Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

BIOLOGIA CELLULARE E MOLECOLARE

Ciclo XXVI

Settore Scientifico Disciplinare: 05/E2
Settore Concorsuale di afferenza: BIO/11

TITOLO TESI

Analysis of the immunological and functional features of the Neisserial Heparin Binding Antigen (NHBA)

Presentata da: **Irene Vacca**

Coordinatore Dottorato

Chiar.mo Prof.
Vincenzo Scarlato

Relatore

Chiar.mo Prof.
Vincenzo Scarlato

Co-relatori
Dott.
Davide Serruto

Dott.ssa
Isabel Delany

Esame finale anno 2014

Attività di ricerca

Il mio progetto di dottorato, presentato in questo lavoro di tesi, si è focalizzato principalmente sulla caratterizzazione delle proprietà immunogeniche e funzionali di Neisserial Heparin Binding Antigen (NHBA), antigene proteico di un nuovo vaccino anti-meningococcico chiamato 4CMenB, che Novartis Vaccines S.r.l. di Siena ha sviluppato contro il sierogruppo B di *Neisseria meningitidis*. In particolare mi sono occupata della generazione di ceppi di meningococco ricombinanti, knockout per NHBA e/o esprimenti diverse varianti dell'antigene, e della caratterizzazione genetica dei ceppi batterici ottenuti. I ceppi ricombinanti sono stati poi utilizzati in saggi di battericidia per valutare le proprietà immunogeniche dell'antigene, la specificità della risposta immunitaria e per studiare la potenziale copertura vaccinale. La seconda parte della tesi si è focalizzata sulla caratterizzazione delle proprietà adesive di NHBA in funzione di studiare l'interazione della proteina con le cellule epiteliali umane. I risultati ottenuti saranno oggetto di due pubblicazioni scientifiche che sono attualmente in preparazione:

- "Evaluation of the immunogenicity and cross-protection of the Neisserial Heparin Binding Antigen (NHBA)" **Vacca I.**, Santini L., Biolchi A., Muzzi A., Siena E., Pizza M., Marzia M. Giuliani MM, Serruto D. and Delany I.

- "Heparin-binding activity of the Neisserial Heparin Binding Antigen (NHBA) contributes to meningococcal adhesion to epithelial cells". **Vacca I.**, Del Tordello E., Pezzicoli A., Serruto D. and Delany I.

Parallelamente all'analisi su NHBA mi sono occupata della caratterizzazione di NalP, una proteasi di *Neisseria menigitidis*, che abbiamo dimostrato contribuire alla resistenza del batterio al siero e al sangue umano, tramite taglio e inattivazione della componente C3 del sistema del complemento. Il lavoro svolto è stato parte di una pubblicazione scientifica:

- "Neisseria meningitidis NalP cleaves human complement C3, facilitating degradation of C3b and survival in human serum". Del Tordello E., **Vacca I.**, Ram S., Rappuoli R., Serruto D. Proc Natl Acad Sci U S A. 2014 Jan 7;111(1):427-32.

Table of Contents

Abstract

Neisserial Heparin Binding Antigen (NHBA) is a surface-exposed lipoprotein ubiquitously expressed by genetically diverse *Neisseria meningitidis* strains and is an antigen of the multicomponent protein-based 4CMenB vaccine, able to induce bactericidal antibodies in laboratory animals and humans. Biochemical approaches showed that it binds to heparin-like molecules through an Arg-rich region. Two proteases, the meningococcal NalP and human lactoferrin, are able to cleave the protein upstream and downstream from the Arg-rich region, respectively. NHBA is genetically structured into a substantial number of alleles that have some association with clonal complexes and Sequence Types (STs) and more than 400 different amino acid sequences have been identified so far. The aim of this thesis was to deeper characterize both the immunological and functional properties of this protein antigen. Moreover, for its possible immunogenic and functional implications, we analysed NHBA NalP-mediated cleavage in different NHBA peptides.

To evaluate immunogenicity and the contribution of amino acid sequence variability to vaccine coverage, we constructed a recombinant isogenic strain that is susceptible to bactericidal killing only by anti-NHBA antibodies and engineered it to express equal levels of selected NHBA peptides. Human sera from different age groups vaccinated with the 4CMenB vaccine were tested in a Serum Bactericidal Assay using human complement (hSBA) against the recombinant strains, and interestingly we observed different titres (negative, low and positive) associated with the different peptide variants, which correlated well with the genetic distance of the peptides from that included in the vaccine. In natural strains, however, anti-NHBA antibodies elicited by 4CMenB were found to be cross-protective against strains expressing genetically distant peptides suggesting that either expression levels, or the contribution of other antigens were important for the observed protection. To demonstrate the specificity of anti-NHBA antibodies in mediating bacterial killing, *N. meningitidis* strains mismatched for the other vaccine antigens were analysed. Specificity was further demonstrated through 1) a competitive hSBA using the NHBA recombinant protein or 2) deletion of the *nhba* gene in the selected strain. Through further engineering of recombinant strains to express different NHBA chimeric proteins, regions containing functional bactericidal epitopes were shown to be present in the N-terminus and central regions of the protein.

