (35). Although 98% of cases occur sporadically, there are several genetic, environmental and behavioural risk factors that

increase the risk of contracting *N. meningitidis* (9). For example, in the sub-Saharan Africa, complement protein 6 (C6) deficiency and the dry weather are important contributing factors (1,36).

1.6.1 Africa

Although incidence of meningococcal disease is difficult to estimate due to variations in surveillance, high incidence countries mostly include countries of the sub-Saharan African meningitis belt. These 25 countries, extending from Senegal in West Africa to Ethiopia in the East, experience superimposed frequent endemics that constitute a major public health problem. The epidemics involve, by WHO definition, >100 cases/ 100000 population, and occurs every 8-10 years and can last for several years. Mostly children less than 5 years of age are affected (37). This pattern of epidemics was firstly described in 1963 by Lapeyssonnie who defined the African 'meningitis belt'. Although the rates of disease vary geographically, the rates also depend on the spread of new strains, vaccination status of the population and environmental factors (6). For example, epidemics usually coincide with the dry season, whereby it is thought that the low humidity and dust-wind blowing from the Sahara affect the mucosa causing damage and produces irritant coughs that aids transmission. Cases can also occur in clusters and localised outbreaks throughout the year. Outbreaks involve three or more confirmed primary cases over a period of less than or equal to 3 months with a primary attack rate of more than or equal to 10 cases per 100000 population (10). In sub-Saharan Africa, serogroup A was most associated with seasonal hyperendemic and epidemic meningitis prior to 2010 (22). The ST5 clone was the first pandemic wave and predominated until 1999. However, in 1995 a new closely related clone, ST-7, emerged in Algeria and has since caused major epidemics and replaced ST5

(38). Since 2003, a major increase in disease rates due to a new clone ST-2859, particularly in Burkina Faso, has been detected (39). Historically serogroup W was responsible for a small subset of meningococcal infections globally; however, the ST-11 complex/ET-37 complex that emerged in 2000 during the Hajj pilgrimage in Saudi Arabia is now causing sporadic disease and epidemics. This complex is commonly associated with serogroup C capsule, thus suggesting there could have been a capsular switch from serogroup C to W. In 2002, the ST-11 clone was responsible for an epidemic in Burkina Faso with about 13,000 cases (40). In May 2012, an outbreak of serogroup W in Burkina Faso contributed to a total of 553 deaths (41). Although outbreaks of serogroup X are not of the same magnitude as serogroup A and W, incidence of meningitis due to X has been on the increase. In 2006, from January to June, an outbreak with over 550 cases infected with serogroup X ST-181 occurred in the south-western region of Niger including Niamey with 27.5 cases per 100,000 population and an incidence of 74.6 cases per 100,000 children between ages of 5-9 years. Delrieu *et al*. (2011) showed an increase in incidence of serogroup X from 2009 to 2010 (4% to 35% of all confirmed cases) in Burkina Faso (42). The authors describe an epidemic involving serogroup X in northern and central regions of Burkina Faso during 2010, which occurred with a seasonal incidence previously only reported in the meningitis belt for W and A. Serogroup X disease has also been reported in Togo accounting for 86% of 92 cases in the Kara Region, and in Ghana and western Kenya. There have also been occasional outbreaks of serogroup C (38).

1.6.2 United States

In comparison to Africa, since the end of Second World War until recently incidence rates in the U.S. fluctuated between 0.5 and 1.5 cases/ 100,000 population/ year.

Surveillance data from 1999-2008 show that the highest rates of meningococcal disease were in children <4 years (~2/100,000) mainly due to serogroup B and adolescents between 15-19 years (~1/100,000) being caused by serogroup C, Y and W. Most outbreaks of serogroup C detected in colleges and schools have been caused by the ST-11 complex/ET-37 complex (9,25). Although there are prominent fluctuations in the meningococcal serogroup distribution, currently serogroup B contributes to 23% of the cases, 31% serogroup C, 35% serogroup Y and 11% serogroup W.

1.6.3 Europe

Serogroup A was the most common cause of invasive disease in Europe before the First and Second World Wars. However, for reasons not fully understood, since 1970's it has ceased to be the primary cause. The vast majority of cases were then caused by serogroup C with an overall incidence of 1.01/100,000, but with higher rates in the UK, the Netherlands, the republic of Ireland and Norway. Since the late 1990s/early 2000s, these countries experienced outbreaks due to the hypervirulent serogroup C clone of ST-11 complex/ET-37 complex. The growing burden of disease prompted the introduction of the meningococcal serogroup C conjugate vaccine in the UK in 1999, which was successful in reducing burden. Ireland and Spain followed this example in 2000, the Netherlands in 2002, Belgium 2002, Iceland 2002, Portugal 2006, Greece 2006, and Germany in 2006 (43). Currently, serogroup B ST-41/44 complex is the most important cause of disease, responsible for about 50% of cases (44). The age distribution of cases varies across countries, however, children under 1 year of age and young adolescents at universities are mostly affected. Disease can occur throughout the year and peak in the winter months and early spring.

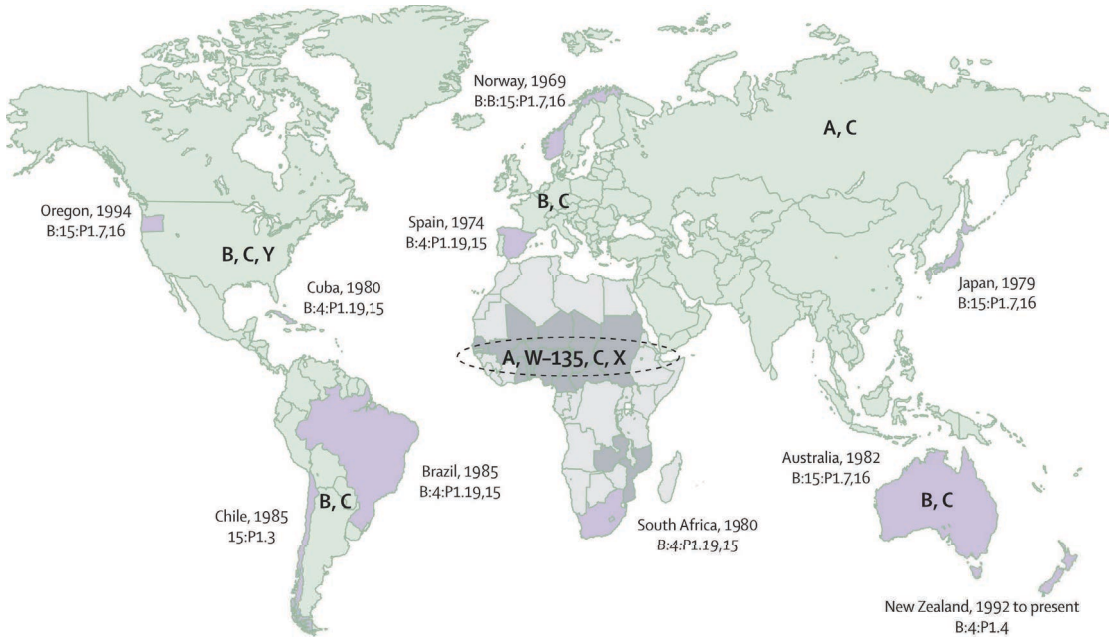

Figure 1.2. Worldwide distribution and prevalence of the major serogroups of *Neisseria meningitidis*.

The sub-Saharan African meningitis belt is marked with dotted lines. Adapted from Stephens *et al*. (2007) (9).

1.7 Meningococcal colonisation and carriage

The natural reservoir of *N. meningitidis* is the human nasopharyngeal mucosa, and colonisation of the nasopharynx is the first step in the establishment of a human carrier state and invasive disease (37). The period of carriage until progression to invasive disease is variable. It can be transient or intermittent and can last for many months and can change in populations over time (23). However, carriage isolates are very diverse in terms of ST and often non-capsulated (50%) (29). Most individuals are colonised with only one strain. Due to the diversity of carriage isolates, rates of carriage are correlated with colonisation with hyper-invasive clones. Rates of carriage

in Europe are low at an early age, increase during adolescence, peak at 20-24 (10-35%) and decrease in older age groups to <10% (29). While rates of carriage in Africa are lower ranging from 3-30% affecting mostly young children in the age group 1-4 years (22). Carriage can be an immunising process allowing for the systemic production of antibodies in response to capsular and non-capsular antigens. Prolonged colonisation does not equate to protection from subsequent colonisation or invasion. (38). Most individuals colonised asymptomatically in non-epidemic situations carry strains that may be capsulated or non-capsulated, whereas during epidemic situations, the carriage strains are often capsulated (26).

The establishment of colonisation and carriage state starts with transmission of the bacteria through respiratory droplets and secretions; however, the required inoculum size is unknown (1). Adhesion to the mucosal surfaces is essential to withstand mechanical forces during nasopharyngeal colonisation when bacteria are exposed to mucus flow, and this is promoted by various adhesins. Common adhesins in *N. meningitidis* are Type IV pili (PilC, PilQ) Opa, Opc, LOS and NadA. Their receptors on the epithelial cells include CD46, CEACAM1, vitronectin and β1-integrins. Meningococcal type IV pili (T4P) initiate the initial adherence to non-ciliated epithelial cells through the receptor CD46 by major PilE and PilX proteins. The pili play an important role in maintaining adherence to the endothelial cells under high flow conditions that poses shear stress on the meningococci. For stronger intimate cellular interactions required for stable colonisation, other adhesins such as Opa and Opc are involved. Although, the capsule is essential for protecting the bacteria from environmental and host immune factors, capsulated bacteria can only adhere using the pili as the capsule shields other adhesins preventing intimate attachment (26). Phase

variation of the capsule, whereby the bacteria can switch capsule production on/off is beneficial following initial attachment. When capsulate meningococci initially adhere to epithelial cells, a two-component regulatory system PhoP-PhoQ can be activated that leads to the loss or down-regulation of the capsule (45). Non-capsulated meningococci can easily engage intimately with cells through Opa and Opc, which promote barrier penetration. Opc is a 10-stranded β-barrel protein that binds host extracellular matrix proteins, and Opa is an eight-stranded β-barrel protein that binds CEACAMs (carcinoembryonic antigen cell adhesion molecules) (1,45). Following Opc engagement, a number of signalling events result in the recruitment of factors that account for the formation of epithelial cell pseudopodia that engulf and internalises the bacterium (1). The meningococci also proliferate on the surface of the epithelial cells forming small microcolonies at the site of attachment (45). Biofilm formation is essential since the bacteria are shielded against the host immune system through a matrix consisting of lipids, DNA and polysaccharides (46).

1.8 Cell invasion and invasive disease

Compared to carriage isolates, invasive strains are from a limited number of hypervirulent lineages (29). As *N. meningitidis* is an obligate human pathogen, there is no 'real' animal model for meningococcal cell invasion. The hypotheses of pathogenesis come from studying post mortem samples and/or biopsies of skin lesions. However, pathology is initiated when the meningococcus crosses the mucosal barrier where it reaches the bloodstream, survives, replicates and disseminates into various tissues and eventually crosses the blood brain barrier (BBB) into the central nervous system (CNS) and cause meningitis (47) (46). The BBB is a specialised structural component that separates the blood from the brain and spinal cord and

protects the CNS from bacteria that have reached the bloodstream through endothelial cells (46,48). Although, the entry of the meningococcus into the CSF is still unclear, there are four possible strategies that the bacterium can use to cross the BBB. Following survival in the bloodstream due to various virulence factors, the blood-borne bacterium can adhere and proliferate in both peripheral and brain blood microvessels (vascular colonisation). Subsequently, the endothelial cells can internalise a small number of bacteria that can then further transcytose (26). The meningococci are able to survive in a membraneous vacuole and translocate through the endothelial layer within 18-40 hours. The survival of the bacteria is determined by factors such as IgA_1 protease, which prevents phagosomal maturation and the up-regulation of expression of capsule. Its ability to replicate intracellularly is facilitated through the acquisition of iron through specialised transport systems such as transferrin binding protein (TbpAB) (1). Secondly, as with adhesion to non-ciliated epithelial cells, adherence to vascular endothelial cells is mediated by T4P and the receptor CD147 (47). Following attachment, the intimate interaction can cause signalling events that lead to disruption of the intercellular junctions enabling paracellular passage. The third route involves endothelial damage by LOS-mediated cytopathic effects. LOS induces inflammation of the endothelial cells of the vasculature, the release of IL-6 and TNF-α and activation of the complement and coagulation cascades that leads to capillary leakage and necrosis facilitating meningococcal penetration through the endothelia into the peripheral cavity. The last possible route involves leukocyte-facilitated transport by infected phagocytes (**Figure 1.3**) (49). All of these options are not exclusive, however, suggestive from *in vitro* data the most likely route is paracellular (46).

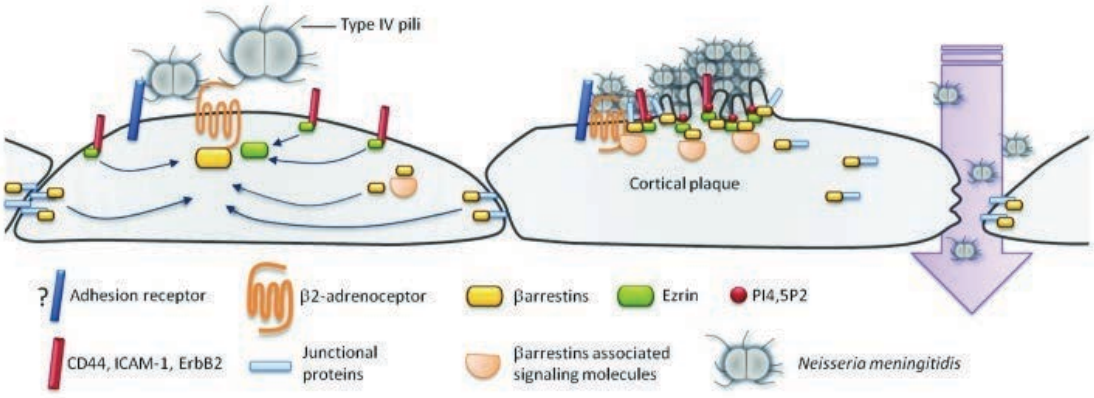

Figure 1.3. Transmigration and formation of cortical plaque by *Neisseria meningitidis.*

The meningococci use Type IV pili to interact and adhere to brain microvascular endothelial cells with CD147 as receptor. Following initial adhesion, the pili recruits and activates the β2-adrenoceptor, which leads to the organisation of specific cytoplasmic molecular complexes referred to as cortical plaques. Local production of PI4,5P2 mediates cortical plaque formation through the accumulation of ezrin and ezrin binding receptor such as ICAM-1 and CD44 and the accumulation of β-arrestins and β-arrestin-binding molecules such as Src, p120-catenin and VE-cadherin. This results in formation of microvilli like protrusions that function to protect the bacterial colonies from blood flow and the opening of the cell-cell junctions, which allows transmigration through the endothelium. Adapted from Coreuil *et al*. (2012) (46).

1.9 *N. meningitidis* and the complement system

The complement system is a major constituent of the immune system and an important element of innate immunity, especially against Gram–negative bacteria. It is composed of about 35 plasma proteins and membrane bound molecules that are mostly produced in the liver, but also from epithelial cells that are active at mucosal surfaces in the nasopharynx (50). The complement system is initiated by the classical pathway (CP, initiated by antigen-antibody interactions) or lectin pathway (LP, binding of mannose-binding lectin (MBL) to microbial carbohydrates) and amplified by the alternative pathway (AP), which involves a series of proteolytic steps (**Figure**

1.4) (51). Engagement of complement protein 1 (C1) complex, which consist of C1q, C1r and C1s, activates the CP by either binding directly to bacteria or to clustered antibody (IgG and IgM) complexes on the surface of bacteria. Upon engagement of C1q, the C1s and C1r proteases become activated and cleave C4 and C2 to yield C4b2a or C3 convertase. C3 convertase cleaves C3 to form C3a and C3b. C3b is an effector protein that covalently binds to the surface of a pathogen resulting in phagocytosis, accentuation of inflammatory responses, chemotaxis and lysis through the insertion of the membrane attack complex (MAC) into the bacterial outer membrane (50). Similarly, LP is initiated upon binding to MBL on the surface of bacteria that result in cleavage of C4 and C2 by the MBL-associated serin protease-2 (MASP-2) to generate C3 convertase. Moreover, C3b can interact with factor B and form C3bBb (C3 convertase of AP), and its activity is stabilised by properdin (36). The convertases formed from CP, LP and AP activation are able to cleave C5 into C5a and C5b that initiates the assembly of the MAC leading to clearance of the bacteria (51). Clearance of *N. meningitidis* through MAC is especially important, which is highlighted by patients who suffer from complement deficiencies such as C5-C9 terminal components of MAC.

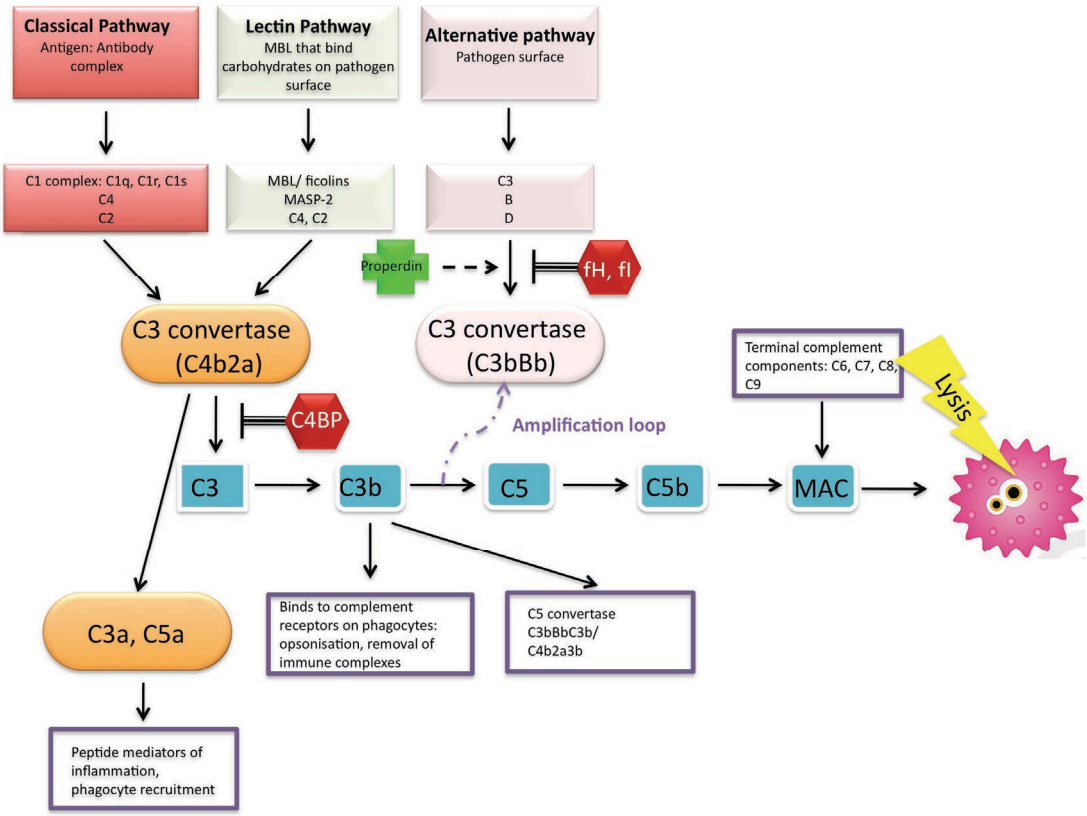

Figure 1.4. The three complement pathways.

The series of proteolytic cleavages all lead to the formation of C3 convertase. Increased cleavage of C3 also contributes to the assembly of C5 convertase that allows for the formation of the MAC that consist of C5b-C9. Polymerisation of C9 takes place inside the bacterial membrane forming a pore that triggers lysis. Arrows indicate activation and T-shapes indicate inhibition.

1.10 Complement deficiencies: genetics of host susceptibility to meningococcal infection

The discovery of individuals with complement deficiencies has contributed to the understanding of the importance of the complement system in host defence against *N. meningitidis*. Complement deficiency can be either inherited or acquired. Inherited deficiencies are rare and occur in about 0.03% of the general population, which also

depends on the complement protein and the ethnicity of the population. For example, hereditary deficiency of C9 in the Japanese population is more common (0.1%) than C7 deficiency (0.005%). Acquired deficiencies are more common and are usually associated with inadequate production, increased consumption and excretion of complement components due to autoimmune disorders (14). For example, about half of patients with systemic lupus erythematosus are deficient in C3 and C4 due to immune complex formation that activates and consumes complement, thus predisposing them to meningococcal disease (52).

Individuals that hereditary lack terminal components of MAC, especially C6, suffer from marked susceptibility of meningococcal infection (53). Due to the inability to form MAC and cause lysis of bacteria, these patients with a late complement component deficiency (LCCD) have a 1,000-10,000-fold higher risk of meningococcal disease and also susceptible to infection by *N. gonorrhoeae*. However, they are not at greater risk of contracting other Gram-negative infections. The increased risk of developing disease affects anyone who has the deficiency, although it is more common for adolescents and young adults due to their increased exposure (14,38). Interestingly, reduced serum lytic activity in LCCD patients results in the lower release of LOS limiting pro-inflammatory cytokine release (TNF-α, IL-6, IL-1) and chemokines, thus endotoxic shock (14). The course of infection in LCCD patients is therefore more benign than immunocompetent individuals (51). In contrast, individuals lacking other complement components such as properdin may be more severe as they are not able to clear bacteria by opsonophagocytosis and lysis, and therefore have a poorer prognosis than MAC deficient persons (51). Properdin deficiency is the most common genetic defect of the AP, which is inherited in an X-

linked manner (14,53). Another common hereditary complement deficiency in the Caucasian population is MBL polymorphism (5-10%). Polymorphism has been associated with predisposition to numerous of infections such as *N. meningitidis* (53). Moreover, factor H (fH) is a soluble negative regulator present in the serum and on mucosal surfaces. fH limits the amplification of complement activation of AP by functioning as a co-factor for factor I that mediates the cleavage of C3b to iC3b. It is also able to dissociate C3bBb. Polymorphism of the promoter of the fH gene can result in an increased production of fH (52). This results in the increased risk of developing invasive meningococcal disease as meningococci can use fH as a mechanism to subvert complement activation.

1.11 Bacterial factors important for the avoidance of complement-mediated lysis

There are two main factors that *N. meningitidis* uses to avoid complement-mediated lysis: capsule and LOS (51). Invasive hypervirulent strains recovered from the bloodstream of patients encompass a capsule. The capsule contains sialic acid (except serogroup A) that on the surface of endothelial cells inhibits complement activation (50). This is by preventing the insertion of MAC, however, the exact mechanism is not fully understood. A study has shown that the capsule of serogroups B, C, W and Y that contain *N*-acetyl neuraminic acid activates the AP and serves as a site for C3 fragment deposition distally from the bacterial membrane (54). Moreover, *N. meningitidis* is able to shed outer membrane vesicles (OMV), which can also initiate

complement activation in the blood remotely, redirecting the immune system away from the meningococci.

N. meningitidis is able to express 13 different LOS types; however, only limited subsets are predominantly disease-causing isolates. LOS plays a role in both adherence of the bacterium and activation of the innate immune system. The LOS of meningococcus has an α and β-chain extending from the oligosaccharide core. The lacto-*N*-neotetraose (LNT) moiety of the α-chain is identical to a human blood group antigen, thus it can avoid the immune system by expressing host structures (52). Invasive strains of L3,7,9 immunotype express the LNT epitope, and can be further modified by sialylation increasing serum resistance.

A known feature of *N. meningitidis* is that it can recruit negative complement regulators to the bacterial surface to promote survival *in vivo*. Negative regulators of complement obstruct excessive complement activation. C4 binding protein (C4bp) and fH are two examples. C4bp is a soluble negative regulator that dissociates C3 convertase of CP and LP, and is also a co-factor for Factor I-mediated C4b to C4c and C4d cleavage (14). On the meningococcus, PorA binds C4bp and strains lacking PorA are more sensitive to serum. The factor H binding protein (fHbp) on the meningococcal surface is able to bind circulating fH in the blood to the bacterial surface protecting itself from complement–mediated killing (14,51).

1.12 Correlation with protection

To support licensure of meningococcal vaccines, safety and efficacy studies demonstrating that serum bactericidal antibodies are elicited against the vaccine antigens that activate complement-mediated bacteriolysis forms such basis (2,32). Serum bactericidal assay (SBA) is the "gold-standard" measurement of such protection. The principle of the assay was established by Goldschneider in 1969 where serological studies in military recruits during a serogroup C epidemic determined that a protective serum SBA titre was conferred at ≥1:4 using human complement as an exogenous source (55). This is currently accepted as a surrogate of vaccine induced protection (36). This applies in particular during evaluation of new antigens and their ability to elicit antibodies that are bactericidal.

1.13 Prevention of meningococcal disease: Vaccine

1.13.1 Polysaccharide vaccines

Meningitis and septicaemia caused by *N. meningitidis* can be treated with antibiotics; however, invasive disease by the meningococcus is feared for its rapid progression and propensity to cause illness. Early diagnosis is pivotal for successful treatment, but it is rarely achieved, therefore, emphasis has been placed on prevention through vaccination (44). Antibiotics are effective, but in the context of recurrent epidemics in the meningitis belt, high disease burden and fragile health care systems, sustained protection is essential (55). The first effective meningococcal vaccine was developed in 1972 in response to meningitis epidemics in military recruits in the U.S. after it was demonstrated by Gotschlich, Goldschneider and Artenstain that extracts of polysaccharide was immunogenic (55). At that time, the polysaccharide was

unconjugated, and such vaccines do not invoke T-cell help. This is because polysaccharides are T-cell-independent antigens, and the antigen is not internalised, processed and presented on major histocompatability class II molecules (MCHII) on B cells (56,57)(57-59). Consequently, CD4+ T helper cells are not recruited, which results in a weak proliferation of B cells to produce IgM and IgG2, preventing development of memory B cells in adults (3,4,55,59). Un-conjugated polysaccharide vaccine only induce short-lived protection, and antibody concentrations wane rapidly (4,57). The highest meningococcal disease burden is seen in infants and young children (5,55). Consequently, one of the most important limitation with polysaccharide vaccines is that children under 2 years of age cannot use these vaccines due to poor responses to T-cell independent antigens in the absence of T-cell help (**Figure 1.5**) (60,61). Further limitations with such vaccines include hyporesponsiveness if given in repeated doses as primary B cells with affinity to the polysaccharide are depleted (2,14,43). The un-conjugated vaccines are also ineffective against carriage with only a short-term effect due to lack of anamnestic response to later doses of the polysaccharide (3).

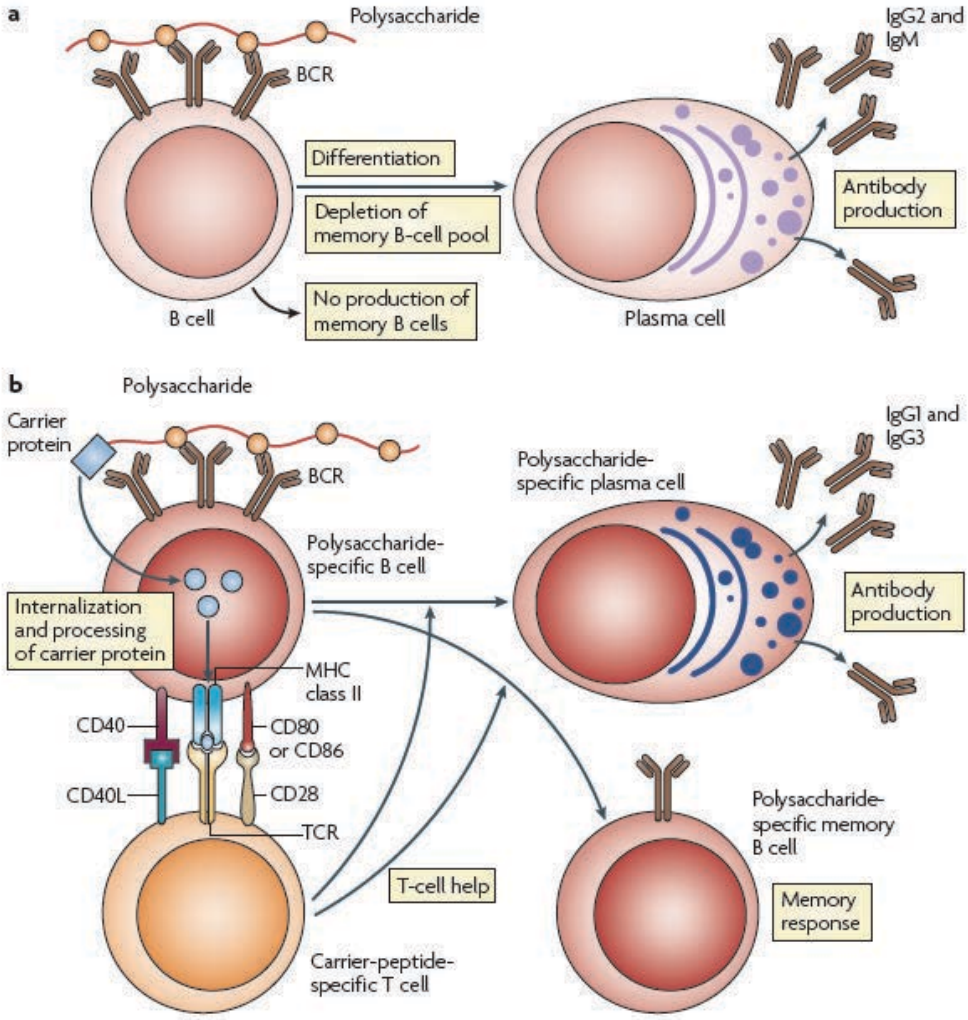

Figure 1.5. Immune responses to polysaccharide and conjugate polysaccharide vaccines.

Panel A: Polysaccharides are T-cell independent antigens, and they can only cross-link B-cell receptors (BCR). Without recruitment of T-helper cells, cross-linking only drives the production of immunoglobulin (IgG) of subclass M and G2, and depletes the memory B cell pool since new memory cells are not augmented. Panel B: Polysaccharide conjugated to a carrier protein allows for the induction of memory B cells and IgG1 and IgG3 antibody production. The polysaccharide cross-links the BCR while the carrier proteins gets internalised, processed and presented on major histocompatability class II molecules (MHC class II), which recruited T-helper cells recognise through T-cell receptor (TCR). CD40-CD40 ligand binding is also initiated along with CD80-CD28 that promotes T-cell help for the production of plasma cells and memory B cells. Adapted from Pollard *et al.* (2009) (61).

1.13.2 Conjugated polysaccharide vaccines

Conjugation of the polysaccharide to a protein carrier that stimulates T-cells such as diphtheria or tetanus toxoid was a major breakthrough in polysaccharide vaccine development in the 1980s. The United Kingdom was the first country to introduce a serogroup C conjugate polysaccharide vaccine (MCC) in 1999 as part of routine scheduled infant immunisation with a catch up campaign for children and adolescents up to 18 years of age (4). Conjugation allows for the carrier protein to be internalised and processed by the polysaccharide specific B cell with the peptide situated on BCR (61). The fragmented carrier protein is subsequently presented on MHCII receptors, which results in T-cell help for the production of plasma cells and memory B cells (**Figure 1.5**) (59). They also provide robust immunity in young children and infants, and the responses have a longer duration of protection than un-conjugated vaccines. Anamnestic responses to further doses is effective against asymptomatic carriage in the nasopharynx, which results in reduced transmission and herd immunity (3). Therefore, they can be used in a population-scale intervention (1,43). Currently available conjugate vaccines are listed in **Table 1.2** (14).

Table 1.2. Conjugate vaccines against *Neisseria meningitidis*.

Adapted from Cohn and Harrison (2013) (63).

Vaccine	Manufacturer	Serogroups	Licensed age group	Protein conjugate
Menveo™	Novartis vaccines	A, C, Y, W	2-55 years	Diphtheria cross-reactive material 197 (CRM_{197})
Menactra™	Sanofi Pasteur	A, C, Y, W	9 months-55 years	Diphtheria toxoid
Meningitec™	Wyeth vaccines	C	>2 months	CRM_{197}
Menjugate®	Novartis vaccines	C	>2 months	CRM_{197}
Neis-Vac-C™	Baxter Bioscience	C	>2 months	Tetanus toxoid (TT)
MenAfriVac™	Serum Institute of India	A	1-29 years	TT
MenHibrix®	GlaxoSmithKline	C, Y[a]	6 weeks-18 months	TT

[a]Includes serotype b *Haemophilus influenzae* polysaccharide.

In 2010, the meningococcal serogroup A polysaccharide glycoconjugate vaccine (PsA-TT) MenAfriVac™ was introduced in three countries in sub-Saharan Africa to prevent invasive meningococcal disease caused by *N. meningitidis* serogroup A as part of a collaborative partnership (Meningitis Vaccine Project) (63). The low-cost conjugate vaccine was first introduced in Burkina Faso, and all individuals of age 1-29 were immunised to ensure maximum effectiveness. Since the roll-out of the vaccine, 94% coverage has been achieved. Extensive surveillance and carriage studies have shown that, although carriage rates were low during epidemics before vaccine introduction, serogroup A can potentially be eliminated. The incidence of meningitis fell by 99.8% in the first year, and there was no case of serogroup A disease among the vaccinated individuals. Moreover, 1 year after the campaign, no asymptomatic carriage was detected in individuals sampled and tested (63). However, 1 year after

vaccine implementation, rates of serogroup W and X disease are increasingly contributing to the burden of disease. With the high costs of conjugate polysaccharide vaccines, an affordable vaccine that provides broad protection in sub-Saharan Africa against all serogroups causing disease is needed (65).

1.14 Vaccines against meningococcus serogroup B

1.14.1 Detergent extracted outer membrane vesicles (dOMV)

Effective conjugated polysaccharide vaccines against serogroups A, C, Y and W are available, but no capsular vaccine exists against serogroup B meningococcus (66). Its polysaccharide is an α(2-8) linked polysialic acid that is chemically identical to the human neural glycoproteins polysialic acids such as neural-cell adhesion molecule (NCAM-1) (5,50). Besides poor immunogenicity of the polysaccharide there is also a safety concern because it could elicit autoantibodies (45). In the absence of an immunogenic polysaccharide vaccine, attempts have been made to develop protein-based vaccines (68). One such strategy is the use of dOMV vaccines from whole bacteria. Treatment with the detergent deoxycholate increases the yield of OMV release, but also decreases endotoxin activity by removing LOS. The dOMVs contain 4-5 major OMPs such as PorA, and various other periplasmic and cytoplasmic proteins (5). dOMV vaccines are safe for use in humans (4,67,69). For example, a dOMV vaccine (MeNZB) was licensed in New Zealand against serogroup B in 2004 when incidence rates had reached 17.4 cases per 100,000 people, and used to control regional and national outbreaks (5,44,69). However, serum bactericidal antibodies produced against dOMV vaccines are directed primarily against immunodominant PorA, which is highly variable in terms of sequence and expression (4,66,68). The

prevalence of certain variants of PorA also changes over time and across geographical regions. To achieve a broad coverage of above 75% of isolates in a country, as many as 9 different serosubtypes of PorA could be necessary (44). Therefore, detergent extracted dOMV is best suited for curtailing epidemic outbreaks caused by a specific predominant strain with conserved PorA rather than in a population-scale intervention (5,14,70).

1.14.2 Protein-based vaccine

The design of a protein-based vaccine is complicated because of the genetic and antigenic variability exhibited by *N. meningitidis*, and its propensity for horizontal gene transfer. Until recently, no broadly protective vaccine against endemic serogroup B was available, although it accounts for more than 50% of meningococcal cases in some industrialised countries (44,63). In an attempt to identify protein antigens against serogroup B, reverse vaccinology approaches have been used. (5). Reverse vaccinology is a genome-based approach where vaccine candidates are identified by *in silico* analysis of a pathogen's genome for potential surface exposed antigens or secreted proteins that could be accessible to antibodies (50,71). The search predicted around 600 novel antigens, which were expressed in *Escherichia coli* and used to immunise mice. Analysis of the sera identified 29 surface-located proteins that elicited bactericidal antibodies (2,63). Of these, the most promising candidate antigens identified included fHbp, previously known as lipoprotein 2086 (LP2086) or genome derived neisserial antigen 1870 (GNA1870), Neisserial heparin binding antigen (NHBA) formerly known as GNA2132 and Neisserial adhesin A (NadA; GNA1994) (66). NadA mediates bacterial cell-adhesion and invasion, and it is classified into five main alleles 1-5 (72,73). The lipoprotein NHBA binds heparin

through arginine-rich regions known to increases serum resistance (30). It is also present in all strains tested to-date and induces bactericidal antibodies if enough protein is expressed on the outer membrane. fHbp is a surface-exposed lipoprotein that binds human complement fH on the bacterial cell surface (71,74,75). The gene is present in most invasive meningococcal isolates independent of serogroup, and can be divided into three main variants (v.1, 2 or 3) (61). Together these antigens, and a dOMV with a PorA P1.7-2,4 from a New Zealand serogroup B strain, are formulated into meningococcal B vaccine called Bexsero® or 4CMenB (**Figure 1.6**).

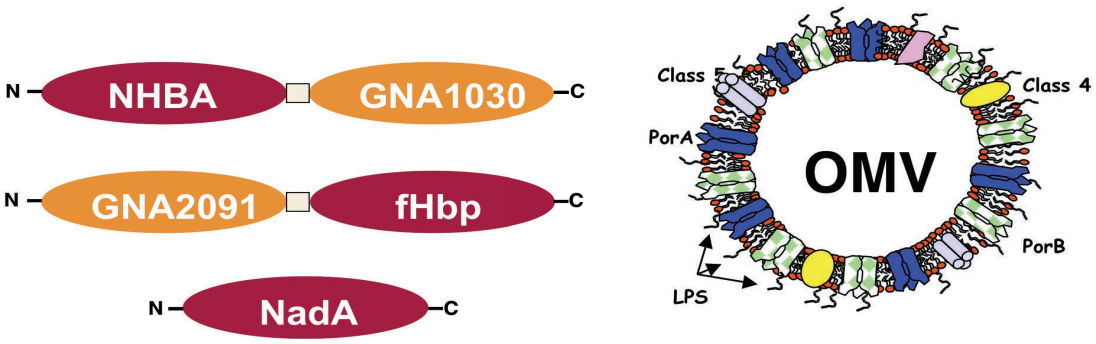

Figure 1.6. Components of the 4CMenB vaccine (Bexsero®).

The vaccine contains four major meningococcal serogroup B components. Two are recombinant fusion proteins: GNA2091 fused with fHbp and NHBA fused with GNA1030, and recombinant NadA. The dOMV with PorA serosubtype P1.7-2,4 was derived from a serogroup B strain-specific outbreak in New Zealand. Adapted from Toneatto *et al*. (2011) (66).

1.14.3 Native Outer Membrane Vesicles (NOMV)

The dOMV are safe for use in humans, but dOMVs induces antibody responses mainly directed to the antigenically variable PorA, limiting its utility (76). Extraction of such vesicles requires the use of detergent that in the process removes desirable antigens that may elicit broader bactericidal antibody responses. Compared to dOMVs, NOMVs are vesicles spontaneously and naturally released from Gram-negative bacteria. Hydrophobic OMPs are retained as there is no detergent involved in the processing, and the antigens are presented on the membrane in their native conformation (76–78). In these vesicles, endotoxicity is reduced by preparing NOMVs from recombinant strains with a deleted *lpxL1* or *lpxL2* genes. These encode for acyltransferases in the LOS biosynthesis pathway, and the mutants have a penta- or tetra-acylated LOS, respectively, instead of wild-type hexa-acylated LOS (76). The mutant forms of LOS are less effective at being recognised by Toll-like receptor 4 (TLR-4) and stimulate the receptor (50). Consequently, the release of pro-inflammatory cytokines by human peripheral blood mononuclear cells is reduced by 100-1000-fold, similar to the level of dOMV vaccines licensed for use in humans (8, 41).

Previous studies have shown that NOMV vaccines with over-expressed fHbp induce broad cross-reactive bactericidal antibodies against a panel of serogroup B strains and diverse African strains (76,78,79). Koeberling *et al*. (2008) prepared a NOMV vaccine from a serogroup B recombinant strain H44/76 with deleted *lpxL1* with over-expressed fHbp 10-fold higher than wild-type expression levels (80). In mice, the vaccine elicited broader bactericidal antibody responses against genetically diverse serogroup B meningococcal isolates with heterologous PorA compared to NOMV

without over-expressed fHbp or recombinant fHbp. Likewise, Pajon *et al*. (2011) showed that the same NOMV as described above induced broadly cross-reactive antibodies that were bactericidal against genetically diverse serogroup A, W and X strains from various countries of the African meningitis belt (79). The recombinant vaccine proteins, however, only elicited anti-fHbp antibodies that killed strains that matched the sequence variant of the recombinant vaccines and not to strains with mismatched fHbp variants. The results suggests that using a vaccine that presents antigens in their native membrane environment is of advantage as they elicit a different antibody repertoire with better and broader bactericidal activity. The greater bactericidal activity could also be attributable to different IgG subclass responses generated or that the NOMV itself act as an adjuvant augmenting immune responses.

1.15 New vaccine strategies

1.15.1 Generalised Modules for Membrane Antigens (GMMA)

In recent years, new vaccine strategies have been employed to expand vaccine protection. One such strategy is GMMA. To have genetically increased release of vesicles, one such method is the deletion of *gna33*. GMMAs are derived from a recombinant strain that has been modified through deletion of the *gna33*. There are proteins that connect the outer membrane and peptidoglycan together. Disruption of this linkage by deletion of the *gna33* gene alters the membrane structure and consequently increasing the budding and vesicle release (81). The final product is an OMV devoid of inner membrane and cytosolic constituents (82). Compared to NOMV, which are naturally released by Gram-negative bacteria in low amounts,

GMMA provide a large-scale source of membrane material for reduced and cost-effective vaccine production platform. Moreover, compared to dOMVs, GMMA are not detergent treated and beneficial detergent soluble antigens such as fHbp are maintained, enriched and presented in their proper structural state and membrane environment (31,68).

The recent study by Koeberling *et al*. (2014) prepared a GMMA vaccine from a strain engineered to over-express fHbp of variant 1 (65). In general, antiserum against fHbp v.1 may be bactericidal against *N. meningitidis* strains expressing fHbp v.1 but has limited cross-reactivity against strains expressing fHbp v.2 or v.3 and vice versa (84,85). Previously, Pajon *et al*. (2011) showed that serogroup W from Africa were conserved in terms of PorA VR P1.5,2 (79). The authors also confirmed that most serogroup A and X isolates have an fHbp of variant 1. Using this information, the serogroup W GMMA produced by Koeberling *et al.* (2014) with over expressed fHbp v.1 was shown to elicit bactericidal antibody response against genetically diverse and invasive heterologous serogroup A, W and X isolates from Africa expressing fHbp v.1 or v.2 respectively (76,79). This illustrates the potential of GMMA as a broadly protective vaccine against African strains potentially suitable and safe for use in humans and potentially affordable (86,87). Unlike conjugate polysaccharide vaccines, the recombinant strains for GMMA production can be engineered to include more than one immunogenic OMP potentially expanding vaccine protection against serogroups that may emerge in the meningitis belt in the future. Moreover, vaccines need to have two basic elements that consist of an antigen that will trigger an immune response and an adjuvant to ensure such response is robust. GMMA are potent immunstimulators of the humoral immune response both as a vaccine and adjuvant

due to its immunogenic properties, whereby production of protective bactericidal antibodies is facilitated (82).

Although, GMMA are an attractive vaccine platform, the exact antigenic content of such vesicles are unknown. Research has combined proteomic analyses, functional genomics and structural vaccinology, to elucidate the envelope components and hopefully identify new target antigens as the probe to exploit the OMV-concept to formulate cross-protective vesicle vaccines. A strategy for that aim, which has not been previously employed, is the use of GMMA as a tool to generate mAbs to specific and unknown antigens on the surface membrane. Analysis of the properties of antigen specific mAbs and their potential bactericidal responses could aid determining the fine specificity of the humoral immune responses elicited by GMMA. Moreover, generation of mAbs to unknown antigens that may be bactericidal could also aid construction of a multi-antigen vaccine platform to broaden protection.

1.16 Thesis objectives

There were three main goals of the thesis. Firstly, we set out to molecularly characterise African serogroup A, W and X isolates. Secondly, to obtain a proof-of-concept in mice for GMMA meningococcal candidate vaccines, we engineered GMMA expressing fHbp v.1 and GMMA expressing NadA. Thirdly, to understand the antigenic specificities and immune responses elicited against GMMA, mAbs were produced against antigens presented on GMMA.

In short the aim of the thesis were as follows:

1. Molecular characterisation of a panel of epidemic meningococcal serogroup A, W and X isolates.
    a. Molecular typing of selected genes *fHbp, porA, nhbA* and *nadA* to determine their genetic variability.
    b. Analysis of fHbp and NadA protein expression levels in serogroup A, W and X isolates.
2. Investigate and characterise the breadth of immune responses elicited by the prototype GMMA-based meningococcal vaccines expressing either fHbp or NadA against genetically diverse *N. meningitidis* serogroups A, W and X isolates.
    a. Evaluation of IgG antibody responses against NadA by ELISA.
    b. Evaluation of serum bactericidal activity of sera from mice immunised with GMMA over-expressing fHbp or NadA against a subset of genetically diverse serogroup A, W and X isolates.
3. To help understand the humoral immune responses elicited, mAbs against GMMA were produced as opposed to the conventional processed recombinant protein.

a. Screening of anti-GMMA antibodies by ELISA and Western blot using GMMA and recombinant protein.
b. IgG isotyping.
c. Evaluation of mAb binding on surface of bacteria by Western blotting, immunofluorescence (IFA) and flow cytometry.
d. Evaluation of the bactericidal activity of mAbs by serum bactericidal assay using baby rabbit and human complement as source.

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CHAPTER 2

CHARACTERISATION OF AFRICAN MENINGOCOCCAL STRAINS AND A PROTOTYPE VACCINE BASED ON GENERALISED MODULES FOR MEMBRANE ANTIGENS (GMMA)

2.1 Introduction

The hallmark of invasive meningococcaemia is rapid progression of disease. Therefore, focus has been situated on developing vaccines to prevent disease. The development of conjugated vaccines against the known virulence factor associated with invasive disease, polysaccharide capsule, provides long-term protection in children and adults (1,2). Existing quadrivalent A, C, W and Y polysaccharide-protein conjugate vaccines are potentially effective at all ages, but the cost of such vaccines deems them unlikely to be affordable for mass distribution in the meningitis belt. Developing an affordable broadly cross-protective vaccine against serogroups causing disease in sub-Saharan Africa has been a major challenge (2).

Generalised modules for membrane antigens (GMMA) is a new affordable protein-based approach towards developing a broadly-protective meningococcal vaccine for Africa. A first step towards facilitating the developing of a comprehensive GMMA vaccine effective against a broad range of serogroups is the selection of a vaccine target/antigen, which requires knowledge of the molecular epidemiology of *N. meningitidis*. Molecular characterisation of subcapsular structures that are important for mucosal colonisation, spread and crossing of the blood-brain barrier can inform what antigen can possibly achieve broad coverage by serum bactericidal antibodies.

2.1.1 Our vaccine strategy

The GMMA vaccines are outer membrane blebs produced from recombinant strains that have deleted *gna33,* and *lpxL1* genes. As discussed in Chapter 1, deletion of *gna33* increases vesicle release by the mutant strain, thus enhancing vaccine

production (3). *LpxL1* codes for a late functioning acyltransferase, and deletion results in penta-acylated lipid A with decreased endotoxic activity compared to wild type hexa-acylated lipid A (4). Therefore, these mutations make GMMA a potential vaccine platform.

NOMV from isogenic mutants with up-regulated expression of fHbp have been shown to provide broad protection against African meningococcal isolates from different serogroups (5). Other OMPs that have been shown to elicit bactericidal antibodies include NadA, NHBA and PorA (6–8). To help determine the potential coverage of these antigens in a GMMA-based vaccine for Africa, we investigated their genetic diversity in two sets of serogroup A, W and X carriage and disease isolates from various countries of the sub-Saharan Africa collected between 1995 and 2011. Focusing on isolates collected over a period of years from both a defined and wide geographic region provides the opportunity to monitor the dynamics and antigenic diversity over time. Here, we also focused on over-expressing fHbp variant 1 in GMMA, and characterising the immune responses elicited.

2.1.2 Factor H binding protein (fHbp)

New vaccine strategies have focused on protein-based vaccines, and the search for proteins was to find a vaccine against serogroup B that elicits serum bactericidal antibodies. The most promising candidate antigen is fHbp previously referred to as GNA1870 and LP2086, identified by two different groups, Novartis and Pfizer, respectively (9,10). FHbp is a 27 kilodalton (kDa) surface-exposed lipoprotein that divided in two subfamilies (subfamily A and B) according to the Pfizer classification system or three variant groups, variant 1 (v.1), v.2 and v.3, based on amino acid

sequence identity and antibody cross-reactivity (Novartis classification) (9,10). It has also been divided into at least nine modular groups based on the combination of five variable α and β fHbp segments (1,11). Variant 1 corresponds to subfamily B while variants 2 and 3 correspond to subfamily A (12) (**Table 2.1**). The variants are further divided into fHbp sub-variants, and each individual sub-variant is given an ID number (13). To date, 715 subvariant peptides have been identified (http://pubmlst.org/neisseria/). The gene has been shown to be present in most strains tested to date, and only a few isolates lacking fHbp gene have been identified (14). Hitherto, fHbp variant and subtype in African meningococcal isolates were shown to be relatively conserved (5).

Table 2.1. Nomenclature of fHbp according to the classification by Pfizer and Novartis.

Pfizer subfamily classification of fHbp was firstly performed by Fletcher *et al*. (2004) (15), variant designation was by Masignani *et al*. (2003) (16) and modular architecture was assigned and Beernink and Granoff (2009) (11). Adapted and modified from McNeil *et al*. (2013) (10).

	Pfizer subfamily designation	**Novartis variant designation**	**Modular architecture**
fHbp	A	v.2	VI
			III
		v.3	V
			II
			IX
			VIII
	B	v.1	IV
			I
	A/B hybrid		VII

One function of fHbp is to bind human complement fH on the bacterial cell surface (18–21). This rapidly sequesters fH and down-regulates complement activation of the alternative pathway through the decay of C3 convertase C3bBb, enhancing the ability

of the bacterium to escape complement-mediated bacteriolysis in human serum or blood (11,22–24). A second function of serum anti-fHbp antibodies is that they can activate both classical and alternative pathway by blocking binding of fH and induce bactericidal activity; however this function is not necessary for complement activation. It has been shown that antiserum against fHbp v.1 (subfamily B) may be bactericidal against *N. meningitidis* strains expressing fHbp v.1 but has limited cross-reactivity against strains expressing fHbp v.2 or v.3 (subfamily A) and vice versa (2,5,25). Previously, a study showed that a mixture of two NOMVs with over-expressed fHbp v.1 and v.2 elicit bactericidal antibody response against genetically diverse and invasive serogroup A, W and X isolates from Africa expressing fHbp v.1 or v.2 respectively. This illustrates the potential of fHbp in a native OMVs as a broadly protective vaccine against African strains (26,27).

Expression level of fHbp is naturally variable, and in most wild-type strains expression is low (10). Oriente *et al*. (2010) showed that fHbp expression is under control of two promoters: a monocistronic at the promoter of fHbp and a bicistronic originating from an upstream gene (28). The monocistronic promoter up-regulates transcription under oxygen limitations, such as in the blood stream, ensuring enhancement of complement resistance. Moreover, Pajon *et al*. (2011) showed that the level of fHbp expression in meningococcal strains determines their susceptibility to killing by anti-fHbp antibodies (5). In general, high expressers of fHbp are more susceptible to killing by anti-fHbp antibodies than low expressers. Low expressers are more resistant to killing because there is not enough correctly spaced fHbp epitopes for IgG antibodies to engage C1 complex (7,10,20). The authors proposed that low expression of fHbp could be defined as ≤33%, intermediate as 33-100% and high

expression as >100% compared to fHbp expressed by the group B reference strains H44/76 or 8047, which are naturally high expressers of fHbp ID 1 v.1 and v.2 ID77, respectively.

2.1.3 Neisserial adhesin A (NadA)

NadA is a surface exposed trimeric protein present in most hypervirulent meningococcal strains and involved in epithelial cell adhesion and colonisation/invasion (29). The gene is well conserved and it clusters into five well-defined alleles. Alleles 1-3 are highly conserved and show cross-reactivity. A more comprehensive introduction to NadA is given in Chapter 3.

2.1.4 Neisserial Heparin Binding Antigen (NHBA)

NHBA is a surface exposed lipoprotein that binds heparin through arginine-rich regions known to increases serum resistance due to the potential interactions of heparin with components of the complement pathway such as fH (30). Most pathogenic meningococci express NHBA, and there are several variants of NHBA that show cross-reactivity (31). Antibody protection is mediated through opsonophagocytocis (32). For example, peptide 2 is most common and included in the 4CMenB vaccine Bexsero® and it is able to induce cross-protective antibodies against many variants (33). Currently 278 peptide amino acid sequences have been identified (http://pubmlst.org/ neisseria/).

2.2 Project objectives

The main objective of this study was to characterise a panel of two sets of African meningococcal strains based on the OMP fHbp, NadA, PorA and NHBA and protein expression level of fHbp. The first set of 50 serogroup A, W and X isolates were received from Swiss Tropical and Public Health Institute isolated from Ghana and Burkina Faso and Norwegian Institute of Public Health (NIPH). The second set of 44 serogroup A, W and X isolates were received from NIPH isolated from various countries of the meningitis belt.

The second objective of the study was to investigate and obtain a proof of principle as to whether over-expression of fHbp v.1 ID1 on GMMA from a serogroup W isolate can induce broadly cross-reactive bactericidal antibodies against epidemic serogroup A, W and X strains. The GMMA vaccine was prepared from a mutant engineered to over-express fHbp v.1 ID1, and with additionally three mutations that will eliminate the capsule (Δ*cps*), detoxify the GMMAs (Δ*lpxL1*) and increase the production of vesicles by the bacteria (Δ*gna33*) (3,4,34). The vaccine is expected to provide protection against the majority of serogroup A and X isolates mediated by antibodies against fHbp v.1.

In short the aims are:

1. Molecular characterisation of a panel of epidemic meningococcal serogroup A, W and X isolates.
    a. Molecular typing of *fHbp, porA, nhbA* and *nadA*.

b. Further analysis of fHbp protein expression levels in serogroup A, W and X isolates.
c. Selection of a representative strain panel including serogroup A, W and X isolates that would be suitable to investigate bactericidal activity induced by prototype GMMA vaccines.

2. Characterisation of antibody responses elicited by GMMA with over-expressed fHbp.
    a. Evaluation of serum bactericidal activity of sera made against GMMA with over-expressed fHbp variant 1 against a subset of genetically diverse serogroup A and X isolates.

2.3 Materials and Methods

2.3.1 *N. meningitidis* isolates

The first set of *N. meningitidis* serogroup A, W and X isolates investigated in this study were kindly provided by Gerd Pluschke at the Swiss Tropical and Public Health Institute (STPHI) and Dominique Caugant at the Norwegian Institute of Public Health (NIPH; 8 serogroup X isolates). Four were of serogroup A (all cases), 31 of serogroup W (10 cases, 21 carriers) and 9 of serogroup X (8 cases; 4869 from STPHI). The A and W strains that had been collected in the Kassena-Nankana District (KND) of Ghana and in the Nouna Health District (NHD) in the Kossi region of Burkina Faso as part of a longitudinal study (**Table 2.2**). Two isolates of serogroup A were isolated in Nouna (Burkina Faso) and two in Navrongo (Ghana) between 2002-2007, 9 of serogroup X in Mali, Uganda, Kenya, Burkina Faso isolated between 1995-2010, and the remaining 31 isolates were of serogroup W isolated in both Ghana (n=23) and Burkina Faso (n=8) between 2003-2009. Case strains were isolated from the CSF of meningitis patients, and carriage strains were isolated from throat swabs collected in the context of longitudinal carriage surveys. Isolation and characterisation of strains has been described previously (35–37). Ethical clearance was obtained from the relevant institutional review boards and informed consent was obtained from all study participants.

The second set of *N. meningitidis* serogroup A, W and X case isolates investigated in this study were kindly provided by Dominique Caugant. The serogroup A (n=13), W (n=20) and X (n=11) isolates were collected from various countries of the sub-

Saharan Africa (**Table 2.3**). The serogroup A strains were isolated between 2001-2010, W between 2001-2011 and X between 2006-2010.

All isolates were molecularly characterised with respect to *fHbp*, *porA* VRs, *nadA* and *nhbA* by gene sequencing and gene typing. A subset of 30 isolates from the first set and 42 isolates from the second set of meningococcal strains were also analysed for their fHbp expression level.

Table 2.2. Characteristics of the first set of serogroup A, W and X isolates used in this study. Molecular characterisation was performed on these isolates by PCR amplification and sequencing of *fHbp*, *porA*, *nadA* and *nhbA*.

Serogroup	Isolate	Source	Origin	Year of isolation	*fHbp* variant	*fHbp* ID	*porA* VRs	*nadA* allele	*nhbA* gene	ST
A	N1361 *	case	Navrongo, Ghana	2002	1	5	P1.20, 9	3	S	ND
A	N2008 *	case	Navrongo, Ghana	2005	1	5	P1.20, 9	3	S	ND
A	N2181 *	case	Nouna, Burkina Faso	2006	1	5	P1.20, 9	3	Y	ND
A	N2602 *	case	Nouna, Burkina Faso	2007	1	5	P1.20, 9	3	Y	2859
W	1485*	carrier	Ghana	2003	2	23	P1.5,2	3	S	11
W	1487	carrier	Ghana	2003	2	23	P1.5,2	3	S	11
W	1489	carrier	Ghana	2003	2	23	P1.5,2	3	S	11
W	1491	carrier	Ghana	2003	2	23	P1.5,2	3	S	11
W	1494*	carrier	Ghana	2003	2	23	P1.5,2	3	S	11
W	1625*	case	Ghana	2003	2	23	P1.5,2	3	Y	11
W	1626	case	Ghana	2003	2	23	P1.5,2	3	S	11
W	1627*	case	Ghana	2003	2	23	P1.5,2	3	S	11
W	1628*	case	Ghana	2003	2	23	P1.5,2	3	S	11
W	1629	carrier	Ghana	2004	2	23	P1.5,2	3	N	11

W	1630*	carrier	Ghana	2004	2	23	P1.5,2	3	S	11
W	1632	carrier	Ghana	2004	2	23	P1.5,2	3	Y	11
W	1634*	carrier	Ghana	2004	2	23	P1.5,2	3	Y	11
W	1636	carrier	Ghana	2004	2	23	P1.5,2	3	Y	11
W	1681*	case	Ghana	2003	2	23	P1.5,2	3	Y	11
W	1682*	case	Ghana	2003	2	23	P1.5,2	3	S	11
W	1683*	case	Ghana	2003	2	23	P1.5,2	3	S	11
W	1846*	carrier	Ghana	2004	2	23	P1.5,2	3	Y	11
W	1848	carrier	Ghana	2004	2	23	P1.5,2	3	Y	11
W	1857*	carrier	Ghana	2004	2	23	P1.5,2	3	Y	11
W	1888	carrier	Ghana	2004	2	23	P1.5,2	3	S	11
W	1903*	case	Ghana	2004	2	23	P1.5,2	3	Y	11
W	1973	carrier	Ghana	2004	2	23	P1.5,2	3	Y	11
W	2039*	case	Burkina Faso	2008	2	22	P1.5,2	3	Y	11
W	2252*	case	Burkina Faso	2008	2	22	P1.5,2	NA	S	11
W	2716	carrier	Burkina Faso	2004	2	22	P1.5,2	3	Y	11
W	2719*	carrier	Burkina Faso	2004	2	22	P1.5,2	3	Y	11
W	2841	carrier	Burkina Faso	2005	2	22	P1.5,2	NA	Y	11
W	2855	carrier	Burkina Faso	2005	2	22	P1.5,2	NA	S	11
W	2882	carrier	Burkina Faso	2009	2	22	P1.5,2	NA	S	11
W	2959*	carrier	Burkina Faso	2009	2	22	P1.5,2	NA	Y	11
X	BF07/ 07[a*]	case	Burkina Faso	2007	1	74	P1.5-1,10-1	N	S	181
X	Ug13/ 07[a*]	case	Uganda	2007	1	74	P1.19, 26	N	Y	5403
X	BF12/ 03[a*]	case	Burkina Faso	2003	1	73	P1.5-1,10-1	N	Y	751
X	Kenya 01/06[a*]	case	Kenya	2006	1	74	P1.19, 26	N	Y	5403
X	BF 15/10[a*]	case	Burkina Faso	2010	1	74	P1.5-1,10-1	N	S	181
X	BF 29/10[a*]	case	Burkina Faso	2010	1	74	P1.5-1,10-1	N	Y	181
X	BF 02/97[a*]	case	Burkina Faso	1997	1	73	P1.5-1,10-1	N	S	751

X	Ug 09/06[a]*	case	Uganda	2006	1	74	P1.19, 26	N	Y	5403
X	4869*	NG	Mali	1995	1	12	P1.19, 15	N	Y	NG

*Isolate used for fHbp protein expression analysis.

ST= sequence type

ND= not defined.

NA: Inactive *nadA* due to insertion sequence *IS1301*.

Y: PCR positive sample

S: NHBA gene with stop codon.

N: PCR negative sample

[a] Isolates from NIPH

Table 2.3. Second set of serogroup A, W and X isolates received from Dominique Caugant at the Norwegian Institute of Public Health.

Molecular characterisation was performed on these isolates by PCR amplification and sequencing of *fHbp*, *porA*, *nadA* and *nhbA*.

Serogroup	Isolate	Origin	Year of isolation	*fHbp* variant	*fHbp* ID	*porA* VRs	*nadA* allele	*nhbA* gene	*fetA*	ST
A	BF8/01	Burkina Faso	2001	1	5	P1.20,9	3	Y	F3-1	7
A	Mk804/03	Ethiopia	2003	1	5	P1.20,9	3	Y	F3-1	7
A	BF3/06	Burkina Faso	2006	1	5	P1.20,9	3	Y	F3-1	7
A	BF6/06	Burkina Faso	2006	1	5	P1.20,9	3	Y	F3-1	2859
A	BuFa20030020	Burkina Faso	2007	1	5	P1.20,9	3	Y	F3-1	6035
A	Mali21/07	Mali	2007	1	5	P1.20,9	3	29	F3-1	2859
A	Su14/07	Sudan	2007	1	5	P1.20,9	3	29	F3-1	7
A	MRS2007446	Togo	2007	1	5	P1.20,9	3	29	F3-1	2859
A	MRS2008162	Niger	2008	1	5	P1.20,9	3	Y	F3-1	2859
A	Niga16/09	Nigeria	2009	1	5	P1.20,9	3	29	F3-1	7
A	BF1/10	Burkina Faso	2010	1	5	P1.20,9	3	29	F3-1	2859
A	Tch44/10	Chad	2010	1	5	P1.20,9	3	29	F3-1	7
A	Mali21/10	Mali	2010	1	5	P1.20,9	3	2	F3-1	8639
W	BF7/01	Burkina	2001	1	9	P1.5,2	3	Y	F1-1	11

		Faso								
W	BF6/02	Burkina Faso	2002	2	23	P1.5,2	3	Y	F1-1	11
W	BF10/02	Burkina Faso	2002	2	23	P1.5,2	3	Y	F1-1	11
W	Nigeria 1/03	Nigeria	2003	2	23	P1.5-1,2-36	3	120	F1-1	2881
W	MRS200 4034	Benin	2004	2	22	P1.5-1,2-36	3	120	F5-1	2881
W	Ghana7/ 04	Ghana	2004	2	23	P1.5,2	3	Y	F1-1	11
W	BF2/06	Burkina Faso	2006	2	22	P1.5,2	3	Y	F3-1	5779
W	Mali1/06	Mali	2006	2	23	P1.5,2	3	29	F1-1	11
W	Sudan4/ 06	Sudan	2006	1	9	P1.5,2	3	29	F1-1	11
W	Uganda 11/06*	Uganda	2006	3	349	P1.5,2	3	Y	F1-1	11
W	Cam2/09	Cameroon	2009	1	9	P1.5,2	3	29	F1-1	11
W	Cam1/09	Cameroon	2009	1	9	P1.5-1,2-36	3	Y	F3-1	2881
W	Tch5/09	Chad	2009	1	9	P1.5,2	3	91	F1-1	11
W	Mali10/0 9	Mali	2009	2	23	P1.5,2	2	Y	F1-1	11
W	Niga85/0 9	Nigeria	2009	1	9	P1.5,2	3	Y	F1-1	11
W	BuFa20/ 10	Burkina Faso	2010	2	23	P1.5-1,2-36	3	Y	F5-1	8638
W	Nigeria 4/10	Nigeria	2010	1	9	P1.5,2	3	Y	F1-1	11
W	BuFa2/1 1	Burkina Faso	2011	1	9	P1.5,2	3	Y	F1-1	11
W	Mali4/11	Mali	2011	1	9	P1.5,2	3	Y	F1-1	11
W	MRS200 8218	Burkina Faso	2008	2	22	P1.5-1,2-36	3	Y	F5-1	2881
X	MRS200 6078	Niger	2006	1	74	P1.5-1,10-1	3	Y	F1-31	181
X	MRS200 6089	Niger	2006	1	74	P1.5-1,10-1	3	Y	F1-31	181
X	MRS200 6093	Niger	2006	1	74	P1.5-1,10-1	3	Y	F4-23	181
X	MRS200 6087	Niger	2006	1	74	P1.5-1,10-1	3	Y	F4-23	5789
X	Uganda 14/06*	Uganda	2006	1	74	P1.19,26-4	3	Y	F3-27	5403
X	MRS200 8223	Burkina Faso	2007	1	74	P1.5-1,10-1	3	118	F1-31	181
X	Uganda 11/07	Uganda	2007	1	74	P1.19,26	3	Y	F3-27	5403
X	MRS200 8112	Benin	2008	1	74	P1.5-1,10-1	3	Y	F1-31	181
X	BuFa9/1 0	Burkina Faso	2010	1	74	P1.5-1,10-1	3	Y	F5-69	181
X	BuFa 16/10	Burkina Faso	2010	1	74	P1.5-1,10-1	3	118	F1-31	181
X	BuFa 24/10	Burkina Faso	2010	1	74	P1.5-1,10-1	3	47	F1-31	181

ST= sequence type

Y: PCR positive sample

*Isolates not used for fHbp protein expression analysis.

2.3.2 Recombinant DNA techniques

The strains were sub-cultured on GC agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) and incubated overnight at 37°C, 5% CO_2. A loop-full of cells was re-suspended in 500µl sterile water and boiled for 10 minutes. The samples were pelleted at 17,900xg for 5 minutes in a microcentrifuge (Eppendorf, Hamburg, Germany). Genomic DNA was purified using the Invitrogen PureLink Genomic DNA kit (Invitrogen, San Diego, California, USA) according to the manufacturer's instructions. The genes *fHbp, porA* VRs*, nadA* and *nhbA* were amplified by PCR from all isolates using the primers described in **Table 2.4**. The final PCR reaction contained: 0.5mM deoxynucleotide triphosphates, 5 U/ml Taq DNA polymerase (New England BioLabs, Ipswich, USA), 1×Thermopol Reaction buffer (New England BioLabs), 1µM primer solution (Sigma-Aldrich, St. Louis, Missouri, USA) and 100ng of genomic DNA quantified by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). The PCR was performed using the Applied Biosystems GeneAmp PCR System 9700 (Applied Biosystems, Foster city, USA) with maximum ramping speeds using conditions described in **Table 2.5**. PCR products were separated by gel electrophoresis using a 0.8% Tris base, acetic acid and EDTA (TAE) agarose gel (Sigma-Aldrich), and purified using the PureLink PCR Purification Kit (Invitrogen) according to the manufacturer's instructions. The DNA amount was measured using the NanoDrop ND-1000 Spectrophotometer.

Table 2.4 Primers used for PCR amplification and sequencing of the genes *fHbp, porA, nadA* and *nhbA*.

Target gene	Primer designation	5'-3' nucleotide sequence	Purpose	Reference
fHbp	A1 (Fw)	GACCTGCCTCATTGAT	PCR, sequencing	(26)
	B2 (Rv)	CGGTAAATTATCGTGTTCGTACGGC	PCR, sequencing	(26)
porA	210 (Fw)	ATGCGAAAAAAACTTACCGCCCTC	PCR, sequencing	(38)
	H (Rv)	CGCATATTTAAAGGCATAG	PCR, sequencing	This study
	EI (Fw)	CCAGCCAGGCCATTGATCC	Sequencing	This study
	103L (Rv)	AACGGATACGTCTTGCTC	Sequencing	(38)
nadA	NadAF (Fw)	AACACTTTCCATCCAAAG	PCR, sequencing	(39)
	NadAR (Rv)	TTACCACTCGTAATTGACG	PCR, sequencing	(39)
nhbA	Fw	GGCGTTCAGACGGCATATTTTTACA	PCR, sequencing	(40)
	Rv	GGTTTATCAACTGATGCGGACTTGA	PCR, sequencing	(40)

Fw: forward; Rv: reverse

Table 2.5. Conditions used for PCR amplification of the genes *fHbp*, *porA*, *nadA* and *nhbA*.

Gene	*fHbp*	*porA*	*nadA*	*nhbA*
PCR profile	94ºC, 4 minutes 35 cycles: 94ºC, 40 seconds 58ºC, 40 seconds 68ºC, 40 seconds Final extension: 72°C, 5 minutes	94ºC, 5 minutes 30 cycles: 94ºC, 1 minute 55ºC, 1 minute 72ºC, 30 seconds Final extension: 72°C, 5 minutes	94ºC, 5 minutes 30 cycles: 94ºC, 1 minute 55ºC, 1 minute 72ºC, 1 minute 30 seconds Final extension: 72°C, 5 minutes	94ºC, 4 minutes 35 cycles: 94ºC, 1 minute 55ºC, 1 minute 72ºC, 1 minute Final extension: 72°C, 5 minutes
Reference	(26)	(38)	(39)	(40)

2.3.3 DNA sequencing

The primers used for *porA* VR1 sequencing were 210 and 103L (**Table 2.4**). We designed primers EI and H for sequencing of the VR2 region, by aligning the conserved regions upstream and downstream of VR2 using the alignment program Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). *PorA* sequences from the following strains of different serogroups were used for the alignment: MC58 (GenBank accession number AE002098.2), Z2491 (AL157959.1), 053442 (CP000381.1), FAM18 (AM421808.1), M6190 (AEQF01000026.1), M13399 (AEQG01000023.1) and alpha 14 (AM889136.1) using Uniprot . The sequences were read at Novartis Vaccines-Cellular Microbiology and Bioinformatics Unit Automated DNA Sequencing Facility, Siena, Italy, on an ABI 3730 DNA Analyzer. Sequences were analysed using the Simmonics program (version 1.6), MEGA software package (version 5) and Chromas (version 2.01). *fHbp* ID, *porA* VR and *nhbA* alleles were identified using the online Neisseria Sequence Typing database (http://pubmlst.org/neisseria).

2.3.4 Western blot analysis of fHbp protein amount

For 30 isolates, labelled with an asterisk in **Table 2.2**, Western blot analysis of the fHbp expression level in whole cell samples was performed as described by Seib *et al.* (2009) (13). The strains were sub-cultured on GC agar plates (Becton Dickinson) and incubated overnight at 37°C with 5% CO_2. 7ml Mueller-Hinton Broth (MH; Becton, Dickinson) supplemented with 0.25% glucose (Sigma-Aldrich) was inoculated with a loop full of single colonies to an optical density at 600 nm (OD_{600}) of 0.12-0.16. The suspensions were incubated at 37°C with 5% CO_2 to an OD_{600} of 0.6 corresponding to approximately 1.8×10^8 colony forming unit (cfu)/ml (exponential growth phase). The

cells from 1ml of culture were collected by centrifugation at 17,900xg for 5 min in a microcentrifuge (Eppendorf), re-suspended in 100µl phosphate buffered saline (PBS) and heat inactivated in a water bath at 56°C for 1 hour. Protein concentrations of the suspensions were determined using Lowry protein assay kit (BioRad Laboratories, Hercules, USA) with bovine serum albumin (BSA; Sigma-Aldrich) as a standard. The samples were adjusted to 2000µg/ml and 10µl was loaded onto the gel (20µg). The fHbp amounts were estimated by SDS-PAGE and Western blotting. Hexa-Histidine tagged recombinant fHbp (rHis-fHbp) v.1 and v.2 of 0.05, 0.025, 0.0125 and 0.006 µg/ml were used as standards. Positive and negative controls were whole cell lysates from *N. meningitidis* group B strain H44/76 and 8047 expressing fHbp v.1 ID1 and v.2 ID77, respectively, and isogenic fHbp knock-out mutants (generated by Oliver Koeberling). Proteins were transferred to a nitrocellulose membrane using the iBlot system (Invitrogen). After blocking overnight in 3% milk powder in 1xPBS (Merck, Whitehouse station, NJ, USA) at 4°C, fHbp proteins were detected with 1µg/ml anti-fHbp mouse monoclonal antibody JAR5 (IgG2b) raised against rHis-fHbp v.1 ID1 (41) and JAR31 (IgG2b) raised against rHis-fHbp v.3 ID 28, which shows cross-reactivity against most fHbp v.2 peptides (18). After washing the membrane four times for 10 minutes each with 1xPBS+0.1% Tween20 (Sigma-Aldrich), secondary antibody horseradish peroxidase-labelled anti-mouse IgG (Invitrogen) in a 1:20000 dilution was added. After another four washes, the membranes were developed using SuperSignal WestPico Chemiluminescent Substrate (ThermoScientific, Waltham, Massachusetts, USA) according to manufacturer's instructions, and the signal was detected with Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, UK). The amount of fHbp expressed by each isolate compared to the standard rHis-fHbp was determined by densitometric analysis for three biological replicates using the

ImageQuant 400 gel documentation system (GE Healthcare). The expression of fHbp by the test isolates was reported as percentages of the amount of fHbp expressed by bacterial cells compared to the reference strains H44/76 and 8047, known to express relatively high amounts of fHbp v.1 and v.2 (5). The levels of fHbp expression by the reference strains were considered to be 100%.

2.3.5 Serum bactericidal assay (SBA) using human complement

Bacteria were sub-cultured on GC agar plate and incubated overnight at 37°C with 5% CO_2 (**Table 2.8,** see result section 2.4.5). Single colonies were used to inoculate 7ml of MH broth (Becton, Dickinson) containing 0.25% (w/v) glucose (Sigma-Aldrich) to an OD_{600} of 0.12-0.15, and incubated at 37°C with 5% CO_2. 1ml of culture at an OD_{600} of 0.6-0.7 (early log-phase) was transferred into warm 6ml MH broth supplemented with 0.02 mM cytidine 5′-monophospho-N-acetylneuraminic acid (CMP-NANA, Sigma-Aldrich) and 0.25% glucose and grown to an OD_{600} of 0.6. The cultures were transferred into a 50ml Falcon centrifuge tube and Dulbecco's saline phosphate buffer (DPBS, Sigma-Aldrich) containing 0.5mM $MgCl_2$ and 0.9mM $CaCl_2$ supplemented with 1% BSA (minimum 96% electrophoresis, Sigma-Aldrich) was added to a final volume of 50ml. The cells were pelleted by centrifugation for 10 minutes at 3500rpm and re-suspended in DPBS+1%BSA to an OD_{600} of 0.7 (approximately $2x10^8$ cfu/ml). The suspensions were subsequently diluted 1:25000 in DPBS+BSA. The 40µl reaction mixture prepared in 96 well plates (Techno Plastic Products (TPP), Trasadigen, Switzerland) contained: 12µl DPBS+BSA, 10µl of 400-500 cfu bacterial cells, 8µl 20% (v/v) final human complement and 10µl diluted mouse sera starting at 1:10 in serial 4 dilutions. The mouse sera were heated at 56°C for 30 minutes to remove endogenous complement activity. The source of

complement, kindly provided by Novartis NV&D (Marburg, Germany), was serum from a donor with no detectable bactericidal activity against the test strains. Five control wells contained cells with buffer only or complement without the test sera. 12µl from the reaction mixture in the wells were plated and the plate was incubated at 37°C with 5% CO_2, for one hour. The bactericidal titre was defined as the reciprocal serum dilution that resulted in a 50% decrease of cfu after 60 minutes compared with the average number of cells in the control wells at time 0.

2.3.6 Mouse immunisation (performed previously)

We generated GMMA from three serogroup W mutant strains: one main vaccine candidate and two controls (**Table 2.6**). All strains had deleted *lpxL1* and *gna33* genes. Over-expression of fHbp in the recombinant serogroup W isolate 1630 with deleted *lpxL1*, capsule, *gna33* genes was done using a multicopy plasmid encoding fHbp ID1. The aim was to compare subsequent groups: group 1 + 2 to evaluate effect of capsule expression on production of anti-fHbp antibodies, groups 1 + 3 to evaluate effect of OE fHbp, and groups 1 + 4 to evaluate the efficacy of antibodies elicited against GMMA versus rHis-fHbp protein. Five weeks old CD-1 female mice (8 mice per group) were obtained from Charles River (Wilmington, MD, U.S.). The mice were immunised intraperitoneally (IP) with three doses of vaccine given at days 0, 14 and 28. The GMMA vaccines were given at 5µg doses based on total protein. Control mice were immunised with 5µg rHis-fHbp ID1 purified from *Escherichia coli* BL21 or aluminium hydroxide (Alum) only (**Table 2.7**). All vaccines were absorbed on 3mg/ml Alum in a 100µl formulation containing 10mM Histidine and 0.9mg/ml NaCl. Blood samples were obtained at day 42 (two weeks after the third dose). The

animal procedures were performed under protocols approved by Novartis NV&D (Siena, Italy) animal care.

Table 2.6. GMMA vaccine strains.

GMMA vaccine strains were previously generated by Oliver Koeberling.

Vaccine strain characteristics	Designation of vaccine strain and GMMA used for immunisation
Prototype vaccine candidate	
Serogroup W, strain 1630 Capsule KO, *lpxL1* KO, *gna33* KO, over-expressed fHbp ID1	Triple KO, OE fHbp
Control vaccines	
Serogroup W, strain 1630 Capsule expressed, *lpxL1* KO, *gna33* KO, over-expressed fHbp ID 1	Double KO, OE fHbp
Serogroup W, strain 1630 Capsule KO, *lpxL1* KO, *gna33* KO	Triple KO

KO= knock-out
OE= over-expressed.

Table 2.7. Groups of animals, dose, route of immunisation and antigens used in the mice immunisation trial.

The isolate used for the production of GMMAs is serogroup W 1630, PorA P1.5,2, OE fHbp v.1 ID1, triple KO (Δ*lpxL1*, Δ*gna33*, Δcapsule). Δ*lpxL1* detoxifies the GMMA due to the production of penta- instead of hexa-acylated lipid A. Δ*gna33* increases blebbing or GMMA release from the bacterial cells. Δcapsule deletes the capsule of the bacterium rendering it non-invasive. Production of His-rfHbp ID1 was presviouslt done by Oliver Koeberling. Following reagents were also used in the vaccine formulations NaCl (Sigma-Aldrich S9888), Histidine (Sigma-Aldrich H6034), Aluminium hydroxide (NV&D Formulation unit). For the production of vaccine candidates and control vaccines, the isolate was grown in as described in Chapter 3 section 3.3.5.

Group (Mouse number)	Antigen	Adjuvant	Dose (µg)	Immunisation route	Volume (µl) per animal
Primary vaccine candidate					
1 (1-8)	Triple KO, OE fHbp	Alum	5	IP	100
Control vaccines					
2 (9-16)	Double KO, OE fHbp	Alum	5	IP	100
3 (17-24)	Triple KO	Alum	5	IP	100
4 (24-32)	His-rfHbp ID1	Alum	5	IP	100
5 (33-40)	None	Alum	NA	IP	100

KO= knock-out, OE= over-expressed, IP= intraperitoneal.

2.3.7 Statistical analysis

Antibody titres were log10 transformed, and SBA titres <10 were assigned the value 5. Data analysis was carried out using Graphpad Prism version 5.01 software. The Mann-Whitney *U* test was used to evaluate the serum bactericidal antibody responses between two vaccine groups. A probability (*P*) value of <0.05 was considered statistically significant.

2.4 Results

2.4.1 The first set of *N. meningitidis* isolates from Africa show conserved *fHbp* variant and *porA* subtypes

From the sequence analysis of the *fHbp* gene, all serogroup A and W isolates expressed *fHbp* v.1 ID 5 and v.2 ID22 (isolates from Burkina Faso) or 23 (isolates from Ghana), respectively, which differ by one amino acid (aa) at position 30 (ID22 serine; ID23 glycine). The serogroup X isolates that encoded an *fHbp* v.1 and an ID of 12 (Mali), 73 (Burkina Faso) or 74 (Burkina Faso, Uganda and Kenya) (**Figure 2.1**). The meningococcal serogroup A and W case and carrier isolates were also conserved in terms of *porA* subtype as serogroup A encoded P1.20,9 and W P1.5,2. Variability was observed among serogroup X case and carrier isolates expressing P1.5-1,10-1, P1.19,26 and P1.19,15 (n=5, 3 and 1 respectively). Despite the limited number of isolates studied, the results show that between Burkina Faso and Ghana, which share a common border, there has been conservation of fHbp and PorA antigens among A and W isolates over a period between 2002-2009. Although greater variation was seen for serogroup X *porA* subtypes probably due to larger geographical are covered, results are consistent with previous studies of isolates from sub-Saharan Africa, which shows limited diversity of serogroup A and W *fHbp* and *porA* over time (5).

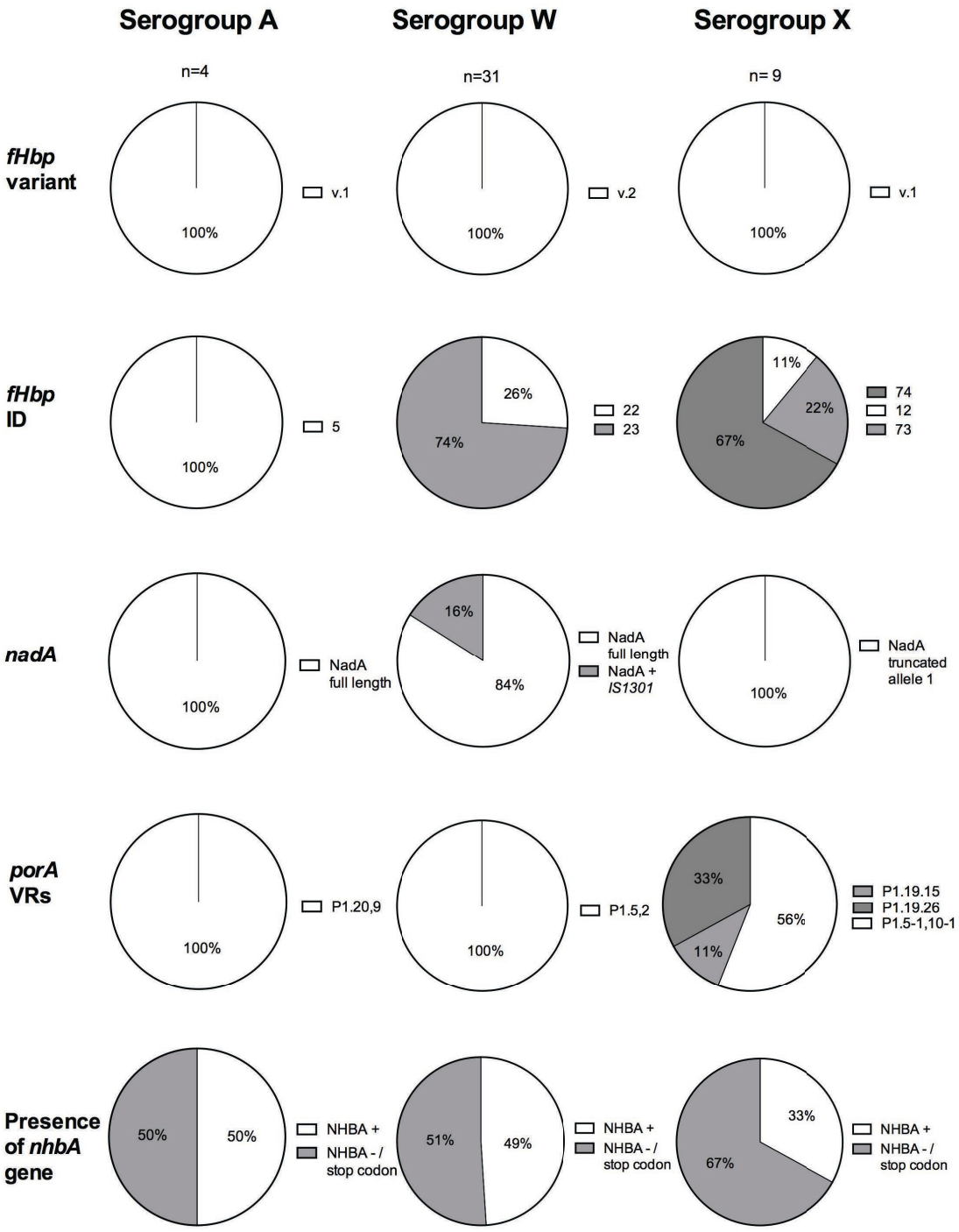

Figure 2.1. *fHbp*, *nad,A porA* and *nhbA* gene-typing analysis of first set of meningococcal serogroup A, W and X isolates collected between 1995-2010.

The fHbp variant group is according to classification proposed by Masignani *et al.* (2003)(26). *fHbp* sequence ID, *nadA, porA* VR and *nhbA* allele was determined by sequence query on http://pubmlst.org/neisseria. Each isolate was typed by PCR amplification of each respective gene, sequenced and analysed using bioinformatics software Simmonics, Mega 5 and Chromas. NadA +

IS1301: strains with *nadA* encoding gene-containing insertion sequence *IS1301*. NHBA-/stop codon: strains lacking NHBA encoding gene or having *nhbA* with stop codon.

2.4.2 Most serogroup A and W isolate genomes from the first set of meningococcal strains contain *nadA* and *nhbA*

NadA and NHBA are two antigens included in the vaccine Bexsero®, and they induce the production of bactericidal antibodies against *N. meningitidis* serogroup B strains (7,8). Among the first set of 50 African A, W and X strains investigated, the *nadA* allele 3 was present in all serogroup A isolates, 26/31 (84%) of serogroup W isolates and a truncated allele 1 of 750bp (Accession number: FJ619647) was detected in the serogroup X strains (**Figure 2.1; Appendix 1**). More carrier isolates (77%, 17/22) compared with case isolates (52%, 13/25) harboured the gene. Previous reports found that *nadA* is present in about 50% of case isolates (non-African), but is underrepresented among carrier isolates (39).

Among the remaining five W isolates (1 case [2252], 4 carriers [2841, 2855, 2882, 2959]), PCR amplification across the *nadA* site gave a 2 kb product instead of the expected 1 kb product on a DNA gel. Western blotting using whole cell lysate and polyclonal mouse anti-rHis-NadA-3 serum indicated that these isolates did not express NadA (**Figure 2.3**, **Panel A**). In the blots, rHis-NadA-3 has a band at 45kDa representing the monomer whereas in whole cells the trimeric form of NadA is seen at around 170kDa. Sequencing of this fragment confirmed the presence of the 842-base pair (bp) mobile genetic element *IS1301* inserted at nucleotide (nt) 470-474 (**Appendix 1**).

The *nhbA* gene was identified in 30/31 (94%) of the serogroup W isolates, all serogroup A and X strains. However, *nhba* sequencing of the PCR positive isolates revealed a stop codon at different nucleotide positions for 15 serogroup A (n=2), W (n=13) and X (n=3) isolates, which has not been previously reported. Western blotting using polyclonal mouse anti-NHBA antibody showed that NHBA protein was not expressed in these isolates. The allele of the W strains was identified as 17 using the Neisseria typing database on http://pubmlst.org/neisseria/NHBA/. For the serogroup A and X strains no definite match was found.

2.4.3 Lack of antigenic diversity of outer membrane proteins in the second set of serogroup A, W and X case isolates from Africa

The diversity of OMP fHbp, NadA and NHBA were investigated in a second set of 44 strains provided by Dominque Caugant at the NIPH. These strains were isolated from a wider geographical range in the meningitis belt over a period of 10 years (2001-2011). Sequence analysis of the *fHbp* gene showed that 100% of the serogroup A and X isolates have a single fHbp peptide belonging to v.1 with an ID of 5 and 74 respectively. Half of the serogroup W isolates encompassed *fHbp* v.2 with a peptide ID of 22 or 23. The remaining 45% (9/20) of the strains were of *fHbp* v.1 (ID 9), and only one isolate Uganda 11/06 was found to encode *fHbp* v.3 ID 349. From the sequence analysis of the *nadA* gene, all serogroup A and X isolates harboured allele 3 and 75% (15/20) of the W isolates (**Figure 2.2**). The *nadA* gene in the isolate Ug11/07 is a pseudogene since several point mutations are observed in the N-terminal domain between aa 5-18 and coiled-coil region between aa 289-300. As observed for five isolates of the first strain panel, PCR amplification across the *nadA* site of these

five W isolates also gave a 2 kb product instead of the expected 1 kb product (Nigeria1/03, MRS2004034, Cam1/09, BuFa20/10, MRS2008218). Likewise, BLAST analysis of this fragment confirmed the presence of the insertion sequence *IS1301* at nt 400-469 (Δ69nt; Nigeria1/03), 470-474 (insertion site CTAAG; MRS2004034, Cam1/09, BuFa20/10) and at nt 472-496 (MRS2008218). Western blotting of these isolates revealed a single band between 49-62kDa (**Figure 2.3, Panel B**).

The *nhbA* gene was identified all of the 44 *N. meningitidis* isolates. Six of the serogroup A isolates could be classified as belonging to subtype 29 and one isolate as 2. Two isolates of serogroup W encode subtype 120, three subtype 29 and one was of subtype 91. Of the serogroup X isolates, two encode subtype 118 and one 47. All other strains were non-typable.

The Norwegian Public Health Institute previously analysed *porA* and *fetA* genes. All A isolates harboured PorA of subtype 20,9 and all serogroup W isolates had a P1.5,2 or P1. 5-1,2-36 subtype. Among the serogroup X isolates, P1.19,26 and P1.19,26-4 accounted for 91% of the isolates, and 9% had a subtype of P1.5-1,10-1. Similarly to the set first of strains analysed, results from the typing analysis of these meningococcal strains isolated from a wider geographical area indicate limited diversity of major OMPs over time.

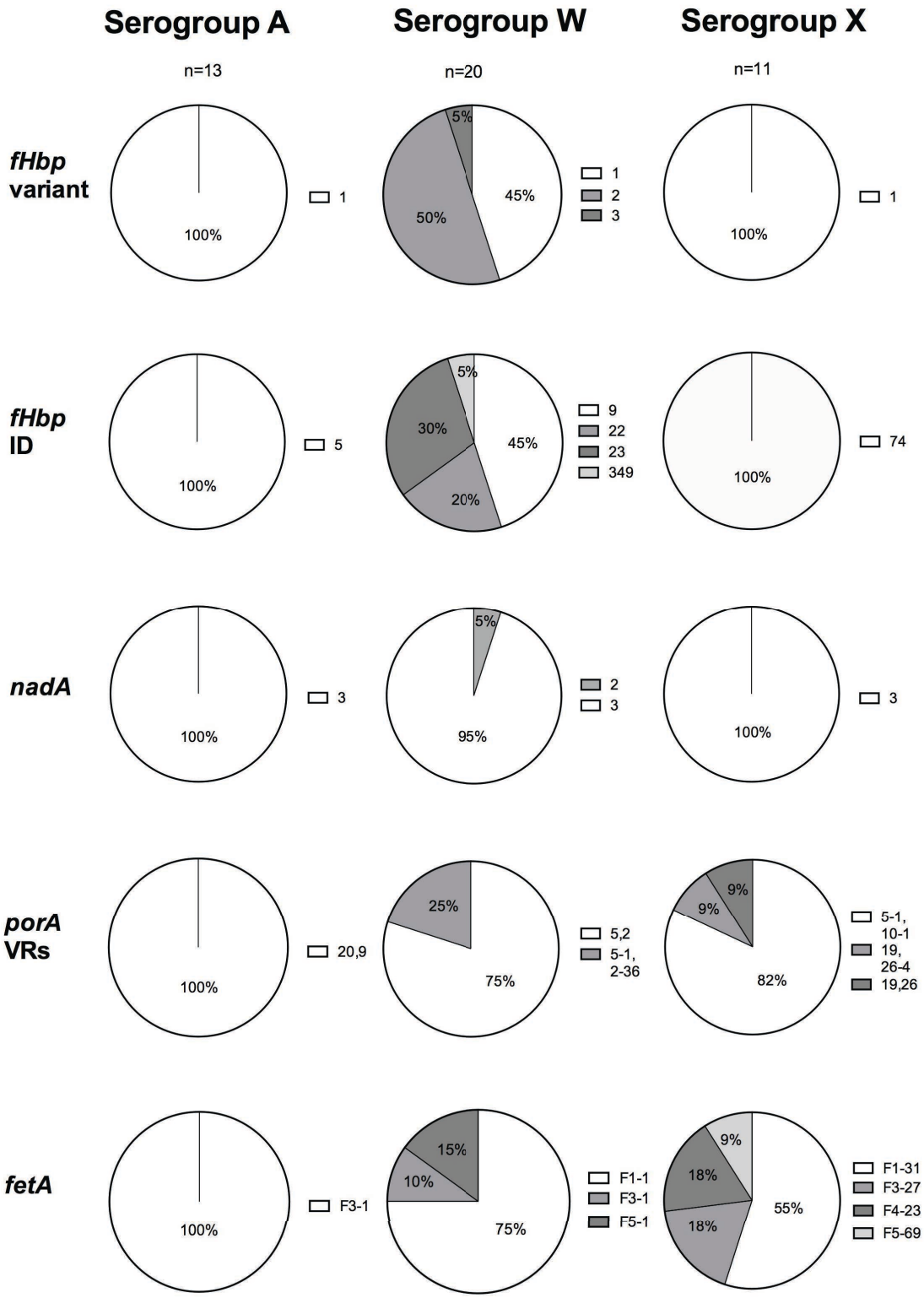

Figure 2.2. Typing analysis of the second set of serogroup A, W and X strains isolated from 12 countries of the sub-Saharan Africa between 2001-2011.