To investigate the functional properties of this antigen and based on its ability to bind heparin and heparin-like molecules, the recombinant purified NHBA protein was tested in *in vitro* binding studies

and was found to be able to bind Hec-1B and CHO epithelial cells. The binding was abolished when these cells were treated specifically with heparinase III, suggesting that the interaction with the cells is mediated by heparan sulfate proteoglycans (HSPG). As expected, mutation of the Arg-rich tract of NHBA abrogated the binding, confirming the importance of this region of the protein in mediating the binding to heparin-like molecules. In a panel of *N. meningitidis* strains, the deletion of *nhba* resulted in a reduction of adhesion with respect to each isogenic wild type strain. Furthermore, the adhesion of the wild-type strain was prevented by using anti-NHBA polyclonal sera, demonstrating the specificity of the interaction. These results suggest that NHBA could have a role in different steps of meningococcal pathogenesis, both contributing to host-cell interaction via the ability to bind to HSPG as well as influencing bacterial survival in blood through the binding to heparin as previously reported.

Finally, the analysis of NHBA NalP-mediated cleavage, revealed that not all NHBA peptides are cleaved by NalP protease. Further studies would be needed to understand the possible functional and immunological implications of the absence of this cleavage event in meningococcal strains.

Introduction

1. Neisseria meningitidis

Neisseria meningitidis (Nm) is a Gram-negative B-proteobacterium, member of the bacterial family of Neisseriaceae. It is a spherical or kidney-shaped aerobic diplococcus, non-motile, non-sporulating, and usually encapsulated and piliated. Humans are the unique reservoir for this bacterium, which is generally a commensal that colonizes the mucosal epithelium of the nasopharinx and only under particular circumstances and in susceptible individuals, can become pathogenic and cause meningitis and septicemia.

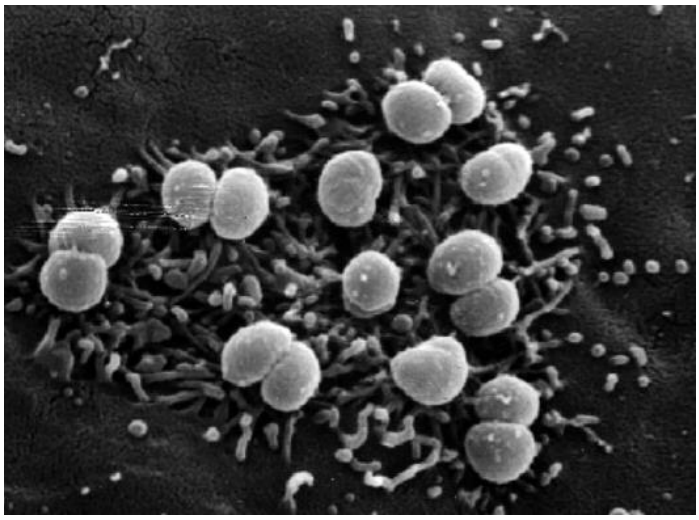


Fig. 1: *Neisseria meningitidis* adhering to of HUVEC cellular monolayers (Eugène E et al, 2002).

On the basis of the immunogenicity and structure of the polysaccharide capsule, *N. meningitidis* can be classified into at least 13 serogroups A, B, C, E-29, H, I, K, L, W-135, X, Y, Z, and 29E (Branham SE, 1953). Among them, only six serogroups (A, B, C, W-135, X, Y) have been associated with meninogococcal disease and are thus considered pathogenic. Further classification into serosubtype, serotype and immunotype is based on class 1 outer membrane proteins (PorA), class 2 or 3 (PorB) outer membrane proteins and lipopoly[oligo]saccharide structure, respectively (Rosenstein NE, 2001), (Stephens DS *et al*, 2007). New methods of classification have been developed more recently, since classification based on variation of few genes that are probably under selective pressure is not suitable

for modern epidemiology. Multi-locus enzyme electrophoresis (MLEE) uses the electrophoretic mobility of various cytoplasmic enzymes to classify meningococci into clonal families with similar characteristics. A genetic typing system based upon polymorphisms in multiple house-keeping genes (Multi-locus Sequence Typing, MLST) is now the gold standard for molecular typing. This technique has shown that the majority of disease-associated isolates cluster into a minority of sequence type (ST) and has thus allowed to define hyper-virulent invasive meningococcal lineages (Maiden M. *et al*, 1998). The bacterium is surrounded by a polysaccharide capsule that is essential for pathogenicity as it confers resistance to phagocytosis and complement-mediated lysis and offers protection against environmental attacks. As a consequence, acapsulated bacteria are attenuated *in vivo* since they cannot persist in the blood. The outer membrane is composed of many proteins, some of which enable the organism to adhere to, and to invade, host cells. Pili, composed of pilin subunits, are filamentous structures essential in mediating the interaction between meningococci and both epithelial and endothelial cells, and are also associated with other phenotypes such as a high level of competence, bacterial auto-agglutination and twitching motility. Pilins are classified into class I and class II based on their differences in antigenicity (Nassif X., 1999). Opa and Opc proteins are capable of mediating adhesion to and invasion into eukaryotic cells, but this effect is visible only in the absence of a capsule (Virji M. *et al*, 1993, 1992). Outer membrane also encloses lipopolysaccharide (LPS), which helps in serum resistance and plays a key role in the pathogenesis of meningococcal disease (Vogel U. and Frosch M., 1999; McNeil G., Virji M. and Moxon ER, 1994).