The fHbp variant group is according to classification proposed by Masignani *et al.* (2003) (26). Each isolate was typed by PCR amplification of each respective gene, sequencing and analysis using bioinformatics software Simmonics, Mega 4 and Chromas.

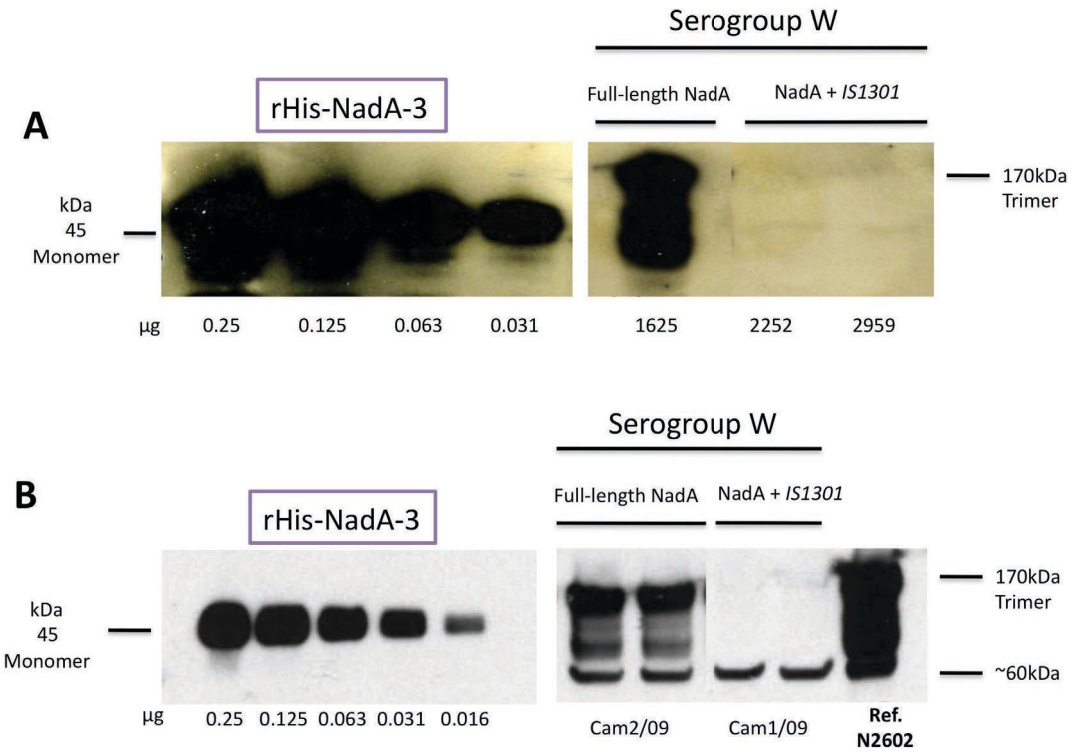

Figure 2.3. NadA protein expression in the African serogroup W isolates measured by Western blotting.

Panel A: NadA protein expression in the **first** set of serogroup W isolates with full-length NadA and NadA with insertion sequence *IS1301*. Isolates were obtained from Gerd Pluschke at STPHI. Panel B: NadA protein expression in the **second** set of serogroup W isolates with full-length NadA and NadA with insertion sequence *IS1301*. Isolates were obtained from Dominique Caugant at NIPH. The strains with the mobile genetic element had the transposon inserted at nt 470-474. Cells were grown to same optical density and whole cell extracts were prepared as described in Materials and Methods. Presence of NadA expression was detected with a polyclonal antiserum raised in mice against recombinant NadA allele 3. Positive controls were recombinant NadA-3 expressed and purified as Hexa-Histidine proteins in *E. coli*.

2.4.4 Majority of the African isolates studied have intermediate or high fHbp expression.

The level of fHbp expression has been shown to be a factor that can affect susceptibility of meningococci to anti-fHbp antibodies. High expressers of fHbp are generally more susceptible to killing than low expressers (5). We measured fHbp expression in whole cell extracts from 30 isolates by Western blot using monoclonal antibodies JAR5 and JAR31 against fHbp v.1 and v.2/3 respectively. Of the serogroup A isolates, we selected all strains from Burkina Faso (n=2, 35%) and 11 of 17 strains from Ghana (50%) for the fHbp expression analysis. Of the W isolates, we selected 6 of 17 strains from Burkina Faso (50%) and all strains from Ghana (65%). All serogroup X strains isolated from various countries of the meningitis belt were selected. The strains were chosen to cover isolates from different years including oldest and newest strains. Within the group of strains selected 72% (n=21) were case isolates and 28% (n=8) were carrier isolates.

We prepared whole cell extracts of all test strains and the serogroup B reference strains and compared fHbp levels with defined amounts of a fHbp v.1 and v.2 protein standard by Western blot and densitometry measurement. Expression level of the reference serogroup B strain H44/76 and 8047 were set to 100% and levels of expression of the test strains were compared with the reference strain. All serogroup A isolates (**Figure 2.4, Panel A**) have intermediate expression of fHbp. Majority of the serogroup X isolates (**Panel C**, n=5, 55%) expressed high amounts of fHbp compared with reference strain H44/76. The expression levels among the W isolates were variable, ranging from 50-152%, with 41% of the isolates expressing >100% of fHbp compared to the reference strain 8047 (**Panel B**). There was no significant

difference in fHbp expression between case and carrier W isolates studied (P=0.74; Mann Whitney U test).

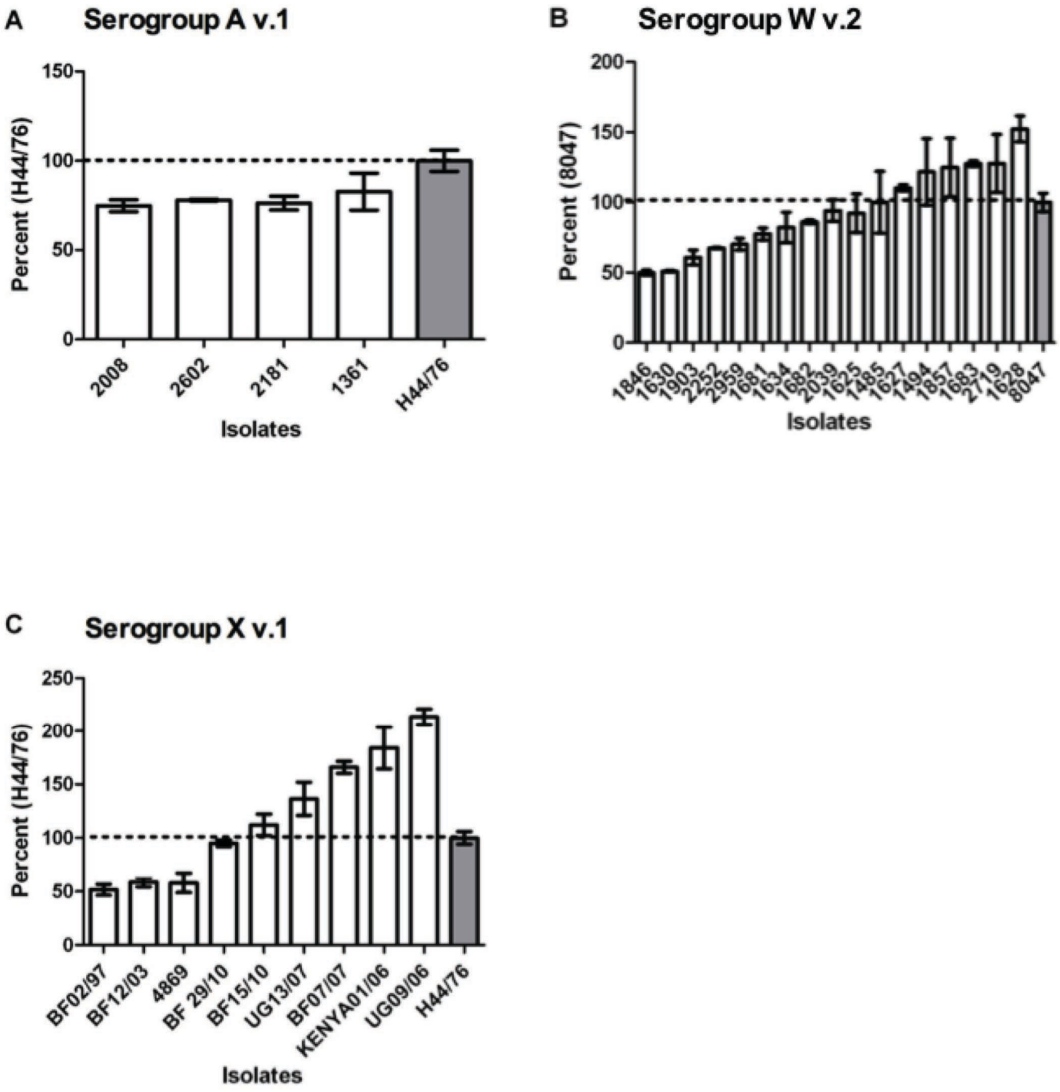

Figure 2.4. Expression of fHbp in the first set of meningococcal serogroup A, W and X isolates assessed by Western blot.

Panel A: serogroup A isolates with fHbp ID 5; Panel B: W isolates with fHbp ID 22 (isolates 2039, 2252, 2719 and 2959) or 23 (13 isolates); Panel C: serogroup X isolates with fHbp v.1 ID 12 (4869), 73 (BF12/03, BF02/97) and 74 (6 isolates). Bars represent the mean percentage from 3 biological replicates compared with the expression of fHbp of the reference serogroup B strains H44/76 and 8047, both high expressers of fHbp variant 1 ID1 and variant 2 ID 77, respectively. Isolates with means below 33% were classified as low expressers while isolates with expression above 100% were categorized as high expressers. Values between 33-100% were considered as intermediate expression (5). Dotted line represents the level of fHbp expression by the reference strains. Error bars represent standard errors.

For the second set of meningococcal serogroup A, W and X isolates, fHbp expression levels in whole cell extracts was measured for 42 isolates by Western blot using polyclonal antibody against rHis-fHbp v.1 and v.2/3 respectively (**Figure 2.5**). Most serogroup A and X isolates (**Panel A** and **C** respectively) have intermediate expression of fHbp. About half of the serogroup X isolates expressed amounts of fHbp lower than 50% compared to H44/76 high expresser of v.1 ID1, and two of those expressed very low levels of fHbp at around 4%. Two-thirds of the W isolates with a *fHbp* of v.1 expressed low amounts of fHbp (**Panel B**). On the contrary, majority of the W isolates with a *fHbp* of v.2 or v.3 were found to express intermediate levels of fHbp compared to reference strain 8047 (**Panel D**). The results suggest that fHbp protein expression on the bacterial surface is variable among strains, serogroup and *fHbp* variant. Representative blot of fHbp expression analysis for both sets of meningococcal isolates is shown in **Figure 2.6.**

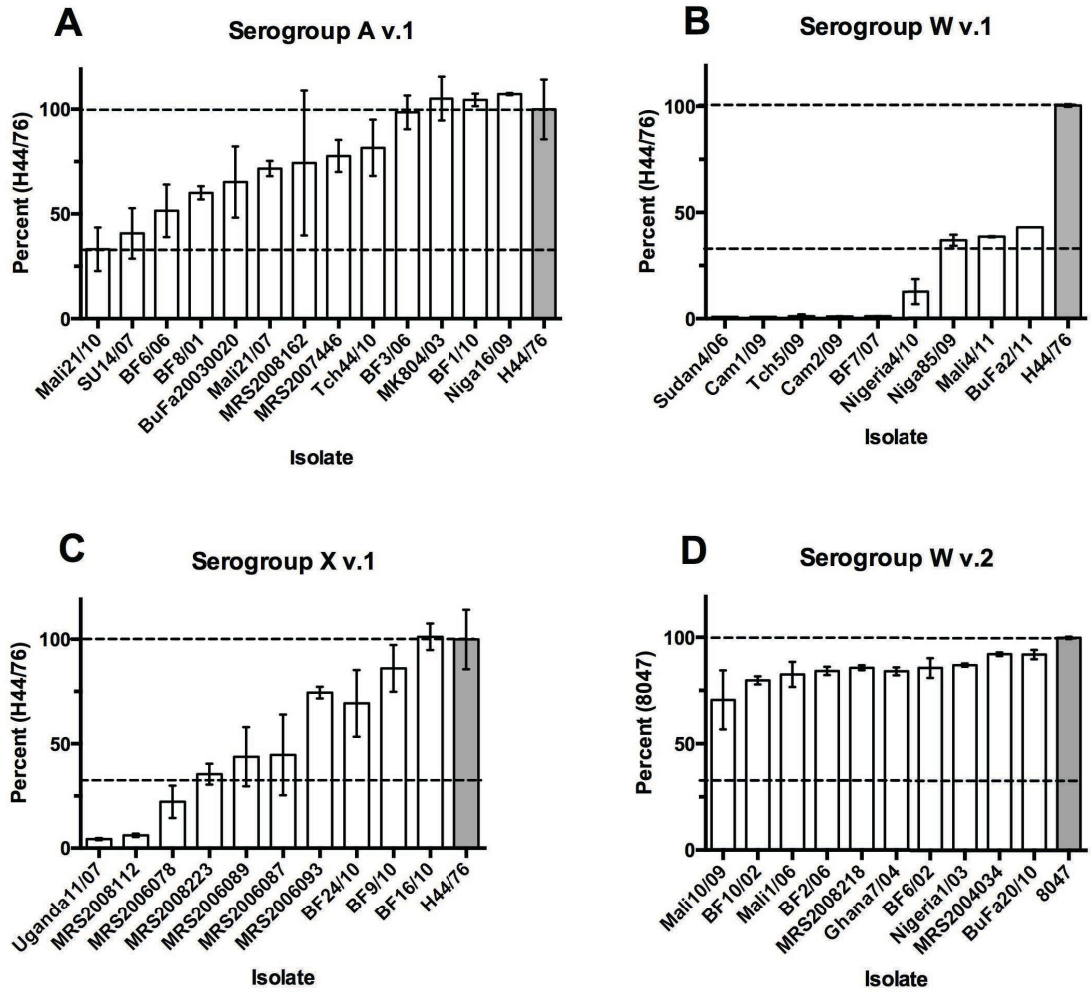

Figure 2.5. FHbp protein expression in the second set of African serogroup A, W and X isolates measured by Western blot.

Panel A: serogroup A isolates with *fHbp* v.1 ID 5; Panel B: serogroup W isolates with *fHbp* v.1 ID 9; Panel C: serogroup X isolates with *fHbp* v.1 ID 74; Panel D: serogroup W isolates with *fHbp* v.2 ID 22 or 23, and *fHbp* v.3 ID 349. Serogroup X Uganda14/06 and serogroup W Uganda11/06 were not included in the analysis since the isolates failed to grow. Bars in panel A-C represent mean percentages of a set of 2 independent experiments compared with expression of fHbp by the reference group B strain H44/76, which is naturally high expresser of fHbp ID 1 v.1. Bars in panel D represent mean percentages of a set of 2 independent experiments compared with expression of fHbp by the reference group B strain 8047, which is naturally high expressers of fHbp ID 77 of v.2. Bottom dotted line represents the low expresser classification threshold. Top dotted line represents the level of fHbp expression by the reference strains. Error bars represent standard errors.

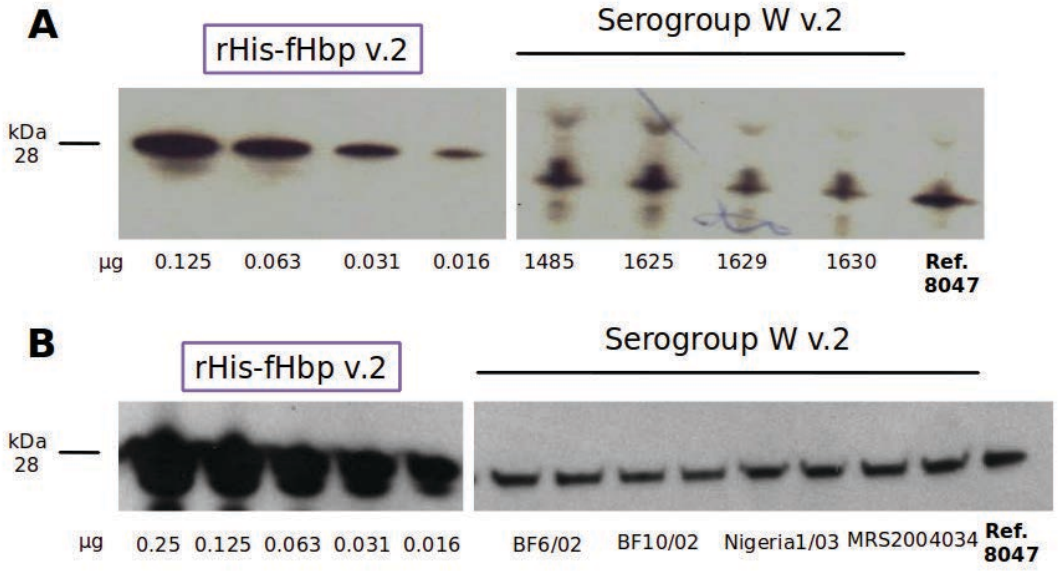

Figure 2.6. Representative analysis of fHbp expression in whole cell extracts of serogroup W isolates from the first and second set of African meningococcal isolates.

Panel A: representative fHbp expression data for four serogroup W isolates (1485, 1625, 1629 and 1630) from the first set of strains; Panel B: fHbp expression data for four serogroup W isolates (BF6/06, BF10/02, Nigeria1/03 and MRS2004034) in duplicate from the second set of strains. Serogroup B 8047 is the reference strain. rHis-fHbp v.2 were loaded as standard. Whole cell extracts were prepared as described in Materials and Methods. fHbp was detected using a monoclonal antibody raised against rHis-fHbp v.3 that shows cross-reactivity with fHbp v.2. Positive controls were recombinant fHbp expressed and purified as Hexa-Histidine proteins in *E. coli*.

2.4.5 Serum bactericidal responses of antibodies elicited by a recombinant serogroup W GMMA vaccine over-expressing fHbp v.1

The SBA responses of the antiserum of mice immunised with the recombinant serogroup W GMMA vaccine over-expressing fHbp v.1 ID1 was assessed against two serogroup A isolates and 3 X isolates expressing heterologous PorA and varying levels of fHbp v.1 (**Table 2.8**). We focussed on A and X strain with heterologous PorA to asses potential cross-protection because strains with homologous PorA are highly susceptible to bactericidal activity of anti-PorA antibodies (5,42).

Table 2.8. Panel of isolates selected for serum bactericidal activity analysis.

Selection included case isolates with variation in fHbp expression level, different sequence types and year of isolation.

Serogroup	Isolate	Origin	Year of isolation	Source	*fHbp* variant	*fHbp* ID	Sequence type (ST)	fHbp expression %[a]
A	N2008	Navrongo, Ghana	2005	case	1	5	ND	75
A	N2602	Nouna, Burkina Faso	2007	case	1	5	2859	78
X	BF 2/97	Burkina Faso	1997	case	1	73	751	52
X	BF 12/03	Burkina Faso	2003	case	1	73	751	58
X	BF 07/07	Burkina Faso	2007	case	1	74	181	131

ND= not defined.

[a]Average level of fHbp expression in percentage compared with expression by reference serogroup B H44/76.

GMMA from the Triple KO, OE fHbp strain induced antibodies that killed only the serogroup A isolate N2602 (geometric mean titre [GMT]=3033), (**Figure 2.7, Panel A** and **B**). The serogroup A strain N2008 isolated from Ghana resistant to killing expressed similar amounts of fHbp (75%) as N2602, which was sensitive to the antiserum. Both strains were readily killed by an anti-A capsular monoclonal

antibody. The Triple KO, OE fHbp GMMA vaccine was also able to kill all serogroup X isolates tested (GMT=11 to 4413) (**Figure 2.7, Panel C-E**). GMMA produced from the W strain that lacked fHbp v.1 over-expression (Triple KO) induced antibodies that were only able to kill the A strain N2602 and X isolate BF7/07 (GMT=135 and 14 respectively), suggesting that majority of the bactericidal antibodies produced are directed against fHbp when measured against strains with a heterologous PorA compared to that included in the vaccine. Antibodies induced by rHis-fHbp ID1 did not elicit a measurable bactericidal antibody responses against the strain tested (Titre <10).

The GMMA vaccines were prepared from a mutant with deleted capsule in order to attenuate virulence. A second motive of this was to reduce the potential generation of serogroup specific anti-capsular antibodies. The effect of the bactericidal antibody responses when maintaining the capsule on the GMMA was tested by immunising mice with GMMA vaccine prepared from isogenic mutants expressing the capsule (Double KO, OE fHbp). The bactericidal activity observed for the Double KO, OE fHbp was lower compared to the Triple KO, OE fHbp vaccine against three of the five strains tested (N2602, BF12/03, BF07/07 [P=0.0119]; GMT 386, 57 and 481 respectively). The results suggest that in accordance with our hypothesis deletion of the capsule increases the antibody responses towards protein-antigens such as fHbp.

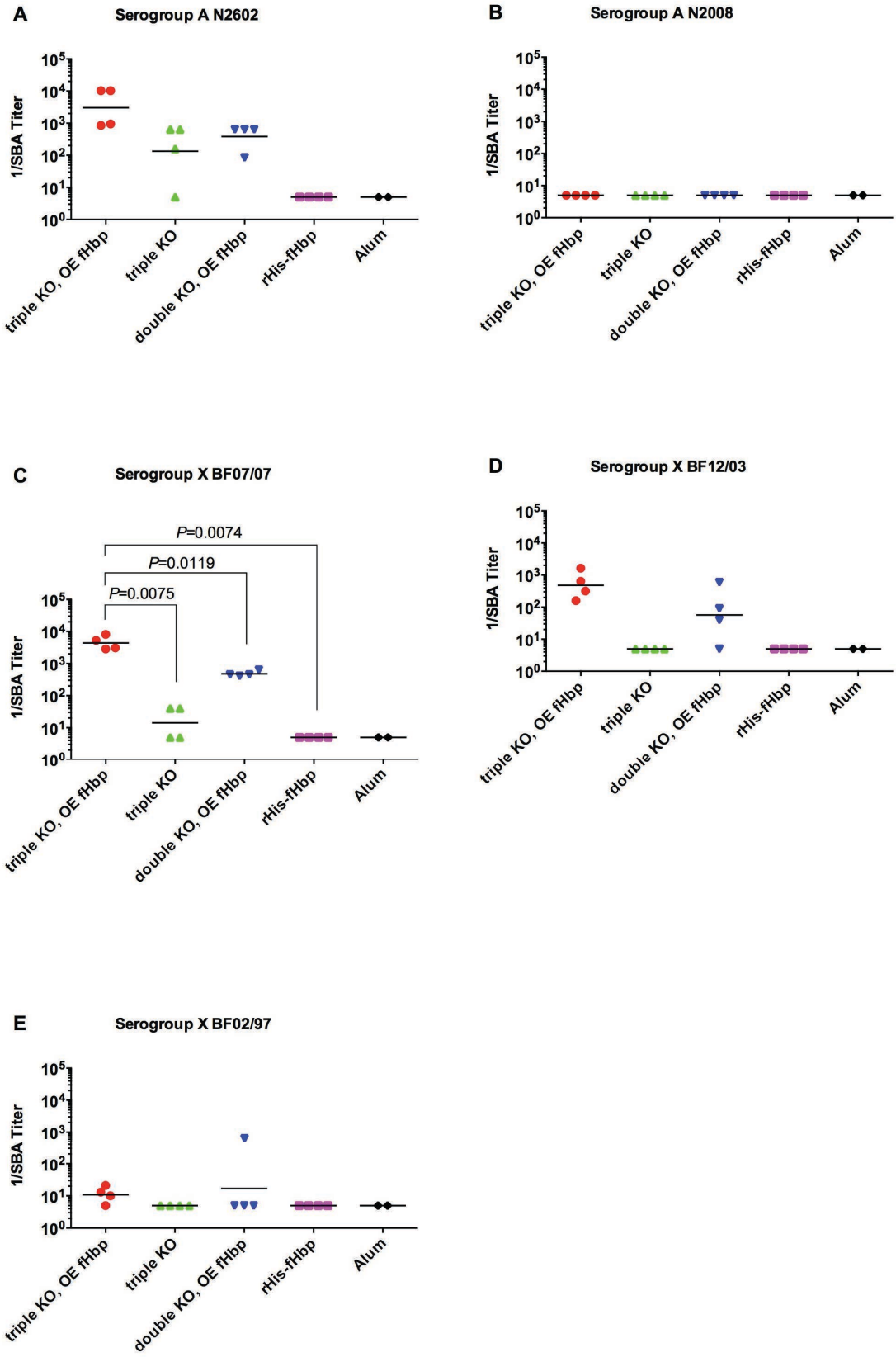
A
Serogroup A N2602
1/SBA Titer
10^5
10^4
10^3
10^2
10^1
10^0
triple KO, OE fHbp
triple KO
double KO, OE fHbp
rHis-fHbp
Alum
B
Serogroup A N2008
1/SBA Titer
triple KO, OE fHbp
triple KO
double KO, OE fHbp
rHis-fHbp
Alum
C
Serogroup X BF07/07
P=0.0074
P=0.0119
P=0.0075
1/SBA Titer
triple KO, OE fHbp
triple KO
double KO, OE fHbp
rHis-fHbp
Alum
D
Serogroup X BF12/03
1/SBA Titer
triple KO, OE fHbp
triple KO
double KO, OE fHbp
rHis-fHbp
Alum
E
Serogroup X BF02/97
1/SBA Titer
triple KO, OE fHbp
triple KO
double KO, OE fHbp
rHis-fHbp
Alum

Figure 2.7. Complement-mediated bactericidal activity responses of mice immunised with prototype GMMA vaccine Triple KO OE fHbp against African serogroup A and X meningococcal strains.

Panel A: strain N2602 serogroup A isolated from Burkina Faso; Panel B: strain N2008 isolated from Ghana; Panel C: serogroup X isolate BF07/07; Panel D: serogroup X isolate BF12/03 isolated from Burkina Faso; Panel E: serogroup X isolate BF02/97 isolated from Burkina Faso. Each symbol represents reciprocal serum titre of pooled sera from two mice (except alum where four mice were pooled to one group) using human complement. The horisontal line represents the geometric mean titre (GMT). Error bars indicate the standard error of the mean. GMMA vaccines: Triple KO, OE fHbp: capsule, *lpxL1* and *gna33* KO with over-expressed fHbp ID1, red dots. Double KO, OE fHbp: *lpxL1* and *gna33* KO with over-expressed fHbp ID1, blue dots. Triple KO: capsule, *lpxL1* and *gna33* KO without over-expressed fHbp, green dots. rHis-fHbp: recombinant hexa-Histidine tagged fHbp ID1, pink dots. Alum: aluminium hydroxide, black dots. Statistical analysis was done by Mann-Whitney *U* test.

2.5 Discussion

In the meningitis belt of sub-Saharan Africa, epidemic meningitis caused by *N. meningitidis* is a severe public health problem. The stepwise introduction of immunisation campaigns with the conjugate polysaccharide vaccine MenAfriVac™ against serogroup A is currently used to control the disease caused by this serogroup (43). Conjugate vaccines do not only protect against disease but also block transmission of carriage strains expressing the same vaccine serogroup. However, meningitis caused by non-serogroup A meningococci, particularly W but also X, are an ongoing problem. There is also a concern regarding the effect of the lack of a stable nasopharyngeal population of apathogenic meningococci due to the neutralising immunity conferred by the conjugate vaccines (43). This could render the population vulnerable to epidemics caused by other serogroups than just W and X. Moreover, the risk of vaccine induced capsule switching to evade killing has been a long standing concern. Evidence of capsule switching between serogroups with a polysialyltransferase gene (*synD*) such as W and C have been reported (44,45). The emergence of the epidemic serogroup W ST-11 strain from Haji in 2000 originated from a serogroup C ST-11 (6). Therefore, an affordable comprehensive vaccine based on OMPs that can provide broad protection against all meningococcal serogroups can be a potential solution to control disease caused by all serogroups in the meningitis belt (46,47). Molecular typing approaches based on DNA sequencing allow the dynamics of the variation and diversification of surface-exposed antigens of meningococci to be monitored over time. Such approaches are also useful to determine whether escape from immune detection by variation of OMPs has occurred. Here, we investigated the putative impact and suitability of the OMPs NadA, fHbp, NHBA and PorA to be used in a GMMA-based vaccine platform for Africa.

We examined the genetic diversity of two sets of 94 meningococcal carriage and disease serogroup A, W and X strains. Both sets of strains studied were isolated from various countries in the meningitis belt between 1995-2011, and contain conserved *fHbp, porA* and *nadA* genes, suggesting little antigenic diversification over time. Previously, Pajon *et al*. (2011) performed a molecular characterization of 106 invasive meningococcal A, W and X isolates from 13 African countries (5). The study showed that 100% of the serogroup A isolates isolated in Burkina Faso and Ghana express fHbp ID 5 and PorA subtype P1.20,9. Most of the serogroup X isolates was shown to have an *fHbp* of ID 73 or 74. In contrast to our findings 58% of the serogroup W were *fHbp* v.2, while 34% were v.1 and 8% v.3. The isolates from Burkina Faso and Ghana expressed fHbp v.1 ID9 or v.2 ID23. Concordant with our findings, 98% of W expressed PorA subtype P1.5,2 or a related subtype indicating a marked homogeneity of PorA type among African serogroup W isolates. A more recent longitudinal study found that a hypervirulent ST-11 serogroup W clone was responsible for most meningococcal disease in 2011 and 2012 (48). All the isolates had PorA P1.5,2. In accordance with our study, these two previous studies emphasise the limited diversification of major OMPs in the meningococcal population in Africa.

NadA has emerged as an important protein for adhesion and invasion, and has been shown to elicit bactericidal antibodies (49). Previous studies have shown that *nadA* is present in 50% of non-African clinical isolates and were highly diversified (32,40). Wang *et al.* (2011) detected *nadA*-1 and 2 in serogroup B isolates from U.S., allele 2>1>3 in serogroup C and alleles 3>2 in serogroup Y (40). Commanducci *et al*. (2004) showed that European serogroup B isolates tested harboured 1>2>3, serogroup C allele 2>3 and serogroup A allele 3 (50). Serogroup A isolates from China were

mainly of allele 3 and 4 (51). To summarise, our results with African strains suggest firstly that there is little variability of *nadA* over time and geographic origin (5,43,52). Secondly, the remarkable antigenic stability also indicates that natural immunity to these OMPs is not a strong driving force for antigenic diversification, making them suitable for use as vaccine candidate antigens for use against African strains. Further analysis of *nadA* sequences showed that 10 (11%) serogroup W isolates had the *nadA* gene disrupter by *IS1301*. Eight of these had the mobile genetic element inserted between nt 470-474 and with a stop codon at nt 166. The others had *IS1301* inserted in nt 400-469 (Δ69nt; Nigeria1/03) and at nt 472-496 (MRS2008218). Previously Comanducci *et al.* (2004) reported such occurrence of interuption of the open reading frame (ORF) by the genetic element at the recognition sequence 5'-ACTAG-3' in position 474 and 583 of *nadA-3* coordinates (50). Compared to our study, it has been described that approximately 30% of meningococcal serogroup B, C and W strains carry the IS, and even higher prevalence was found in Y isolates (86.7%) (54,55). Nevertheless, Western blotting of the first set of isolates generated no bands while for the second set of isolates revealed a single band between 49-62kDa. As these proteins lack most of the C-terminal coiled-coil region and anchor we predict that there is some transcript produced, which could account for the band visualised, but not inserted into the membrane because the protein is devoid of the predicted anchor domain (39,56,57). One way to test this could be by flow cytometry staining of live cells using a anti-NadA mAb. However, the benefit of *IS1301* insertion into *nadA* is a conondrum. The 842-bp mobile genetic element can cause various effects upon insertion such as deletions, knockout of gene expression and regulation of downstream located genes (55). For example, previous studies have shown that insertion of *IS1301* into the *siaA* gene of the capsule biosynthesis locus is associated

with phase variation of capsule expression, therefore conferring fitness to the meningococci. The exact excision of the transposon allows for reversible on/off capsule synthesis, where expression is on during transmission and switched back to off during colonisation (58). Possibly antibodies elicited against NadA protein in the host population may give selective advantage to strains with no or decreased expression levels establishing immune escape variants. Moreover, such insertion is reversible upon precise excision and may predispose a strain to transmission or colonisation (54,55,59). Since NadA is involved in colonisation/adhesion and invasion, the transposon might render these states interchangable creating diversity in a bacterial population pre-adapted to environmental changes by providing another mechanism of *nadA* phase variation.

The GMMA approach is based on OMVs that have already been licensed for use in humans. However, the GMMA vesicles are not extracted using detergent rather produced from recombinant meningococcal strains that are engineered with a deleted *gna33* gene, which up-regulates vesicle release and retains hydrophobic OMPs like fHbp (1,2,60). Studies with isogenic mutants with different expression levels of fHbp suggested that low fHbp expression contributes to resistance to anti-fHbp bactericidal activity (5). High expressers of fHbp are generally more susceptible to killing by anti-fHbp antibodies than low expressers. It has been suggested that sparse distribution of antigens on the bacterial surface impedes cross-linking of two IgG anti-fHbp antibodies to correctly spaced epitopes (61). Consequently, the antibodies cannot engage the complement protein C1q by adjacent Fc domains, preventing subsequent C4 activation and C4b deposition of the classical complement pathway (27). It has been shown that mAbs that bind abundant antigens such as PorA, which is a trimer,

are more effective at engaging C1q (62). In the present study, for the first set of isolates tested, the serogroup A strains were found to express medium levels of fHbp and the W isolates expressed medium to high levels. There was no difference in expression between case and carrier isolates indicating that both could be targets of vaccine-induced anti-fHbp antibodies. For the second set of isolates we found that serogroup A and X isolates varied in fHbp expression with most isolates expressing intermediate levels of fHbp. Two-thirds of the W isolates with an *fHbp* of v.1 expressed low levels of fHbp. While majority of the W isolates with *fHbp* v. 2 or 3 expressed intermediate levels of fHbp. There is a chance that developing a vaccine based on one antigen were the level expression of the protein on the surface of bacteria is important could select for escape mutants. Consequently, developing a vaccine with additional antigens could possibly prevent this.

For the second objective of the study, we tested the bactericidal activity of sera from mice immunised with GMMA OE fHbp v.1 against five serogroup A and X isolates with varying expression levels of fHbp and with a heterologous PorA compared to the vaccine W strain. We generated the main vaccine GMMA from a recombinant serogroup W strain engineered with deleted capsular biosynthesis genes, *lpxL1* and *gna33* genes and up-regulation of fHbp v.1 ID1 (Triple KO, OE fHbp). The Triple KO OE fHbp vaccine induced bactericidal antibodies in four serogroup A and X isolates tested (80%) compared to none for the rHis-fHbp vaccine. This suggests that anti-fHbp antibodies induced by GMMA are of greater functional activity than those induced by rHis-fHbp. In the case of serogroup X isolates BF02/97 and BF12/03, which have similar levels of fHbp expression, higher titre was obtained for BF12/03 suggesting that fHbp expression level do not always account for resistance/sensitivity

to bactericidal activity. BF12/03 could potentially bind fH more strongly than BF02/97. Stability of fH-fHbp binding to the meningococcal surface has been shown to be a crucial factor for evading complement killing (63). Moreover, other proteins such as antigen NspA (Neisserial surface protein A) and PorB2 could also bind fH. PorB2 and NspA are another ligands for fH, and could be responsible for enhancing meningococcal resistance to complement (13,62).

An additional meningococcal factor important for evasion of complement-mediated killing is the capsule. In this study the serogroup A isolate N2008 was resistant to killing by the Triple KO, OE fHbp. However, both N2008 and N2602 were sensitive to anti-capsular A mAb. One possible reason for this observation could be that N2008 produces higher amounts of capsule than N2602 of polysaccharide length or of different chemistry. Future experiments that could investigate this could involve analysis of the amount of capsule expression by flow cytometry, and more in-depth analysis of the polysaccharide length and chemistry by Dionex, HPLC-SEC, NMR (currently in progress). Moreover, the capsule could act as an antigenic competitor interfering and decreasing the immune response to common protein antigens. This is also supported by the lower GMT observed for the Double KO, OE fHbp vaccine generated from a recombinant strain expressing the capsule against all strains tested compared to the Triple KO, OE fHbp without a capsule. The higher amount of capsule could also prevent accessibility of the fHbp antigen on the meningococcal surface to C1q complement protein and anti-fHbp antibodies (64,65).

2.6 Conclusion

This study of meningococcal serogroup A, W and X isolates from several African countries, together with the findings of other studies, suggests that there is limited antigenic variation of meningococcal OMPs that induce bactericidal antibodies. Moreover, the presence of the *nadA* gene in most case and carrier strains isolated from African countries suggests that NadA could be a potentially important vaccine antigen to be included in a GMMA vaccine for Africa. The GMMA vaccine with over-expressed fHbp v.1 generated here provides a proof-of-concept in mice that it can elicit antibodies that are bactericidal against majority of the serogroup A and X isolates testes with heterologous PorA and homologous fHbp. These findings support a strategy of using protein-based vaccines, such as GMMA, to prevent meningococcal meningitis in Africa caused by serogroup A, W and X.

2.7 List of publications

1. Koeberling O, **Ispasanie E**, Hauser J, Rossi O, Pluschke G, Caugant DA, et al. A broadly-protective vaccine against meningococcal disease in sub-Saharan Africa based on generalized modules for membrane antigens (GMMA). Vaccine. 2014; 32:2688–95.

2. **Ispasanie E**, Pluschke G, Hodgson, Sie A, MacLennan C, Koeberling O. Characterization of vaccine antigens of meningococcal serogroup W isolates from Ghana and Burkina Faso from 2003 to 2009. F1000Research. 2014; 3:264.

2.8 Appendix 1

Example DNA sequences:

fHbp

ID9_strain BF7/01 (second set)

GCGGCGGAAGCGGAGGCGGCGGTGTCGCCGCCGACATCGGTGCGGGGCT
TGCTGATGCGCTAACCGCACCGCTCGACCATAAAGACAAAGGTTTGCAGT
CTTTGACGCTGGATCAGTCCGTCAGGAAAAACGAGAAACTGAAGCTGGCG
GCACAAGGTGCGGAAAAAACTTATGGAAACGGCGACAGCCTCAATACGG
GCAAATTGAAGAACGACAAGGTCAGCCGCTTCGACTTTATCCGTCAAATC
GAAGTGGACGGGCAGCTCATTACCTTGGAGAGCGGAGAGTTCCAAGTGTA
CAAACAAAGCCATTCCGCCTTAACCGCCCTTCAGACTGAGCAAGTACAAG
ACTCGGAGGATTCCGGGAAGATGGTTGCGAAACGCCAGTTCAGAATCGGC
GACATAGCGGGCGAACATACGTCTTTCGACAAGCTTCCCAAAGGCGGCAG
TGCGACATATCGCGGGACGGCGTTCGGTTCAGACGATGCTGGCGGAAAAC
TGACCTATACTATAGATTTCGCCGTCAAACAGGGACACGGCAAAATCGAA
CATTTGAAATCGCCCGAACTCAATGTCGACCTGGCCGCCGCCTATATCAA
GCCGGATAAAAAACGCCATGCCGTCATCAGCGGTTCCGTCCTTTACAACC
AAGACGAGAAAGGCAGTTACTCCCTCGGCATCTTTGGCGGGCAAGCCCAG
GAAGTTGCCGGCAGCGCGGAAGTGGAAACCGCAAACGGCATACACCATA
TCGGTCTTGCCGCCAAGCA

ID12_strain 4869 (first set of meningococcal isolates)

TATATTATCTANNTCTGCGTATGACTAGGAGCAAACCTGTGAACCGAACT
GCCTTCTGCTGCTTTTCTCTGACCGCCGCCCTGATTCTGACCGCCTGCAGC
AGCGGAGGCGGCGGTGTCGCCGCCGACATCGGCGCGGGGCTTGCCGATGC
ACTAACCGCACCGCTCGACCATAAAGACAAAAGTTTGCAGTCTTTGACGC
TGGATCAGTCCGTCAGGAAAAACGAGAAACTGAAGCTGGCGGCACAAGG
TGCGGAAAAAACTTATGGAAACGGCGACAGCCTCAATACGGGCAAATTG
AAGAACGACAAGGTCAGCCGCTTCGACTTTATCCGTCAAATCGAAGTGGA
CGGGCAGCTCATTACCTTGGAGAGCGGAGAGTTCCAAGTGTACAAACAAA
GCCATTCCGCCTTAACCGCCCTTCAGACCGAGCAAGTACAAGACTCGGAG
GATTCCGGGAAGATGGTTGCGAAACGCCAGTTCAGAATCGGCGACATAGC
GGGCGAACATACATCTTTTGACAAGCTTCCCAAAGGCGGCAGTGCGACAT
ATCGCGGGACGGCGTTCGGTTCAGACGATGCTGGCGGAAAACTGACCTAT
ACTATAGATTTCGCCGCCAAGCAGGGACACGGCAAAATCGAACATTTGAA
ATCGCCCGAACTCAATGTCGAGCTTGCCACCGCCTATATCAAGCCGGATG
AAAAACGCCATGCCGTTATCAGCGGTTCCGTCCNTTTACAACCAAGACGA
NAAANGGCAGTTACTCCCTCGGTATCTTTGGGCGGGCAAGCCCAGGAAGT
TGCCNGGCAGCNNNGNAAGNNNAANCGCAAACGGCATACACCNNATCGG
NTCTTGCNGCCNNGCAGTNNNNNNNGAAAANGCCGNACGA

ID22_strain 2039 (first set of meningococcal isolates)

TGCAGCAGCGGAGGCGGCGGTGTCGCCGCCGACATCGGCGCGGGGCTTGC
CGATGCACTAACCGCACCGCTCGACCATAAAGACAAAAGTTTGCAGTCTT
TGACGCTGGATCAGTCCGTCAGGAAAAACGAGAAACTGAAGCTGGCGGC
ACAAGGTGCGGAAAAAACTTATGGAAACGGCGACAGCCTCAATACGGGC

AAATTGAAGAACGACAAGGTCAGCCGCTTCGACTTTATCCGTCAAATCGA
AGTGGACGGGCAGCTCATTACCTTGGAGAGCGGAGAGTTCCAAATATACA
AACAGGACCACTCCGCCGTCGTTGCCCTACAGATTGAAAAAATCAACAAC
CCCGACAAAATCGACAGCCTGATAAACCAACGCTCCTTCCTTGTCAGCGG
TTTGGGTGGAGAACATACCGCCTTCAACCAACTGCCCAGCGGCAAAGCCG
AGTATCACGGCAAAGCATTCAGCTCCGACGACCCGAACGGCAGGCTGCAC
TACTCCATTGATTTTACCAAAAAACAGGGTTACGGCAGAATCGAACACCT
GAAAACGCCCGAGCAGAATGTCGAGCTTGCCTCCGCCGAACTCAAAGCAG
ATGAAAAATCACACGCCGTCATTTTGGGCGACACGCGCTACGGCGGCGAA
GAAAAAGGCACTTACCACCTCGCCCTTTTCGGCGACCGCGCCCAAGAAAT
CGCCGGCTCGGCAACCGTGAAGATAAGGGAAAAGGTTCACGAAATCGGC
ATCGCCGGCAAACAG

ID23_strain 1630 (first set)
TGCAGCAGCGGAGGCGGCGGTGTCGCCGCCGACATCGGCGCGGGGCTTGC
CGATGCACTAACCGCACCGCTCGACCATAAAGACAAAGGTTTGCAGTCTT
TGACGCTGGACCAGTCCGTCAGGAAAAACGAGAAACTGAAGCTGGCGGC
ACAAGGTGCGGAAAAAACTTATGGAAACGGCGACAGCCTCAATACGGGC
AAATTGAAGAACGACAAGGTCAGCCGCTTCGACTTTATCCGTCAAATCGA
AGTGGACGGGCAGCTCATTACCTTGGAGAGCGGAGAGTTCCAAATATACA
AACAGGACCACTCCGCCGTCGTTGCCCTACAGATTGAAAAAATCAACAAC
CCCGACAAAATCGACAGCCTGATAAACCAACGCTCCTTCCTTGTCAGCGG
TTTGGGTGGAGAACATACCGCCTTCAACCAACTGCCCAGCGGCAAAGCCG
AGTATCACGGCAAAGCATTCAGCTCCGACGACCCGAACGGCAGGCTGCAC
TACTCCATTGATTTTACCAAAAAACAGGGTTACGGCAGAATCGAACACCT
GAAAACGCCCGAGCAGAATGTCGAGCTTGCCTCCGCCGAACTCAAAGCAG
ATGAAAAATCACACGCCGTCATTTTGGGCGACACGCGCTACGGCGGCGAA
GAAAAAGGCACTTACCACCTCGCCCTTTTCGGCGACCGCGCCCAAGAAAT
CGCCGGCTCGGCAACCGTGAAGATAAGGGAAAAGGTTCACGAAATCGGC
ATCGCCGGCAAACAG

ID73_strain BF12/03 (first set)
ATGTATTATCTATTCTGCGTATGACTAGGAGCAAACCTGTGAACCGAACT
GCCTTCTGCTGCCTTTCTTTGACCGCCGCCCTGATTCTGACCGCCTGCAGC
AGCGGAGGCGGCGGTGTCGCCGCCGACATCGGCGCGGGGCTTGCCGATGC
ACTAACCGCACTGCCCGACCATAAAGACAAAGGTTTGCAGTCTTTGACGC
TGGATCAGTCCGTCAGGAAAAACGAGAAACTGAAGCTGGCGGCACAAGG
TGCGGAAGAAACTTATGGAAACGGCGACAGCCTCGATACGGGCAAATTG
AAGAACGACAAGGTCAGCCGCTTCGACTTTATCCGTCAAATCGAAGTGGA
CGGGCAGCTCATTACCTTGGAGAGCGGAGAGTTCCAAGTGTACAAACAAA
GCCATTCCGCCTTAACCGCCCTTCAGACCGAGCAAGTACAAGATTCGGAG
CATTCCGGGAAGATGGTTGCGAAACGCCGGTTCAGAATCGGCGATATAGC
GGGCGAACATACATCTTTTGACAAGCTTCCCGAAAGCGACAGGGCGACAT
ATCGCGGGACGGCGTTCAGTTCAGACGATGCTGGTGGAAAACTGACCTAC
ACCATAGATTTCGCCGCCAAGCAGGGACACGGCAAAATTGAACATTTGAA
ATCGCCAGAACTCAATGTTGACCTGGGCCGCCGCCGATATCAAGCCCGGA
TGAAAAACACCATGCCCGTCATCAGCNGNTNCCGNCCCTTTANNNCAAGA
CGANNNNGCANNNCTCCCNNNGNATCTTTNGNGGNANCCNGNAGTNGCC
GGCAGC

ID74_strain Kenya01/06 (second set)
CATGATGCGGTANGTNAAAAAAANGNNTNCANTNANTAGGGNGNTATAT
NTNTCTATATCTGCGTATGANTNGNAGTAAACCTGTGAACCCGAACTNNN
NCTGCTGCTTTTCTCTGACCGCCGCCCNGNTTCTGACCGCCNGCAGCAGCG
GAGGGGGCGGTGTCGCCGCCGACATCGGTGCGGGGCTTGCCGATGCACTA
ACCGCACCGCTCGACCATAAAGACAAAGGTTTGCAGTCTTTAACGCTGGA
TCAGTCCGTCAGGAAAAACGAGAAACTGAAGCTGGCGGCACAAGGTGCG
GAAAAAACTTATGGAAACGGCGACAGCCTTAATACGGGCAAATTGAAGA
ACGACAAGGTCAGCCGTTTCGACTTTATCCGTCAAATCGAAGTGGACGGG
CAGCTCATTACCTTGGAGAGCGGAGAGTTCCAAGTGTACAAACAAAGCCA
TTCCGCCTTAACCGCCCTTCAGACCGAGCAAGAACAAGATCCAGAGCATT
CCGGGAAGATGGTTGCGAAACGCCGGTTCAAAATCGGCGACATAGCGGG
CGAACATACATCTTTTGACAAGCTTCCCAAAGACGTCATGGCGACATATC
GCGGGACGGCGTTCGGTTCAGACGATGCCGGCGGAAAACTGACCTATACT
ATAGATTTTGCTGCCAAACAGGGACACGGCAAAATCGAACATTTGAAATC
GCCGGAACTCAATGTCGATCTGGCCGTCGCCTATATCAAGCCGGATGAAA
AACACCATGCCGTCATCAGCGGTTCCGTTCTTTACAACCAAGACGAGAAA
GGCAGTTACTCCCTCGGTATCTTTGGCGAAAAAGCCCAGGAAGTTGCCGG
CAGCGCGGAAGTGAAAACCGCAAACGGCATACACCATATCGGCCTGCC

ID349_strain Uganda11/06 (second set)
AAGAATGGTTGTTTATATTATCTATTCTGCGTATGACTAGGAGTAAACCTG
TGAATCGAACTGCCTTCTGCTGCCTTTCTCTGACCACTGCCCTGATTCTGA
CCGCCCCCCCCCCCCTGCAGCAGCGGAGGCGGCGGTGTCGCCGCCGACATC
GGCACGGGGCTTGCCGATGCACTAACTGCGCCGCTCGACCATAAAGACAA
AGGTTTGAAATCCCTGACATTGGAAGACTCCATTCCCCAAAACGGAACAC
TGACCTTGTCGGCACAAGGTGCGGAAAAAACTTTCAAAGCCGGCGACAAA
GACAACAGCCTCAACACGGGCAAACTGAAGAACGACAAAATCAGCCGCT
TCGACTTTGTGCAAAAAATCGAAGTGGACGGACAAACCATCACGCTGGCA
AGCGGCGAATTTCAAATATACAAACAGGACTACTCCGCCGTCGTTGCCCT
ACAGATTGAAAAAATCAACAACCCCGACAAAATCGACAGCCTGATAAAC
CAACGCTCCTTCCTTGTCAGCGGTTTGGGCGGAGAACATACCGCCTTCAAC
CAACTGCCCGGCGGCAAAGCCGAGTATCACGGCAAAGCATTCAGCTCCGA
CGACCCGAATGGCAGGCTGCACTACTCCATTGATTTTACCAAAAAACAGG
GTTACGGCAGAATCGAACACCTGAAAACGCCCGAGCAGAATGTCGAGCTT
GCCGCCGCCGAACTCAAAGCAGATGAAAAATCACACGCCGTCATTTTGGG
CGACACGCGCTACGGCAGCGAAGAAAAAGGCACTTACCACCTCGCCCTTT
TCGGCGACCGCGCCCAAGAAATCGCCGGCTCGGCAACCGTGAAGATAGG
GGAAAAGGTTCACGAAATCGGCATCGCCGGCAAACAG

PorA
PorA, P1.5,2 strain 1630 (first set)
TTACCGCCCTCGTATTGTCCGCACTGCCGCTTGCGGCCGTTGCCGATGTCA
GCCTGTACGGCGAAATCAAAGCCGGCGTGGAAGGCAGGAACATCCAGCT
GCAGTTGACCGAA**CCGCTCCAAAATATTCAACCTCAGGTTACTAAGCG**
CAAAAGCCGCATCAGGACGAAAATCAGCGATTTCGGCTCGTTTATCGGCT
TTAAGGGGAGCGAGGATTTGGGCGAAGGGCTGAAGGCTGTTTGGCAGCTT
GAGCAAGACGTATCCGTTGCCGGCGGCGGCGCGACCCAGTGGGGCAACA
GGGAATCCTTTATCGGCTTGGCAGGCGAATTCGGCACGCTGCGCGCCGGT

CGCGTTGCGAATCAGTTTGACGATGCCAGCCAAGCCATTGATCCTTGGGA
CAGCAATAATGATGTGGCTTCGCAATTGGGTATTTTCAAACGCCACGACG
ATATGCCGGTTTCCGTACGCTACGATTCTCCGGAATTTTCCGGTTTCAGCG
GCAGCGTCCAATTCGTTCCGGCCCAAAACAGCAAATCCGCCTATACACCG
GCT**CATTTTGTTCAGCAGACTCCTAAAAGTCAGCCTACTCTCGTTCCG**
GCTGTTGTCGGCAAGCCGGGGTCGGATGTGTATTATGCCGGTCTGAATTA
CAAAAATGGCGGTTTTG

Bold base pairs and highlighted in grey: PorA variable region (VR) 1 corresponding to allele 5.

Bold base pairs underlined: PorA VR 2 corresponding to allele 2.

NadA

NadA truncated allele 1 strain Ug09/06 (first set)

ATGAAACACTTTCCATCCAAAGTACTGACCACAGCCATCCTTGCCACTTTC
TGTAGCGGCGCACTGGCAGCCACAAGCGACGACGATGTTAAAAAAGCTG
CCACTGTGGCCATTGTTGCTGCCTACAACAATGGCCAAGAAATCAACGGT
TTCAAAGCTGGAGAGACCATCTACGACATTGGTGAAGACGGCACAATTAC
CCAAAAAGACGCAACTGCAGCCGATGTTGAAGCCGACGACTTTAAAGGTC
TGGGTCTGAAAAAAGTCGTGACTAACCTGACCAAAACCGTCAATGAAAAC
AAACAAAACGTCGATGCCAAAGTAAAAGCTGCAGAAACTGCAGCAGGCA
AAGCCGAAGCTGCCGCTGGCACAGCTAATACTGCAGCCGACAAGGCCGA
AGCTGTCGCTGCAAAAGTTACCGACATCAAAGCTGATATCGCTACGAACA
AAGCTGATATTGCTAAAAACTCAGCACGCATCGACAGCTTGGACAAAAAC
GTAGCTAATCTGCGCAAAGAAACCCGCCAAGGCCTTGCAGAACAAGCCGC
GCTCTCCGGCCTGTTCCAACCTTACAACGTGGGTCGGTTCAATGTAACGGC
TGCAGTCGGCGGCTACAAATCCGAATCGGCAGTCGCCATCGGTACCGGCT
TCCGCTTTACCGAAAACTTTGCCGCCAAAGCAGGCGTGGCAGTCGGCACT
TCGTCCGGTTCTTCCGCAGCCTACCATGTCGGCGTCAATTACGAGTGGTAA

NadA allele 2 strain Mali10/09 (second set)

TGGCAGCCACAAAGGGCGGTGGATTTAAAAAAGCTGCCAATGTGGCCATT
GGTGCTGCCTACAACAATGGCCAAGAAATCAACGGTTTCAAAGCTGGAGA
GACCATCTACGACATTGATGAAGACGGCACAATTACCAAAAAAGACGCA
ACTGCAGCCGATGTTGAAGCCGACGACTTTAAAGGTCTGGGTCTGAAAAA
AGTCGTGACTAACCTGACCAAAACCGTCAATGAAAACAAACAAAACGTC
GATGCCCAAGTAAAAGCTGCAGAATCTGAAATAGAAAAGTTAACAACCA
AGTTAGCAGACACTGATGCCGCTTTAGCAGATACTGATGCCGCTCTGGAT
GCAACCACCAACGCCTTGAATAAATTGGGAGAAAATATAACGACATTTGC
TGAAGAGACTAAGACAAATATCGTAAAAATTGATGAAAAATTAGAAGCC
GTGGCTGATACCGTCGACAAGCATGCCGAAGCATTCAACGATATCGCCGA
TTCATTGGATGAAACCAACACTAAGGCAGACGAAGCCGTCAAAACCGCCA
ATGAAGCCAAACAAAACGTCGATGCCAAAGTAAAAGCTGCAGAAACTGC
AGCAGGCAAAGCCGAAGCTGCCGCTGGCACAGCTAATACTGCAGCCGAC
AAGGCCGAAGCTGTCGCTGCAAAAGTTACCGACATCAAAGCTGATATCGC
TACGAACAAAGATAATATTGCTAAAAAAGCAAACAGTGCCGACGTGTAC
ACCAGAGAAGAGTCTGACAGCAAATTTGTCAGAATTGATGGTCTGAACGC

TACTACCGAAAAATTGGACACACGCTTGGCTTCTGCCGAAAAATCCATTA
CCGAACACGGTACGCGCCTGAACGGTTTGGATAGAACAGTGTCAGACCTG
CGTAAAGAAACCCGCCAAGGCCTTGCAGAACAAGCCGCGCTCTCCGGTCT
GTTCCACCTTACAACGGTGGGTCGGTTCAATGTAACGGCTGCAGTCGGCG
GCTACAAATCCGAATCGGCAGTCGCCATCGGTACCGGCTTCCGCTTTACC
GAAAACTTTGCCGCCCAAGCAGGGCGTGGCAGTCGGCACTTCGTCCGGGT
CTTCCGCACTTA

NadA allele 3 strain N2602 (first set)
ATGAAACACTTTCCATCCAAAGTACTGACCACAGCCATCCTTGCCACTTTC
TGTAGCGGCGCACTGGCAGCCACAAACGACGACGATGTTAAAAAAGCTG
CCACTGTGGCCATTGCTGCTGCCTACAACAATGGCCAAGAAATCAACGGT
TTCAAAGCTGGAGAGACCATCTACGACATTGATGAAGACGGCACAATTAC
CAAAAAAGACGCAACTGCAGCCGATGTTGAAGCCGACGACTTTAAAGGTC
TGGGTCTGAAAAAAGTCGTGACTAACCTGACCAAAACCGTCAATGAAAAC
AAACAAAACGTCGATGCCAAAGTAAAAGCTGCAGAATCTGAAATAGAAA
AGTTAACAACCAAGTTAGCAGACACTGATGCCGCTCTGGATGCAACCACC
AACGCCTTGAATAAATTGGGAGAAAATATAACGACATTTGCTGAAGAGAC
TAAGACAAATATCGTAAAAATTGATGAAAAATTAGAAGCCGTGGCTGATA
CCGTCGACAAGCATGCCGAAGCATTCAACGATATCGCCGATTCATTGGAT
GAAACCAACACTAAGGCAGACGAAGCCGTCAAAACCGCCAATGAAGCCA
AACAGACGGCCGAAGAAACCAAACAAAACGTCGATGCCAAAGTAAAAGC
TGCAGAAACTGCAGCAGGCAAAGCCGAAGCTGCCGCTGGCACAGCTAAT
ACTGCAGCCGACAAGGCCGAAGCTGTCGCTGCAAAAGTTACCGACATCAA
AGCTGATATCGCTACGAACAAAGATAATATTGCTAAAAAAGCAAACAGTG
CCGACGTGTACACCAGAGAAGAGTCTGACAGCAAATTTGTCAGAATTGAT
GGTCTGAACGCTACTACCGAAAAATTGGACACACGCTTGGCTTCTGCCGA
AAAATCCATTACCGAACACGGTACGCGCCTGAACGGTTTGGATAGAACAG
TGTCAGACCTGCGTAAAGAAACCCGCCAAGGCCTTGCAGAACAAGCCGCG
CTCTCCGGTCTGTTCCAACCTTACAACGTGGGTCGGTTCAATGTAACGGCT
GCAGTCGGCGGCTACAAATCCGAATCGGCAGTCGCCATCGGTACCGGCTT
CCGCTTTACCGAAAACTTTGCCGCCAAAGCAGGCGTGGCAGTCGGCACTT
CGTCCGGTTCTTCCGCAGCCTACCATGTCGGCGTCAATTACGAGTGGTAA

NadA allele 3+*IS1301* strain BuFa20/10 (second set)
CGATGTTAAAAAAGCTGCCACTGTCGGCCATTGCTGCTGCCTACAACAA
TGGCCAAGAAATCAACGGTTTCAAAGCTGGAGAGACCATCTACGACATT
GATGAAGACGGCACAATTACCAAAAAAGACGCAACTGCAGCCGATGTTG
AAGCCGACGACTTTAAAGGTCTGGGTCTGAAAAAAGTCGTGACTAACCT
GACCAAAACCGTCAATGAAAACAAACAAAACGTCGATGCCAAAGTAAAA
GCTGCAGAATCTGAAATAGAAAAGTTAACAACCAAGTTAGCAGACACTG
ATGCCGCTTTAGCAGATACGGATGCCGCTCTGGATGCAACCACCAACGC
CTTGAATAAATTGGGAGAAAATATAACGACATTTACTGAAGAGACTAGG
GCGTG**NNNTCCTCAATTTAACTTAGCATGAATAATAGAGCAAGCCAAG**
TAAAGCATCGATTTAAAATTACGAGCTAATTTCTCATAGCGAGTAGCA
ATACTACGAAACTGCTTTAATCGTGCAAATGCATTCTCGACTAAGTGG
CGTAATTTATAAAGGTAGCTATCAAAATCAGGGTTAGGTTTCTTGGCA
TTTTTACGCTTTGGTATAATAGCTTTCATACCGTGTTCTATCGCTTTAT
CTCTGATTTCTTGCGAGTCATACCCTTTGTCAGCGATAAAATACTGGG
CTTCTTGTATGACTTCTATCAAGTCGTTTGCAACTTGACCGTCGTGCA

CGTTACCCCCAGTGACTTTAAAATCGAGCGGATTTCCATGCGAGTCCA
CACATAGGTGTATTTTTTGTCGTGTTTCCGCCACGGCTTTGTCCAATT
GCTCTATCGAAACCACGCCGAGCTCCACTTGCATGTTGATGACACCG
TACATAACTTCCGTCGATGAATACCCATTCTTTGTCAATTTCTTTTCGT
AGATCAAAAAAAAATTCTGCCACAAGCCTTTTTTAGACCATCGGTTAA
AGCGGTTATAAGCGGTTTTCCATGACCCTAGCTCAATAGGTATGTCTC
GCCATGGTGCACCTGTTCTTAGCTTCCATAGTAAGGCTTCCATCACGG
TACGGTCGTTCTTCCATTGATGACAGCCGTGCGCTTTCATGGTTGTTT
GTAATTGTTCCCATATGTTGTCAGTTATTGCGGTTCTCGCCATTGTTT
GTGTTTGCCTATATTTCGTTGGAATATAGGGTTAAAATAGGGCNNNTC
TTTTGGGCTTATTCAAATTAGGGACACGCCTTAAGACAAATATCGTAAAA
ATTGATGAAAAATTAGAAGCCGTGGCTGATACCGTCGACAAGCATGCCGA
AGCATTCAACGATATCGCCGATTCATTGGATGAAACCAACACTAAGGCAG
ACGAAGCCGTCAAAACCGCCAATGAAGCCAAACAGACGGCCGAAGAAAC
CAAACAAAACGTCGATGCCAAAGTAAAAGCTGCAGAAACTGCAGCAGGC
AAAGCCGAAGCTGCCGCTGGCACAGCTAATACTGCAGCCGACAAGGCCG
AAGCTGTCGCTGCAAAAGTTACCGACATCAAAGCTGATATCGCTACGAAC
AAAGATAATATTGCTAAAAAAGCAAACAGTGCCGACGTGTACACCAGAG
AAGAGTCTGACAGCAAATTTGTCAGAATTGATGGTCTGAACGCTACTACC
GAAAAATTGGACACACGCTTGGCTTCTGCTGAAAAATCCATTGCCGATCA
CGATACTCGCCTGAACGGTTTGGATAAAACAGTGTCAGACCTGCGCAAAG
AAACCCGCCAAGGCCTTGCAGAACAAGCCGCGCTCTCCGGTCTGTTCCAA
CCTTACAACGTGGGTCGGTTCAATGTAACGGCTGCAGTCGGCGGCTACAA
ATCCGAATCGGCAGTCGCCATCGGTACCGGCTTCCGCTTTACCGAAAACTT
TGCCGCCAAAGCAGGCGTGGCAGTCGGC

The insertion sequence *IS1301* is in bold letters and highlighted in grey.

NHBA

NHBA allele 17 strain (first set)

ATGTTTAAACGCAGTGTGATTGCAATGGCTTGTATTGTTGCCCTTTCAGCC
TGTGGGGGCGGCGGTGGCGGATCGCCCGATGTTAAGTCGGCGGACACGCT
GTCAAAACCTGCCGCCCCTGTTGTTACTGAAGATGTCGGGGAAGAGGTGC
TGCCGAAAGAAAAGAAAGATGAGGAGGCGGTGAGTGGTGCGCCGCAAGC
CGATACGCAGGACGCAACCGCCGGAAAAGGCGGTCAAGATATGGCGGCA
GTTTCGGCAGAAAATACAGGCAATGGCGGTGCGGCAACAACGGATAATC
CCGAAAATAAAGACGAGGGACCGCAAAATGATATGCCGCAAAATGCCGC
CGATACAGATAGTTCGACACCGAATCACACCCCTGCACCGAATATGCCAA
CCAGAGATATGGGAAACCAAGCACCGGATGCCGGGGAATCGGCACAACC
GGCAAACCAACCGGATATGGCAAATGCGGCGGACGGAATGCAGGGGGAC
GATCCGTCGGCAGGGGAAAATGCCGGCAATACGGCAGATCAAGCTGCAA
ATCAAGCTGAAAACAATCAAGTCGGCGGCTCTCAAAATCCTGCCTCTTCA
ACCAATCCTAACGCCACGAATGGCGGCAGCGATTTTGGAAGGATAAATGT
AGCTAATGGCATCAAGCTTGACAGCGGTTCGGAAAATGTAACGTTGACAC
ATTGTAAAGACAAAGTATGCGATAGAGATTTCTTAGATGAAGAAGCACCA
CCAAAATCAGAATTTGAAAAATTAAGTGATGAAGAAAAAATTAATAAAT
ATAAAAAAGACGAGCAACGAGAGAATTTTGTCGGTTTGGTTGCTGACAGG
GTAGAAAAGAATGGAACTAACAAATATGTCATCATTTATAAAGACAAGTC
CGCTTCATCTTCATCTGCGCGATTCAGGCGTTCTGCACGGTCGAGGCGGTC
GCTTCCGGCCGAGATGCCGCTGATTCCCGTCAATCAGGCGGATACGCTGA
TTGTCGATGGGGAAGCGGTCAGCCTGACGGGGCATTCCGGCAATATCTTC
GCGCCCGAAGGGAATTACCGGTATCTGACTTACGGGGCGGAAAAATTGTC
CGGCGGATCGTATGCCCTCAGTGTGCAAGGCGAACCGGCAAAAGGCGAA
ATGCTTGCGGGCACGGCCGTGTACAACGGCGAAGTGCTGCATTTCCATAT
GGAAAACGGCCGTCCGTCCCCGTCCGGAGGCAGGTTTGCCGCAAAAGTCG
ATTTCGGCAGCAAATCTGTGGACGGCATTATCGACAGCGGCGATGATTTG
CATATGGGTACGCAAAAATTCAAAGCCGTTATCGATGGAAACGGCTTTAA
GGGGACTTGGACGGAAAATGGCGGCGGGGATGTTTCCGGAAGGTTTTACG
GCCCGGCCGGCGAAGAAGTGGCGGGAAAATACAGCTATCGCCCGACAGA
TGCGGAAAAGGGCGGATTCGGCGTGTTTGCCGGCAAAAAAGAGCAGGAT
TGA

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CHAPTER 3

IMMUNE RESPONSES TO PROTOTYPE GMMA VACCINE WITH DELETED *NADR* AGAINST *NEISSERIA MENINGITIDIS* IN AFRICA

3.1 Introduction

Vaccines against meningococcal serogroup B disease have proven difficult to produce because the capsular polysaccharide is similar to human neural antigens. With the quest of developing a broadly protective vaccine against serogroup B through the identification of OMP using reverse vaccinology, Neisserial Adhesin A (NadA) was discovered (NMB1994) (1). Significantly, Wang *et al*. (2011) showed that in U.S serogroup B, C and W strains this antigen is not specific for serogroup B (2,3). Results from Chapter 2 indicated that good immunogenicity and cross-protection of fHbp in GMMA. The *nadA* gene was also detected in 89% (84/94) of the African meningococcal strains characterised. NadA is able to elicit bactericidal antibodies in mice and humans and is included as recombinant protein in a vaccine licensed for use in humans (3–5). Therefore, we set out to investigate the immunogenicity induced by NadA in GMMA.

3.1.1 Overview of NadA

Neisseria meningitidis colonises the nasopharynx of 10-35% of healthy individuals. The first step of the bacterium to cause invasive disease is to colonise the epithelium through multistage adhesion cascades followed by invasion of the cell, intracellular persistence and transcytosis (6,7). These events are modulated by the interaction of virulence factors with host cell receptors. A new type of antigen described as NadA has emerged as an important protein involved in epithelial cell adhesion and invasion (8). NadA KO mutants of *N. meningitidis* show a significantly reduced cell adhesion and invasion compared to wild-type strains (9). NadA protein has also been shown to stimulate monocytes, macrophages and monocyte-derived dendritic cells during

infection (10,11). Previous typing studies have shown that approximately 50% of clinical isolates and about 5% of carrier strains from healthy individuals harbour the gene; however, it is absent in commensal *Neisseria* species (7,8).

3.1.2 Structure and nomenclature

On the basis of structure prediction and analysis of sequence homology, NadA protein presents a tripartite structural organisation. The peptide has a N-terminal globular domain with a possible leader peptide of 23 amino acids (aa; head domain), an intermediate α-helix region that forms coiled-coil structures (coiled-coil stalk) and a conserved C-terminal membrane anchor domain (**Figure 3.1**) (1,11–13). The anchor domain has a conserved COOH-terminal membrane anchor, which with its amphipatic β-barrel structure and a terminal aromatic aa allows for autotransport of the remaining part of the adhesin, insertion into the outer membrane (OM) and oligomerisation of the monomer (9). The aa 88-150 of the stalk domain is important for adhesion, and aa 90-146 and 183-288 have a propensity to form coiled coils. Residues 122-143 contain four leucine residues in the heptad form that forms a leucine zipper domain (L-x[6]-L-x[6]-L-x[6]-L) (13). Together with the coiled-coils, they are able to mediate oligomerisation of monomers through the association of two or more α-helices (1). The 45kDa NadA protein mostly exist in the membrane as a trimer (~170kDa), which is stable in SDS-PAGE and following treatment with β-mercaptoethanol (5,10). The NH_2-terminal region has a globular structure that is essential for binding to host cell receptor. The aa 24-88 have been shown to be part of the region responsible for interaction with the host receptor (9). Due to the structure and ability to form oligomers, NadA has been classified as a non-fimbrial adhesin related to other adhesin-invasin pathogenicity factors such as UspA2 of *Moraxella*

catarrhalis and YadA of *Yersinia*, which are part of the family oligomeric coiled-coil adhesin (OCA) (8). The 1086-1215bp ORF of *nadA* shares approximately 32-34% identity to *yadA* and *uspA2*.

The *nadA* gene is conserved in *N. meningitidis* (89.2%), and the independent genetic unit consist of a promoter region, coding sequence and a terminator region (13). Analysis of the G+C content of the ORF and its upstream region showed that it is lower than average (45% against 51.5% of the genome), suggesting acquisition of the gene by horizontal gene transfer. The exact origin has not been established, but homologous artefacts of the gene has been found in *N. cinerea* (13). *nadA* has been divided into five defined alleles 1-5 based on the different PCR sizes obtained with primers binding externally to the gene (3). Currently, there are 98 distinct gene sequences classified into these alleles based on sequence similarity, which then code for 43 distinct aa sequences differing in length mostly between and slight variations within an allele (http://pubmlst.org/ neisseria/). The aa sequences are then grouped into peptide 1-5 subvariants. The nomenclature scheme is as follows: *e.g.* NadA-1.3, which refers to allele 1 and peptide subvariant 3. The three alleles 1-3 share 84-99% homology and induce antibodies with cross-bactericidal activity. They are also present in most clinical isolates and hyperinvasive isolates belonging to cc ST-8, 11, 32 and 213 (1,8). In particular, *nadA-2* and *nadA-3* are very similar with ≥97% shared identity, and are associated with ST-8 and ST-11 while *nadA-1* is connected with ST-32. Although, these alleles differ in length, deletions and substitutions in the gene do not affect the overall structure of the protein rather the number of turns of the α-helix in the stalk domain (15). Allele 1 codes for a protein of 362aa, allele 2 398aa and allele 3 405aa. The *nadA-4* gene was first described in carriage isolates. The protein is

shorter than NadA-1-3 (323aa), but has the same secondary structure and domain organisation. *nadA-5* was identified through analysis of isolates belonging to the ST-213 cc associated with serogroup B. The gene is usually switched off due to an internal poly(C) tract unique to this variant, however, "on" variants of this gene have been found. Antibodies against NadA-4 and NadA-5 peptides show limited cross-reactivity against each other and to the alleles 1-3 (16). Nevertheless, the recent study by Bambini *et al*. (2014) revised the nomenclature of NadA by taking into account the dataset found in http://pubmlst.org/ neisseria/. The phylogenetic analysis grouped nucleotide sequences into two clusters (I, II) and 6 NadA variants: group I consist of *nadA*-1-3 and group II of *nadA*-4-6. The variants of group II were found to share 75-99% homology, but only 46-50% with group I. The new variant 6 belonged to ST-11 cc of isolates from Spain; however, the gene was inactivated by frame shift mutations. The authors proposed a new nomenclature, and the protein variants are: NadA-1, NadA-2/3, NadA-4/5 and NadA-6 grouped into two groups. The high level homologous variants were grouped together. A subvariant is followed by a dot *e.g*. NadA-3.2. However, for simplicity the original classification system of NadA "alleles" will be followed here.

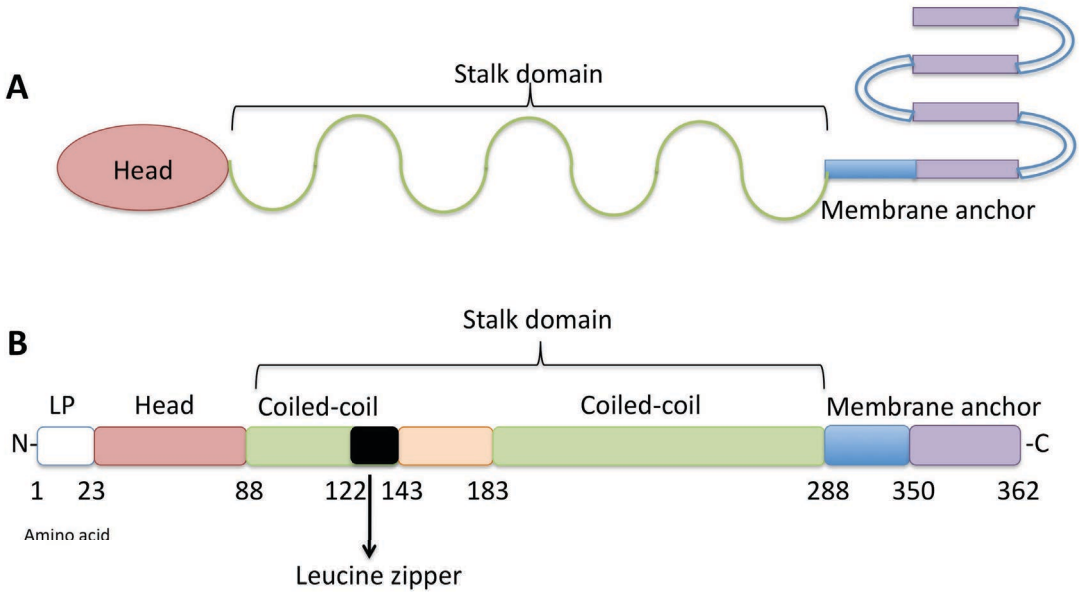

Figure 3.1. Structure and domain organisation of Nesserial adhesin A (NadA).

Panel A: Schematic diagram of NadA protein topology. Panel B: *nadA* domain structure. The N and C represent the N- and COOH-termini of the protein. The leucine zipper is indicated by a black box. The numbers represent the amino acid number in the peptide sequence. Adapted and modified from Comanducci *et al*. (2002) (1).

3.1.3 Regulation of NadA expression

To facilitate adaptation of bacterial population in different microenvironments and ensure effective colonisation and at the same time avoid the host immune system, the meningococcus must undergo frequent and reversible phenotypic changes resulting from regulated and stochastic expression of virulence genes. NadA expression is phase variable, which means that expression is mediated by high frequency of reversible changes (12). Frequency is estimated to be $4.4x10^{-4}$ with promoters of low, medium and high activity.

The main regulator of NadA expression is Neisserial adhesin regulator (NadR, NMB1843), a member of the MarR family of regulators, which in prokaryotes is

critical for control of virulence factor production, response to antibiotics and catabolism of environmental aromatic compounds (16). It has been shown through microarray analysis that NadR is able to co-regulate over 30 genes, however, *nadA* being the strongest repressed (16). NadR binds to the *nadA* promoter, and other gene promoters, and strongly represses the transcription through a looping mechanism that sterically hinders RNA polymerase (RNAP) access to the promoter (13,14). More specifically, NadR binds the Growth Phase Regulatory region (GPR), which is a distal cis-acting region upstream of the promoter region of about -170- -112 nucleotides (nt), also called operator I (OpI). Adjacent to the GPR is the low NadR binding affinity OpIII spanning the TAAA repeat tract (-85--50) and the high affinity OpII overlapping -10 region of the promoter. NadR normally binds OpII site, obstructing access of RNAP to the promoter. The transcriptional regulator IHF (integration host factor) binds OpIII looping of the DNA allowing NadR to interact with the other operators resulting in more efficient repression of *nadA* promoter. Moreover, the aromatic metabolite 4-hydroxyphenylacetic acid (4HPA) of aa tyrosine and phenylalanine, present in saliva, has been shown to relieve DNA binding activity of NadR increasing NadA expression (18). It does so by stabilising NadR in a conformational state preventing effective binding to the *nadA* operators, thus inducing NadA protein expression (16). Fagnocchi *et al.* (2011) proposed that this metabolite can act as a relevant niche signal for meningococci present in the nasopharynx for the induction of NadA expression and other co-regulated genes under NadR control during exponential growth phase (16,19). Moreover, the authors observed that 4HPA added in the growth medium increased the expression of NadA, but not all isolates responded in a similar fashion with partial or full NadR de-repression. 4HPA is an

inducer or co-repressor while NadR represses all genes in its regulon, and deletion of *nadR* results in a 60-fold transcriptional increase of NadA expression (19).

Moreover, expression is also controlled by variation of the TAAA tetranucleotide repeats 130 nt upstream of the core promoter preceded with a second promoter with -10 and -35 regions, which suggests that *nadA* is phase variable (1). 4, 9 and 12 repeats result in low transcript level and 7, 8 and 10 repeats result in high transcript level. 5, 6, 11 and 13 repeats and a promoter mutant lacking TAAA give varying intermediate levels. The number of repeats is modified during replication through slipped strand misspairing. Loss or gain of TAAA repeats affects the differential spacing between the Ops, thus the suboptimal or optimal configuration of protein complexes that affect the efficiency of NadR repression (12).

Expression of NadA has been shown to exhibit growth-phase dependent behaviour with levels maximal in the stationary growth phase of strains tested to date (12). Metruccio *et al.* (2009) deleted the GPR region between -170 and -108, which resulted in increased expression during log phase of growth. The authors suggested that this region is responsible for repression of expression of *nadA* promoter in log phase probably due to binding of NadR protein.

3.1.4 NadA as vaccine antigen

A large part of the serum antibodies in normal individuals with bactericidal activities against *N. meningitidis* is directed against non-capsular antigens (20). In particular, children recovering from invasive disease produce specific antibody responses against NadA (3,21). Tavano *et al*. (2009) investigated the activity of NadA expressed in an

OMV compared to rNadA$_{\Delta 351\text{-}405}$ missing the OM anchor sequence (22). On the surface of monocytes, rNadA$_{\Delta 351\text{-}405}$ binds heat-shock protein 90 (hsp90) that recruits hsp70 and TLR-4 into a transducing signal complex, which stimulates the production of heat shock related cytokines such as IL-1, TNF-α (14). Compared to the recombinant protein, NadA presented in a serogroup B OMV had an increased ability to present the antigen to macrophages without the induction of shock-related cytokines by monocytes (14). This pro-immune action of wild-type NadA argues for the use of NadA in an OMV platform as a vaccine candidate.

Studies have looked into the bactericidal responses elicited by OMV vaccines over-expressing NadA. Norheim *et al*. (2012) tested a conventional bivalent MenA and MenW OMV vaccine extracted by detergent, which express NadA-3, fHbp v.1 ID5, PorA P1.20,9 and NadA-3, fHbp v.2 ID23, P1.5,2, respectively, against strains from Burkina Faso and Ethiopia (20). Zollinger *et al*. (2010), Pinto *et al*. (2011) and Moran *et al*. (2012) investigated immunogenicity of a trivalent MenB vaccine over-expressing fHbp v.1,/2, NadA-3 and three PorA variants against serogroup A, B, C, Y, W and X strains from Africa, Cuba, Norway and Germany (5,21,23). The vaccine groups, which were used to immunise mice and rabbits, induced broadly cross-reactive bactericidal antibodies against homologous and heterologous strains. Antibodies to LOS, PorA and NadA were responsible for most of the bactericidal activity seen. The results of these studies highlight the suitability of using an OMV approach for expanding vaccine protection. Given that the set of strains in this study showed antigenic stability, the usage of specific OMPs as vaccine candidate antigens is potentially a viable option.

3.2 Project objectives

There are several attributes to NadA being a good vaccine candidate. Firstly, it is an outer membrane antigen and a virulence factor. Secondly, it is present in most hypervirulent lineages. Thirdly, alleles 1-3 found in clinical isolates elicit cross-protective bactericidal antibodies and when presented in OMVs, it has a pro-immune action (1). However, the potential of NadA to control outbreaks of disease has not been evaluated for African strains.

GMMAs include the *gna33* deletion, which offers an alternative way of vesicle production, protein expression and presentation compared to the vaccine strategies employed by previous studies. The recombinant strains from which GMMA are produced can be engineered to include additional mutations to over-express candidate vaccine antigens of choice. To attempt to over-express NadA in our GMMA vaccines, we aimed to knock-out its regulator *nadR* instead of growing the recombinant strains for GMMA production in medium supplemented with the inducer 4HPA. This is because deletion of NadR has been shown to increase NadA transcription by 60-fold, while 4HPA differentially induces NadA expression in meningococcal isolates.

For this project, we aimed to characterise the breadth of immune responses elicited by a prototype serogroup A and W GMMA vaccine expressing NadA-3 against genetically diverse African *N. meningitidis* serogroup A, W and X isolates from a wide geographical range. The strains were characterised based on their NadA expression level. These data could then aid selection of a panel of isolates for the analysis of serum bactericidal activity.

In short the aims are:

1. Characterisation of African meningococcal serogroup A, W and X isolates.
    a. Analysis of NadA expression level during growth with and without the presence of 4HPA in order to investigate the level of increase that 4HPA could provide in an "*in vivo*" condition.
    b. Selection of a representative strain panels including serogroup A, W and X isolates suitable to investigate bactericidal activity induced by the prototype GMMA vaccines.

2. Characterisation of antibody responses elicited by GMMA with deleted NadR.
    a. Evaluate IgG antibody responses against NadA by ELISA.
    b. Evaluation of serum bactericidal activity of sera made against GMMA with deleted *nadR* against a subset of genetically diverse serogroup A, W and X isolates.

3.3 Materials and Methods

3.3.1 *Neisseria meningitidis* isolates

In this study, the second set of clinical *N. meningitidis* case isolates (A=13, W=20, X=11) received from Dominique Caugant at NIPH were used in subsequent analyses (Chapter 2, **Table 2.3**). All isolates were analysed for their NadA expression level by Western blot and comparing the levels to known amounts of recombinant Hexa-Histidine tagged NadA allele 3 (rHis-NadA-3; produced in this study). In proceeding experiments for testing the antisera raised against GMMA expressing NadA-3 made from a mutant serogroup A and W strain, sets of meningococcal isolates were selected based on genetic diversity and different levels of NadA-3 expression.

3.3.2 Western blot analysis of NadA protein amount

A subset of 37 isolates were analysed for their NadA expression level during growth with and without the addition of 4HPA compared to known amounts of rHis-NadA-3 produced in this study. Five serogroup W isolates that had *IS1301* in the *nadA* gene were not included in the analysis. Preparation of the samples without 4HPA was performed as described in Chapter 2 section 2.3.4 on page 62. For the preparation of cultures grown in the presence of 4HPA, a loop-full of single *N. meningitidis* colonies from overnight GC plate cultures were resuspended in 7ml MH broth at an OD_{600} of 0.12-0.16 and grown until mid-log phase (OD_{600} = 0.6), harvested, and resuspended in 7ml MH broth + 5mM 4HPA (Invitrogen). The cultures were then incubated for 1 hour with agitation at 37°C and 5% CO_2, harvested and resuspended in 200µl 1xPBS and heat inactivated at 56°C for 1 hour. Preparation of Western blot membranes was performed as previously described in Chapter 2. 0.0156, 0.031, 0.063, 0.125µg/ml of

rHis-NadA-3 was used as standards for estimating NadA protein amounts expressed by all isolates. Positive control used was serogroup A strain N2602 expressing NadA-3. NadA proteins were detected with a polyclonal serum against Hexa-Histidine tagged NadA allele 3 (rHis-NadA-3; NV&D). The serum was diluted 1:5000 in 1xPBS + 1% milk and incubated for 2 hours at room temperature. After washing the membrane four times 10 minutes each with 1xPBS+0.1% Tween20 (Sigma-Aldrich), membranes were incubated with secondary antibody horseradish peroxidase-labeled goat anti-mouse immunoglobulin G λ1 chain (Southern Biotech, Birmingham, Alabama, U.S.A) for 1 hour at a dilution of 1:20000 in 1xPBS+0.1% Tween20. After another four washes, membranes were developed using SuperSignal® WestPico Chemiluminescent Substrate (Thermofisher Scientific) according to manufacturers' instructions, and developed using Amersham Hyperfilm™ ECL (GE Healthcare). The amount of NadA expressed by each isolate compared to the standard rHis-NadA-3 was determined by ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012). NadA expression by the test isolates was reported as percentages of the amount of NadA expressed by bacterial cells compared to the corresponding reference strain N2602 expressing high amounts of NadA-3. NadA expression of N2602 was considered to be 100%.

3.3.3 Cloning, expression and purification of recombinant NadA-3

The *nadA* sequence (aa 24-350) of allele 3 was amplified by PCR from chromosomal DNA of serogroup A strain N2602 (Chapter 2, **Table 2.2**). The PCR product was ligated with pET21^{+}b vector (Invitrogen) digested with restriction enzymes *XhoI* and *NdeI* (New England BioLabs). The ligation product was transformed in *E. coli* DH5α

(Invitrogen), and the plasmid was purified by QIAprep spin Miniprep kit (Qiagen, Venlo, Limburg, Netherlands). The purified plasmid was subsequently transformed in *E. coli* BL21 (DE3, Invitrogen) to express the protein as COOH-terminal Hexa-Histidine fusion. Protein expression was induced at 30°C by the addition of 1mM IPTG (New England BioLabs) at OD_{600} 0.3 and growing the bacteria for additional 3 hours. The cultures were subsequently pelleted by centrifugation at 1500rpm for 10 minutes and re-suspended in 60ml Buffer 1 per 1 litre culture (**Table 3.1**). 0.2mg/ml lysozyme (New England BioLabs) was added and incubated on shaker on ice for 30 minutes. The cell suspension was sonicated on ice with six-30 seconds bursts in 30 seconds intervals. Cell debris was centrifuged at 10000xg for 30 minutes at 4°C. Supernatants were then transferred into 50ml Falcon tubes, 2ml of 50% slurry Ni-charged Sepharose (GE Healthcare) and equilibrated with Buffer 1. After over-night incubation at 4°C on shaker, the tubes were centrifuged at 2000rpm for 5min at 4°C, supernatants were discarded and pellet re-suspended in 5ml Buffer 1. The wash step was repeated twice and the resin was loaded into a column (Biorad, Hercules CA, USA). The column was then washed twice with 5ml Buffer 2, and the recombinant protein eluted by adding 3x1ml fractions of Buffer 3. Protein concentration of each fraction was measured, and the fractions that contained the protein were pooled and dialysed using a slide-A-lyzer® dialysis cassette G2 10000 MWCO (Thermofisher Scientific) and 2l 1xPBS overnight at 4°Cunder stirring.

Table 3.1. Buffers used to purify recombinant NadA from *Escherichia coli.*

All buffers are adjusted to pH8. All chemicals were obtained from Sigma-Aldrich.

	Buffer 1	**Buffer 2**	**Buffer 3**
NaCl (mM)	300	300	300
NaH_2PO_4 (mM)	50	50	50
Imidazole (mM)	10	20	250

3.3.4 Transformation of *N. meningitidis*

N. meningitidis knock-out strains generated by transformation with plasmids and replacement of target gene with antibiotic resistant markers are described in **Table 3.2**. A few single bacterial colonies were added to GC plates, and 1µg plasmid was mixed with the bacteria and allowed to grow for 6 hours at 37°C with 5% CO_2. The cells were then plated on GC plates supplemented with respective antibiotics, and grown over night (**Table 3.3**). Single transformants were sub-cultured on GC plates supplemented with antibiotics. Correct transformants were confirmed by Western blotting and PCR (**Table 3.4**).

Table 3.2. Plasmids used to generate knock-out mutations.

Each knock out (KO) mutation was generated in ascending order as indicated in the table starting from number 1. Δ*nadR* with pGEM1843 leads to the de-repression of *nadA* transcription/expression. Δ*lpxL1* with pUC18::lpxl1 detoxifies the GMMA due to the production of penta- instead of hexa-acylated lipid A. Δ*gna33* with pBSUDGNA33 increases blebbing or GMMA release from the bacterial cells. Δcapsule with either p_cpsW or p_cpsA deletes the capsule of the bacterium rendering it non-invasive (25).

Number	Plasmid	Gene target	Resistance marker	Reference
1	pGEM1843	*nadR*	Chloramphenicol (Cm)	(12)
2	pBS961	*nadA*	Kanamycin (Kan)	(12)
3	p_cpsW	Capsule serogroup W	Spectinomycin (Spec)	This study
4	p_cpsA	Capsule serogroup A	Spec	This study
5	pUC18::lplx1	*lpxL1*	Tetracycline (Tet)	This study
6	pBSUDGNA33	*gna33*	Erythromycin (ErmC)	(24)

Table 3.3. Concentration of antibiotics in GC media for growth of *Neisseria meningitidis* mutants.

Antibiotics were obtained from NV&D.

Antibiotic	Concentration (µg/ml)
Kanamycin	80
Chloramphenicol	5
Erythromycin	5
Spectinomycin	60
Tetracycline	1

Table 3.4. Primers for construction of plasmids and for confirming knock-out mutants.

Monoclonal antibodies JW-W1b-IgG2b and JW-A2-IgG2b were also used to confirm capsular W and A deletion, respectively (kindly provided by Dan Granoff). The capsular primers contained restriction enzyme sites. Abbreviations: KO= knock out, Fw= forward primer, Rv= reverse primer.

Target gene	Primer designation	5'-3' nucleotide sequence	Purpose	Reference
nadA	NadAF (Fw)	AACACTTTCCATCCAAAG	Confirming KO	(8)
	NadAR (Rv)	TTACCACTCGTAATTGACG	Confirming KO	(8)
nadR	1843-F (Fw)	ATGCCATCCCAATCAAAACATGCG	Confirming KO	(12)
	1843-R (Rv)	CGGCGTATTACGAGTTCAACGCATCC TCG	Confirming KO	(12)
lpxL1	htrBU (Fw)	GGCACGCGTCCGCTGATCAGTATGT	Confirming KO	(26)
	htrBL (Rv)	GGCACGCGTAAATTGATTTCGCCGAT A	Confirming KO	(26)
gna33	33FOR (Fw)	CGCGGATCCCATATGCAAAGAAAGAG AATCCCAA	Confirming KO	(24)
	33REV (Rv)	GCTCTGAGGGCGACGACAGGCGG	Confirming KO	(24)
tetracycline	tetF (Fw)	GCTCTAGAGCAGTTCGAGCGCGGTTC GG	Construction of pUC18::lplx1	This study
	tetR (Rv)	GCTCTAGATTCTCATGTTTGACAGCTT ATC	Construction of pUC18::lplx1	This study
Serogroup A capsule*	mynf_Kpn (Fw)	CGGGGTACCCAAGAAGAAGCTCCATC CC	Construction of cpsA	This study
	mynr_Spe (Rv)	GGACTAGTGGCTTAAGAGTAAGATTA GAAGG	Construction of cpsA	This study
Serogroup W capsule*	synf_Kpn (Fw)	CGGGGTACCCGTGGAATGTTTCTGCT CAA	Construction of p_cpsW	This study
	synr_Spe (Rv)	GGACTAGTCCATTAGGCCTAAATGCC TG	Construction of p_cpsW	This study
Capsule	ctrAf_Xma (Fw)	CCCCCCGGGCAGGAAAGCGCTGCATA G	Confirming KO	This study
	ctrAr_Xba (Rv)	CGTCTAGAGGTTCAACGGCAAATGTG C	Confirming KO	This study

*Back-bone pSL1190 (Pharmacia, Kalamazoo, MI, USA)

We generated five serogroup A and three serogroup W mutant strains (**Table 3.5**). All strains had deleted *lpxL1* and *gna33* genes. Four serogroup A mutants (strains 1, 3-5) had deleted capsule biosynthesis genes and two had deleted *nadR* gene (strains 1 and 2). We also prepared a serogroup A control strain that had the capsule biosynthesis intact (strain 2), and a control strain that had *nadR* intact (strain 3). Additional control strains were generated that lacked *nadA* (strain 4) or both *nadA* and *nadR* (strain 5). Serogroup W mutant strains with deleted capsule biosynthesis genes (strains 6-8) were also prepared. One mutant strain had *nadR* deleted (strain 6), one control strain had intact *nadR* (strain 7) and one was generated with deleted *nadA* (strain 8). The order of mutation/transformation of each recombinant strain produced is shown in **Figure 3.2.**

Table 3.5. GMMA vaccine strains.