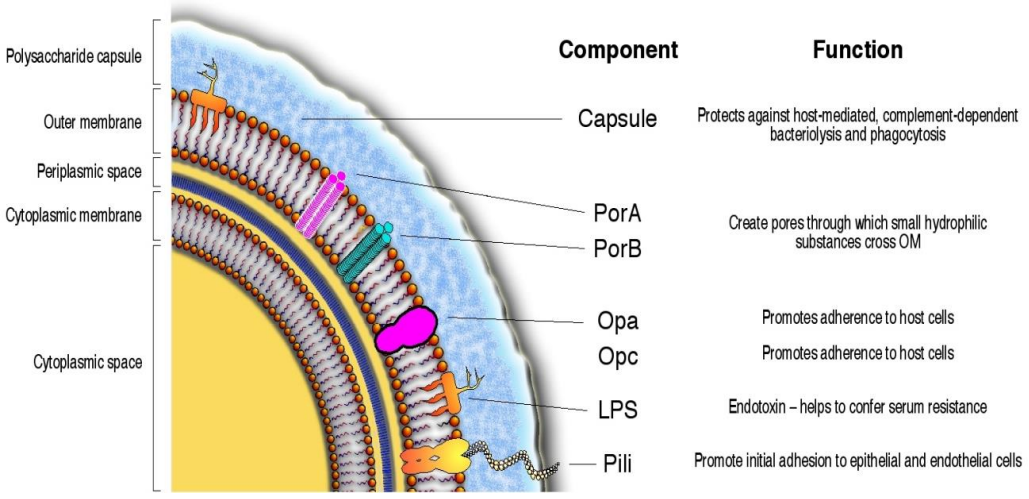


Fig. 2: Schematic representation of the different bacterial compartments and of the main components of the outer membrane, together with their known function (image from Serruto *et al* 2004).

Based on sequencing of eight genomes, the chromosome is between 2.0 and 2.1 megabases in size and contains over 2000 genes (Parkhill J., Achtman M. *et al*, 2000; Tettelin H., Saunders NJ *et al*, 2000; Schoen *et al*, 2008). The core meningococcal genome that encodes for essential metabolic functions represents about 70% of the genome. Each new strain sequenced has identified 40–50 new genes and the meningococcus shares about 90% homology at the nucleotide level with either *N. gonorrhoeae* or *N. lactamica*. Mobile genetic elements including IS elements and prophage sequences make up ~10% of the genome (Parkhill J., Achtman M. *et al*, 2000). Other than the capsule locus, no core pathogenome has been identified suggesting that virulence may be clonal group dependent or polygenic in nature. Given that transformation is an efficient mechanism of genetic exchange and that meningococci have acquired DNA from commensal *Neisseria* spp. and other bacteria (e.g. *Haemophilus*) as well as phages, the gene pool for adaptation and evolution is quite large. Genome plasticity and phenotype diversity through gain and loss of DNA or, for example, through DNA repeats, is a characteristic of meningococcal evolution. The acquisition of the capsule locus by horizontal transfer possibly from *P. multoacida* or *P. hemolytica* (Schoen C. *et al* 2008) appears to be a major event in the evolution of the pathogenicity of the meningococcus. Another characteristic of the meningococcal genome is the presence of multiple genetic switches (e.g., slipped-strand misparing, IS element movement), contributing to the expression of pathogen-associated genes (Hilse R. *et al* 1996).

2. Meningococcal disease epidemiology

Invasive meningococcal disease results from the interplay of microbial factors influencing the virulence of the organism, environmental conditions facilitating exposure and acquisition, and host susceptibility factors. In the pre-serum therapy and pre-antibiotic eras, 70– 85% of meningococcal disease cases were fatal; today, the overall mortality rate in invasive meningococcal disease still remains high, at between 10 and 15% (Sharip A. *et al* 2006). Meningococcal disease is also associated with marked morbidity including limb loss, hearing loss, cognitive dysfunction, visual impairment, educational difficulties, developmental delays, motor nerve deficits, seizure disorders, and behavioral problems (Rosenstein NE

et al 2001). Although rates of sporadic disease can reach ~5–10/100,000 population, a key characteristic of meningococcal disease are epidemic outbreaks. Seasonal epidemics (usually due to serogroup A) occur yearly in sub-Saharan Africa and cyclical pandemics have occurred there every 8–10 years for the last 100 years. During seasonal epidemics and cyclical pandemics the incidence can climb to >1/1000 population for weeks before the frequency of disease declines in the immediate outbreak area. Serogroups B, C and Y are associated with sporadic disease, case clusters and outbreaks seen in the United States, Canada, New Zealand, South America, Europe and other parts of the world (Rosenstein NE *et al* 2001). Serogroup W-135 is responsible for recent worldwide outbreaks associated with pilgrims returning from the Hajj (Aguilera JFJ *et al* 2002). The different characteristics of outbreaks are caused by hypervirulent lineages as defined by MLST. The worldwide W-135 outbreaks were caused by W-135 strains of the ST-37 clonal complex most often associated with serogroup C disease and outbreaks. The introduction of new virulent clones into a population can change the epidemiology and the clinical spectrum of meningococcal disease and the recent emergence of serogroup X meningococci in Niger (Boisier P. *et al* 2007) highlights the need for continued surveillance for new clonal complexes. Meningococcal disease has the highest incidence in infants and children aged <4 years and adolescents (Stephens DS *et al* 2007). Two-thirds of meningococcal disease in the first year of life in the US occurs in infants less than 6 months of age (Shepard CW *et al* 2003). Worldwide, the rates of meningococcal disease are also highest for young children due to waning protective maternal antibody, but in epidemic outbreaks, older children and adolescents can have high rates of disease. Even though peak incidence occurs among infants and adolescents; one-third to one-half of sporadic cases are seen in adults older than 18 years. The early stages of disease can mimic viral infections such as influenza, but the disease course may be fulminant. Thus, it can be difficult to identify and treat the disease quickly. Rapid progression of the disease from bacteremia and/or meningitis to life-threatening septic shock syndrome or meningitis can occur within the first few hours after initial symptoms appear. Because of these parameters, prevention through vaccination is the best option for the control of this disease in a community. While significant progress is being made in understanding meningococcal pathogenesis and in new meningococcal vaccines and vaccine strategies, challenges remain (Stephens DS *et al* 2009).