Strain NVGH0348 is serogroup A N2602 and NVGH0385 is serogroup W 1630 (see **Table 2.2**, Chapter 2). Abbreviations: KO= knock-out, cps= capsule.

Mutant strain number	**Serogroup, strain (genotype)**	***fHbp* variant**	***fHbp* ID**	***porA* VRs**	***nadA* allele**	**GMMA designation**
1	A, NVGH0348_001 (LpxL1, cps, NadR, gna33)	1	5	P1.20,9	3	MenA NadR KO cps-
2	A, NVGH0348_002 (LpxL1, NadR, gna33)	1	5	P1.20,9	3	MenA NadR KO cps+
3	A, NVGH0348_003 (LpxL1, cps, gna33)	1	5	P1.20,9	3	MenA WT NadA cps-
4	A, NVGH0348_004 (NadA, LpxL1, cps, gna33)	1	5	P1.20,9	3	MenA NadA KO cps-
5	A, NVGH0348_005 (NadA, NadR, LpxL1, cps, gna33)	1	5	P1.20,9	3	MenA NadA and NadR KO cps-
6	W, NVGH0385_001 (LpxL1, cps, NadR, gna33)	2	23	P1.5,2	3	MenW NadR KO cps-
7	W, NVGH0385_002 (LpxL1, cps, gna33)	2	23	P1.5,2	3	MenW WT NadA cps-
8	W, NVGH0385_003 (NadA, LpxL1, cps, gna33)	2	23	P1.5,2	3	MenW NadA KO cps-

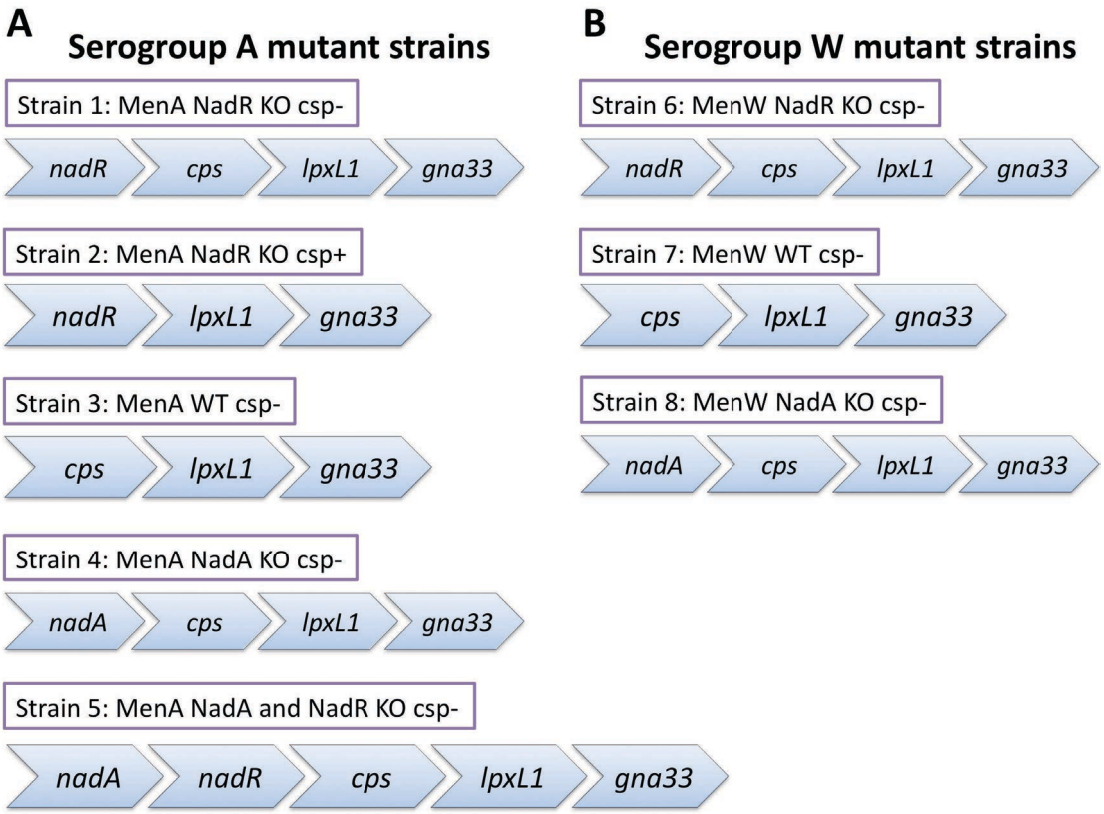

Figure 3.2. Flow diagram of the order of mutations introduced for generating the recombinant vaccine strains.

The order of transformation and mutation introduced in the recombinant serogroup A N2602 strains (panel A) and serogroup W 1630 strains (panel B) are indicated by the direction of the arrows in the flow chart. For example, for strain 1, deletion of *nadR* was performed first followed by deletion of capsule (cps), *lpxL1* and lastly *gna33*. Mutations cannot be introduced after deletion of *gna33* because the bacteria cannot subsequently take up DNA. The integrity of join between inner and outer membrane is affected (24).

3.3.5 GMMA preparation

The vaccine strains were sub-cultured on GC agar plates with antibiotics. 7ml Meningitis Chemically Defined Medium I (MCDMI) medium was inoculated to OD_{600} 0.15-0.2 and incubated with shaking at 37°C and 5% CO_2 until OD_{600} 0.6 was reached (**Table 3.6**). This culture medium was used to inoculate 50ml MCDMI in 250ml Erlenmeyer flasks (Thermofisher Scientific) and incubated over night at 37°C, 5% CO_2 and 185rpm. Subsequently, the cultures were centrifuged at 3500rpm for 10 minutes, and supernatant was collected and filtered using a vacuum filter device with 0.22µm pore size membrane (Millipore, Billerica MA, USA). 100µl of the filtrated supernatant was plated on GC agar and the plates were incubated at 37°C, 5% CO_2. After 48 hours when the plates tested sterile, the culture supernatants were transferred into 50ml ultracentrifuge tubes (Beckman, Brea, California, U.S., rotor Ti45) and centrifuged at 35000rpm, 2 hours, 4°C to collect the GMMA. Supernatants were subsequently discarded, and the membrane pellet was washed once with 1xPBS and centrifuged as before, supernatant discarded and the pellet soaked over night and re-suspended in 500µl 1xPBS. GMMA preparations were sterilised by filtration (0.22µm pore sized syringe filter, Millipore) Concentration of the protein content in the GMMA was measured by Pierce BCA protein assay kit (Thermofisher Scientific).

Table 3.6. Constituents of the growth medium Meningitis Chemically Defined Medium (MCDMI).

All chemicals were obtained from Sigma-Aldrich.

Chemicals	Amount (g/l)
Soy peptone	15
NaCl	5.8
K_2SO_4	1
K_2HPO_4	4
L-glutamic acid	5
L-arginine	0.3
L-serine	0.5
L-cysteine	0.23
$MgCl_2$ x $6H_2O$	0.19
$CaCl_2$ x $2H_2O$	0.021
Fe(III) Citrate	0.002
Glucose	10

3.3.6 Mouse immunisations

Five weeks old CD-1 female mice (8 mice per group) were obtained from Charles River (Wilmington, MD, U.S.). The mice were immunised intraperitoneally with three doses of vaccine given at days 0, 14 and 28 (**Table 3.7**). All GMMA vaccine strains were given at 5µg doses based on total protein. Control mice were immunised with 5µg rHis-NadA-3 or Alum only. All vaccines were absorbed on 3mg/ml Alum in a 100µl formulation containing 10mM Histidine and 0.9mg/ml NaCl. Collection of blood was done two weeks after the second dose and two weeks after the third dose. Terminal blood samples were obtained at day 42. The animal procedures were performed under protocols approved by Novartis NV&D (Siena, Italy) animal care.

Table 3.7. Mouse immunisation scheme.

ACWY conjugate polysaccharide vaccine is conjugated to a diphtheria CRM_{197}, developed by Novartis vaccines (NV&D Vaccine Chemistry). NaCl (Sigma-Aldrich S9888), Histidine (Sigma-Aldrich H6034), Aluminium hydroxide (NV&D Formulation unit), KO= knock out, IP= intraperitoneal, PS= polysaccharide.

Group (Mouse number)	Antigen	Adjuvant	Dose (µg)	Immunisation route	Volume (µl) per animal
Primary vaccine candidates					
1 (1 - 8)	MenA NadR KO cps-	Alum	5	IP	100
2 (9 - 16)	MenW NadR KO cps-	Alum	5	IP	100
Control vaccines					
3 (17-24)	MenA WT NadA cps-	Alum	5	IP	100
4 (25-32)	MenW WT NadA cps-	Alum	5	IP	100
5 (33-40)	MenA NadR KO cps+	Alum	5	IP	100
6 (41-48)	MenA NadA KO cps-	Alum	5	IP	100
7 (49-56)	MenW NadA KO cps-	Alum	5	IP	100
8 (57-64)	MenA NadA and NadR KO cps-	Alum	5	IP	100
9 (65-72)	rHis-NadA-3	Alum	5	IP	100
10 (73-80)	ACWY conjugate	Alum	2, MenA PS 1, MenC PS 1, MenY PS 1, MenW PS	IP	100
11 (81-88)	None	Alum	NA	IP	100

We focused on the primary vaccine candidate group 1 and 2 and the control groups to evaluate the potential and coverage of our two vaccine candidates. The aim was to compare subsequent groups: group 1 + 3 and group 2 + 4 to evaluate effect of *nadR* KO, group 1 and 5 to evaluate effect of capsule expression on production of anti-NadA antibodies, group 1+ 6 and group 2 + 7 to compare the contribution of NadA antigen on antibody responses, group 1 and 8 to see if there are other antigens than NadA possibly regulated by NadR that contribute to immunogenicity, group 1 and 9 to evaluate the efficacy of antibodies elicited against GMMA versus rHis-NadA-3

protein, group 1 and 10 to determine the efficacy of the candidate vaccine compared to the ACWY conjugate vaccine.

3.3.7 Enzyme-linked immunosorbent assay (ELISA) screening of anti-NadA antibody responses

4HBX (NUNC-IMMUNOPLATE, Penfield, New York, U.S.A) plates were coated with 2µg of rHis-NadA-3 over night. Subsequently, the plates were washed with wash buffer (1xPBS + 0.1% Tween20 (Sigma-Aldrich)) three times, blocked with blocking buffer (1xPBS + 2% milk + 0.5% Tween 20) for 1 hour and then washed again. After 2 hours of incubation with primary antibody (mouse sera) starting from 1:10 dilution in serial 4 dilutions, secondary antibody 230 (goat anti-mouse IgG γ-chain horseradish peroxidase-labelled, Sigma-Aldrich) was added for 1 hour at 1:20000 dilution. The reaction was developed by the addition of P-Nitrophenyl phosphate substrate (PNP; Sigma-Aldrich) in PNP substrate buffer at pH9.6 diluted 1:100 (for 1 litre: 1.4g sodium carbonate, 3.0g sodium bicarbonate, 0.2g magnesium chloride-hydrate (Sigma-Aldrich)), and read at 405nm after 30-60 minutes of incubation with an ELISA reader (Sunrise Tecan).

3.3.8 SBA using baby rabbit complement

The assay was performed as previously described for SBA using human complement in Chapter 2 section 2.3.5 page 63. Isolates used are listed in **Table 3.9** (Result section 3.4.4). The source of complement was serum from baby rabbit (Cedarlane, Burlington, Ontario, Canada) with no detectable bactericidal activity against the test strains.

3.3.9 Statistical analysis

Data analysis was carried out as described in Chapter 2 section 2.3.7 page 67. For the Western blot results, multiple group comparison (ANOVA) was utilised to evaluate the increased expression of NadA upon addition of 4HPA.

3.4 Results

3.4.1 Majority of the serogroup A, W and X isolates expressed intermediate to high levels of NadA protein

Fagnocchi *et al.* (2012) previously showed that the small molecule 4HPA is able to relieve NadR-mediated repression of NadA expression in meningococcus (16). Unlike fHbp, it has not been ascertained whether the level of NadA expression is an additional factor for determining the susceptibility of a strain to anti-NadA antibodies. To establish this and to understand the effect of 4HPA on the NadA expression *in vitro*, we investigated the expression of NadA with and without the presence of 5mM 4HPA. We compared the level of NadA protein expression to the vaccine serogroup A strain N2602. Most serogroup A and W isolates express intermediate to high levels of NadA compared to the reference strain N2602 (**Figure 3.3**, **Panel A** and **B**). However, selected X isolates expressed low amounts of NadA (**Panel C**). When the strains were grown in the presence of 4HPA, analysis of the whole cell extracts suggested an increased level of NadA expression for 31/37 isolates. Statistical analysis using two-way ANOVA showed that this difference was statistically significant ($P<0.001$).

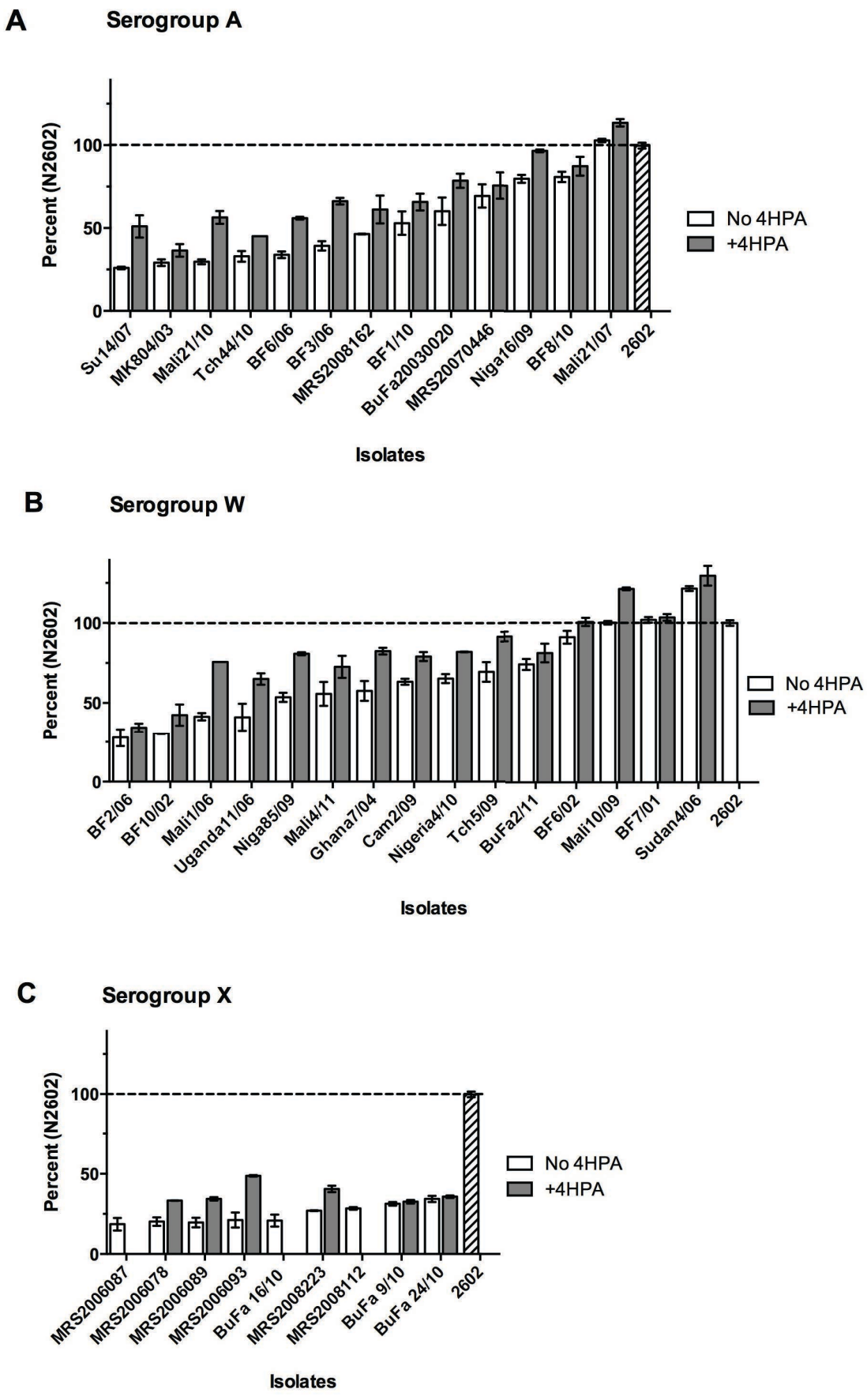
A
Serogroup A
Percent (N2602)
100
50
0
No 4HPA
+4HPA
Su14/07
MK804/03
Mali21/10
Tch44/10
BF6/06
BF3/06
MRS2008162
BF1/10
BuFa20030020
MRS20070446
Niga16/09
BF8/10
Mali21/07
2602
Isolates
B
Serogroup W
Percent (N2602)
100
50
0
No 4HPA
+4HPA
BF2/06
BF10/02
Mali1/06
Uganda11/06
Niga85/09
Mali4/11
Ghana7/04
Cam2/09
Nigeria4/10
Tch5/09
BuFa2/11
BF6/02
Mali10/09
BF7/01
Sudan4/06
2602
Isolates
C
Serogroup X
Percent (N2602)
100
50
0
No 4HPA
+4HPA
MRS2006087
MRS2006078
MRS2006089
MRS2006093
BuFa 16/10
MRS2008223
MRS2008112
BuFa 9/10
BuFa 24/10
2602
Isolates

Figure 3.3. NadA protein expression in African serogroup A, W and X isolates grown with and without the presence of the metabolite 4HPA as measured by Western blotting.

Panel A: serogroup A isolates; Panel B: serogroup W isolates; Panel C: serogroup X isolates. All serogroups were of NadA-3 except for Mali10/09, which expresses NadA-2. Serogroup X isolates Uganda14/06 and Ug11/07 were not included in the analysis since the isolates failed to grow. Bars in panel A-D represent mean percentages of a set of 2 independent experiments compared with expression of NadA by the reference serogroup A vaccine strain N2602. Dotted line represents the level of NadA expression by the reference strain. Error bars represent standard errors. Statistical analysis was calculated using multiple group comparison [ANOVA].

3.4.2 Quantification of NadA protein amount on GMMA from serogroup A and W mutant strains

To attempt to over-express NadA, we deleted *nadR* in the serogroup A N2602 and W 1630 isolates by transforming them with plasmid pGEM1843 that contains flanking regions of the *nadR* gene. NadA protein expressions in the GMMAs were measured by Western blotting using polyclonal anti-rHis-NadA-3 antibody (**Figure 3.4**, **Panel A**). Densitometry indicated that the GMMA produced from the recombinant serogroup A and W strains with deleted capsule and *nadR* (MenA NadR KO cps- and MenW NadR KO cps-) have lower NadA amounts than isogenic control strains with the capsule expressed (MenA NadR KO cps+, MenW NadR KO cps+) or wild-type NadA expression (MenA WT NadA cps-, MenW WT NadA cps-) (**Table 3.8**). We expected that the MenA NadR KO cps- and MenW NadR KO cps- GMMAs would contain the highest amount of NadA since *nadR* was deleted in the respective recombinant strains for that purpose. During development of the recombinant vaccine strains, Western blotting of whole cell lysates of all serogroup A and W NadR KO

mutants were assessed prior to introducing subsequent mutations. The NadR KO mutants with capsule expressed the highest amount of NadA compared to the wild-type strains with capsule (**Figure 3.4**, **Panel B**). Plausible explanation for the lack of over-expression of NadA in MenA NadR KO and MenW NadR KO could be that the introduction of subsequent cps, *lpxL1* and *gna33* deletions affect the trafficking of proteins, membrane environment and stability of the anchoring of the NadA in the OM (25). It could be that the NadA protein produced does not get transported out to the OM for GMMA formation, but rather stuck in the whole cell biomass.

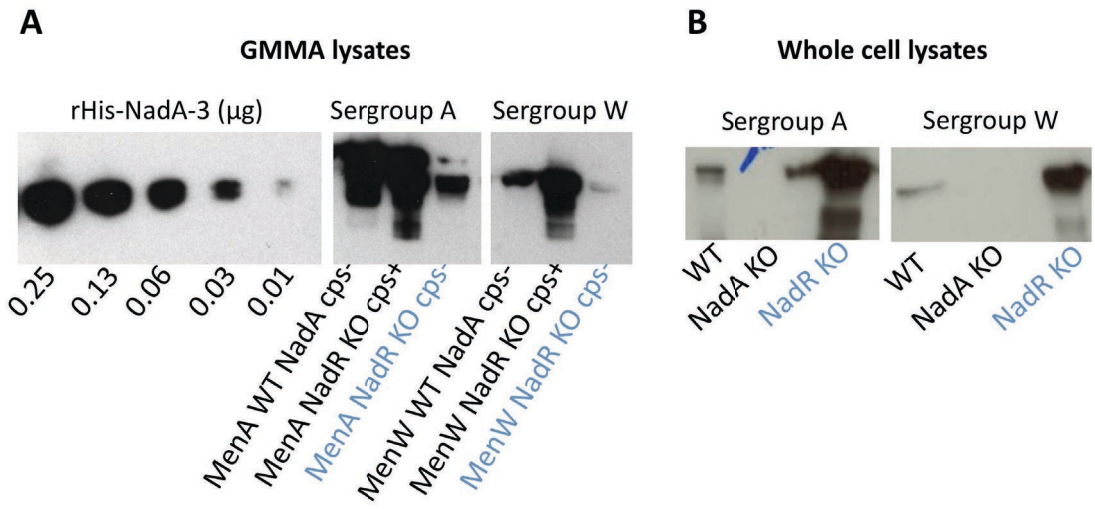

Figure 3.4. Expression of NadA-3 in GMMA and whole cell lysate prepared from serogroup A N2602 and serogroup W 1630 recombinant vaccine strains measured by Western blotting.

Panel A: Expression of NadA in serogroup A N2602 and W 1630 GMMA lysates; Panel B: amount of NadA expression in whole cell lysates from serogroup A N2602 and W 1630 mutant strains with *nadA* and *nadR* knock-out only. The serogroup W GMMA with deleted *nadR* and expressing the capsule (MenW NadR KO cps+) was produced during the generation of vaccine serogroup W GMMAs, but not included in the mouse study. This is because the corresponding GMMA made from the serogroup A strain N2602 (MenA NadR KO cps+) will evaluate effect of capsule expression on production of anti-NadA antibodies. The rHis-NadA-3 standard is in µg, and 4µg of GMMA and 10µg of whole cell lysate sample were loaded. The primary vaccine candidates are highlighted in blue.

Table 3.8. Quantification of expression of NadA-3 in GMMA vaccines prepared from serogroup A and W recombinant strains.

Each GMMA preparation were from mutants with Δ*lpxL1* that detoxifies the GMMA due to the production of penta-acylated lipid A, and Δ*gna33* that increases GMMA release from the bacterial cells. Δcapsule (cps-) deletes the capsule, and in two preparations the capsule was retained (cps+). The amount of NadA on GMMA was assessed based on total protein concentration of the GMMA preparations. Total protein amounts were determined by Lowry protein assay kit (BioRad DC™ Protein Assay, BioRad Laboratories, Hercules, California, U.S.), and the GMMA preparations were adjusted to the same protein concentration. The primary vaccine candidates are highlighted in blue.

	Serogroup A			**Serogroup W**		
GMMA vaccine group	MenA WT NadA cps-	MenA NadR KO cps+	MenA NadR KO cps-	MenW WT NadA cps-	MenW NadR KO cps+	MenW NadR KO cps-
% of total protein	24	26	9	7	19	3

3.4.3 Antibody responses elicited in mice immunised with GMMA

We measured anti-NadA IgG antibody responses in serum samples containing sera from two mice each by ELISA. GMMA from recombinant serogroup A strains with deleted capsule (MenA WT NadA cps-) and deleted *nadR* but expressing the capsule (MenA NadR KO cps+) elicited significantly higher GMT compared to the serogroup A GMMA with deleted capsule and *nadR* genes (MenA NadR KO cps-; **Figure 3.5, Panel A**; GMT= 463410, 463410 and 5120 respectively; *P*=0.0052). Possibly corresponding to the higher amount of NadA in MenA WT NadA cps-. The rHis-NadA-3 also induced more bactericidal anti-NadA antibodies than MenA NadR KO cps- (GMT= 463410 and 40960 respectively; *P*=0.0147). In contrast, MenW NadR KO cps- and MenW WT NadA cps- GMMAs elicited low anti-NadA-3 IgG antibody responses (**Figure 3.5, Panel B**; GMT=160 and 226 respectively). GMMA from both

groups without NadA expression induced no measurable anti-NadA antibody responses.

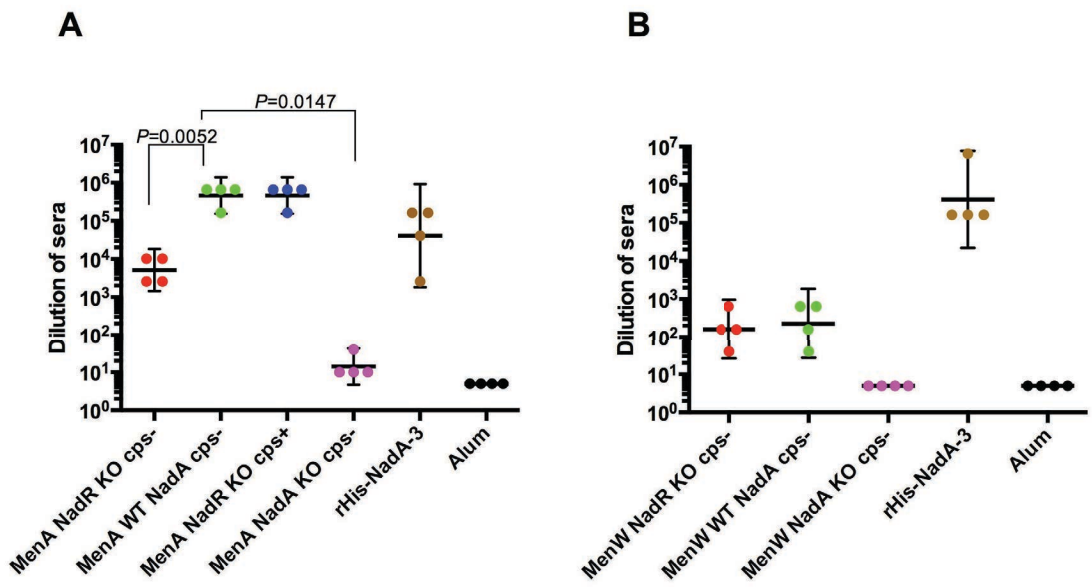

Figure 3.5. Anti-NadA IgG antibody responses elicited in mice immunised with serogroup A and W GMMA vaccines as measured by ELISA.

Panel A: anti-NadA IgG responses elicited by GMMA from serogroup A isogenic mutants, Panel B: anti-NadA IgG responses elicited by GMMA from serogroup W recombinant strains. The ELISA plates were coated with 2µg of rHis-NadA-3. For each vaccine group, eight mice were immunised with three doses, 2 weeks apart. The serum samples analysed were obtained 2 weeks after the third dose. Each symbol represents sera of two mice pooled. Of each vaccine group the line indicates the GMT. GMMA used for immunisation: MenA NadR KO cps- and MenW NadR KO cps- (*nadR*, capsule biosynthesis locus, *lpxL1* and *gna33* KO); MenA WT NadA cps- and MenW WT NadA cps- (capsule, *lpxL1* and *gna33* KO); MenA NadR KO cps+ (*nadR*, *lpxL1* and *gna33* KO); MenA NadA KO cps- and MenW NadA KO cps- (*nadA*, capsule, *lpxL1* and *gna33* KO); rHis-NadA-3: recombinant hexa-histidine tagged NadA allele 3; Alum: aluminium hydroxide. Statistical analysis between pairs of groups was performed using Mann–Whitney *U* test.

3.4.4 SBA responses of sera from mice immunised with GMMA from serogroup A and W mutant strains

The SBA responses of the antiserum of mice immunised with GMMA from the recombinant serogroup A and W strains were assessed against three serogroup A isolates, three W and one X isolate expressing low, intermediate and high levels of NadA (**Table 3.9**).

Table 3.9. Panel of isolates selected for serum bactericidal activity analysis.

Serogroup	Isolate	Origin	Year of isolation	*fHbp* variant	*fHbp* ID	*porA* VRs	*nadA* allele	NadA expression (%)	Sequence type (ST)
A	Su14/07	Sudan	2007	1	5	P1.20,9	3	25.9	7
A	BuFa20030020	Burkina Faso	2007	1	5	P1.20,9	3	60.2	6035
A	Mali21/07	Mali	2007	1	5	P1.20,9	3	102.9	2859
W	BF 10/02	Burkina Faso	2002	2	23	P1.5,2	3	30.4	11
W	Ghana 7/04	Ghana	2004	2	23	P1.5,2	3	57.6	11
W	Mali 10/09	Mali	2009	2	23	P1.5,2	2	100.0	11
X	MRS2006087	Niger	2006	1	74	P1.5-1,10-1	3	18.6	5789

3.4.4.1 Isolates expressing low amounts of NadA

The MenW NadR KO cps- GMMA from the recombinant serogroup W strain 1630 (NVGH0385) with PorA P1.5,2 and fHbp v.2 ID23 induced bactericidal antibodies that were able to kill the serogroup A isolate Su14/07, expressing heterologous PorA P1.20,9 and fHbp v.1 ID5 (GMT=3822) (**Figure 3.6, Panel A**). Higher titres were observed for MenW WT NadA cps- (GMT=26425), which contained slightly higher amounts of NadA (3 and 7% respectively). GMMA from the MenW NadA KO cps- strain was also able to induced bactericidal antibodies (GMT= 2053) of similar level

as MenW NadR KO cps-. The results suggest that majority of the bactericidal antibodies induced by the GMMA vaccine are being directed against other OMP than NadA, fHbp and PorA. Moreover, rHis-NadA-3 also elicited high titres with equal levels to the ACWY vaccine (GMT=85334 and 163840).

The MenA NadR KO cps- and MenA WT NadA cps- GMMA from the recombinant serogroup A strain N2602 (NVGH0348) with NadA-3, PorA P1.20,9 and fHbp v.1 ID5 elicited bactericidal titres that killed the serogroup W isolate BF10/02 expressing heterologous PorA P1.5,2, fHbp v.2 ID23 and low amounts of NadA-3 (30%; **Figure 3.6, Panel B**; GMT=10328 and 14382 respectively). GMMA from the isogenic mutant expressing the capsule MenA NadR KO cps+ induced a lower titre (GMT=380, not significant). Possible explanation could be that the capsule masks OMP preventing accessibility of the antigens on the meningococcal surface to C1q complement protein and bactericidal antibodies. Antibodies induced by rHis-NadA-3 elicited a measurable bactericidal antibody responses against the strain tested (GMT=915). Three pools of sera for the control groups MenA NadA and NadR KO cps- and MenA NadA KO cps- could kill BF10/02 indicating that other OM than NadA, fHbp and PorA, some possibly regulated by NadR, are inducing bactericidal antibodies (GMT=3947 and 544 respectively). For reasons not fully understood, neither GMMA from the recombinant serogroup A and W strains elicited any bactericidal titres against serogroup X strain MRS2006087 expressing NadA-3 and heterologous PorA P1.5,10 and fHbp v.1 ID74 (**Figure 3.6, Panel C**). Possible explanations could be the low amount of NadA or other immunogenic antigens on the meningococcal surface or high capsule amount preventing antigen accessibility.

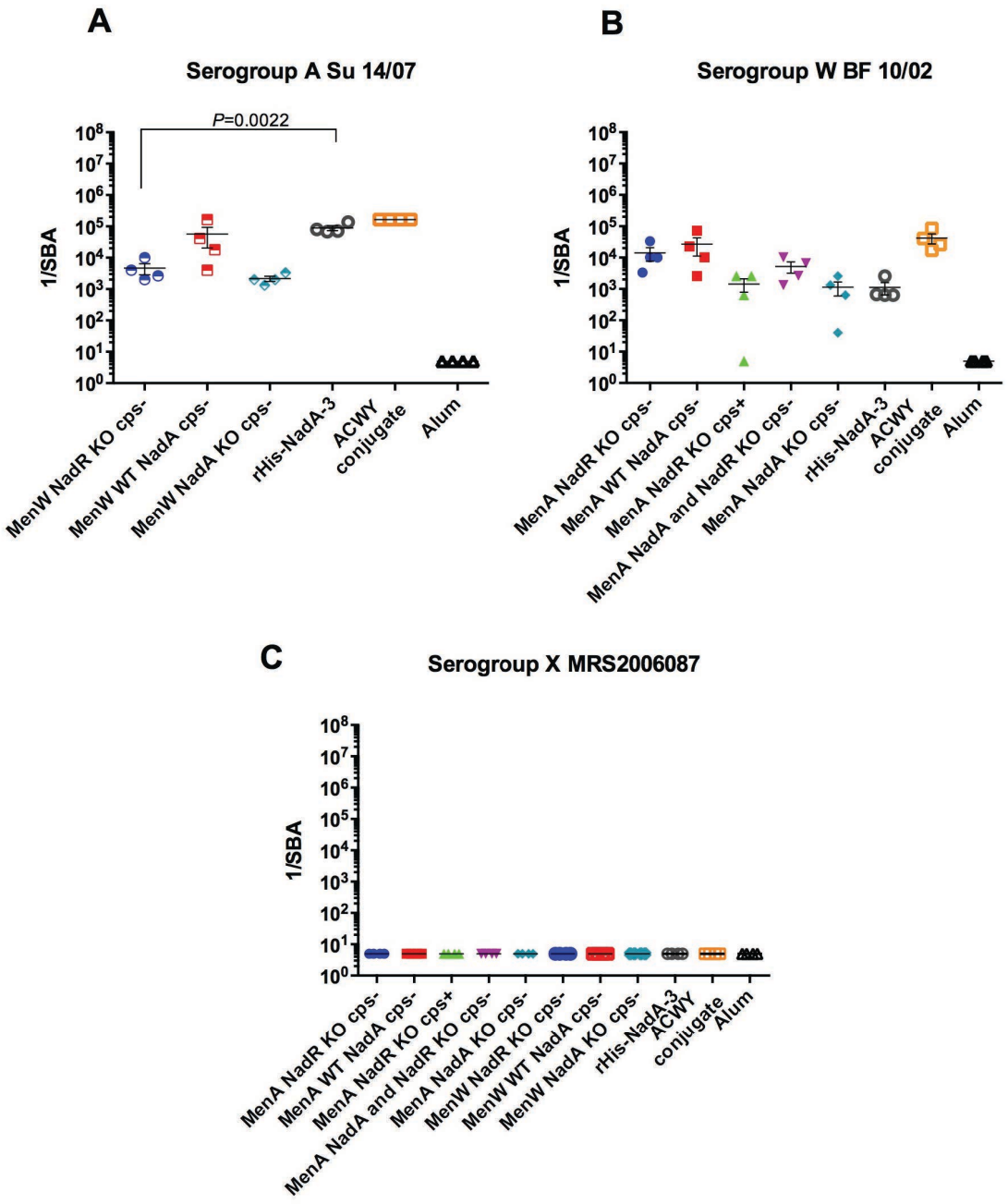

Figure 3.6. Serum bactericidal antibody responses of mice immunised with MenA and MenW GMMA against African meningococcal strains expressing low NadA amounts.

The sera from immunisation with the serogroup A and W vaccine groups were tested against serogroup A Su14/07 (panel A), serogroup W BF10/02 (panel B), respectively, and both sets of sera against serogroup X MRS2006087 (panel C). The isolates expressed low amounts of NadA (~19-30%). Serum bactericidal responses were measured with baby rabbit complement. The serum samples used in the analyses were obtained 2 weeks after the third vaccine dose. Each symbol represents a sample of sera from two mice pooled, and the line of each vaccine group indicates the GMT. Half open symbols

represent the sera from the MenW vaccine groups. Error bars indicate ±95% confidence interval of the GMT. GMMA used for immunisation: MenA NadR KO cps- and MenW NadR KO cps- (*nadR*, capsule biosynthesis locus, *lpxL1* and *gna33* KO); MenA WT NadA cps- and MenW WT NadA cps- (capsule, *lpxL1* and *gna33* KO); MenA NadR KO cps+ (*nadR*, *lpxL1* and *gna33* KO); MenA NadA KO cps- and MenW NadA KO cps- (*nadA*, capsule, *lpxL1* and *gna33* KO); MenA NadA and NadR KO cps- (*nadA, nadR*, capsule biosynthesis locus, *lpxL1* and *gna33* KO); rHis-NadA-3: recombinant hexa-histidine tagged NadA allele 3; Alum: aluminium hydroxide. Statistical analysis between pairs of groups was performed using Mann–Whitney *U* test.

3.4.4.2 Isolates expressing intermediate amounts of NadA

GMMA from the MenW NadR KO cps- strain induced bactericidal antibodies that were able to kill the serogroup A isolate BuFa20030020 with heterologous PorA P1.20,9 and fHbp v.1 ID5 (**Figure 3.7, Panel A**; GMT=4207). Significantly higher titres were observed for MenW WT NadA cps- and rHis-NadA-3 (GMT=70336 and 163840 respectively). The MenW NadA KO vaccine group also induced titres (GMT=498), showing that a fraction of bactericidal antibodies were directed against other immunogenic OMPs.

GMMA from the MenA NadR KO cps- recombinant strain elicited bactericidal titres that killed the serogroup W isolate Ghana7/04 expressing heterologous PorA P1.5,2 and fHbp v.2 ID23 (**Figure 3.7, Panel B**; GMT=72843). Significantly higher titres were generated for MenA WT NadA cps- and MenA NadR KO cps+ (GMT=$6.9x10^6$ and $4.8x10^6$ respectively). This could be due to higher levels of NadA in both the GMMA vaccine strains MenA WT NadA cps- and MenA NadR KO cps+ and the strain tested compared to MenA NadR KO cps-. Low but measurable titres were also induced by the MenA NadA and NadR KO cps- and MenA NadA KO cps- vaccine groups (GMT=24 and 76 respectively) indicating the presence of other immunogenic

antigens on GMMA. However, the lower titres suggest most were directed against NadA.

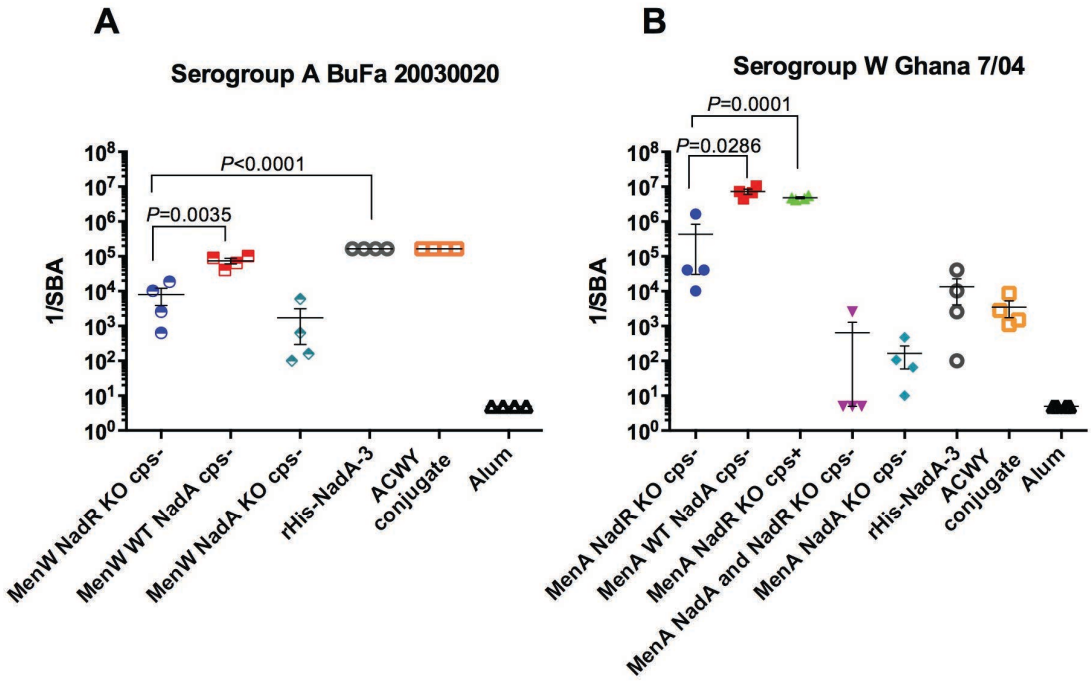

Figure 3.7. Serum bactericidal antibody responses of mice immunised with MenA and MenW GMMA against African meningococcal strains expressing intermediate NadA amounts.

The sera from immunisation with the serogroup A and W vaccine groups were tested against serogroup A BuFa20030020 (panel A) and serogroup W Ghana7/04 (panel B), respectively, expressing intermediate amounts of NadA (60 and 58% respectively). Serum bactericidal responses were measured with baby rabbit complement (see legend of Figure 3.6). Statistical analysis between pairs of groups was performed using Mann–Whitney *U* test.

3.4.4.3 Isolates expressing high amounts of NadA

The antibody responses elicited following immunisation with GMMA from the MenW NadR KO cps- strain did not kill the serogroup A isolate Mali21/07 with heterologous PorA P1.20,9 and fHbp v.1 ID5, despite it expressing high levels of NadA-3 (103%) (**Figure 3.8, Panel A**). Only MenW WT NadA cps- and rHis-NadA-3 elicited measurable bactericidal titres (GMT=13898 and 19837 respectively) possibly, differences in fine antigen specificities and capsular amount.

All vaccine groups, except Alum, induced bactericidal antibodies against the high NadA-2 expresser Mali10/09 (100%) with heterologous PorA P1.5,2 and fHbp v.2 ID23 (**Figure 3.8, Panel B**). Serum from the MenA NadR KO cps- GMMA vaccine group was able to kill the strain, and the MenA WT NadA cps- significantly more so (GMT=$1.3x10^6$ and $1.1x10^7$ respectively). The presence of the capsule on the GMMA seemed not to affect the bactericidal antibody activities as similar GMT was obtained as for the capsular negative group MenA NadR KO cps- (GMT=$1.3x10^6$). Antibody responses generated by MenA with deleted NadA+NadR and NadA (GMT=7750 and 194156 respectively) also killed the isolate. The data are consistent with the hypothesis that there are additional antigens on the GMMA that can elicit bactericidal antibodies. Antibodies induced by rHis-NadA-3 induced bactericidal antibody responses against the strain tested (GMT=$1.3x10^6$), which shows that cross-protection is possible between the *nadA* alleles 2 and 3.

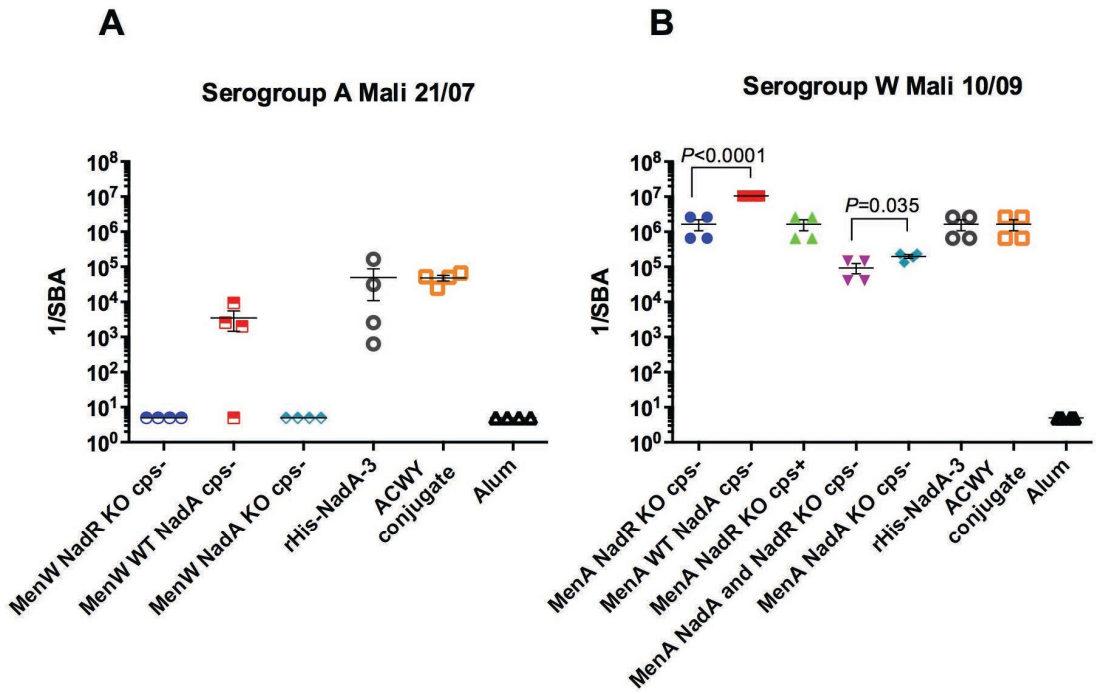

Figure 3.8. Serum bactericidal antibody responses of mice immunised with serogroup A and W GMMA against African meningococcal strains expressing high NadA amounts.

The sera from immunisation with the MenW and MenA vaccine groups were tested against serogroup A Mali 21/07 (panel A) and serogroup W Mali10/09 (panel B), respectively, expressing high amounts of NadA (103 and 100% respectively) (see legend of Figure 3.6). Mali10/09 express NadA-2.

3.5 Discussion

Phase variation often results in ON/OFF changes of expression or graduated alterations. The mechanism of NadA expression is complex, and the many ways of regulation result in different levels of expression (27). Although, results from the typing analyses in Chapter 2 suggests that natural and herd immunity is not a strong driving force for antigenic variability, it could be so for antigenic expression. Selection for the ON phenotype or high expression involves gain of function such as adhesion and invasion to epithelial cells, but at the same time it also involves potential susceptibility to anti-NadA antibodies (12). While the OFF phenotype or decreased protein expression reduces adhesion to epithelial cells, perhaps promoting spread of the bacterium, and allowing escape of killing by bactericidal antibodies due to a reduction in the concentration of surface-bound antibody and hence reduced complement activation (28). The correlation between the level of expression of NadA on bacterial OM, and the susceptibility and production of bactericidal antibodies has not been established. In this study, majority of the serogroup A and W isolates were found to express intermediate to high levels of NadA while the X isolates investigated showed low expression of NadA. Although the regulation of gene expression is bifunctional, low expressers still have the potential to respond to proper niche signals such as 4HPA and express higher levels under certain environmental conditions such as during adhesion (19). Here, we saw that when the bacterial cells were grown in the presence of 4HPA, the molecule differentially induced a significant increase in NadA expression (p<0.001). Fagnocchi *et al.* (2011) also showed that 4HPA and human saliva increased NadA expression. This suggesting that 4HPA could mimic physiologically relevant signalling molecules *in vivo* promoting the bacteria to switch to the “ON” phenotype (16). However, at this point it not clear as to how the increase

of NadA expression by 4HPA or other niche signals *in vivo* translates into *e.g.* increased adhesion capability. Further adhesion assay experiments on Chang cells is warranted (9).