3. Anti-meningococcal vaccines

Different meningococcal vaccines have been developed against the distinct serogroups (reviewed in Zahlanie YC *et al* 2014).

There are a number of polysaccharide and conjugate meningococcal vaccines in use against serogroups A, C, Y and W135. The tetravalent vaccine composed of purified capsular polysaccharides, although efficacious in adolescent and adults, is poorly immunogenic in infants and fails to induce immunological memory (Joshi VS, et al. 2009).

Conjugate vaccines against meningococcus are available as monovalent (A or C) or as different tetravalent formulations (A, C, W-135 and Y). Conjugate vaccines against meningococcal serogroups A and C have been developed and tested in clinical trials (Costantino P. *et al*, 1992; Anderson EL *et al*, 1994; Fairley CK *et al*, 1996; Lieberman JM *et al*, 1996). The first trials conducted in the United Kingdom with the meningococcus C conjugate showed a dramatic decline in the incidence of serogroup C disease in all age groups that received the vaccine (Borrow R. *et al*, 2000; Miller E., Salisbury D. and Ramsay ME, 2001) with an efficacy of 97 and 92 per cent for teenagers and toddlers, respectively (Ramsay ME *et al*, 2001).

The most critical target for vaccination is meningococcus B, which is responsible for 32 per cent of all cases of meningococcal disease in the United States and for 45–80 per cent or more of the cases in Europe (Scholten RJ *et al*, 1993), and for which conventional biochemical and microbiological approaches have so far failed to produce a vaccine able to induce broad protection. A polysaccharide-based vaccine approach could not be used for group B meningococcus because the MenB capsular polysaccharide is a polymer of *Æ*(2-8)-linked N-acetylneuranimic acid that is also present in mammalian tissues. This means that it is almost completely non-immunogenic as it is recognized as a self-antigen. Attempts to break tolerance to induce immunity to this polysaccharide are likely to lead to autoimmunity (Hayrinen *et al*, 1995; Finne *et al*, 1987). An alternative approach to MenB vaccine development is based on the use of surface-exposed proteins contained in outer membrane preparations (outer membrane vesicles, OMVs). The first OMV vaccines were developed in Norway and Cuba and showed efficacy in humans ranging from 50 to 80 per cent (Tappero JW *et al*, 1999). However, while each vaccine was shown to induce good protection against the homologous strain, it failed to induce protection against heterologous strains (Rosenstein, Fischer and Tappero, 2001). The major protective antigen in both these vaccines is PorA, the most abundant outer membrane protein, which is known to be highly variable across different isolates of serogroup B *N. meningitidis*.

To overcome these limitations, a novel multicomponent recombinant protein-based vaccine, named 4CMenB or Bexsero®, has been recently developed against capsular group B meningococcal strains and approved in Europe and Australia.

4CMenB is based on novel antigens identified through an innovative genetic approach termed reverse vaccinology and combines OMVs from the New-Zealand epidemic strain (NZ98/254) with three major protein antigens: factor H-binding protein (fHbp), Neisserial Heparin-Binding Antigen (NHBA) and Neisserial adhesin A (NadA) (Pizza M and Scarlato V. 2000, Giuliani MM et al 2006). Two of these main antigens, fHpb (sub-variant 1.1) and NHBA (peptide 2), are present as fusion proteins to two minor antigens, GNA2091 and GNA1030, respectively.

Another vaccine against serogroup B, developed by Pfizer, is in phase III clinical trials and contains two alleles of the recombinant fHbp (Anderson AS *et al* 2012).

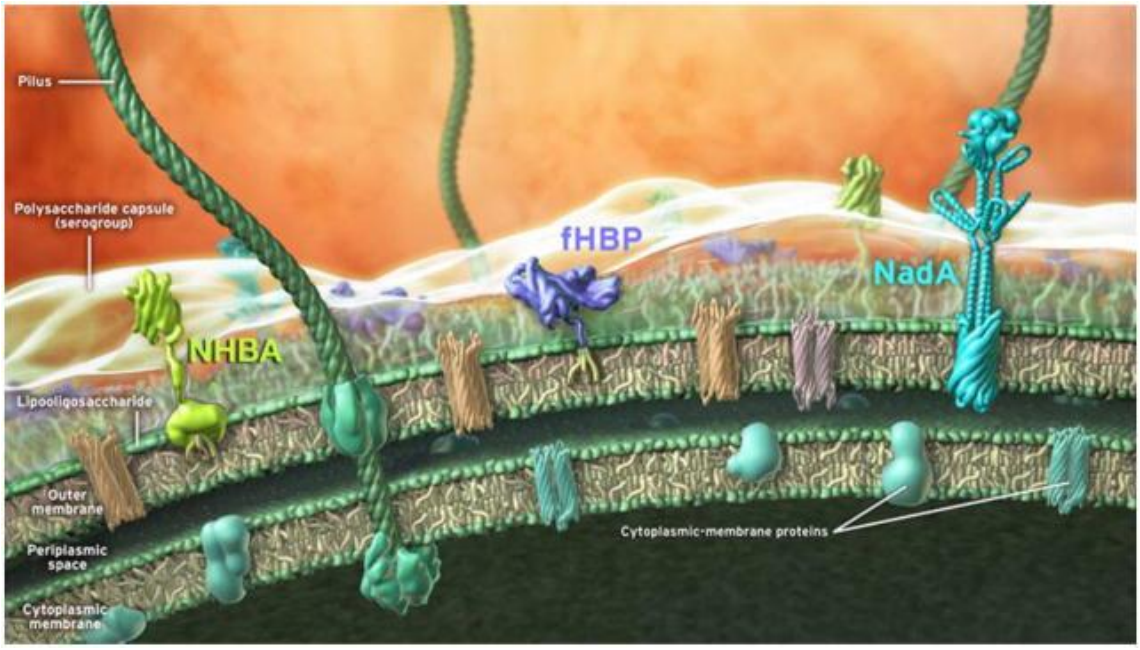


Fig.3: Schematic representation of the 4CMenB vaccine antigens on the surface of *N. meningitidis* (Serruto D. *et al* 2012). The different bacterial compartments (outer membrane, periplasmic space, cytoplasmic membrane) and the main antigens identified through reverse vaccinology approach (NHBA, fHbp and NadA) are depicted. Other components of the meningococcal membranes are also shown (pilus, polysaccharide capsule, lipooligosaccharide and integral inner and outer membrane proteins).

4. Colonization and carriage

Colonization of the upper respiratory mucosal tract by *N.meningitidis* is the first step in establishment of carriage and invasive meningococcal disease. *N. meningitidis* may be acquired through the inhalation of respiratory droplets and secretion. Acquisition is generally asymptomatic, but infrequently may result in local inflammation, invasion of mucosal surfaces, access to the bloodstream and fulminant sepsis or focal infections such as meningitis (Stephens DS *et al* 2007). Meningococcal disease usually occurs 1–14 days after acquisition of the pathogen (Rosenstein NE et al 2001). Acquisition may also result in upper respiratory and pharyngeal meningococcal carriage. The duration of carriage can vary from days to months. The probability of meningococcal disease after the acquisition of *N. meningitidis* declines very sharply, such that invasive disease becomes unlikely 10–14 days after acquisition (Stephens DS *et al* 2009). From an evolutionary perspective, the interactions of meningococci and the human nasopharynx are key events. Meningococcal carriage and transmission, not disease, determine the global variation and composition of the natural population of meningococci.

5. Meningococcal adhesion and cell invasion

Adhesion to the respiratory epithelium is regarded as the primary and principal event in colonization of the nasopharynx by *N. meningitidis*. This is essential for bacterial survival, colonization and transmission, and is also a prerequisite for invasive meningococcal disease. Upon contact with human cells, the meningococcus forms microcolonies and adheres using filamentous structures named type IV pili (T4P), forming a layer tightly attached to host cells (Nassif, X. *et al* 1997).

N. meningitidis has evolved numerous surface-exposed adhesive structures that facilitate interactions with human cells. Bacterial host-specificity resides in the structural specificity of the meningococcal ligands for human molecules, which can range from nutrients, to serum/secreted proteins and surface-located adhesion receptors. Mechanisms of meningococcal adhesion are multifactorial, dynamic and display temporal changes during the course of infection. After the initial colonization, there is a loss or down-regulation of the capsule, which sterically masks the outer membrane proteins. This event can occur both via regulatory system upon cell contact (Deghmane AE, Giorgini D. *et al* 2002), and/or by the selection of low or no-capsule expressing bacteria, due to phase variation (Hammerschmidt S., Muller A. *et al* 1996). Adhesion to the human epithelium is indeed mediated by a variety of possible redundant adhesins, previously masked by the capsule. Antigenic and phase variation of meningococcal

outer membrane proteins (OMPs) also play a very important role, and the presence of multiple adhesins compensates for phase variation and may lead to an altered tissue tropism. Close adherence of meningococci to the host epithelial cells results in the appearance of cortical plaques and the recruitment of factors leading to the formation and extension of epithelial cell pseudopodia that engulf the bacteria (Stephens DS 2009). This intracellular lifestyle can give the bacteria the opportunity to evade host immune response, find more available nutrients and is also a way to further cross the epithelium and enter the blood stream (Stephens DS 2009). Intracellular meningococci reside inside membranous vacuoles and are capable of translocating through basolateral epithelial tissues by transcytosis within 18-40 hours.

Within the cells, meningococcus has to express again the capsule, which can prevent antibody and complement deposition (Achtman M. 1995), is anti-opsonic and anti-phagocytic and therefore aids survival in blood (Virji M. 2009). Once access to the bloodstream is obtained, meningococci may multiply rapidly to high levels and may also translocate across the blood-brain barrier, proliferate in the central nervous system and cause meningitis. These later steps in invasion of the blood stream and the possible subsequent crossing of the blood-brain barrier are still poorly understood. The ability to cause invasive disease is also multifactorial and depends on environmental factors, microbial virulence factors and lack of a protective immune response.