GMMA is a new vaccine strategy recently employed for the development of a broadly protective vaccine against disease causing serogroups in Africa (29). This vaccine strategy involves the deletion of the *gna33* gene, which differentiates GMMA from NOMV vaccines. During normal bacterial cell growth, osmotic stability is maintained by a network of glycan strands linked with peptides (peptidoglycans) (30). The protein Genome Derived *Neisseria* antigen 33 (GNA33) was identified through reverse vaccinology screening of serogroup B genome. GNA33 is a membrane-bound lipoprotein with murine hydrosylase activity that is present in all *Neisseria* species (31–33). In this study, *gna33* gene was knocked-out to increase membrane blebbing for efficient vaccine production. Adu-Bobie *et al*. (2004) have previously shown that deleting the gene not only increases vesicle release through physiological dissociation of the membrane, but also affects cell separation, membrane assembly and architecture (24). Quantification of NadA on GMMA from serogroup A and W isogenic mutant strains showed that control strains with the capsule expressed (MenA NadR KO cps+, MenW NadR KO cps+) or wild-type NadA expression (MenA WT NadA cps-, MenW WT NadA cps-) expressed the highest amount of NadA. We anticipated that GMMA from the serogroup A and W recombinant strain MenA NadR KO cps- and MenW NadR KO cps- would contain the highest amount of NadA since *nadR* was deleted for that purpose. Western blotting of whole cell lysates of *nadR* KO serogroup A and W mutants showed that NadA was over-expressed as a result of *nadR* KO. However, the NadA protein does not seem to have been transported to the

OM for GMMA formation. There are several plausible explanations for this. The mutations introduced for the generation of the recombinant vaccine strains are not neutral and their cumulative effect could affect the trafficking of NadA and other proteins to the OM. NadA could be accumulated in inclusion bodies or possibly NadA is over-expressed in the "final" strain MenA NadR KO cps-. To determine the localisation of NadA in the "final" recombinant strains one could do FACS and Western blot analysis of NadA expression in the isogenic mutants. Further electron or fluorescence microscopy experiments could be done on cell fractionations of the mutant strain MenA NadR KO cps- using an anti-NadA mAb to determine NadA protein location. Moreover, deletion of *gna33* by itself has been shown to affect the membrane architecture. It could be that deleting *gna33* and in combination with deletion of *nadR*, capsule and *lpxL1* in one strain (*e.g.* MenA NadR KO cps-) affects assembly and insertion of NadA into the OM more profoundly than the isogenic strains with fewer mutations (*e.g.* MenA WT NadA cps-). NadA has to go through the transmembrane domain for insertion into the OM, and this could be impeded since the integrity of the membrane is impaired. NadA could be in the cytoplasm, and microscopy studies with cell fractionation of the different recombinant strains could aid prove this hypothesis. Therefore, to perform these experiments it would also be beneficial to develop a mAb against native NadA on GMMA. The Western blot results of the GMMA lysates showed that the mutant strains with a capsule (MenA NadR KO cps+, MenW NadR KO cps+) contained more NadA than the mutants without the capsule (MenA NadR KO cps-, MenW NadR KO cps-). It could be that the capsule somehow helps to stabilise the NadA trimer in the OM. Further investigations are also needed to look into the individual effect of the mutations on the membrane composition of the isogenic mutants, and if the cell membrane

composition is reflected by the GMMA. The protein composition of GMMA is unknown and GMMA may not represent the entire cell membrane composition. A more in-depth mass spectrometry analysis such as MALDI-TOF of the GMMA and membrane fraction of the mutant strains is necessary.

The expression of vaccine candidate antigens in a GMMA format allows for their representation in the correct orientation and conformation that resembles whole cell vaccine, which is naturally presented to the host upon infection. Here, we generated GMMA from serogroup A and W recombinant strains with deleted *nadR,* capsule, *lpxL1* and *gna33*. The effectiveness of these vaccine groups were evaluated at inducing an antibody response (ELISA) that also showed serum bactericidal activity against African meningococcal strains of serogroup A, W and X expressing low, intermediate and high levels of the vaccine antigen NadA. The ELISA results showed that the MenA WT NadA cps- and MenA NadR KO cps+ GMMA elicited significantly higher anti-NadA IgG titres compared to MenA NadR KO cps- and rHis-NadA-3. While all MenW GMMAs elicited measurable but low anti-NadA antibody responses. A similar trend was seen when MenA and MenW vaccine groups were assessed based on their ability to kill a low, intermediate and high expressing serogroup W and A isolates, respectively. For all strains except serogroup W BF10/02 MenA WT NadA cps-, MenA NadR KO cps+ and MenW WT NadA cps- GMMA elicited the highest bactericidal titres that killed the test strains. The results suggest that the higher the NadA expression in the GMMA in combination with other immunogenic antigens contribute to an increase in bactericidal responses. The results do not, however, indicate whether there is a cut off level of NadA expression on the surface of the meningococcus needed to cross-link IgG antibodies and activate the

complement cascade. This is because one cannot exclude bactericidal activity against other target antigens. Such experiments require an anti-NadA mAb or the rHis-NadA-3 serum to determine the minimum level of NadA on the surface of meningococcus that is necessary to kill the bacteria. Moreover, for reasons not fully understood lack of bactericidal antibody responses by MenW NadR KO cps- and a low titre generated by MenW WT NadA cps- did not effectively kill the serogroup A strain Mali21/07 despite it expresses high amounts of NadA-3. Sera from mice immunised with rHis-NadA-3 killed the isolate. In addition, low titres for the MenW NadR KO cps- were elicited when tested against serogroup W isolate Mali21/07, and X isolate was not killed by any of the vaccine groups. The results could be due to the difference in epitope specificity of the NadA and other immunogenic proteins of isolates MRS2006087 and Mali21/07 compared to N2602 and rHis-NadA-3. More functional antibodies could have been generated against recombinant NadA than that in GMMA. The isolates may also express more capsule preventing accessibility of OMPs on the meningococcal surface. The capsule may also be of different chemistry and length. To assess this, FACS analysis using mAb against the serogroup A capsule could determine if a higher capsule amount is expressed by these isolates. If it the length and chemistry is a factor, Dionex, HPLC-SEC and NMR provides a more in-depth analysis.

Nonetheless, the fact that sera from the NadA KO cps- GMMA vaccine groups from both serogroup A and W recombinant strains killed the test strains highlights the advantage of using GMMA as a vaccine strategy. MenA NadA and NadR KO cps-, MenW NadA and NadR KO cps-, MenA NadA KO cps- and MenW NadA KO cps- vaccine groups elicited bactericidal antibodies to antigens other than NadA, fHbp and

PorA. The data indicate even strains that express low levels of certain target surface antigen, antibodies to other but shared OM antigens along with antibodies to the main target bactericidal antigens may provide protection (23). Thereby dependency on a single antigen or antigen type is avoided. A second advantage is that there is a potential for synergistic function of antibodies to different antigens in cases where expression level of a particular antigen is low (20,23,34). To identify such immunogenic antigens, one could mine for them by generating mAbs against GMMA. The bactericidal mAbs can then be used to immunopresipitate the antigens from whole cell lysates and analyse the immunopresipitates by Mass spectrometry.

In this study we also showed that removal of capsule does not significantly enhance the level of bactericidal activity induced against strains with heterologous fHbp and PorA compared to the isogenic non-encapsulated GMMAs. In Chapter 2, we saw that removal can significantly affect antibody responses towards fHbp. Possibly, it could be that the capsule is not able to fully mask the NadA trimer on the surface. An alternative explanation is that the high amount of NadA on the GMMA from serogroup A isogenic mutant expressing the capsule (MenA NadR KO cps+) compared to the wild-type GMMAs and GMMAs with deleted *nadR* and capsule biosynthesis locus (MenWT NadA cps-, MenW WT NadA cps-, MenA NadR KO cps-, MenW NadR KO cps-) interferes with the capsule being an antigenic competitor (29). In principle, one could test this by performing a FACS analysis on the mutant strains using anti-NadA mAbs and compare the results with FACS analysis against fHbp mutant strains using anti-fHbp mAbs. In combination with SBA analysis of the mutant strains using the mAbs, this could also give a clearer indication than using mouse sera as to whether masking of the antigens affects bactericidal activity.

Previous studies have shown that serum antibodies produced against recombinant proteins have either no or limited bactericidal activity against meningococcal isolates expressing the same fHbp variant (29,35,36). This was confirmed in Chapter 2 where antibodies induced by rHis-fHbp ID1 did not elicit a measurable bactericidal antibody responses against the strain tested. It could be that bactericidal antibodies are induced towards conformational epitopes. Compared to the rHis-fHbp antibody responses, rHis-NadA-3 induced bactericidal antibodies that killed all strains tested. The results suggest that immunogenicity to NadA is less dependent on the three-dimensional conformation of the protein than other OMPs such as fHbp. To test this, one could map the epitopes on linear rHis-NadA fragments responsible for eliciting bactericidal antibodies using bactericidal anti-NadA mAb generated towards NadA expressed in GMMA. The results can be compared with the eiptopes mapped on rHis-NadA using bactericidal anti-NadA mAbs generated against rHis-NadA-3.

3.6 Conclusion

To conclude, the GMMA vaccine strategy offers an additional mode of expressing vaccine candidate antigens compared to recombinant, dOMV and NOMV approaches. Here, we investigated the bactericidal antibody responses elicited by NadA expressed in GMMA. The results show that NadA in GMMA provides a broad protection against African serogroup A and W isolates expressing low, intermediate and high amounts of NadA. Despite some of its current limitations, such a vaccine strategy could serve as an alternative or a supplement to the existing conjugate vaccines against epidemics in the meningitis belt.

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CHAPTER 4

GENERATION OF MONOCLONAL ANTIBODIES AGAINST NADA, FHBP AND OTHER OUTER MEMBRANE ANTIGENS ON GMMA

4.1 Introduction

The existence of antibodies or immunoglobulins (Ig) in blood were firstly described in 1890 by von Behring and Kitasato. In the following years, the classical tautology of antibodies or "antikörper" and the substance that induced the production of them (antigen) was discriminated. After more than 100 years of investigation into the structure and function, the eponymous description of antibodies is their classical heterodimeric composition of two heavy and two light chains, which consist of either a κ or ι chain. Each chain has a NH_2-terminal variable domain and one or more COOH-terminal constant domain (1). The chains are held together by disulfide bridges between two conserved cysteine residues. Pepsin digestion of IgG has shown that the Ig can be split into a Fc fragment and a single dimeric $F(ab)_2$ that can bind antigens. Moreover, the Ig can be separated into five isotypes based on the heavy chain (IgD, IgG, IgM, IgA, IgE). IgG is the most predominant subclass with longest half-life, and investigation into the functional difference of IgG has identified 4 subclasses with highest levels in serum ranked accordingly IgG1>IgG2>IgG3>IgG4. In mice the subclasses are IgG1, IgG2a, IgG2b and IgG3 (1). The Fc portion of each of these subclasses affects antibody flexibility (hinge region), and each Ig exhibit different functional activity. For *N. meningitidis,* activation of the complement cascade is an important means of clearance of the pathogen through bacteriolysis. Human IgG3 and IgG1 and murine IgG2a are the most prominent subclasses that fix C1q through binding of constant domain (2). The purpose of antibodies is to bind pathogen surface components and promote cell signalling and activation, but also bind soluble effector molecules for neutralisation. Antibodies are able to bind almost any ligand through the diversity and alteration of the DNA encoding antigen binding regions of individual B cells (affinity maturation). This has been exploited for the

development of single specific (monoclonal) antibodies that recognise specific targets for diagnostic, therapeutic and research purposes.

4.1.1 Monoclonal antibody production and their applications

Monoclonal antibodies (mAb) are specific and homologous antibodies to a particular target antigen or epitope produced from a single B cell clone. The first successful generation of mAbs *in vitro* was in 1975 by Kohler and Milstein (3). The spleen of a mouse immunised with an antigen was fused with immortal myeloma cancer cell line (4). The classical hybridoma method involves cloning the fusion mix into 96 well plates and screening of the supernatant for target antigen specific antibodies. Positive cell lines are cloned further to generate monoclonal immortalised B cell lines, which produce unlimited amounts of antibody that have a specific heavy and light chain derived variable regions (**Figure 4.1**).

To date, mAb can be produced in a variety of expression systems and cell lines, which has increased the usability and application of mAbs (5). Development and production of mAb now include DNA based recombinant technologies, expression in bacterial systems, phage display libraries, chimerisation and humanisation strategies based on transgenic mice. This has revolutionised, for example, therapeutic treatment where mAbs have been produced for the treatment of cancer and autoimmune diseases (6). Moreover, due to their high-specificity and sensitivity of binding antigens, mAb have become a well established tool in research for detecting antigens in their native conformation, Western blotting or in immune-affinity based assays (3). Recently, the utility of mAb has been expanded to proteomic and recombinant protein structure

analysis. This application has been employed for the characterisation of vaccine candidate antigens in *N. meningitidis*.

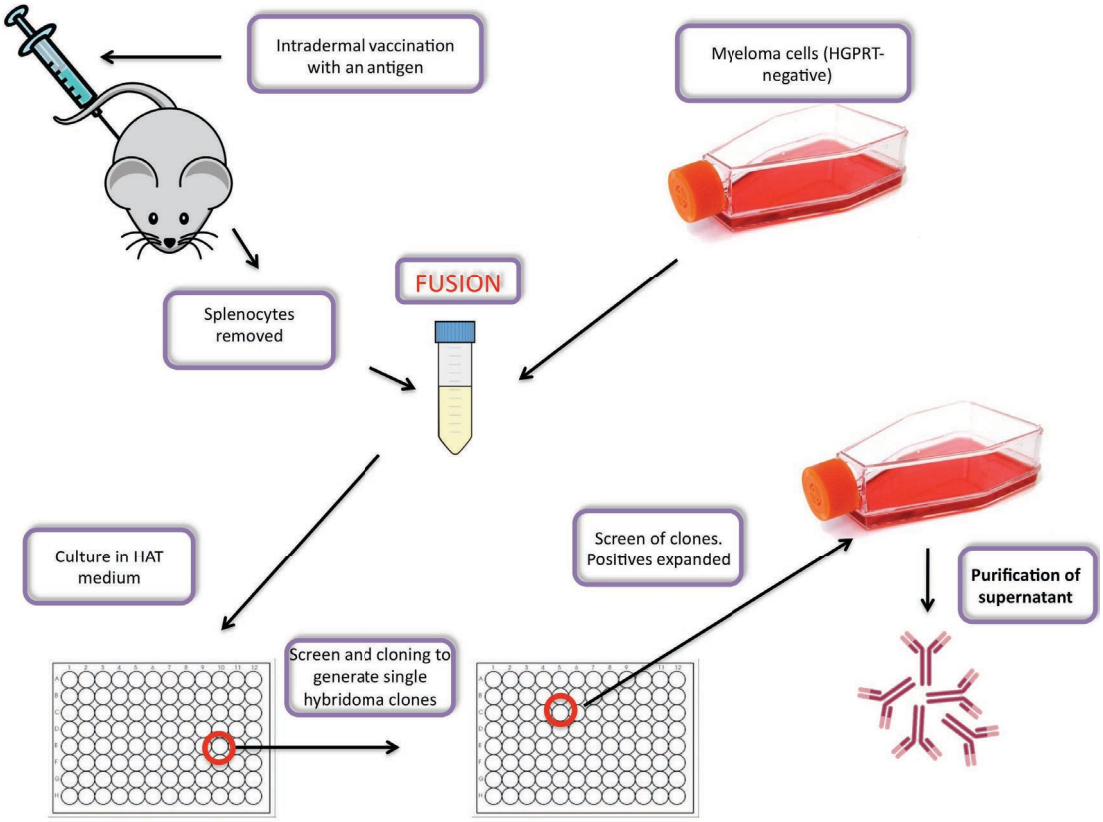

Figure 4.1. Hybridoma technology for production of murine monoclonal antibodies.

A mouse is immunised with a desired antigen. B cells from the spleen of this mouse are fused with immortal myeloma cells that have lost the ability to synthesis hypoxanthine-guanine-phosphoribosyl transferase (HGPRT), an enzyme that salvages synthesis of nucleic acids. The HAT medium contains hypoxanthine, aminopterin and thymidine that is selective for fused hybridoma cells. Un-fused myeloma cells cannot grow because they lack HGPRT and cannot replicate DNA, and unfused B cells die after a few cell cycles. The fusion mix is plated onto 96 well titre plates, and after 7-10 days screened. Positive hybridomas are selected and cloned again into 96 well plates to generate single cell derived lines. Positive clones are subsequently selected for expansion and large-scale production of monoclonal antibodies.

4.1.2 Monoclonal antibodies against *Neisseria meningitidis* antigens

N. meningitidis is a global pathogen responsible for local to epidemic outbreaks. As previously mentioned, mAbs have been used in serological studies to characterise meningococcal strains based on their capsule (serogroup), OMP PorB (serotype) and PorA (serosubtype). The information gained has been pivotal in monitoring and epidemiologic surveillance of disease-causing strains (7). However, serological methods have been complicated by the numerous variants of these OMP, which show strain-to-strain variation limiting sensitivity and specificity (7). Serological typing has been mostly replaced by PCR-based genotyping techniques and recently genomics. MAbs are now being primarily prepared against specific vaccine antigens to characterise the fine specificity of immune responses towards the protein.

In the case of fHbp, many studies have generated mAbs against the recombinant protein to elucidate the specific epitopes involved in generating bactericidal antibodies and binding of fH (8–10). Beernink *et al*. (2008) generated a panel of mAbs from mice immunised with recombinant fHbp v.2 and v.3 to determine the regions on fHbp in these variant groups that are recognised by bactericidal antibodies, and the regions that are involved in binding of fH (8). The authors found that none of the mAbs were bactericidal individually, only in combination with each other. This was firstly shown by Welsch *et al*. (2004), and was likely to be due to the sparse distribution of fHbp on the surface of the meningococci that prevents cross-linking of two IgG anti-fHbp antibodies to correctly spaced epitopes and engagement of complement protein C1q (11). However, alignment of fHbp sequences together with site-specific mutagenesis of fHbp genes confirmed that residues between aa 174-216 of fHbp v.2 and v.3 are recognised by bactericidal antibodies. Giuntini *et al*. (2012)

compared two mAbs against fHbp v.1 that share overlapping epitopes in relation to their different effects on fH binding and bactericidal activity, as they expected that blocking of binding of fH renders the pathogen more susceptible to bacteriolysis (9). Yeast display and site-specific mutagenesis showed that binding of antibodies JAR1 (IgG3) and mAb502 (IgG2a) was eliminated by a single aa substitution, R204A. However, only JAR1 inhibited binding of fH to fHbp and had human complement dependent bactericidal activity. Although, susceptibility to anti-fHbp antibodies is influence by IgG subclass and amount of fHbp expressed by the strain, their data illustrates that subtle differences in fine antigenic specificity affects fH binding and mAb-induced protection. Studies performed so far have been focused on generating mAbs against recombinant fHbp and not against the antigen in its native membrane environment.

Far fewer studies have looked at the epitopes of NadA. Tavano *et al.* (2011) developed deletion mutants devoid of sequences distributed in the coiled-coil domain and progressively closer to the anchor domain (12). Together with polyclonal antibodies (Fab fragments), the mutants were analysed for their ability to form surface oligomers on their *E.coli* model, and their ability to promote bacterial adhesion to Chang cells. The authors showed that two critical regions present in the globular head domain and intra-chain coiled-coil stalk were responsible for NadA-cell receptor binding with Chang cells. Fukusawa *et al*. (2003) developed a mAb against NadA expressed in serogroup B OMV (13). The mAb showed bactericidal activity and cross-reactivity to serogroup B and C strains from Brazil. It also recognised approximately 60% of the samples from both serogroups in dot-blot analyses.

4.2 Project objectives

Antigens presented on GMMA are in their native conformation, and this is what is presented to the host during an infection. As discussed in Chapter 2, recombinant fHbp induces limited cross-reactivity against fHbp from different sub-families or variant groups compared to fHbp natively presented on NOMV or GMMA. While recombinant NadA is able to induce cross-protective antibodies against NadA of different peptides similarly to NadA natively presented in GMMA. We set out to determine the difference of such immunological responses by generating mAbs against antigens on GMMA in their native conformation and membrane environment as approaches so far have focused on generating mAbs against recombinant fHbp. The specific focus was on developing mAbs targeting vaccine candidate antigens fHbp and NadA in GMMA in order to better understand the antigenic specificities of fHbp and NadA. We also aimed at investigating the antibody repertoire induced by GMMA when given with or without Alum. Three fusions were made, and the first two fusions were performed using mice immunised with MenA NadR KO cps- (generated in Chapter 3) formulated on Alum. The third fusion was performed using a mouse immunised with GMMA Triple KO OE fHbp ID9 R41S absorbed to Alum (see Materials and Methods 4.2.1). The expansion and production of mAb against the fHbp protein is currently underway. Therefore, the focus of this chapter is on the results from the first two fusions. The initial characterisations of the mAbs were as follows:

1. Production of monoclonal antibodies against NadA allele 3 and fHbp v.1 ID9 expressed natively in GMMA and other potential immunogenic proteins on GMMA.

a. Screening of anti-GMMA antibodies by ELISA and Western blot using GMMA and recombinant protein.
b. IgG isotyping.
c. Evaluation of mAb binding to bacterial cells by immunofluorescence (IFA) and flow cytometry.
d. Evaluation of the functional bactericidal activity of monoclonal antibodies by serum bactericidal assay against serogroup A, W and X African isolates.

4.3 Materials and Methods

4.3.1 Vaccine GMMA groups used for immunisation

Vaccines used for immunisations were GMMA from a *Neisseria meningitidis* serogroup A ST2859 case strain N2602 (NVGH0348_001), PorA P1.20,9, fHbp v.1 ID5 expressing NadA-3 (MenA NadR KO cps- [Chapter 3]) and GMMA from serogroup W carrier strain 1630 ST11, PorA P1.5,2 over-expressing fHbp v.1 ID9 (Triple KO OE fHbp [generated by Oliver Koeberling]). The fHbp had the R41S mutation, which is a single aa substitution Arg at residue 41 replaced by Ser. This results in decreased binding of fH (10). Both strains had deleted capsule biosynthesis genes, *lpxL1*, and *gna33*. We also deleted the gene encoding for the *nadA* repressor *nadR*. The third vaccine group received rHis-NadA-3 produced and described in Chapter 3 section 3.3.3 on page 117.

4.3.2 Monoclonal antibody production

Eight weeks old Balb/c female mice (3 mice per group) were obtained from Charles River. The mice were immunised intradermally with three doses of vaccine given at days 1, 21 and 35 (**Table 4.1**). Two groups of mice received 5µg of GMMA MenA NadR KO either absorbed to Alum or not. The third group was immunised with 5µg of Triple KO OE fHbp GMMA. These three groups were used for mAb production. Two control groups were immunised with 30µg of recombinant rHis-NadA-3 given with CpG or SIGMA adjuvant. Blood samples were obtained prior to the first immunisation and 14 days after the third immunisation. Three days prior to cell-fusion (day 49) a final boost was given intravenously with 5µg pure non-adjuvated antigen. Vaccines received by two groups of mice were absorbed on 3mg/ml Alum in

a 100µl formulation containing 10mM Histidine and 0.9mg/ml NaCl. Terminal blood samples were obtained at the day of fusion (day 49). Vaccines were prepared as described in Chapter 3 and sterilised prior to formulation by filtration (0.22µm). Sterility of the formulations was confirmed by plating 100µl on GC agar (Becton Dickinson) and growing over night at 37°C.

Table 4.1. Mouse immunisation scheme for monoclonal antibody production.

CpG and SIGMA adjuvants were obtained from the Swiss Tropical and Public Health Institute. NaCl and Histidine were obtained from Sigma-Aldrich and Alum from NV&D.

Group (Mouse number)	Antigen	Adjuvant	Dose (µg)	Volume per animal	Route	Formulation
Fusion 1 1 (1 - 3)	MenA NadR KO cps- GMMA	Alum	5	100 µl	ID (IV)[b]	MenA NadR KO cps- + NaCl 9 mg/ml Histidine 10 mM Alum hydroxide (3 mg/ml)
Fusion 2 2 (4 - 6)	MenA NadR KO cps- GMMA	-	5	100 µl	ID (IV)	MenA NadR KO cps-
3 (7 - 9)	His-rNadA-3	CpG	30	100 µl	ID	rHis-NadA-3 1:1 CpG
4 (10-12)	His-rNadA-3	SIGMA[a]	30	100 µl	ID	rHis-NadA-3+ 1:1 SIGMA
Fusion 3 (1-3)	GMMA, triple KO OE fHbp GMMA	Alum	5	100 µl	ID (IV)	GMMA, Triple KO OE fHbp NaCl 9 mg/ml Histidine 10 mM aluminium hydroxide (3 mg/ml)

[a] 2% squalene oil-in-water emulsion, 0.2% Tween 80, LipidA from *Salmonella Minnesota* and 0.5mg synthetic trehalose dicorynomycolate.

[b] Two days before fusion with hybridomas, an Intravenous booster (IV) is given to the mice. All other immunisations are performed intradermal (ID).

One week before the fusion, PAI myeloma cells (obtained in-house from STPHI) were cultivated to approximately $1x10^8$ cells in myeloma cell medium (**Table 4.2**). All cells were combined with the same amount of spleen cells from sacrificed mouse (1:1 ratio) in a 50ml falcon tube with the addition of PEG 1500 (Roche, Basel,

Switzerland) over 1 minute. After 10 minutes incubation at room temperature the fused cells were harvested by centrifugation for 10 minutes 1500 rpm and resuspended in 150ml HAT medium. The cells were then distributed in 12x96 well plates (Costar) with 100µl of cell suspension in each well, and incubated for 10 days before screening for GMMA specific IgG producing hybridomas (positive) by ELISA coated with 2µg MenA NadR KO cps- or Triple KO OE fHbp GMMA. After the initial screening, positive clones were selected and re-cloned in order to obtain individual clones in each well. Re-cloning was done by adding 0.3 cell/well (limiting dilution) into 10ml HAT medium each and distributed in 96 well plates. The re-cloned hybridomas were subsequently screened for single positive clones by ELISA coated with 2µg MenA NadR KO cps- or Triple KO OE fHbp. Positive clones were transferred into a 24 well plate (Techno Plastic Products (TPP), Trasadigen, Switzerland). After screening of the clones in the 24 well plates, positive IgG producing clones were selected and transferred to 6 well plates (TPP). Subsequently, the clones were screened by Western blotting using 10µg MenA NadR KO cps- and Triple KO OE fHbp GMMA. Further testing involved screening of positive hybridomas growing in the 6 well plates with ELISA and Western blotting using rHis-NadA-3 or rHis-fHbp v.1 ID9. The hybridoma cells were isotyped to test if they were monoclonal. Positive cell lines were subsequently selected for production and grown in HAT medium in 150cm^2 vented flasks (TPP). The supernatant of the hybridoma cells that contain the mAbs were collected by centrifugation for 10 minutes at 1500rpm and purified as described below (4.2.4).

Table 4.2. Medium for cell cultivation.

	Myeloma medium	HAT medium
Iscove's modified Dulbecco's medium (IMDM) (Sigma-Aldrich)	500ml	500ml
FBS (foetal bovine serum) 10% (Sigma-Aldrich)	50ml	50ml
L-glutamin 200mM (Sigma-Aldrich)	5.5ml	5.5ml
2-mercaptoetanol 50mM 1000x (GIPCO, Invitrogen)	550µl	550µl
Penicillin/Streptomycin antibiotics 100µg/ml stock (Sigma-Aldrich)	-	5.5ml
H2062 HAT medium supplement 50x (Sigma-Aldrich)	-	Resuspend in 10ml

4.2.3 ELISA screening of sera and hybridoma supernatant

Screening and isotyping of sera from mice immunised with MenA NadR KO cps- and Triple KO OE fHbp GMMA and mAbs was performed as previously described in Chapter 3 section 3.3.7 page 126. 4HBX (NUNC-IMMUNOPLATE) plates were coated with 2µg of MenA NadR KO cps- and Triple KO OE fHbp GMMA or 5µg rHis-NadA-3 and rHis-fHbp v.1 ID9 overnight. Secondary antibodies used for the screening and IgG isotyping are listed in **Table 4.3**.

Table 4.3. Secondary antibodies for ELISA and for IgG isotyping.

Antibody	Dilution	Source
Goat anti-mouse γ (230)	1:20000	Sigma-Aldrich
Goat anti-mouse γ1 (2400)	1:3000	Southern Biotech (Birmingham, Alabama, U.S.A)
Goat anti mouse γ2a (209)	1:1000	Southern Biotech
Goat anti-mouse γ2b (2403)	1:1000	Southern Biotech
Goat anti-mouse γ3 (208)	1:1000	Southern Biotech
Goat anti-mouse µ (207a)	1:1000	Southern Biotech
Anti IgM (AP labelled) (2404)	1:1000	Southern Biotech

4.3.4 Monoclonal antibody purification and quantification

Antibody purification was performed following mAb production using affinity chromatography over a 5 ml HiTrap Staphylococcal rProtein A FF (for mouse IgG2 and IgG3 subclass antibody) or Streptococcal protein G column (IgG1) (GE Healthcare). Buffers used for the purifications were prepared as listen in **Table 4.4**. Before purification, the BioRad pump system was washed with 20% 50ml ethanol and 50ml 1xPBS (not filtered). For purification of IgG1 subclass, the filtered supernatant was premixed 1:1 with High salt binding buffer to promote binding of the mAbs to the column. The filtered supernatant was subsequently loaded with a flow rate of 3.5ml/min (amplitude sensitivity 1.0). Subsequently, 1xPBS was used to wash the system until the base level of absorption (OD) was reached. Elution buffer of pH 3.5 was used to elute IgG1 subclass and elution buffer pH 2.7 was used for IgG2 and IgG3. The elution-fraction was collected and the pH adjusted to 7.0 with Tris-HCl buffer. To remove excess antibody, the column was washed with elution buffer pH 2.2. The eluted fractions were dialysed using a slide-A-lyzer® dialysis cassette G2 10000 MWCO (Thermofisher Scientific) and 2L 1xPBS overnight at room temperature under stirring. Antibody concentration was measured using Pierce BCA protein assay kit (Thermofisher Scientific).

Table 4.4. Buffers used for monoclonal antibody purification.

All buffers were filtered through a sterile filter before purification. The chemicals were purchased from Sigma-Aldrich unless stated otherwise.

High salt buffer pH 8.9	Elution buffer pH 2.2	Elution buffer pH 2.7	Elution buffer pH 3.5	Collection buffer pH 9.0
113.4g Glycine (Merck)	495ml 0.1M Citric acid	3.75g Glycine	360ml 0.1M Citric acid	15.8g Tris HCl
175.32g NaCl (Merck)	5ml 0.2M Na_2HPO_4 (Merck)	4.38g NaCl	140ml 0.2M Na_2HPO_4	100ml dH_20
dH_20 1 liter		500ml dH_20		

4.3.5 Immunofluorescence (IFA)

Strains were cultured as previously described in Chapter 2 for Western blot analysis section 2.3.4 page 62 before diluting the cell suspension 1:100 in 1xPBS+1% BSA (Sigma-Aldrich; **Table 4.5**). 10µl of the cell suspension was added to each well on the 10 well diagnostic microscope slide (Thermofisher Scientific) and mixed with 30µl fixing solution (4% neutral buffered formalin; DiaPath, Martinengo, Italy). After 30 minutes incubation in a humid chamber at room temperature, the solution was removed and washed 5 times with 50µl 1xPBS. Following blocking of the wells with 50µl 1xPBS+1%BSA for 30 minutes, the blocking solution was removed and 30µl/ well of 1mg/ml mAb or sera from mice immunised with MenA NadR KO cps- were added and incubated in a humid chamber for 1 hour before washing 5 times with 50µl blocking buffer 1xPBS+1%BSA. Subsequently, 30µl of 1:400 diluted secondary antibody Alexa Fluor 568 donkey anti-mouse antibody (emission 578⁄603nm; Sigma-Aldrich) was added. After 1 hour, the wells were washed 5 times with 50µl 1xPBS, and mounting solution (Prolong Gold antifade reagent with DAPI staining, Invitrogen) was added to the slides before fitting with a glass slide cover.

Fluorescence microscopy was performed on a Leica DM-5000B using a X60 oil immersion objective lens and documented with a Leica DFC300FX digital camera system. Images were processed using the Leica Application Suite.

4.3.6 Flow cytometry

Strains were cultured as previously described in Chapter 2 for Western blot analysis section 2.3.4 page 62 (**Table 4.5**). 1ml of the culture was diluted with 5ml Dulbecco's phosphate buffered saline (DPBS; Sigma-Aldrich), centrifuged 5 minutes at 5000rpm, pellet resuspended in 1ml 4% formalin and incubated for 30 minutes at room temperature to inactivate the bacteria. After inactivation, the suspension was centrifuged for 5 minutes at 5000rpm and the pellet re-suspended with 5ml 0.5% BSA-DPBS (Sigma-Aldrich) and stored at 4°C for 30 minutes. 100µl of the cells were incubated with mAb at a concentration of 25mg/ml for 1 hour at room temperature before the cells were collected. Subsequently, 100µl of the secondary antibody Alexa Fluor 488-conjugated goat anti-mouse heavy and light chain IgGs specificity (emission 488/519nm; Invitrogen) at a 1:200 dilution was added for 1 hour at room temperature. After collection, the cell pellets were resuspended in 400µl FACS flow (Beckton Dickinson (BD)) and surface staining was detected by FACS calibur (BD) FL2-H channel and analysed using the software Cell quest pro version 5.2.1 (BD).

To ensure that the experiments were correctly performed, initially unlabelled cells were run through the flow cytometer. Following this the FS and SS parameter voltages were adjusted to enable appropriate gating of cells so at least 30000 events were recorded within the gated region. To ensure consistency of the data, each isolate preparation was labelled with secondary antibody alone, to ensure there was no

staining of the cells with the dye. The two controls were also used to ensure the reading was placed in the first decade for each experiment. The same protocol and controls was used for all experiments in this study ensuring intra-experimental consistency. The data were plotted in a one-dimensional histogram format.

4.3.7 Bactericidal assay

The assay was performed as previously described for SBA using human complement in Chapter 2 section 2.3.5 page 63 and baby rabbit complement in Chapter 3 section 3.3.8 page 126. Isolates used are listed in **Table 4.5**. The source of complement was human serum from a healthy individual with no detectable bactericidal activity against the test strains. The serum was depleted of IgG over a 5 ml HiTrap Staphylococcal rProtein G FF column and displaced with equal amounts of 1xPBS, and filter sterilised. Antibody depletion was confirmed using Pierce BCA protein assay kit (Thermofisher Scientific). Total haemolytic activity before and after depletion of IgG was measured (Binding Site, Birmingham, U.K.).

Table 4.5. Panel of isolates selected for IFA, flow cytometry and serum bactericidal activity analysis.

Serogroup	Isolate	Origin	Year of isolation	*fHbp* variant	*fHbp* ID	*porA* VRs	*nadA* allele	NadA expression (%)	Sequence type (ST)
A	MK804/ 03*§	Ethiopia	2003	1	5	P1.20,9	3	29.1	7
A	N2602*§	Nouna, Burkina Faso	2007	1	5	P1.20,9	3	100.0	2859
W	Ghana 7/04	Ghana	2004	2	23	P1.5,2	3	57.6	11
W	Mali 10/09*§	Mali	2009	2	23	P1.5,2	2	100.0	11
X	MRS200 8223*§	Burkina Faso	2007	1	74	P1.5-1,10-1	3	27.0	181

* Strains used in IFA analysis.

§ Strains used in flow cytometry analysis.

4.4 Results

4.4.1 Selecting mice suitable for fusion based on the nature of their immune response

In this study, three fusions were performed to generate mAb against natively expressed NadA, fHbp and other OMP in GMMA. We also included two control groups: rHis-NadA given with CpG or SIGMA adjuvant. These two control groups were used to compare the immune responses elicited against recombinant NadA and that towards GMMA. The mice were bled 14 days after the third immunisation, and the sera were characterised by ELISA and SBA to determine the mouse with the highest antibody titres to GMMA (MenA NadR KO cps-, Triple KO OE fHbp) and recombinant protein (rHis-NadA-3, rHis-fHbp ID9). We also characterised the sera by Western blotting to determine if a band was seen for the corresponding vaccine antigens fHbp or NadA. With these tests, we also wanted to understand the effect of adjuvant on the immune responses induced to GMMA alone and GMMA formulated with Alum in terms of Western blotting profile and titres obtained in an ELISA and SBA measurement.

ELISA analysis showed that the MenA NadR KO cps- GMMA preparation not absorbed to Alum elicited higher specific antibody titres (not significant) compared to the respective Alum absorbed group when the ELISA plate was coated with this vaccine (**Figure 4.2, Panel B**). On the contrary, when the plate was coated with rHis-NadA-3, the mice immunised with rHis-NadA-3+CpG and rHis-NadA-3+SIGMA generated the highest titres most likely due to dosage effect (**Figure 4.2, Panel A**). These two adjuvants are known to induce high antibody titres in mice when measured

in an ELISA compared to recombinant proteins absorbed on Alum (14). When anti-NadA antibody responses were measured, MenA NadR KO cps- GMMA alone elicited higher titres (not significant) than MenA NadR KO cps-+Alum (**Panel B**). ELISA analysis of the sera generated from all three mice immunised with Triple KO OE fHbp v.1 ID9 absorbed to Alum showed that all of the mice had similar antibody titres (**Figure 4.3, Panel A** and **B**).

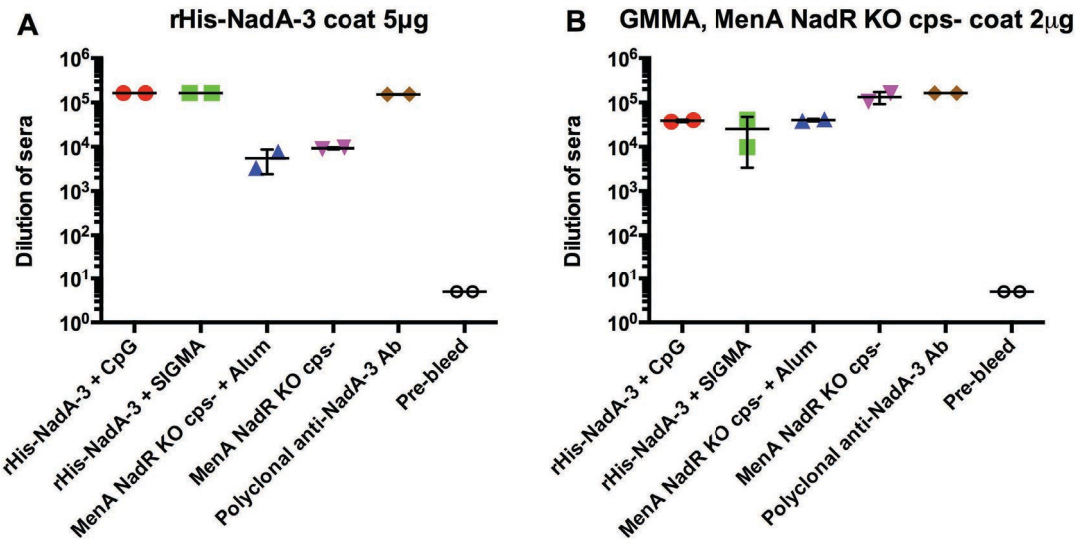

Figure 4.2. ELISA analysis of sera from mice immunised with rHis-NadA-3 formulated with CpG or SIGMA adjuvant and MenA NadR KO cps- GMMA absorbed to Alum or without.

ELISA plates were coated with 5µg of rHis-NadA-3 (Panel A) and 2µg of MenA NadR KO cps- GMMA (Panel B). Pre-bleed serum was used as negative control and polyclonal rHis-NadA-3 serum (NV&D) as positive control. Each symbol represents a mouse serum sample. GMMA used for immunisation: MenA NadR KO cps- and MenW NadR KO cps- (*nadR*, capsule, *lpxL1* and *gna33* KO); recombinant Hexa-Histidine tagged rNadA allele 3. Error bars represent standard error. Statistical analysis was done using the Mann-Whitney *U* test.

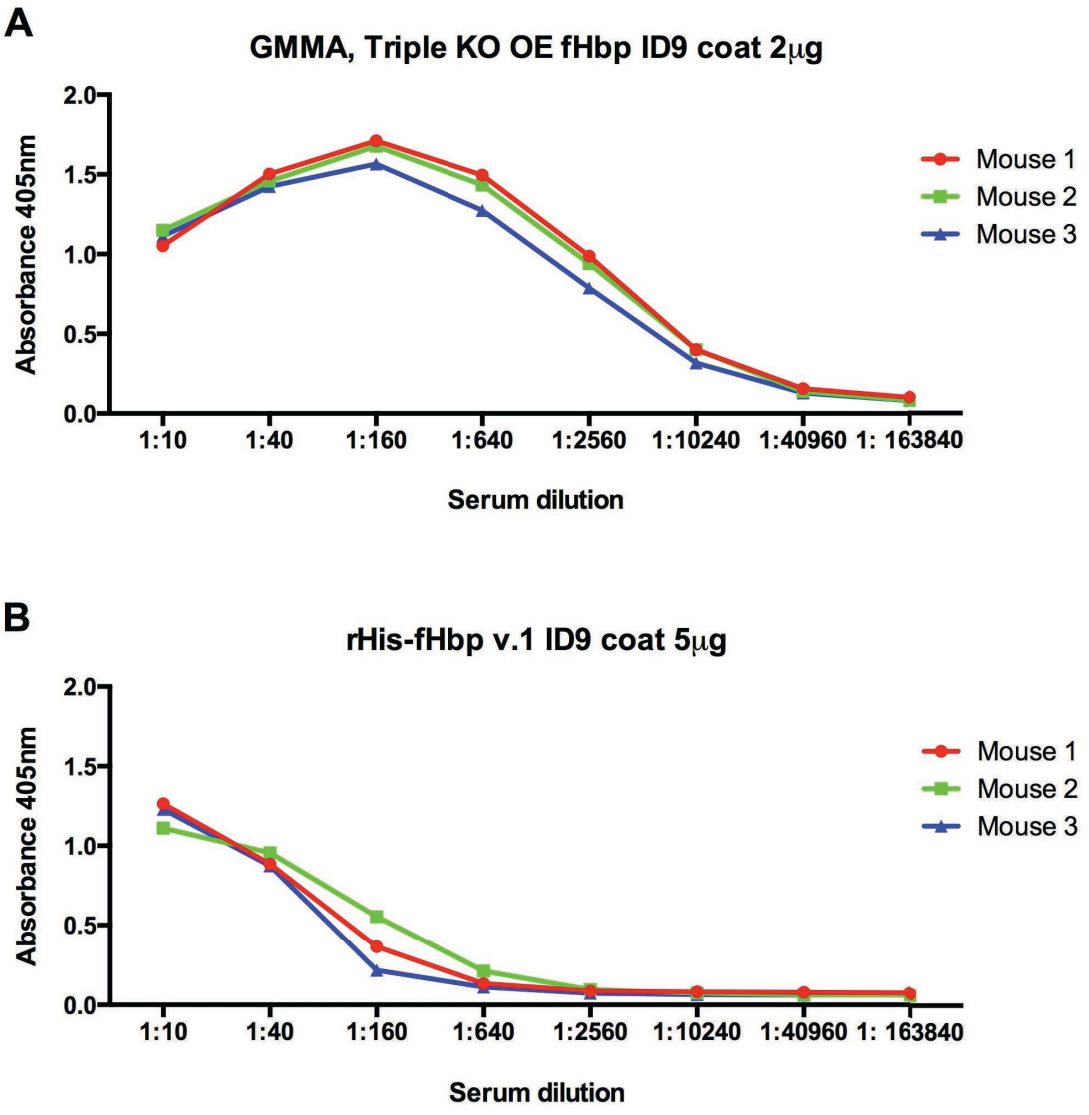

Figure 4.3. ELISA analysis of sera from mice immunised with Triple KO OE fHbp variant 1 ID9 R41S GMMA absorbed to Alum.

ELISA plates were coated with 2μg of Triple KO OE fHbp GMMA (Panel A) or 5μg of rHis-fHbp v.1 ID9 (Panel B). Each symbol represents one mouse labelled 1, 2 and 3. GMMA used for immunisation: Triple KO OE fHbp (capsule, *lpxL1* and *gna33* KO, over-expressed fHbp variant 1 ID9). Error bars represent standard error.

To further investigate the Th antibody responses generated by the vaccine groups, IgG isotyping was performed on mouse serum. Antibodies of subclass IgG2a were most predominant in mice immunised with rHis-NadA-3+CpG (**Figure 4.4, Panel A**) while the vaccine group rHis-NadA-3+SIGMA generated mostly IgG1 subclass antibodies (**Panel B**). This has previously been reported for these adjuvants (16,17). In mice immunised with GMMA MenA NadR KO cps-+ Alum, antibodies of IgG2b and IgG1 subclass were mostly predominant (**Panel C**), while mice immunised with MenA NadR KO cps-GMMA elicited antibodies of subclass IgG2b, IgG2a and IgG3 (**Panel D**).

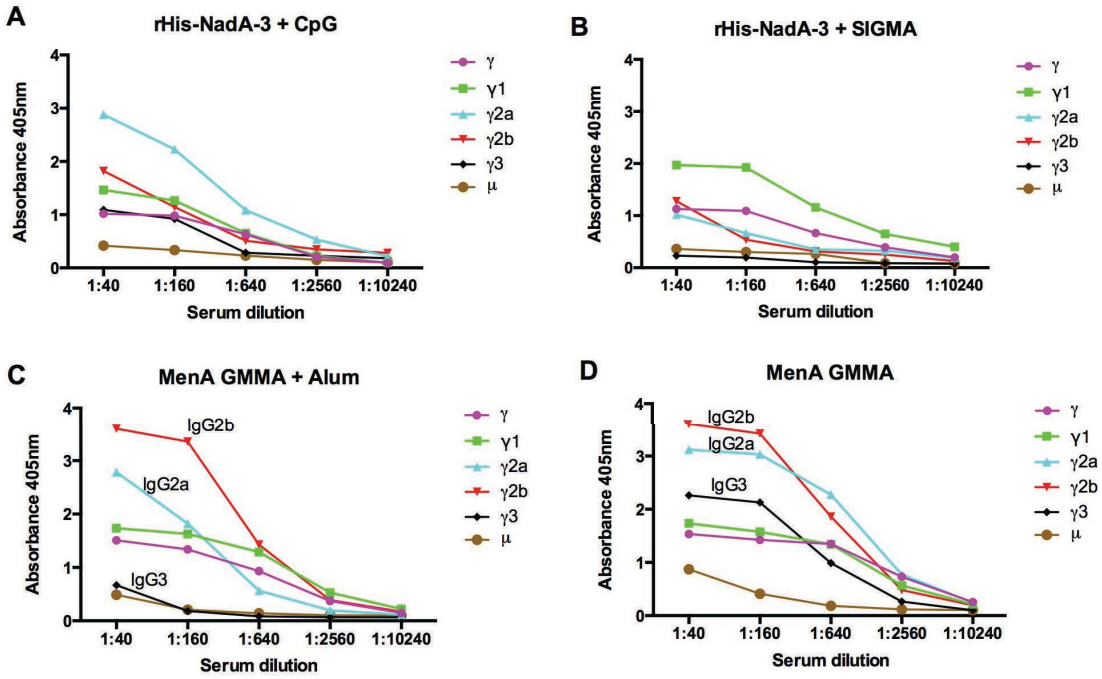

Figure 4.4. IgG isotyping of mouse sera from mice immunised with rHis-NadA-3 formulated with CpG and SIGMA adjuvant and MenA NadR KO cps- GMMA formulated with Alum and without.

The ELISA plates were coated with 4μg of MenA NadR KO cps- GMMA.

Western blotting analysis of the individual mouse sera from mice immunised with MenA NadR KO cps- formulated with Alum and without generated two different immune response profiles in terms of the bands visualised. Strongest bands for the MenA NadR KO cps- + Alum group were around 38-62kDa, while for the MenA NadR KO cps- alone bands at 170kDa were most prominent. A common band for all vaccine groups was observed at around 170kDa that represents oligomeric NadA (**Figure 4.5**). In comparison, Western blot analysis of the sera of mice immunised with Triple KO OE fHbp revealed a single strong band at 28kDa both in the blot with GMMA (**Figure 4.6, Panel A**) and the blot with recombinant fHbp (**Panel B**). This band represents fHbp as the polyclonal anti-fHbp antibodies showed a band at this size. The mouse number 2 showed the strongest band for the recombinant fHbp blot (Panel B). Therefore, this mouse was selected for fusion, which is currently under progress. Western blot results show that immune responses elicited by the Triple KO OE fHbp GMMA were mostly towards fHbp, while the MenA NadR KO cps- GMMA elicited a wider immune response to NadA and other antigens.

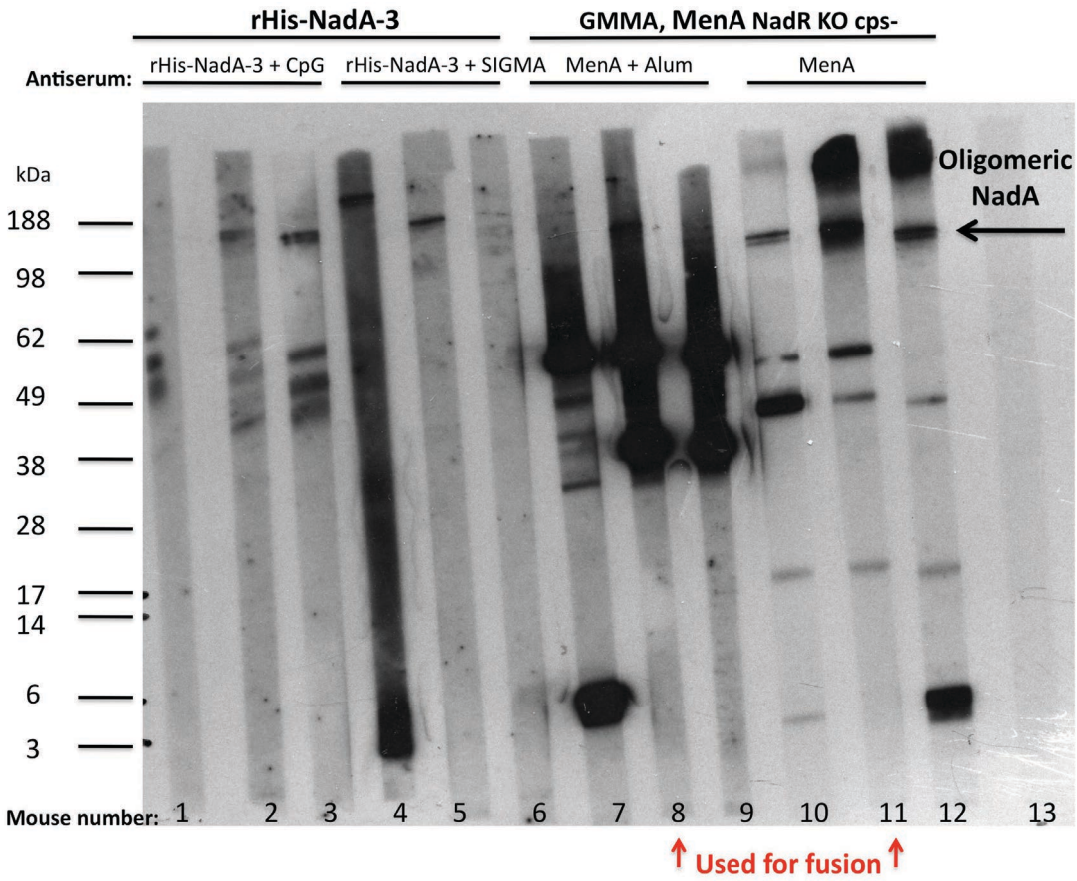

Figure 4.5. Western blot analysis of sera from mice immunised with rHis-NadA-3 formulated with CpG or SIGMA adjuvant and MenA NadR KO cps- GMMA absorbed to Alum (MenA + Alum) or without (MenA).

The blot was prepared with 5µg of MenA NadR KO cps- GMMA. Each strip 1-13 represent serum from individual mice [mouse 1 rHis-NadA-3 + CpG group (strip 1), mouse 2 rHis-NadA-3 + CpG2 (strip 2), rHis-NadA-3 + CpG3 (3), rHis-NadA-3 + SIGMA1 (4), rHis-NadA-3 + SIGMA2 (5), rHis-NadA-3 + SIGMA3 (6), MenA NadR KO cps- + Alum1 (7), MenA NadR KO cps- + Alum2 (8), MenA NadR KO cps- + Alum3 (9), MenA NadR KO cps- 1 (10), MenA NadR KO cps- 2 (11), MenA NadR KO cps- 3 (12) and pre-bleed (13)]. Sera were diluted 1:100 in 1% milk + 1xPBS.

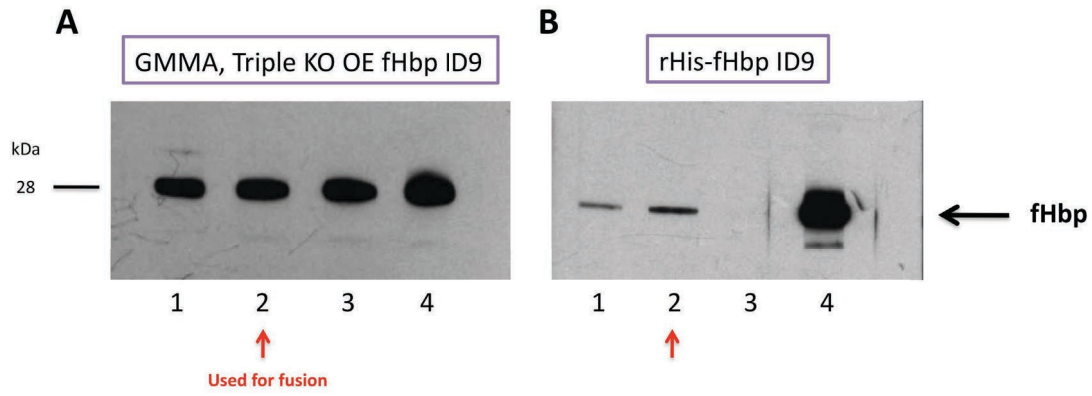

Figure 4.6. Western blot analysis of sera from mice immunised with Triple KO OE fHbp ID9 R41S GMMA absorbed to Alum.

The blots were prepared with 10µg of Triple KO OE fHbp ID9 R41S GMMA and 10µg of rHis-fHbp ID9. Each number represents mouse 1, 2 and 3. Lane 4 for panel A, poly-GMMA OE fHbp v.1 was added while for panel B poly rHis-fHbp v.1 was added. Sera were diluted 1:100 in 1% milk + 1xPBS. Blots were exposed for 5 minutes.

The SBA responses of the antisera of mice immunised with MenA NadR KO cps- and rHis-NadA3 was assessed against the serogroup W isolate Ghana7/04 with heterologous PorA P1.5,2 and fHbp v.2 ID23, grown with and without the presence of 4HPA. 4HPA is an aromatic metabolite in the human saliva that relieves NadR repression of NadA expression. Using 4HPA in the growth medium simulates *in vitro* the niche signal for meningococci present in the nasopharynx. GMMA from the MenA NadR KO cps- recombinant strain induced bactericidal antibodies that were able to kill the serogroup W isolate expressing intermediate amounts of NadA (geometric mean titre [GMT]=4008) (**Figure 4.7, Panel A**). Lower titres, but not significant, were seen for the MenA NadR KO cps- GMMA group formulated with Alum (GMT=391). When 4HPA was added to increase NadA expression from 58% to 82%, again the MenA NadR KO cps- GMMA group elicited the highest bactericidal titres (GMT=121874) compared to the MenA NadR KO cps-+Alum

group (GMT=76528) (**Figure 4.7, Panel B**). From these results, mouse number two of both MenA NadR KO cps- alone and MenA NadR KO cps-+Alum group were selected for fusion as they showed highest bactericidal titres in each group (number 8 and 11 in **Figure 4.5**).

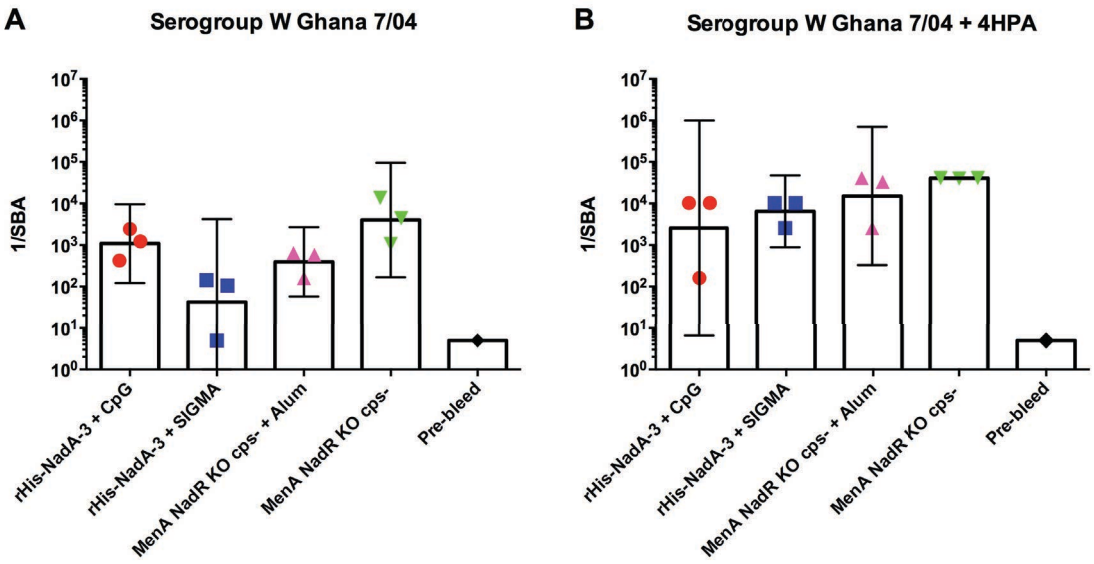

Figure 4.7. Complement-mediated bactericidal activity of sera from mice immunised with rHis-NadA-3 formulated with CpG or SIGMA adjuvant and MenA NadR KO cps- GMMA absorbed to Alum or without.

Each symbol represents reciprocal serum titre (50% killing of bacteria) of serum from a mouse using baby rabbit complement. Panel A: isolate Ghana7/04 grown without 4HPA; Panel B: isolate Ghana7/04 grown in the presence of 4HPA. The serum samples used in the analysis were obtained 2 weeks after the third vaccine dose. The line indicates the GMT of each vaccine group. Error bars indicate ±95% confidence interval of the GMT. GMMA used for immunisation: MenA NadR KO cps- (*nadR*, capsule, *lpxL1* and *gna33* KO); recombinant Hexa-Histidine tagged rNadA allele 3. Statistical analysis was done using the Mann-Whitney *U* test.

4.4.2 Summary of mAbs generated from fusion with NadA and fHbp GMMA

We obtained 25 hybridoma clones producing GMMA-binding mAbs from fusions 1 and 2 of mice immunised with MenA NadR KO cps- GMMA formulated with Alum and without Alum. From fusion 1 and 2, 4 mAbs were specific for NadA and they were of the immunoglobulin subclasses IgG1, IgG2a/b and IgG3 (3 from fusion 1). The other 21 mAbs were specific for seven other unknown surface exposed antigens on GMMA (2 from fusion 1 of IgG1 and IgG2b subclass (**Table 4.6** [fusion 1], **Table 4.7** [fusion 2]). These antigens were numbered according to the number of bands seen in the Western blot analysis with MenA NadR KO cps- GMMA (**Figure 4.8**) starting from the top (1:NadA) to the last band at 3 kDa (10). From fusion 3 with Triple KO OE fHbp ID9 R41S GMMA, we obtained eight hybridoma clones, of which 4 were specific for fHbp of IgG subclass IgG1 as determined by ELISA and Western blotting (**Table 4.8**). The other four mAbs of the IgG2a subclass were specific for two other GMMA antigens. The results suggest that GMMA alone generates a wider immune response and antibody repertoire compared to GMMA formulated on Alum.

Table 4.6. Fusion 1 monoclonal antibodies generated from a mouse immunised with MenA NadR KO cps- GMMA formulated with Alum.

Molecular weights are given as approximations from Western blot results.

Monoclonal antibody	Protein number	Molecular weight (kDa)	IgG isotype
3B10/F8	1: NadA	170	IgG3
5A5/F3	1: NadA	170	IgG2b
11H6/E9	1: NadA	170	IgG1
1D11/C3	4	45	IgG1
1G10/D6	5	38	IgG2b

Table 4.7. Fusion 2 monoclonal antibodies generated from a mouse immunised with MenA NadR KO cps- GMMA.

The vaccine was not formulated with Alum. Molecular weights are given as approximations from Western blot results.

Monoclonal antibody	Protein number	Molecular weight (kDa)	IgG isotype
1F12/F6	1: NadA	170	IgG2a
10F9/H9	2	100	IgG2b
10A6/B7	3	49	IgG2a
6H9/F3	4	45	IgG1
6H9/D6	4	45	IgG1
6H9/D6	4	45	IgG1
7F11/F2	4	45	IgG2a
2C8/F6	5	38	IgG2b
2F12/A1	5	38	IgG2a
4G1/D3	5	38	IgG2b
4H5/F6	5	38	IgG2b
8D3/D1	5	38	IgG2a
5H4/G7	5	38	IgG1
6G9/G10	5	38	IgG3
4H5/F9	5	38	IgG2b
10E2/E7	5	38	IgG3
4G1/A7	8	8	IgG2b
6H9/C2	8	8	IgG1
3E7/D7	9	6	IgG2a
2B9/F4	9	6	IgG2a
4H5/D2	10	3	IgG2b

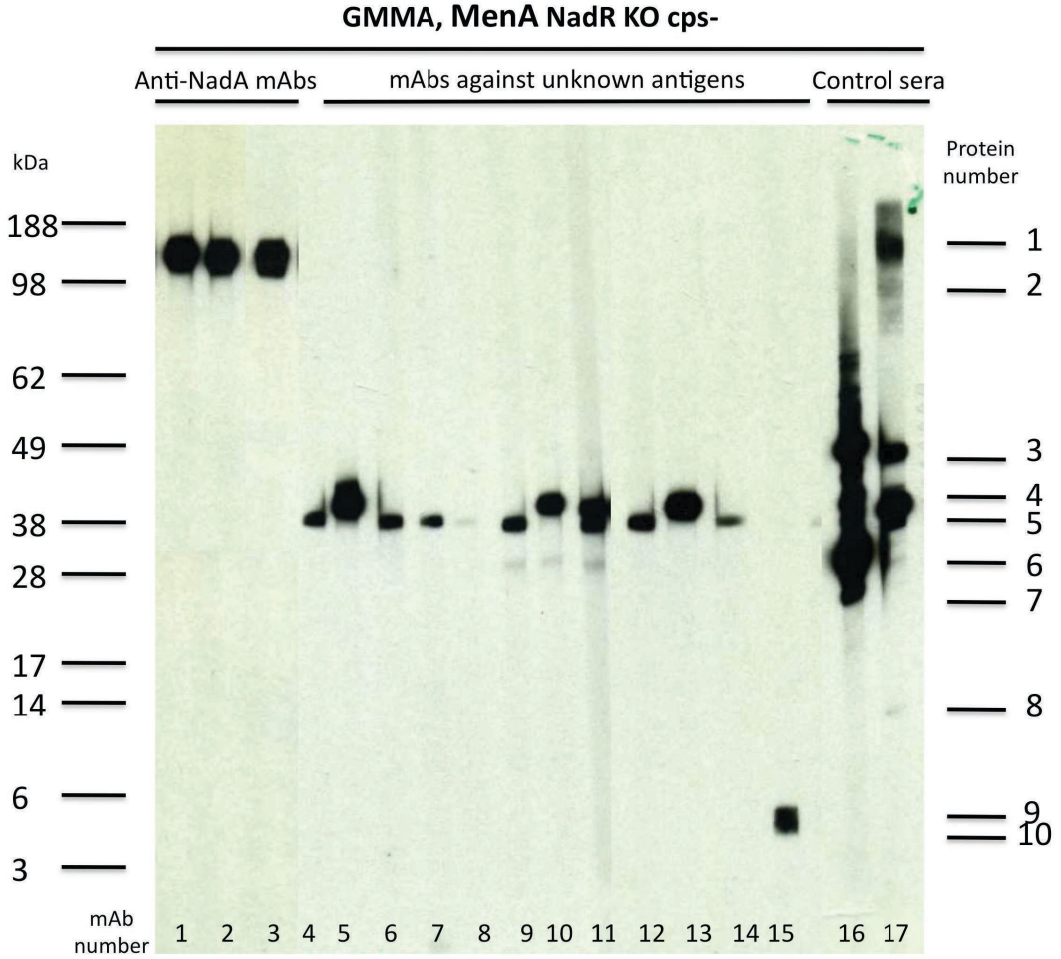

Figure 4.8. Representative Western blot analysis of monoclonal antibodies generated from fusion 1 and 2 of splenocytes from mice immunised with MenA NadR KO cps- + Alum and MenA NadR KO cps- GMMA, respectively.

The blots were prepared with 10µg of MenA NadR KO cps-. 1µg/ml mAbs were added to 1% milk + 1xPBS. Exposure of the blot was for 5 minutes. Each strip 1-17 represents a mAb or serum from individual mice [3B10/F8 (strip 1), 5A5/F3 (2), 11H6/E9 (3), 1G10/D6 (4), 1D11/C3 (5), 2C8/F6 (6), 2F12/A1 (7), 4G1/D3 (8), 4H5/F6 (9), 6H9/F3 (10), 7F11/F2 (11), 8D3/D1 (12), 6H9/D6 (13), 5H4/G7 (14), 2B9/F4 (15), MenA NadR KO cps- + Alum serum (16), MenA NadR KO cps- serum (17)].

Table 4.8. Fusion 3 monoclonal antibodies generated from a mouse immunised with Triple KO OE fHbp v.1 ID9 R41S GMMA.

Monoclonal antibody	Protein number	Molecular weight (kDa)	IgG isotype
9F1/A3	1	38	IgG2a
10D3/H11	1	38	IgG1
9G5/E5	1	38	IgG2a
9H4/F3	2: fHbp	28	IgG1
9H5/H5	2: fHbp	28	IgG1
9F7/B11	2: fHbp	28	IgG1
11C6/C2	2: fHbp	28	IgG1
9A1/A3	3	17	IgG2a

4.4.3 Binding specificity of mAbs from fusion 1 and 2 of mice immunised with MenA NadR KO cps- GMMA

From fusion 1, the three anti-NadA mAbs of IgG1, IgG2b and IgG3 subclass showed broad reactivity with differences in binding in Western blotting analyses using a panel of 44 *N. meningitidis* serogroup A (n=13), W (n=20) and X (n=11) strains (**Table 4.9, Figure 4.9**). Most isolates were of NadA-3 except the sergroup W isolate Mali 10/09, which expresses NadA-2. The isolates exhibited varying NadA expression from low to high as determined in Chapter 3 **Figure 3.3**. The 3B10/F8 mAb of IgG3 subclass showed specific binding to two serogroup A and four W isolates while the other two anti-NadA mAbs both reacted with 87 % of A strains and 65% of W strains. None of the anti NadA mAbs reacted with serogroup X strains that expressed NadA-3. The mAbs against protein 4 or 5 only bound to whole cell lysates prepared from the vaccine serogroup A strain N2602.

Table 4.9. Specificity of monoclonal antibodies generated from fusion 1 from a mouse immunised with MenA NadR KO cps- +Alum GMMA.

Summary of Western blotting results with whole cell lysates. Preparation of whole cell lysates was done as previously described in Chapter 2 Materials and Methods section 2.3.4.

Monoclonal antibody	Protein number	IgG isotype	Serogroup A (n=15)	Serogroup W (n=20)	Serogroup X (n=11)
3B10/F8	1: NadA	IgG3	2 (13%)	4 (20%)	-
5A5/F3	1: NadA	IgG2b	13 (87%)	13 (65%)	-
11H6/E9	1: NadA	IgG1	13 (87%)	13 (65%)	-
1D11/C3	4	IgG1	1 (6%)	-	-
1G10/D6	5	IgG2b	1 (6%)	-	-

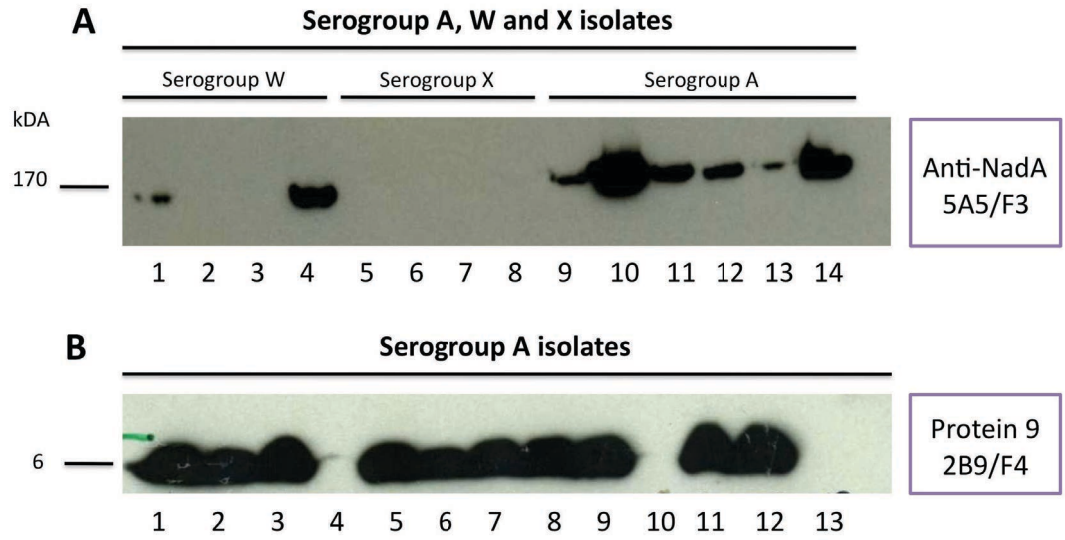

Figure 4.9. Representative Western blot of mAbs 5A5/F3 and 2B9/F4 generated from fusion 1 and 2 of mice immunised with MenA NadR KO cps- + Alum and MenA NadR KO cps- GMMA, respectively, against African serogroup A, W and X isolates.

The blots were prepared with 20µg of whole cell lysate per lane. 1µg/ml mAbs were added to 1% milk + 1xPBS. Exposure of the blot was for 5 minutes. Panel A: anti-NadA 5A5/F3 mAb produced from a spleen of the mouse immunised with MenA NadR KO cps- + Alum against serogroup W [Ghana 7/04 (1), BF10/02 (2), Nigeria1/03 (3), Mali 10/09 (4)], X [MRS2006078 (5), MRS2006087 (6), Uganda11/07 (7), MRS2008223 (8)] and A [Su14/07 (9), Mali21/07 (10), BF6/06 (11), BuFa20030020 (12), Mali21/10 (13), N2602 (14)] isolates. Panel B: anti-protein 9 2B9/F4 mAb produced from the spleen of a mouse immunised with MenA NadR KO cps- against serogroup A [BF8/01 (1), MK804/03

(2), BF3/06 (3), BF6/06 (4), BuFa20030020 (5), Mali21/07 (6), Su14/07 (7), MRS2007446 (8), MRS2008162 (9), Niga16/09 (10), BF1/10 (11), Tch44/10 (12), Mali21/10 (13)] isolates.

From fusion 2 the anti-NadA mAb 1F12/F6 of IgG2a subclass showed reactivity only against serogroup A strains (**Table 4.10**). The other mAbs directed against unknown antigens also showed binding specificity to serogroup A isolates only. The mAbs 2F12/A1 (IgG2a, protein 5), 4H5/F9 (IgG2b, protein 5), 4G1/A7 (IgG2b, protein 8) and 4H5/D2 (IgG2b, protein 10) did not bind to any whole cell lysates, which could suggests that the mAbs are very specific or the target conformational epitopes may have been destroyed upon preparation of the lysates with SDS-PAGE. The mAbs 4G1/A7 and 4H5/D2 did show surface binding by IFA analysis (**Figure 4.10, Panel B**). Future analysis of their binding to live bacteria by flow cytometry was performed to confirm the hypothesis (section 4.3.4). Moreover, Western blotting as a technique may not be sensitive enough for certain mAbs targeting unknown antigens if the expression level of these proteins is too low.

Table 4.10. Specificity of monoclonal antibodies generated from fusion 2 from a mouse immunised with MenA NadR KO cps- GMMA.

Monoclonal antibody	Protein number	IgG isotype	Serogroup A (n=15)	Serogroup W (n=20)	Serogroup X (n=11)
1F12/F6	1: NadA	IgG2a	11 (73%)	-	-
10F9/H9	2	IgG2b	1 (6%)	-	-
10A6/B7	3	IgG2a	13 (87%)	-	-
6H9/F3	4	IgG1	12 (80%)	-	-
6H9/D6	4	IgG1	12 (80%)	-	-
7F11/F2	4	IgG2a	13 (87%)	-	-
2C8/F6	5	IgG2b	13 (87%)	-	-
2F12/A1	5	IgG2a	-	-	-
4G1/D3	5	IgG2b	13 (87%)	-	-
4H5/F6	5	IgG2b	1 (6%)	-	-
8D3/D1	5	IgG2a	1 (6%)	-	-
5H4/G7	5	IgG1	6 (40%)	-	-
6G9/G10	5	IgG3	7 (47%)	-	-
4H5/F9	5	IgG2b	-	-	-
10E2/E7	5	IgG3	13 (87%)	-	-
4G1/A7	8	IgG2b	-	-	-
6H9/C2	8	IgG1	12 (80%)	-	-
3E7/D7	9	IgG2a	12 (80%)	-	-
2B9/F4	9	IgG2a	10 (67%)	-	-
4H5/D2	10	IgG2b	-	-	-

4.4.4 Immunofluorescence assay binding of monoclonal antibody to panel of African serogroup A, W and X isolates

To determine whether the mAbs generated from fusion 1 and 2 bind to whole bacteria, we analysed their immunofluorescence assay (IFA) binding to a set of meningococcal serogroup A (n=1), W (n=2) and X (n=1) strains from Africa. All anti-NadA mAbs showed surface binding to vaccine serogroup A N2602, expressing NadA-3 (**Figure 4.10, Panel A**) and serogroup W strain Mali10/09 expressing NadA-2, suggesting that the mAbs are cross-reactive between NadA encoded by these alleles (**Figure 4.11, Panel A**). However, the anti-NadA mAbs did not bind to the serogroup X isolate MRS2008223, which expresses NadA-3 (**Figure 4.11, Panel B**). These

results have two possible explanations; firstly MRS2008223 is a low expresser of NadA (27%) compared to N2602 and Mali10/09, which are both high expressers (both 100%). Therefore, the amount of NadA on the surface of MRS2008223 might not be sufficient to generate fluorescence due to the lack of sensitivity of IFA. Secondly, the anti-NadA mAbs may be directed to specific epitopes shared by only some subtypes of NadA-3 and not present in the serogroup X isolate. The mAbs 1D11/C3 (IgG1, protein 4), 4H5/F6 (IgG2b, protein 5), 4G1/A7 (IgG2b, protein 8), 2B9/F4 (IgG2a, protein 9), 4H5/D2 (IgG2b, protein 10) targeting unknown antigens also showed surface binding against vaccine serogroup A N2602 strain, highlighting that these proteins are surface located and distributed across the bacterial surface (**Figure 4.10, Panel B**).

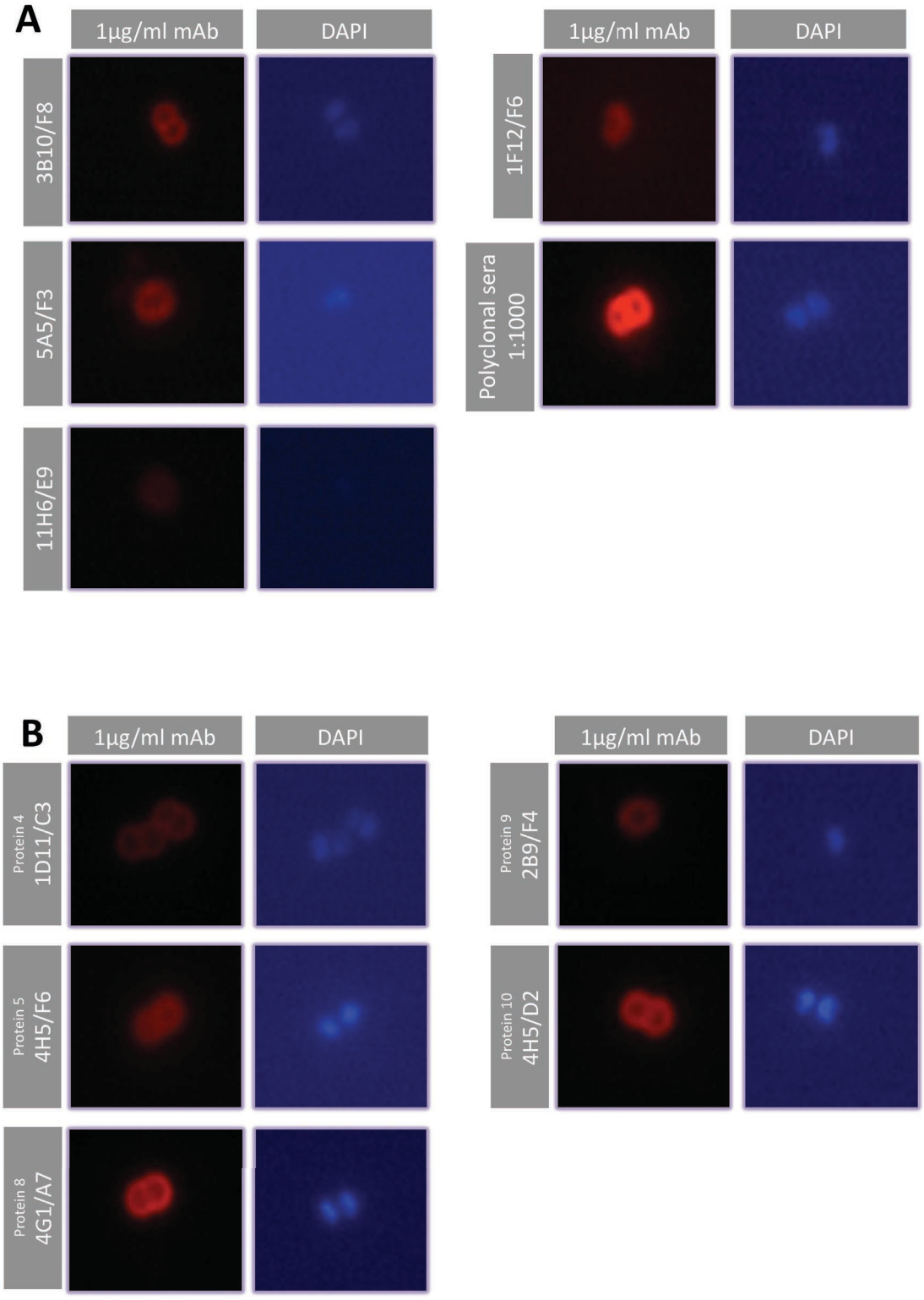

Figure 4.10. Immunofluorescence staining of formalin fixed serogroup A N2602 bacteria with anti-NadA mAbs and mAbs against protein 4, 5, 8, 9 and 10.

Panel A: binding of anti-NadA mAbs against NadA-3 on sergogoup A isolate N2602 expressing fHbp v.1 ID5, PorA P1.20,9; Panel B: binding of mAbs against unknown proteins on N2602. Polyclonal

serum used was from mice immunised with MenA NadR KO cps- GMMA. Exposure times were identical for all pictures of the same channel. Nuclei were stained with DAPI (blue). Original magnification ×1008.

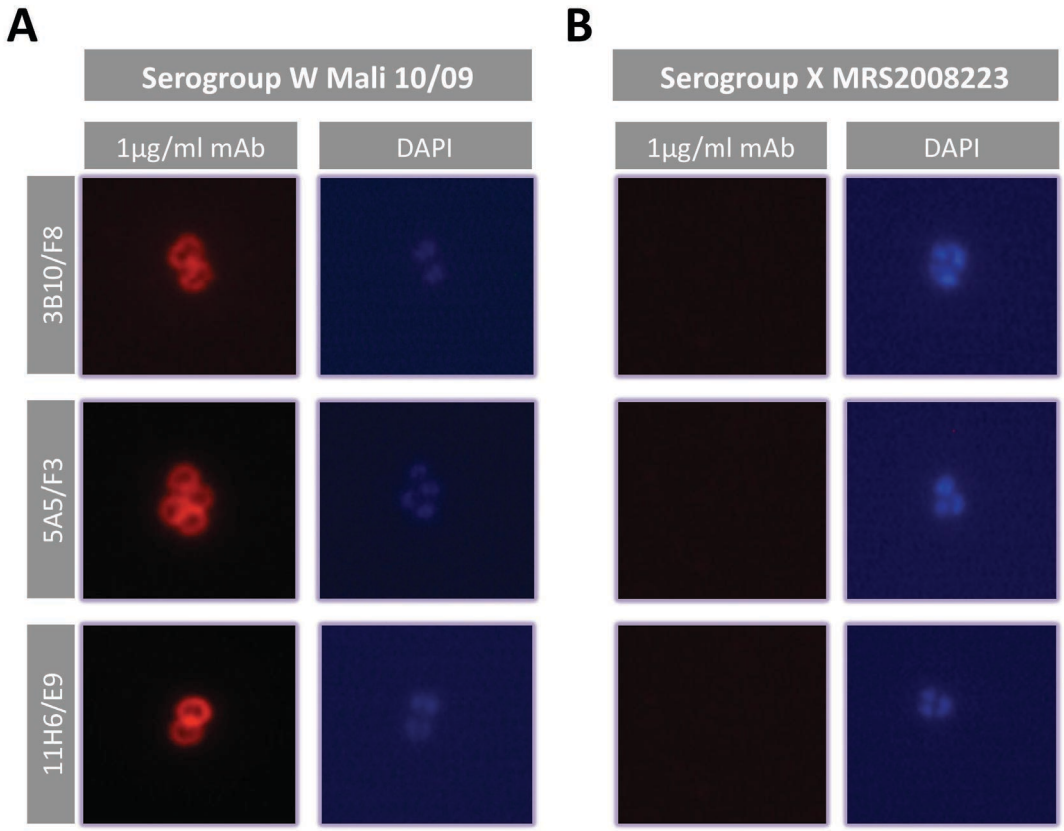

Figure 4.11. Immunofluorescence staining of formalin fixed serogroup W Mali10/09 and X MRS2008223 bacteria with anti-NadA mAbs.

Panel A: binding of anti-NadA mAbs against NadA-3 on sergogoup W isolate Mali10/09 expressing NadA-2, fHbp v.2 ID23, PorA P1.5,2; Panel B: binding of mAbs against serogroup W MRS2008223 expressing NadA-3, fHbp v.1 ID74, PorA P1.5-1,10-1. Exposure times were identical for all pictures of the same channel. Nuclei were stained with DAPI (blue). Original magnification ×1008.

4.4.5 Flow cytometric assessment of monoclonal antibody binding to African *N. meningitidis* serogroup A, W and X isolates

To investigate whether the anti-NadA mAbs generated bind to live bacteria, we stained two serogroup A, one W and one X isolates with all mAbs from fusion 1 and 2 and analysed the population of cells positively or negatively stained by FACS. Anti-NadA mAbs of subclass 5A5/F3, 11H6/E9 and 1F12/F6 showed better binding to the vaccine strain serogroup A N2602 and serogroup W isolate Mali10/09 than 3B10/F8 (**Figure 4.12, Panel A and C**). MK804/03 is, like the serogroup X isolate MRS2008223, a low expresser of NadA, which may be one reason for decreased binding (**Panel B**). None of the anti-NadA mAbs showed binding to serogroup X MRS2008223 (**Panel D**). Only mAb 1F12/F6 stained the second serogroup A isolate tested MK804/03 (**Panel B**). The results suggest that the ability of the different anti-NadA mAbs are dependent upon the NadA expression level on the bacterial surface, and the epitopes required for binding.

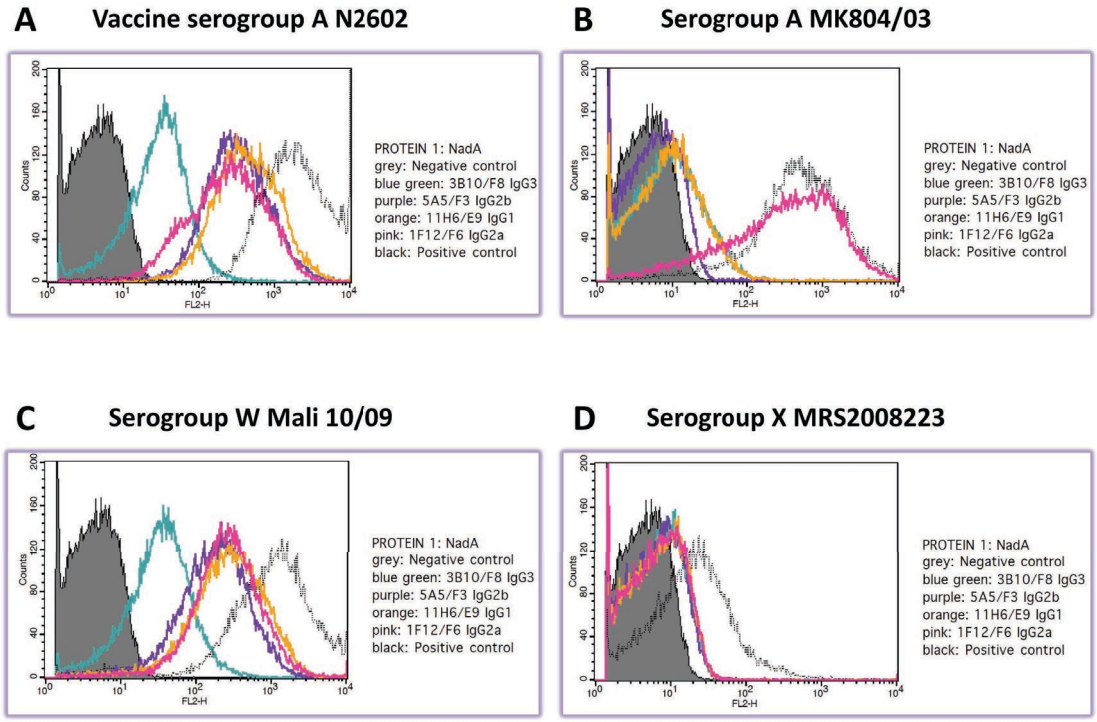

Figure 4.12. Histogram plots of serogroup A, W and X isolates stained with anti-NadA mAbs.

Alexa Fluor 488 secondary antibody was used to label the cells. Gating was set at 30000 events.

To better understand the properties of protein 4, we stained bacterial cells with 4 mAbs (3 from fusion 2 [GMMA]) directed against this protein. All mAbs were able to bind serogroup A isolates N2602 and MK804/03 (**Figure 4.13, Panel A** and **B**). No binding was observed for the serogroup W isolate Mali10/09 and serogroup X MRS2008223. Further flow cytometry analysis was performed with 10 mAbs (4 from fusion 2 [GMMA alone]) directed against protein 5. MAbs 8D3/D1, 4H5/F6, 1G10/D6 (fusion 1 GMMA+Alum) were able to bind to N2602 and MK804/03 serogroup A isolates (**Figure 4.14, Panel A** and **B**). In comparison, both 5H4/G7 and 6G9/G10 mAbs from fusion 2 had lower ability to bind both serogroup A isolates. Likewise, the anti-protein 5 mAbs 2C8/F6, 2F12/A1, 4G1/D3 and 4H5/G9 bound N2602 and MK804 except 10E2/E7 (**Figure 4.15, Panel A** and **B**). The mAbs directed against protein 2, 3 and 10 were able to bind the serogroup A isolates

(**Figure 4.16, Panel A** and **B**), and only one mAb 4H5/D2 stained the serogroup W cells (**Panel C**). MAbs against protein 8 bound both serogroup A isolates, and mAb 4G1/A7 was also able to bind serogroup W Mali10/09 (**Figure 17, Panel A-C**). Overall, only mAbs 4G1/A7, 4H5/D2, 2F12/A1, 2C8/F6, 4H5/G9, 4H5/F6, 8D3/D1, 2B9/F4, 3E7/D7 directed against unknown surface antigens showed binding to the serogroup W isolate. The anti-protein 9 mAb 2B9/F4 was the only mAb that bound all isolates (**Figure 4.18, Panel A-D**). None of the other mAbs tested that were directed at non-NadA antigens bound to the serogroup X isolate tested, suggesting that either the genetic diversity of these proteins affects cross-reactivity or the expression level of them in the X isolate is not sufficient to stain cells to detectable levels by flow cytometry.

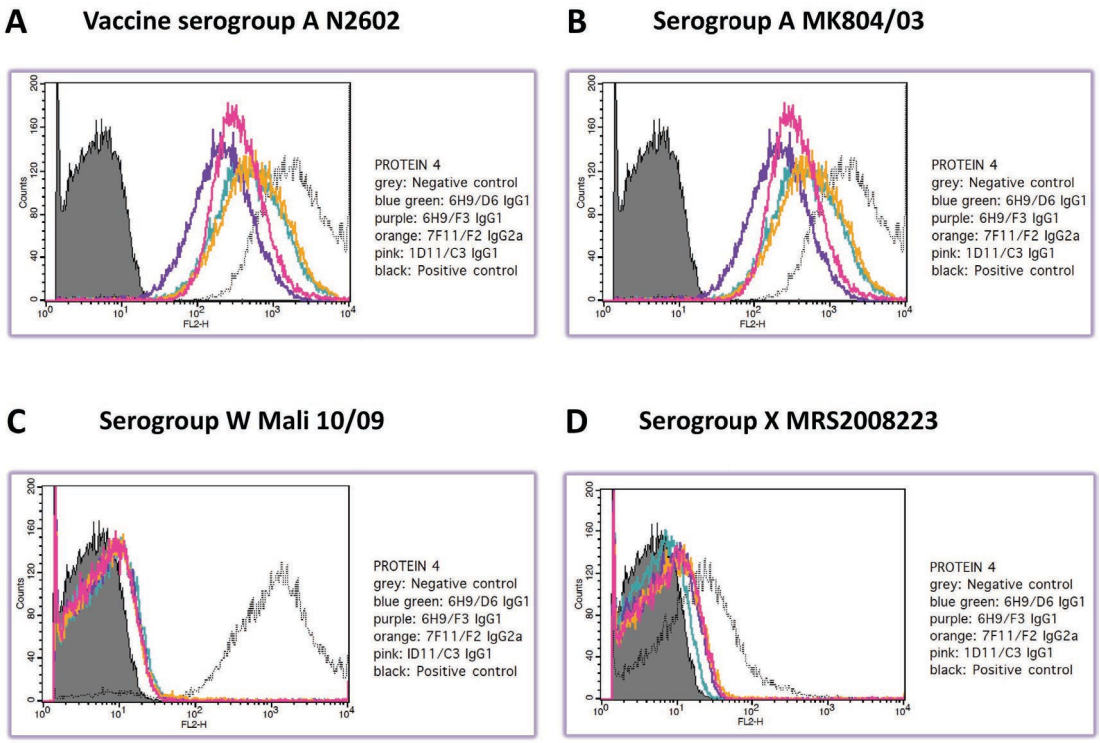

Figure 4.13. Histogram plots of serogroup A, W and X isolates staining with anti-protein 4 mAbs.

Alexa Fluor 488 secondary antibody was used to label the cells. Gating was set at 30000 events.

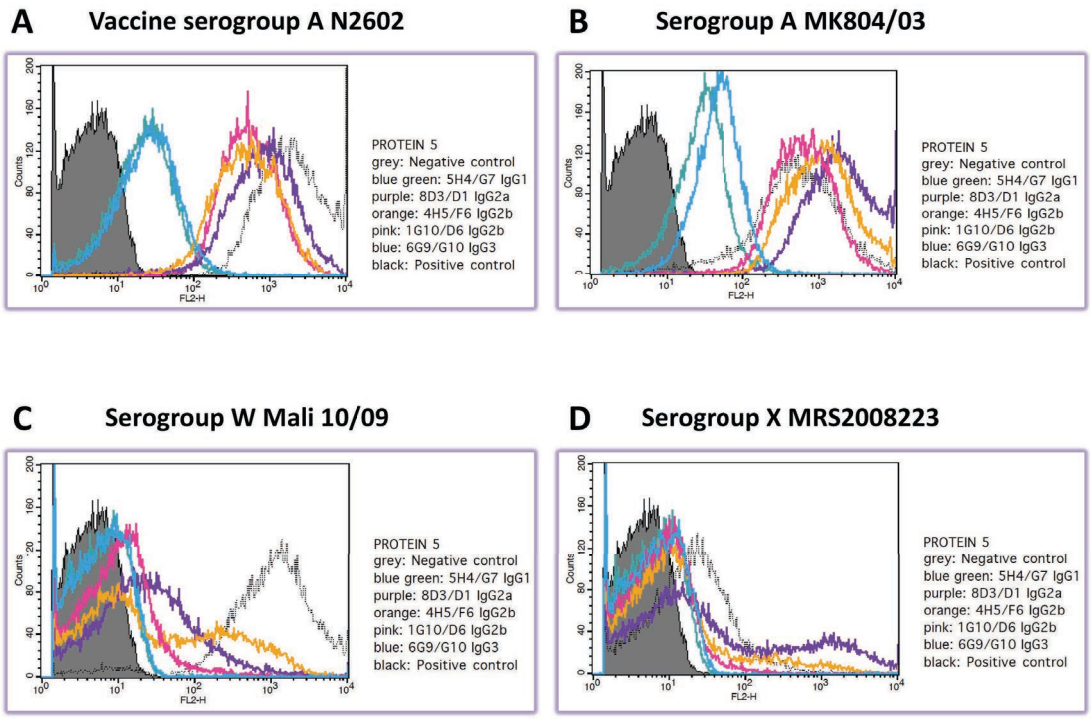

Figure 4.14. Histogram plots of serogroup A, W and X isolates staining with anti-protein 5 mAbs.