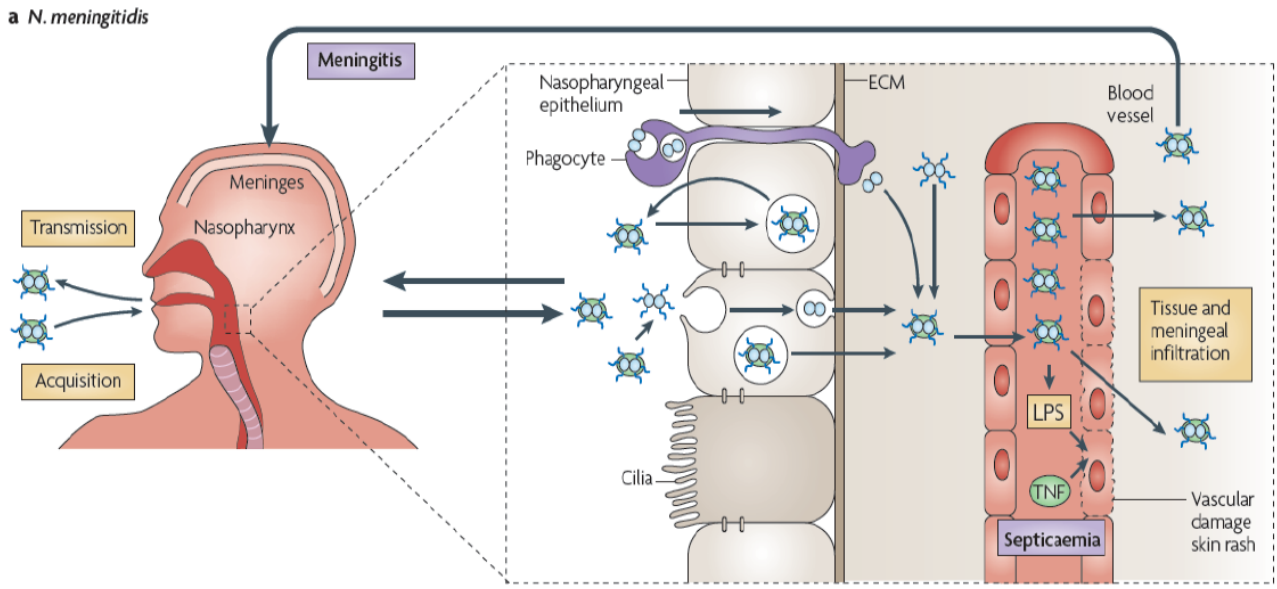


Fig. 4: Stages in the pathogenesis of *N. meningitidis* (image from Virji M. 2009). *N. meningitidis* may be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-

ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells briefly before migrating back to the apical surfaces of the cells for transmission to a new host. Besides transcytosis, *N. meningitidis* can cross the epithelium either directly following damage to the monolayer integrity or through phagocytes in a 'Trojan horse' manner. In susceptible individuals, once inside the blood, *N. meningitidis* may survive, multiply rapidly and disseminate throughout the body and the brain. Meningococcal passage across the brain vascular endothelium (or the epithelium of the choroid plexus) may then occur, resulting in infection of the meninges and the cerebrospinal fluid (Nassif X. et al 1999).

6. Adhesins and other important virulence factors

Several adhesins may be present simultaneously and often cooperate to increase avidity of binding, necessary for the bacterial internalization within host cells. Meningococcal adhesins that enable bacteria to localize on specific host cells can be divided into major and minor ones (Hill DJ, Virji M. 2012, Hill DJ *et al* 2010).

The major adhesive molecules of *N. meningitidis* are the outer membrane opacity proteins, Opa and Opc, and pili. Opa and Opc proteins are integral outer membrane proteins which can be partially or totally masked by the capsule and LPS sialic acids, whereas type IV pili are able to traverse the capsule and are generally considered the most important adhesins in the initial attachment to many host cells. Moreover, pili often act synergistically with the major outer membrane proteins. Both Opa and Opc are able to bind to serum and extracellular matrix components. In particular, Opa proteins may bind to CEACAMs and heparan sulphate proteoglycans (HSPGs), and Opc proteins can interact with integrin receptors, trough the binding of fibronectin and activated vitronectin (Sa E Cunha C. *et al* 2010).

Other minor surface-exposed proteins have been implicated in adhesion such as NadA, Apc, NspA, NhhA, App, MspA. NhhA has been shown to interact with HSPGs and laminin (Sjölinder H. *et al* 2008), NadA to bind to beta-1 integrins and extracellular HSP90 (Nägele V. *et al* 2011, Cecchini P. *et al* 2011), while the receptors targeted by the other factors have not been identified yet. Moreover, whether *in vivo* they act in concert or independently from the major adhesins remains to be determined.

Major meningococcal contributors to the invasive meningococcal disease include: capsular polysaccharide, other surface structures such as pili, OMPs (e.g. PorA, PorB, Opa, Opc), lipooligosaccharide (LOS) and genotype. Resistance to complement-mediated lysis and phagocytosis is determined by the expression of the capsule and lipooligosaccharide (Kahler CM *et al* 1998). Meningococcal endotoxin released in blebs also plays a major role in the inflammatory events of

meningococcemia and meningococcal meningitis (Takahashi H. *et al* 2008). LOS plays a role in the adherence of the meningococcus (Takahashi H. *et al* 2008) and activation of the innate immune system. Severity of meningococcal sepsis has been correlated with circulating levels of meningococcal LOS (Parkhill J. *et al* 2000). In conclusion, multiple meningococcal invasion virulence factors influence invasive disease and some are the focus of new vaccines (Holst J *et al* 2005).

7. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and HSPGs

Cell surface adhesins that mediate microbial adherence to various components of the extracellular matrix of host tissues are called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules, Patti JM *et al* 1994). To be classified as a MSCRAMM, the molecule must be localized on the bacterial surface, recognize a macromolecular ligand that can be found within the ECM and this interaction should be of high affinity and exhibit a high degree of specificity. A single MSCRAMM can also bind several ECM ligands. In addition, a microorganism can express several MSCRAMMs that recognize the same matrix molecule (Patti JM *et al* 1994).