Alexa Fluor 488 secondary antibody was used to label the cells. Gating was set at 30000 events.

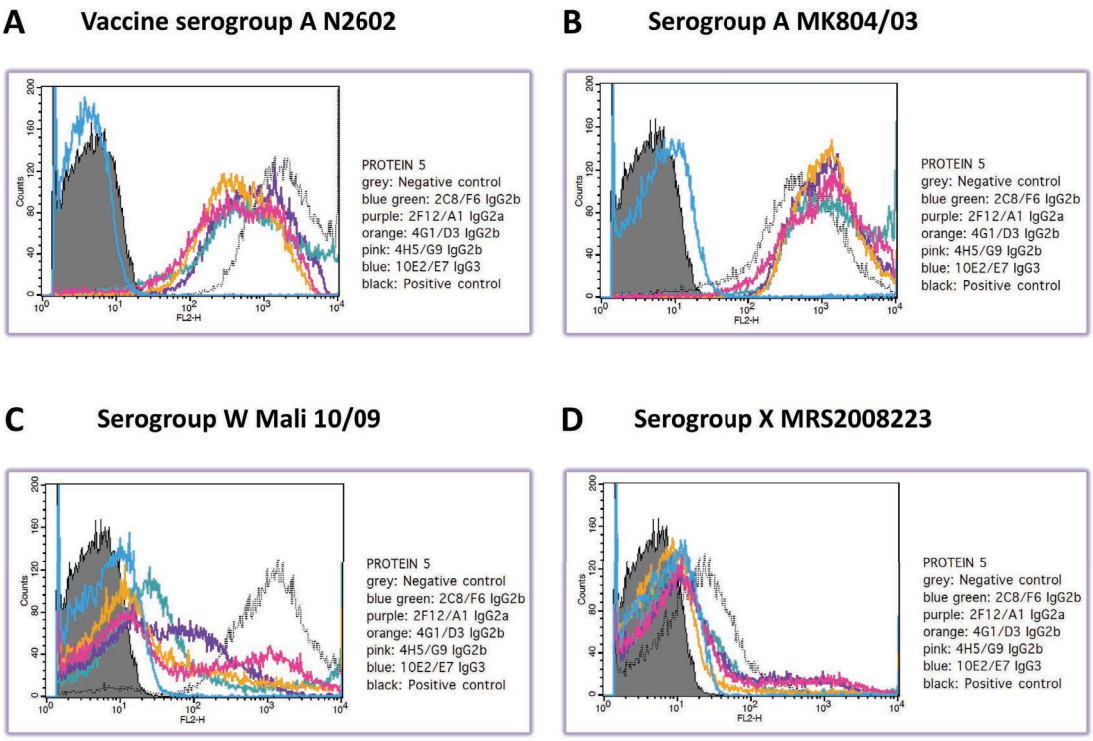

Figure 4.15. Histogram plots of serogroup A, W and X isolates staining with anti-protein 5 mAbs.

Alexa Fluor 488 secondary antibody was used to label the cells. Gating was set at 30000 events.

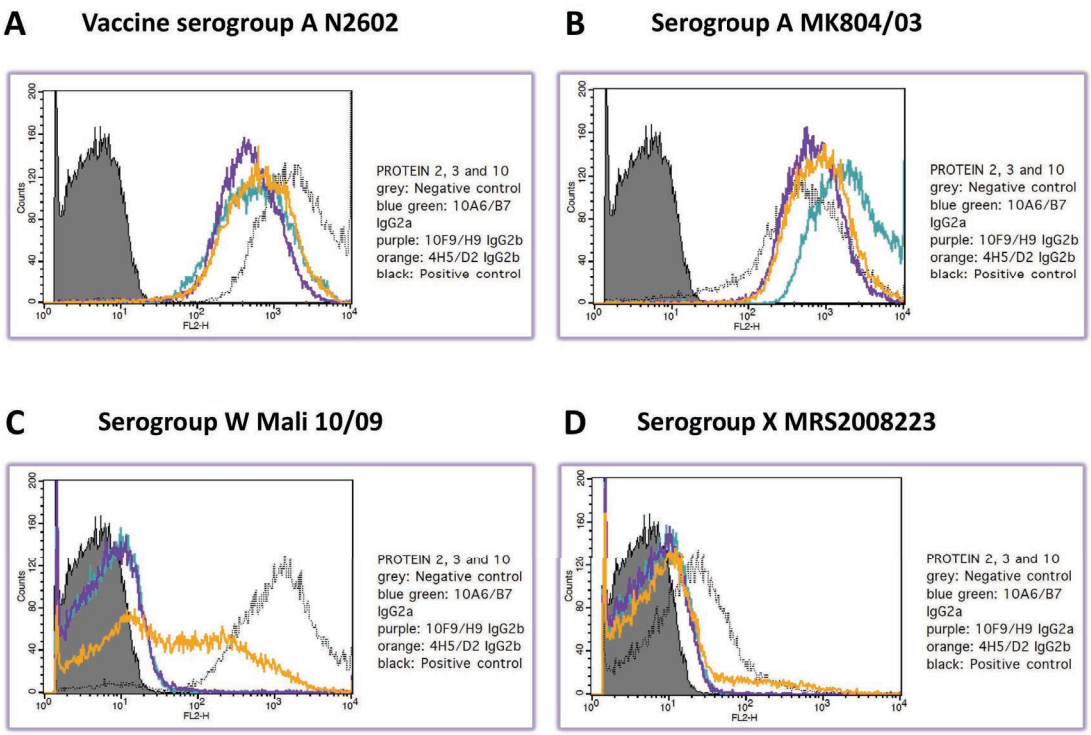

Figure 4.16. Histogram plots of serogroup A, W and X isolates staining with anti-protein 2, 3 and 10 mAbs.

Alexa Fluor 488 secondary antibody was used to label the cells. Gating was set at 30000 events.

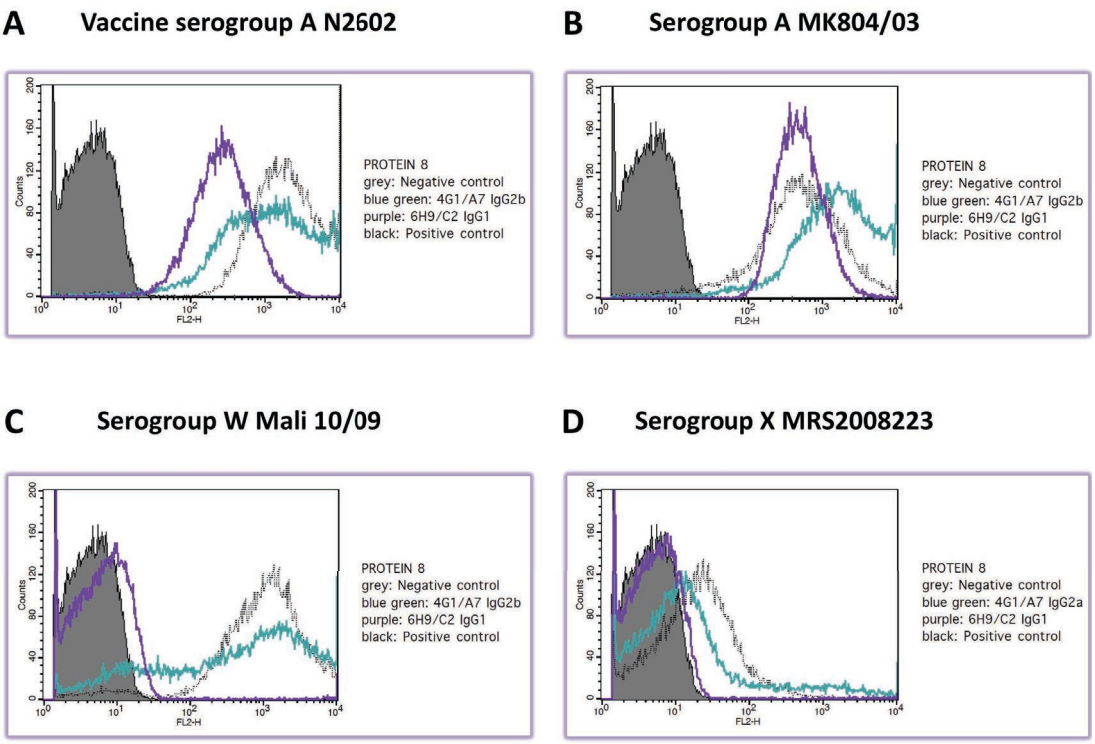

Figure 4.17. Histogram plots of serogroup A, W and X isolates staining with anti-protein 8 mAbs.

Alexa Fluor 488 secondary antibody was used to label the cells. Gating was set at 30000 events.

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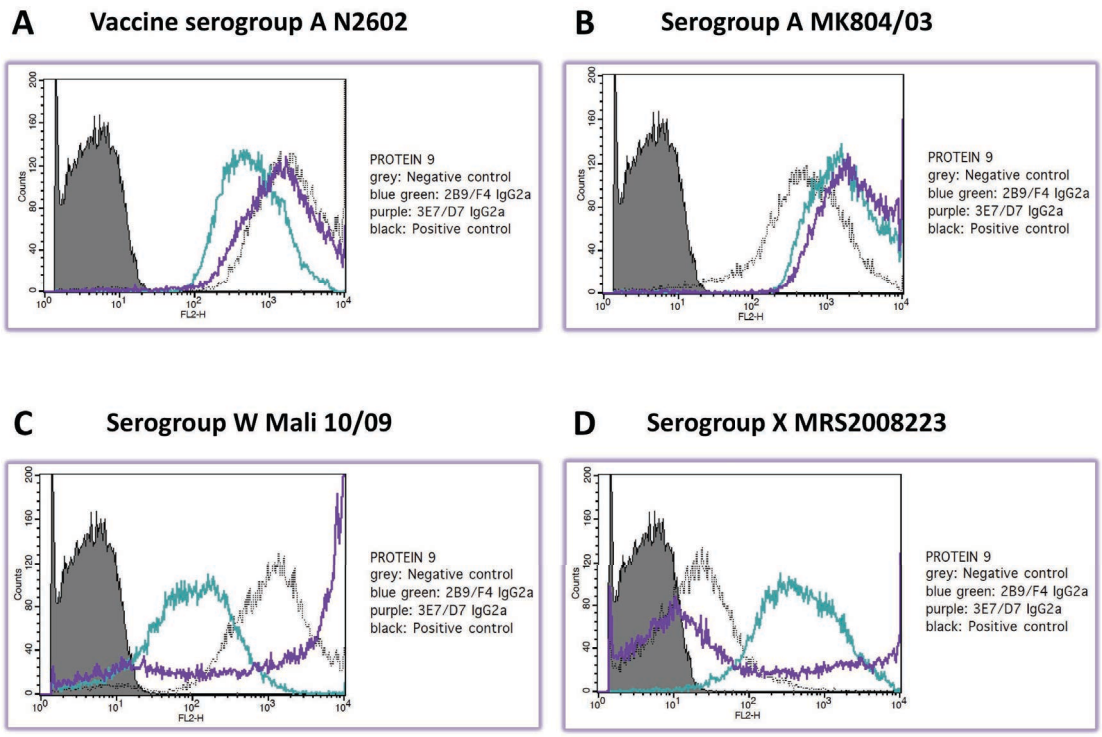

Figure 4.18. Histogram plots of serogroup A, W and X isolates staining with anti-protein 9 mAbs.

Alexa Fluor 488 secondary antibody was used to label the cells. Gating was set at 30000 events.

4.4.6 Bactericidal activity of mAbs from fusion 1 and 2 against African meningococcal A, W and X isolates

The SBA responses elicited by the mAbs generated from fusion 1 and 2 were firstly assessed against the vaccine serogroup A strain N2602 using baby rabbit complement (**Figure 4.19**). The results showed that out of the 25 mAbs generated, all four anti-NadA mAbs of IgG1, IgG2a/b and IgG3 subclass were bactericidal against the serogroup A isolates N2602 (**Panel A**). Only mAbs 10A6/B7 targeting protein 3 (IgG2a), 1D11/C3 and 7F11/F2 specific for protein 4 (IgG1 and IgG2a) and 1G10/D6 and 2B9/F4 (IgG2b and IgG2a) specific for proteins 5 and 9, respectively, were bactericidal against the second serogroup A isolate MK804/03 (**Panel B**). mAbs 10/A6/B7, 7F11/F2 and 2B9/F4 also killed the serogroup W isolate Mali10/09 (**Panel C**). None were bactericidal against the X isolate MRS2008223 (**Panel D**). The bactericidal activity of these mAbs was further assessed using human complement against the same serogroup A, W and X isolates. Likewise, all the mAbs were able to kill the vaccine strain N2602 with human complement as source (**Figure 4.20, Panel A**). Against serogroup A isolate MK804/03, bactericidal titres were again elicited by mAbs 10A6/B7, 1D11/C3, F11/F2, 1G10/D6 and 2B9/F4 (**Panel B**). None of the anti-NadA mAbs showed bactericidal activity against MK804/03 consistent with results each of binding obtained from the IFA and flow cytometry analyses. The low NadA expression of the strain may account for the inability of the mAbs to cross-link IgG antibodies and activate the complement cascade. Lack of bactericidal activity of all mAbs was also seen against the serogroup W Mali10/09 and serogroup X MRS2008223 isolates expressing high and low amounts of NadA-2 and 3, respectively (**Panel B** and **D** respectively). This is despite the fact that the mAbs bind to NadA of the serogroup W isolate in Western blotting, flow cytometry and IFA

analyses. The results suggest that the ability of the mAbs to trigger serum bactericidal activity is specific with regards to the presence of specific epitopes and the expression level of NadA. mAbs of murine IgG subclass IgG2a produced the highest bactericidal titres in this study compared to the other subclasses. This may have implications for future vaccine design.

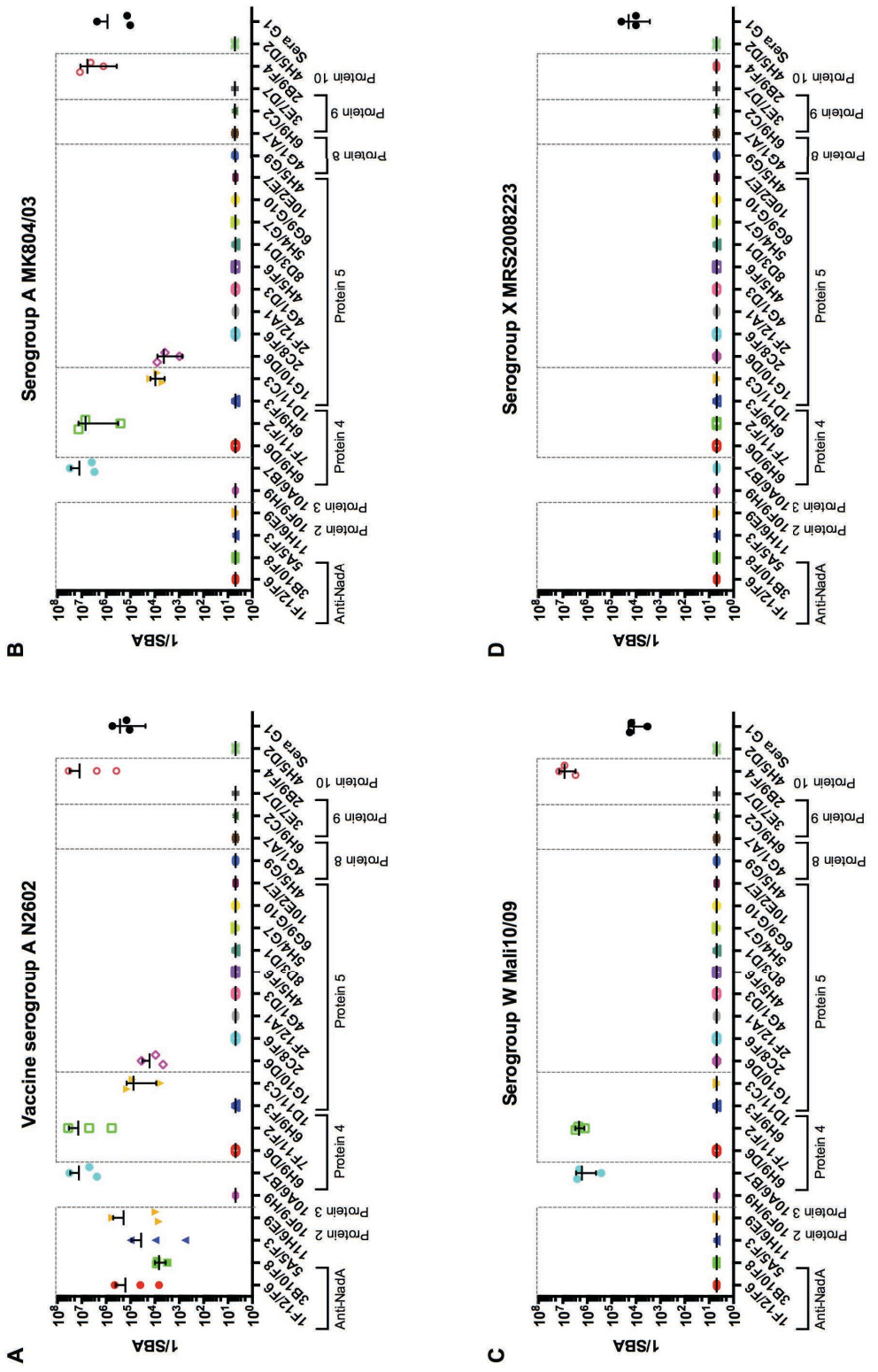
A
Vaccine serogroup A N2602
B
Serogroup A MK804/03
C
Serogroup W Mali10/09
D
Serogroup X MRS2008223
1/SBA
Anti-NadA
Protein 2
Protein 3
Protein 4
Protein 5
Protein 8
Protein 9
Protein 10
Sera G1

Figure 4.19. Bactericidal responses using baby rabbit complement and monoclonal antibodies generated from mice immunised with MenA GMMA NadR KO cps- GMMA formulated with and without Alum against African meningococcal strains.

The mAbs generated from fusion of the spleenocytes of mice immunised with with the MenA NadR KO cps- GMMA formulated with Alum and without any adjuvant were tested against serogroup A vaccine strain N2602 (panel A) expressing high amounts of NadA-3, serogroup A MK804/03 (panel B) isolate expressing low amounts of NadA-3, serogroup W Mali10/09 (panel C) expressing high amounts of NadA-2 and serogroup X MRS2008223 (panel D) expressing low levels of NadA-3. The bactericidal responses were measured using baby rabbit complement as source (see Materials and Methods Chapter 3). Each symbol represents a SBA measurement of a mAb. Sera G1 was obtained from the final bleed of the mouse used for Fusion 2. The line indicates the geometric mean titre (GMT) of each group. Error bars indicate ±95% confidence interval of the GMT.

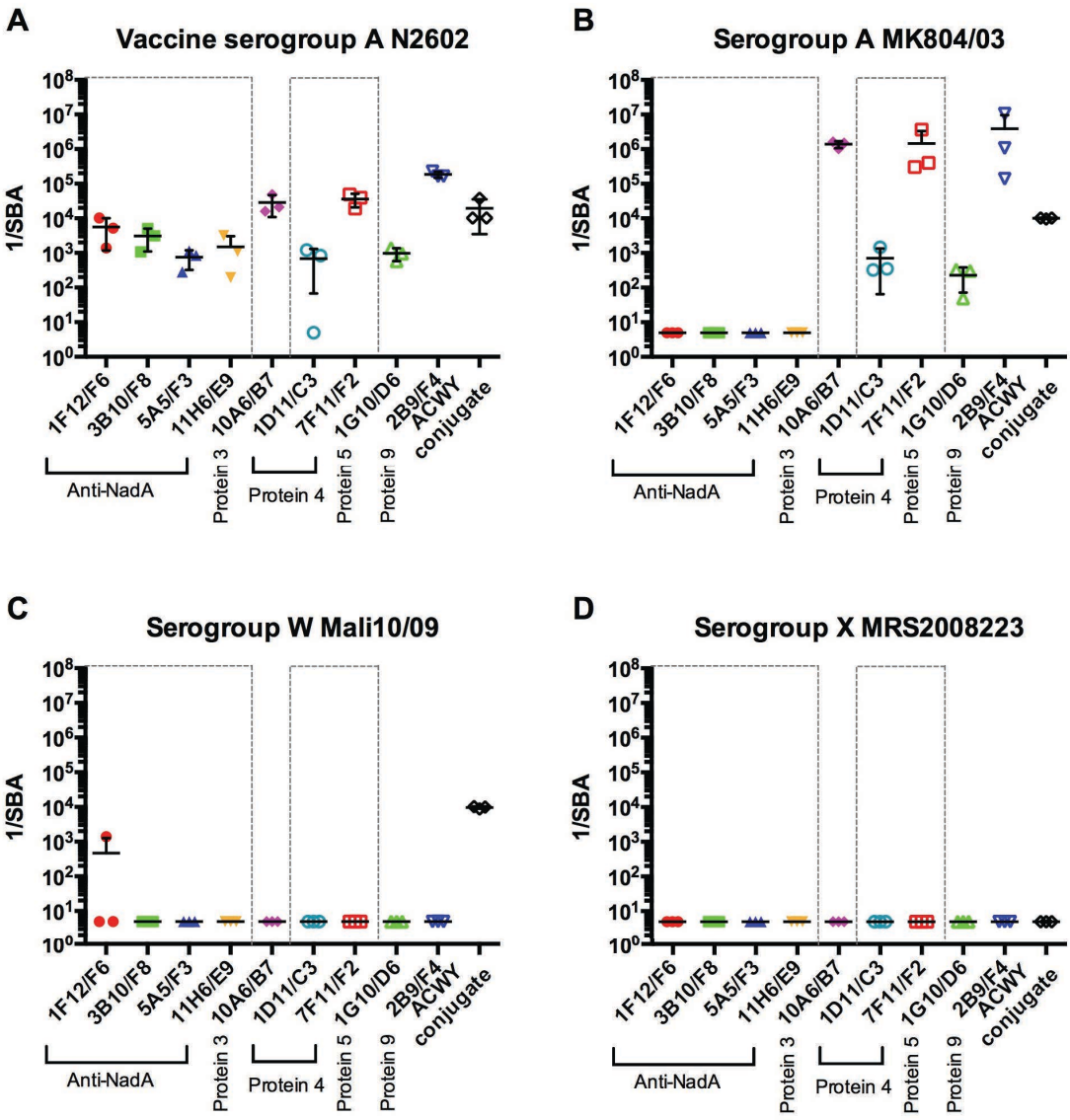

Figure 4.20. Complement-mediated bactericidal responses of monoclonal antibodies generated from fusion 1 and 2 against African meningococcal strains.

The mAbs generated from fusion of the spleenocytes of mice immunised with the MenA NadR KO cps- GMMA formulated with Alum and without any adjuvant were tested against serogroup A vaccine strain N2602 (panel A) expressing high amounts of NadA-3, serogroup A MK804/03 (panel B) isolate expressing low amounts of NadA-3, serogroup W Mali10/09 (panel C) expressing high amounts of NadA-2 and serogroup X MRS2008223 (panel D) expressing low levels of NadA-3. The bactericidal responses were measured with human complement. ACWY conjugate polysaccharide vaccine is coupled to diphtheria CRM_{197} and developed by Novartis vaccines (NV&D Vaccine Chemistry). Each symbol represents a SBA measurement of a mAb. The line indicates the GMT of each group. Error bars indicate ±95% confidence interval of the GMT.

4.5 Discussion

GMMA is a new vaccine platform currently under investigation as approach against meningococcal disease in Africa. An in-depth evaluation of the immunological responses generated against GMMA and the antigens presented on it have not been performed. As an approach to better understand this, we generated mAbs against surface exposed antigens from Balb/C mice immunised with MenA NadR KO cps- formulated with Alum and without, and GMMA from a group W strain with deleted *lpxL1*, *gna33*, capsule and over-expressed fHbp ID9 R41S (Triple KO OE fHbp). The aim was to generate mAbs against NadA-3, fHbp v.1 ID9 and other antigens on GMMA, characterise the fine specificity and activity of NadA and fHbp specific antibodies and mine for other immunogenic/immunodominant antigens that elicit bactericidal antibodies for future vaccine design. The sera generated from the immunisations described above showed different immune profiles when antigen specificities were visualised on Western blot loaded with MenA NadR KO cps- and Triple KO fHbp GMMA. Analysis of the sera from the MenA and MenW GMMA groups formulated with Alum showed that the immune responses were mostly targeted towards the main vaccine antigen NadA or fHbp. The MenA NadR KO cps- GMMA alone group had a wider spectrum of bands on the Western blot, and elicited highest titres in an ELISA when assessed against MenA NadR KO cps- GMMA. Similarly, the MenA NadR KO cps- serum was more bactericidal than the sera of mice immunised with GMMA group absorbed to Alum. This was also observed when the serogroup W isolate was grown in the presence of 4HPA. There could be several explanations for this observation. For example, the absorption of GMMA on Alum could affect the structure of the GMMA thus leading to the generation of a different antibody repertoire compared to GMMA alone. Adjuvants are known to modulate the

immune response against antigens by creating a depot effect, targeting immune cells or increasing the production of certain cytokines. Although aluminum salts are extensively used in vaccine formulations, their mechanism of action and effect on GMMA structure and stability has not been explored. Jones *et al.* (2005) observed, based on spectroscopic and differential scanning calorimetry studies, that the structure of proteins absorbed to Alum were altered rendering the proteins less thermally stable (18). The authors also observed that the absorptive strength and amount of Alum affected protein structure. GMMA consists mostly of lipids and outer membrane proteins (19). Thus, hypothetically GMMA absorbed to Alum may make the proteins on the vesicle surface less accessible to antigen presenting cells or more destabilised upon absorption. Chemical changes of the proteins such as deamidation and oxidation may also be a possibility. This could in turn explain the different pattern visualised on the Western blots. Moreover, GMMA is likely to be used in man in conjunction with Alum for safety reasons, as Alum is also an absorbant for free LPS, which with Alum is released more slowly. GMMA without Alum could present more LPS to the immune system, thus enhancing the immune response, which could be an additional explanation for the ELISA and Western blot results.

We generated 25 mAbs from the spleens of mice immunised with MenA NadR KO cps- formulated on Alum and without, and Triple KO OE fHbp + Alum. Western blotting, IFA and flow cytometry analysis with these mAbs showed limited cross-reactivity of the anti-NadA mAbs against other meningococcal serogroup A isolates than the vaccine A strain N2602. Some cross-reactive binding of the anti-NadA mAbs was seen against serogroup W strains, but none against serogroup X strains. The other mAbs specific for unknown surface antigens showed a broader reactivity against both serogroup A isolates N2602 and MK804/03 isolates. A few mAbs also cross-reacted

with the serogroup W isolate Mali/10/09. Only one mAb 2B9/F4 specific for protein 9 showed staining in the flow cytometry analysis against the serogroup X isolate MRS2008223. When human complement was used as a source, the anti-NadA mAbs were only bactericidal against the vaccine serogroup A strain N2602, while the other 5 mAbs showed cross-reactivity against a second A strain MK804/03. The results suggest that there is microheterogeneity within the subtypes of NadA. Some mAbs bind to specific epitopes, while others bind to epitopes shared across several subtypes that may or may not kill the meningococcus. The specificity of bactericidal anti-NadA mAbs can be used to map epitopes on the NadA protien and compare these with epitopes that elicits bactericidal antibodies on recombinant NadA (20). Such results are beneficial in the understanding of the immune responses against GMMA.

Th1/Th2 patterns coexist in the immune response but one often predominates over the other, depending on the antigenic stimulation and vaccine formulation. In mice, a Th1 response is associated with the induction of IgG2a/b and IgG3 antibodies while IgG1 is associated with a Th2-like response. IgG2a and 2b exhibit strongest binding to Fc receptors and are able to activate complement efficiently, while in humans IgG1 and IgG3 provide protection (21,22). In this study, the results suggest that GMMA without Alum generate mAbs that are more diverse in terms of protein target and IgG subclass. IgG isotyping of the serum of the mouse immunised with GMMA without Alum and with a mAb antibody repertoire of predominantly IgG2a/b subclass, the immune response is suggestive of a Th1 type. We obtained only one mAb against NadA while GMMA formulated on Alum seems to direct the immune responses more specifically towards NadA skewed towards Th2 with predominantly IgG1 and IgG2b subclass. Despite this, the lack of bactericidal activity seen for the mAbs produced

against other strains than the vaccine strain N2602 and the second serogroup A strain MK804/03 could be related to the epitopes recognised and their specificity, and maybe un-related to the adjuvant effect. To obtain a clearer picture of the Th type of response that the GMMA elicit, cytokine release in response to GMMA alone and GMMA+Alum immunisation could give additional indications (Chapter 5, section 5.1.2) (23).

Bactericidal activity is dependent on Fc-mediated differences in complement activation, amount and density of surface protein on the pathogen and epitope specificity of the mAbs. For example, Giuntini *et al*. (2012) constructed chimeric mAbs with three different murine fHbp-specific binding domains paired with human IgG1, IgG2 or IgG3 (21). The authors concluded that IgG3 mAbs had bactericidal activity >5-fold higher than IgG1 mAbs, and IgG2 mAbs had least activity when assessed against serogroup B isolates. However, the bactericidal activity was dependent on epitope density where IgG3 anti-fHbp mAbs had greatest activity at low epitope density while IgG1 had highest bactericidal activity at high epitope density. The IgG3 subclass has an extended hinge region allowing greater flexibility of the Fc portion promoting more efficient engagement of C1q during low fHbp density. These observations highlight the importance of isotype when comparing the relative effectiveness of mAbs in a bactericidal assay, but also the quantity of antigen. The ability of a vaccine to elicit protective antibodies regardless of adjuvant effect, is therefore likely enhanced if the antibody repertoire is of high avidity (6,19). The mAbs produced here may thus be bactericidal if enough antigens are present on the pathogen surface. For example, we observed that all anti-NadA mAbs killed the serogroup A isolate N2602 expressing high amounts of NadA-3, while the anti-NadA

mAbs did not kill the second serogroup A isolate MK804/03 expressing low levels of NadA-3. Similarly, flow cytometry showed that the anti-NadA mAbs bound the isolate N2602 and only one bound to MK804/03. For low expressing strains the mAbs may show synergism if combination of 2 non-bactericidal mAbs bind to non-overlapping epitopes engage C1q. Welsch *et al*. (2008) observed that JAR 3 (IgG3) mAb and JAR 4 (IgG2a) mAb independently did not elicit SBA against high fHbp expressing strains (25). Together the mAbs increased C4b binding and elicited SBA titres. The results suggest that the failure of an individual mAb to activate complement may not be exclusive to IgG subclass or antigenic specificity. Further studies on the C3b deposition activated by the mAbs could elucidate whether the mAbs are able to cause killing by opsonophagocytic bactericidal activity via Fc and iC3b receptors.

In the case of NadA, Ciabattini *et al*. (2008) immunized Balb/c mice intranasally with recombinant strains (26). Assessment of the local and systemic immune responses revealed that immunisation with live bacteria expressing NadA induced a Th1 response with a dominance of IgG2a. However, unlike the monomeric fHbp, NadA exists in an oligomeric state in the cell membrane, and the dependence of antigenic density and epitope specificity need to be further assessed.

Unlike the studies using recombinant protein to generate mAbs, our approach reflects the overall response against GMMA including antigens that are not over-expressed. With respect to over-expressed antigens, native proteins imbedded in their natural environment are primarily B cell epitopes presented to the B cell that are exposed on the outside of GMMA. mAbs made against antigens on GMMA may thus be

primarily directed against epitopes that are present on the surface of live bacteria and may, therefore, bind the same epitope during meningococcal infection. In contrast, recombinant proteins are usually linear may not have adopted entirely their native conformation. Thus, a smaller proportion of mAbs may be generated against bactericidal epitopes and may have reduced SBA activity. The generation of mAbs against GMMA may prove to be a useful tool to better understand and dissect the humoral immune responses towards *N. meningitidis.*

4.6 Conclusion

To conclude, the overall results reconfirm that immune response against a vaccine depend on multiple factors that influence the immune response. The route of immunisation, adjuvant, antigen dose, time and interval between immunisations all contribute towards the type of immune response and hence antibody repertoire produced (3). We generated 25 mAbs from fusions of spleen of mice immunised with MenA NadR KO cps- GMMA. The anti-NadA mAbs were only bactericidal against the vaccine strain, while 5 mAbs targeting unknown antigens were also bactericidal against a second A strain. Future studies should, however, identify the unknown antigens that the mAbs bind from fusion 1 and 2. This will lead to the identification of new target antigens that elicit bactericidal antibodies. Expressing them in GMMA as a "multi-protein" delivery system may have massive synergistic effects and enhance the protective potential of the vaccine strategy. Other potential future investigations could involve elucidating the epitopes on the antigens that the mAbs bind, and to test whether synergies of antibodies against the same antigen and different target antigens induces bactericidal activity.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

5.1.1 Summary of findings

In the sub-Saharan Africa, high disease burden of *Neisseria meningitidis*, especially in young children, is a public health problem. Despite the availability of effective antibiotics for treatment, mortality is still high due to fragile health care systems, affordability and distribution issues. Effective polysaccharide conjugate vaccines are available, but they are unlikely to be affordable. Hong *et al*. (2013) showed that the bactericidal antibody responses induced by 4CMenB vaccine Bexsero® suggested coverage of serogroup X isolates tested (1). An affordable serogroup A specific conjugate polysaccharide vaccine was rolled out in 2010 in several countries of the sub-Saharan Africa. However, there is a need for an inexpensive vaccine that covers other serogroups that cause disease such as W and X, and those that may potentially do so in the future (2,3).

GMMA is a new protein-based approach towards developing a broadly-protective meningococcal vaccine for Africa, which we employed here. To be able to obtain a proof-of-principle in mice of the effectiveness of such a vaccine for the use in Africa, we firstly set out to determine the genetic variability of potential OMP vaccine candidate antigens fHbp, NadA, NHBA and PorA of strains isolated from various countries in Africa (Chapter 2). Secondly, we generated GMMA from a serogroup A and a serogroup W strain. Both strains had deleted capsule biosynthesis genes, *lpxL1*, and *gna33*. The candidate antigen fHbp of variant 1 was over-expressed in the serogroup W strain 1630 by a multicopy plasmid. The gene encoding for the *nadA* repressor *nadR* was deleted in the serogroup A isolate (N2602) and the W isolate

(1630) in order to attempt to increase expression of NadA-3. In the latter two GMMAs, fHbp was not over-expressed. The first immunogenicity study evaluated the GMMA over-expressing fHbp (Chapter 2). The second mouse immunogenicity study evaluated GMMA from the serogroup A and W recombinant strains expressing NadA-3 (Chapter 3). Moreover, to obtain additional insights into the immune responses towards GMMA, we generated mAbs against fHbp, NadA and other proteins natively presented on the GMMA as opposed to traditional methods of mAbs developed against processed recombinant proteins (Chapter 4).

Molecular characterisation of 94 meningococcal serogroup A, W and X isolates from sub-Saharan Africa isolated between 1995-2011 showed limited antigenic variability of fHbp, NadA, PorA and NHBA across the serogroups. The results also suggest that natural herd immunity against these protein target antigens does not seem to be a strong driving force for allelic replacement. Rather, variation was seen for the expression level of the fHbp and NadA antigens, and the isolates analysed expressed intermediate to high amounts of NadA and fHbp. From our immunogenicity studies, serum from the mice immunised with GMMA from a recombinant strain with over-expressed fHbp was able to kill 3 out of 4 group A isolates, and one X isolate. The ability of the sera to induce bactericidal antibody responses was dependent upon level of fHbp expression on the surface of the isolates. Strains expressing low levels of fHbp were resistant to killing by anti-fHbp antibodies. Bactericidal antibodies generated from serogroup A GMMA with deleted *nadR* and capsule killed isolates expressing low, intermediate and high NadA of serogroup A and W. Serum from mice immunised with wild-type serogroup W GMMA without capsule induced bactericidal antibodies against a low and intermediate NadA expresser. We also observed that

GMMA from mutant strains with NadA KO and NadA + NadR KO also induced measurable bactericidal titres, which were directed against other OMPs than NadA, fHbp and PorA. The results from the typing and immunogenicity studies highlight the advantage of using fHbp and NadA in GMMA as a vaccine strategy. The data indicate that antibodies to other but shared OM antigens along with antibodies to the main target antigens may provide broad cross-protection (4). With GMMAs, dependency on a single antigen or antigen type is avoided. Moreover, antibodies induced by a control recombinant fHbp v.1 vaccine group only induced a low but detectable SBA response suggesting that fHbp expressed in the native membrane environment induces antibodies with greater functional activity. On the contrary, rHis-NadA elicited bactericidal titres comparable to those induced by GMMA indicating that immunogenicity is less dependent on the three-dimensional conformation of the protein than fHbp. One possibility for this difference could be that rHis-fHbp induces antibodies against "non-functional" epitopes on parts of the protein that are not naturally surface exposed, which may not be the case for NadA that is more exposed. While the GMMA may allow more antibodies to be induced against conformational parts of the protein that are also surface exposed, thus there may be a greater proportion of "functional" anti-fHbp antibodies. This also highlights the advantage of using GMMA as a vaccine strategy.

From the results of the immunogenicity studies, we wanted to further understand the immune responses towards GMMA by generating mAbs against GMMA antigens, in particular focusing on NadA and fHbp as targets. We performed three fusions, whereby the first two fusions were performed using mice immunised with GMMA from a serogroup A recombinant strain with deleted *nadR* and capsule (generated in

Chapter 3) absorbed to Alum and without. The third fusion was recently performed using a mouse immunised with GMMA from a mutant serogroup W strain engineered to over-express fHbp v.1 absorbed to Alum, which is currently in progress. In total, 25 mAbs were generated from the first two fusions. The anti-NadA mAbs were only bactericidal against the vaccine strain, while 5 mAbs targeting unknown antigens were also bactericidal against a second A strain. Seven mAbs of subclass IgG2a were produced in the NadA GMMA alone group and two mAbs of subclass IgG2a against GMMA with over-expressed fHbp formulated on Alum. The results suggest that GMMA alone induced IgG subtypes with a Th1 type response generating antibodies of subclass IgG2a, while the use of Alum favours a Th2 type immune response (5). The results also indicate that there could be more genetic diversity in NadA than would be indicated by the serotypes (*i.e.* microheterogeneity within the subtypes). mAbs may bind to such "private" epitopes, or epitopes shared by all members of a subtype and potentiall also across several subtypes.

5.1.2 Implications for vaccine design against the meningococcus

An effective vaccine needs to fullfill a number of criteria. Firstly, since there is a high disease burden in the meningitis belt, especially in young children, a vaccine against meningoccus needs to be immunogenic in all age groups and be affordable by these countries. Secondly, a vaccine that induces long-term protection is important, which is maintained by three mechanisms: antibody persitence, B cell memory and herd immunity (6–9).

The results from our study point towards the investigation of a future GMMA vaccine over-expressing fHbp and NadA for the use in Africa, as these antigens appear to be

relatively conserved among African meningococci. Moreover, when natively expressed in GMMA, the antigens elicit broadly protective antibody responses, and the vaccine is cheap to manufacture. In principle, fHbp is a good vaccine candidate since it is found in more than >99% of both invasive and carriage genomes sequenced to date, and has been shown to be important for survival of *N. meningitidis* in *ex vivo* models (10). Therefore, a vaccine with fHbp may have an impact on carriage, interupt transmission and promote herd immunity. A recent evidence for this was seen by Read *et al*. (2014) where the 4CMenB vaccine, which contains recombinant fHbp, was able to reduce meningococcal carriage rates during 12 months after vaccination (11). Antibodies towards fHbp can both activate the classical and alternative complement pathaway and block fH binding (12). However, the results from this and other studies show that induction of bactericidal antibodies is dependent on several factors. Primarily the expression level of the antigen is important as it is sparsley distributed on the surface of meningococci. A vaccine based only on fHbp may thus decrease survival of the meningococci but not exclude the possibility of escape mutants expressing low levels of fHbp. Moreover, the three-dimensional structure of the fHbp protein and the epitope surface exposed on GMMA to the immune system are two other factors affecting the induction of "functional" bactericidal anti-fHbp antibodies (2,13–15). There is cross-reactivity between variant 2 and 3 but not between variant 1 and 2/3. For future vaccine design against the meningococcus, the solution could be to include a second fHbp variant or to engineer the recombinant strain to include another OMP.

Looking at NadA as a vaccine candidate, results from the immunogenicity study suggest that antibodies towards NadA are less dependent on the three-dimensional

structure of the protein. Similarly to fHbp, bactericidal activity is dependent on NadA expression level, which in turn is phase variable. Highest expression of NadA is resported to be during adhesion/invasion of the nasopharynx. We also observed that the gene in several sergroup W isolates was interupted by *IS1301*, which could have implications for vaccine coverage. Although, all isolates in this study had the *nadA* gene, several other studies have shown that the gene is less present in carriage isolates (16–18).

It is important to determine the host immune response towards a vaccine and the effect of adjuvant used to boost cellular and humoral immune responses to promote future vaccine design. Adjuvants may induce T-cell response that favours Th1 or Th2 responses, which may have profound influence on the outcome of a vaccination. In this study, we present a novel approach whereby mAbs were generated against various natively surface exposed antigens on GMMA, as opposed to processed recombinant proteins. We used these mAbs to investigate the immune responses generated by GMMA alone, formulated on Alum and by the individual antigens themselves to better understand the immune responses against GMMA. One of the observations made was that the use of Alum in the vaccine formulations elicited an immune response that favoured a Th2 response, which is also widely known in the literature (19–21). MAbs generated from mice immunised with GMMA alone showed increased bactericidal activity and a Th1 response. It is known for polysaccharide conjugate vaccines that protection against *N. meningitidis* needs to involve presentation of the vaccine antigen through MHCII on B cells to Th1 in combination with co-stimulatory molecules CD40-CD40L and CD80-CD28. This interaction generates plasma cells that produce and secrete antibodies of subclass IgG1 and IgG3

in humans and IgG2a in mice. These IgG subclasses are the most effective at fixing complement necessary for clearance of the meningococcus through classical and alternative complement pathways (19). Therefore, the desirable immune response against the meningococcus appears to be a Th1-polarised response. GMMA itself contains LOS and is a TLR2 and TLR4 agonist and autoadjuvant that favours the adjuvant action of $CD4^+$ T Th1 lymphocytes (22). As GMMA has been proven safe for use in mice due to the deletion of capsule biosynthesis genes and *lpxL1* that reduces endotoxicity, Alum may not be necessary for future use in humans for the induction of appropriate immune response for the development of protective immunity. Alum is used in many vaccine formulations to reduce reactogenicity in man, occasionally associated with tissue reactions such as erythema (20). Future investigations into the Th type responses by GMMA alone and GMMA formulated on Alum would help improve the understanding of these results. Cytokine release in response to GMMA alone and GMMA+Alum immunisation would give additional indications as to whether a Th1 or Th2 response is induced. Th1 cells are characterised by their secretion of interleukin-2 (IL-2) and gamma interferon (IFN-γ), whereas Th2 cells produce IL-4, IL-5 and IL-10 (20,21,23–26).

Moreover, with the novel mAb approach used here we generated mAbs against natively presented antigens on GMMA. The anti-NadA mAbs showed limited cross-protective bactericidal responses to other serogroups than the serogroup A vaccine strain N2602. For future vaccine design, the novelty of this approach allows for mapping of the epitopes on the NadA protein in GMMA that induce production of bactericidal antibodies and the epitopes that can induce cross-protection across serogroups. Compared to mapping of epitopes using mAbs generated from mice

immunised with recombinant NadA, mapping using mAbs against NadA on GMMA could give an added comprehensive picture of the actual immune responses to NadA during invasive meningococcal disease. From fusion 1 and 2, most of the mAbs produced were directed against unknown antigens. Several of these mAbs were of IgG2a subclass, and for some of these mAbs cross-protection was observed against a second serogroup A isolate when human complement was used as complement source. Elucidating their identity could have an impact on future vaccine design as some of these antigens may be novel and could potentially be used in a future GMMA vaccine. Previous methods for identifying novel immunogenic antigens in *N. meningitidis* was by reverse vaccinology. We show that generation of mAbs against surface proteins on GMMA can be used to mine for new immunogenic antigens.

5.1.2 Future directions

Following on from the thesis, there are several potential future projects that could be undertaken to understand immune responses to GMMA better. Firstly, identification of the unknown antigens that the mAbs from fusion 1 and 2 bind would help to get a better overview of the immune responses induced by GMMA. Such a path was started, but halted due to time limitations. The experiments would immunopresipitate the soluble antigens using the mAbs generated and mass spectrometry to identify the extracted antigens. Whole cell lysates are prepared with a lysis buffer and the lysate is incubated with a mAb bound to protein G. The antigen-antibody complex is purified through washes with lysis buffer, and the immunopresipitate run on SDS-PAGE gel. The correct band is excised, digested and analysed by MALDI-TOF.

A second project would involve characterising the anti-fHbp mAbs generated from fusion 3. This would follow the same direction and experiments as for fusion 1 and 2 (Western blotting, IFA, flow cytometry, SBA). Alternatively, another fusion with GMMA over-expressing fHbp can be made in order to try and expand the anti-fHbp antibody repertoire. Further experiments to characterise these anti-fHbp mAbs could be undertaken by comparing the results generated with those from previous studies using recombinant fHbp mAbs. Such experiments could include mapping of the GMMA-fHbp or GMMA-NadA epitopes important for generating bactericidal anti-fHbp antibodies by hydrogen/deuterium exchange mass spectrometry and using yeast display (27–29). Further studies could also investigate mAb functions such as avidity, affinity, ability to deposit C3b/C4b and block fH binding (30,28,31,32). Comparing the results obtained from previous studies using recombinat fHbp mAbs would give a better understanding of the advantageous immunogenicity induced by GMMA. The study can be further extended by investigating the cooperative bactericidal activities of the GMMA anti-fHbp mAbs with the anti-NadA mAbs generated here, as the bactericidal activity of anti-fHbp mAbs are known to depend on the density of fHbp on the bacterial surface. This could help determine whether expressing both fHbp and NadA in GMMA increases bactericidal responses.

The anti-NadA mAbs generated here can also be used for determining the minimum level of NadA expression on the meningococcal surface that is required to cross-link two IgG anti-NadA antibodies to correctly spaced epitopes. Such experiments could involve generating isogenic mutant strains of different NadA expression. The ability of a bactericidal anti-NadA mAb to kill these strains can be assessed by SBA. Another potential project could involve over-expression of fHbp and NadA in the

same GMMA, or other immunogenic antigens identified from the mass spectrometry analysis. Similarly to the study undertaken in Chapter 3, the multi-antigen GMMA could be assessed if this genetic approach broadens protection.

5.2 Conclusion

To conclude, the findings of our study provide support for a GMMA-based vaccine approach as an affordable and broadly-protective vaccine strategy. The results also highlight the fact that inclusion of more vaccine antigens could impove breadth of coverage. Generation of mAbs against the antigens on GMMA indicate that there are additional immunogenic antigens on GMMA that could be potential future vaccine candidates.

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