Between epithelial and endothelial cells, the meningococcus encounters and crosses the ECM in order to adhere and invade target cells. Fibronectin, collagen types I, II and V have been shown to be the ECM components that interact with adherent bacteria. It was also discovered that bacterial binding to ECM components occurs regardless of the Opa and Opc protein expression.

Studies on several bacterial adhesins that bind to ECM proteins have shown that proteoglycans (PGs) may also be important since many ECM proteins bind to heparin or heparan sulfate proteoglycans (HSPGs, Rostand KS and Esko JD 1997).

HSPGs are ubiquitously expressed on the cell surface of adherent cells and in the ECM. They are composed of one or several heparan sulfate (HS) glycosaminoglycan chains covalently attached to specific core proteins. Many viruses, bacteria and parasites express adhesins that bind to cell surface HSPGs to facilitate their initial attachment and subsequent cellular entry (Rostand KS and Esko JD 1997). The interaction of specific adhesins with carbohydrate chains on the glycoconjugate enables the microbes to take their first step towards establishing an infection. In pathogens, mutant strains lacking HS adhesins are viable and show normal growth rates, suggesting that the capacity of interaction with HSPGs is strictly a virulence activity (Barlett AH and Park PW 2011).

Cell surface HSPGs mainly function as initial low-affinity co-receptors for HS-binding ligands, serving as a scaffold that localizes the ligands to the cell surface, enabling them to interact more efficiently

with their respective signaling receptors. Thus, they concentrate pathogens on host cells surfaces, increasing binding to specific secondary receptors. For several pathogens they can also act as direct internalization receptors (Barlett AH and Park PW 2011). For targeting glycosaminoglycans (GAGs), bacterial ligands must have defined clustered sequences of basic aminoacids (Patti JM *et al* 1994).

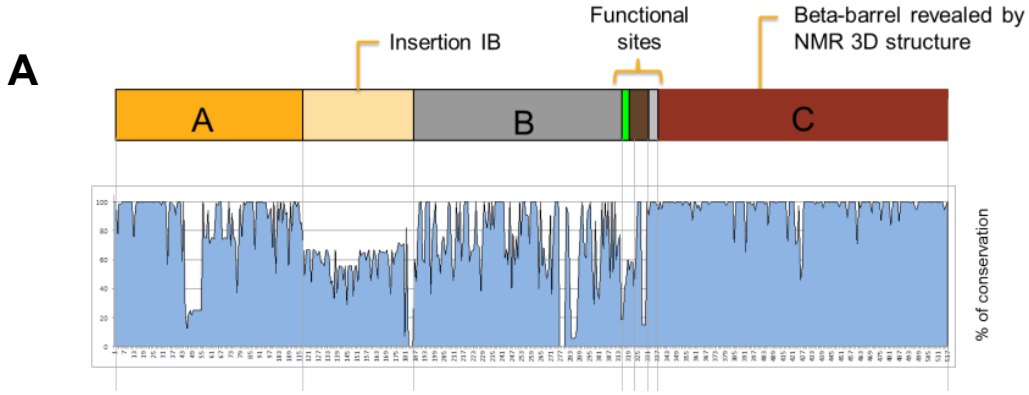
8. NHBA: Neisserial Heparin Binding Antigen

NHBA (Neisserial Heparin Binding Antigen, or GNA2132), is a surface-exposed lipoprotein and it is able to induce antigen-specific bactericidal antibodies in animals and humans immunized with 4CMenB (Serruto D. *et al* 2010, Giuliani MM *et al* 2010, Welsch JA *et al* 2003).

The protein is specific for *Neisseria* species and the gene is ubiquitous in all neisserial group B strains examined thus far (Bambini S. *et al* 2009; Jacobsson S *et al* 2006; Lucidarme J. *et al* 2009; Lucidarme J. *et al* 2010, Muzzi A. *et al* 2013). The NHBA-encoding gene is also found in *N. gonorrhoeae* and the commensal species *N. lactamica*, *N. polysaccharea*, and *N. flavescens* and with the exception of *N. gonorrhoeae* it is highly conserved among them (Muzzi A. *et al* 2013).

Gene sequences from genetically diverse group B strains reveal the existence of a number of more than 400 peptides that have some association with clonal complexes and sequence types (Muzzi A. *et al* 2013, Bambini S. *et al* 2009). Variable segments of NHBA are present at the level of the primary aminoacid sequence. In particular, most variability is observed at the level of the amino-terminal region, annotated as intrinsically unfolded by commonly used structure prediction algorithms, while the carboxyl-terminal region, which is represented by a single 8-stranded anti-parallel beta-barrel structure, is highly conserved (Esposito V. *et al* 2011).

Protein length can span from approximately 430 to 500 residues, depending on the different peptide expressed by each strain.



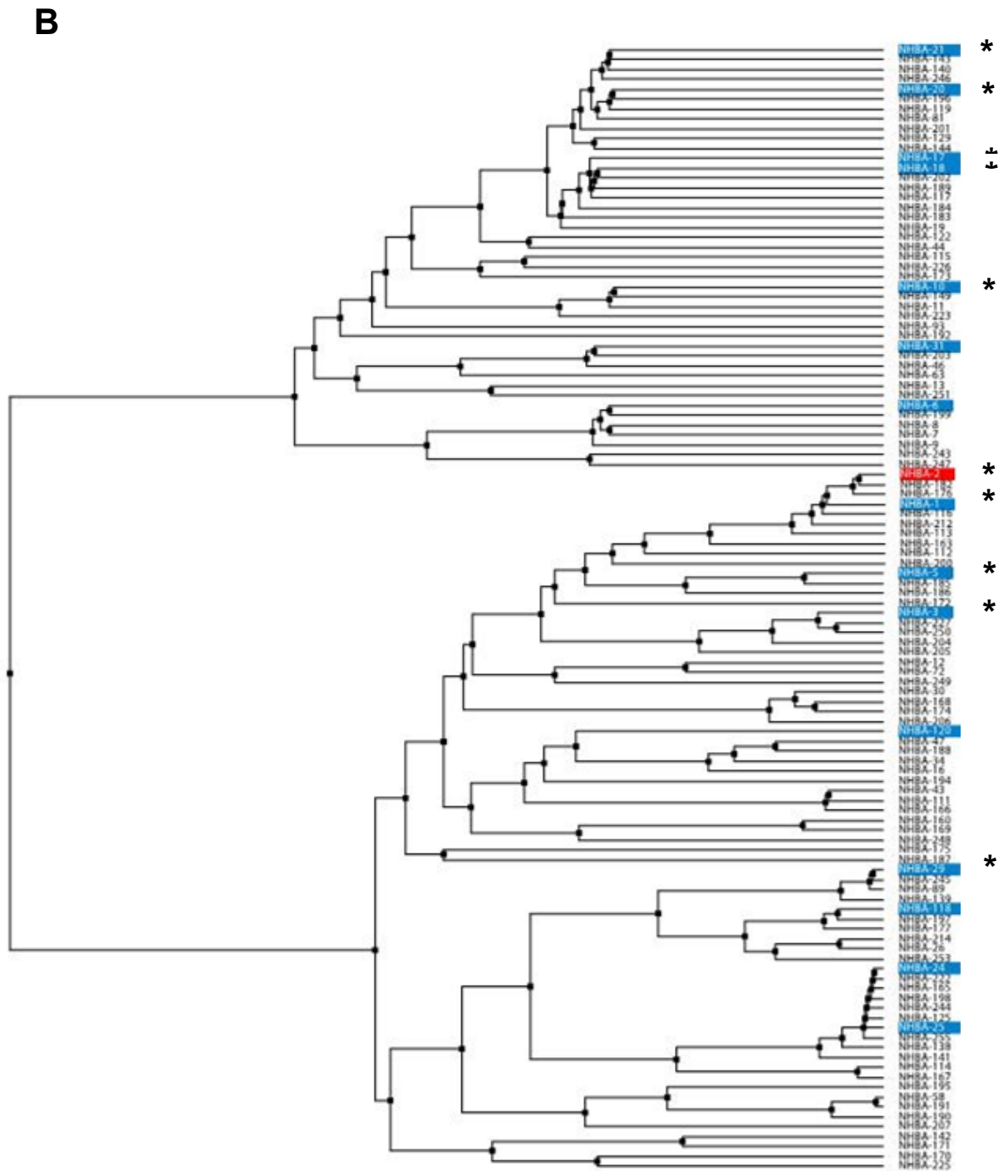


Fig. 5: NHBA variability and modular structural organization. A) NHBA protein sequence reflects a modular structural organization, where it is possible to recognize 3 main domains (A, B and C). Highlighted is the presence of an insertion sequence of 60 amino acids, present only in some of the NHBA peptides (Insertion IB). Functional sites are represented by the Arg-rich region (in brown), by the NalP cleavage site (in green) and by the human lactoferrin cleavage site (in grey). The C-term of the protein, corresponding to module C is highly

conserved and is represented by a beta-barrel structure. The lower graph shows the percentage of amino acid conservation between the different peptides along the protein sequence. B) Phylogenetic tree showing all different NHBA peptides identified so far. Highlighted in blue are the most frequent peptides and in red the peptide NHBA-2 present in the vaccine formulation. Asterisks indicate those peptides that will be analysed in this study.

The specific genotype of NHBA included in the multicomponent MenB vaccine is peptide 2. Based on molecular epidemiology studies recently conducted in different countries, this is the one most frequently found in Europe and was identified in 24.7% of strains circulating in Europe (Vogel U. *et al* 2013). Peptides 21, 2 and 17 were found in almost half of the European isolates, while peptides 3 and 20 are the ones most commonly found in France, Germany and Italy and peptide 5 is the most frequent in US (Wang X. *et al 2011*).

Serum antibodies from mice immunized with recombinant NHBA are able to bind to the surface of diverse *N. meningitidis* strains and elicit deposition of human C3b on the bacterial surface and complement-mediated bactericidal activity (Pizza M. *et al* 2000, Giuliani MM *et al* 2006, Welsch JA *et al* 2003). Moreover, anti-NHBA antibodies elicited and protected infant rats against meningococcal bacteremia in a passive protection model (Welsch JA *et al* 2003). NHBA is recognized by sera of patients after meningococcal disease (Serruto D. *et al* 2010) and previous studies reported that antibodies against NHBA may confer protection in humans based on opsono-phagocytic immunity and passive protection in an *ex vivo* model of meningococcal bacteremia (Plested JS and Granoff DM 2008; Plested JS *et al* 2009).

Recently it was also demonstrated that a cooperative serum bactericidal activity exists between human antibodies against fHbp and NHBA (Vu DM *et al* 2011).

Although preclinical studies suggest that antibodies elicited against the vaccine peptide are cross-protective against meningococcal strains expressing different NHBA peptides (Giuliani MM *et al* 2006), the impact of sequence diversity of NHBA in relation to vaccine coverage is yet to be established.

NHBA has been shown to bind to heparin *in vitro* through an arginine-rich region and this property correlates with increased survival of the unencapsulated bacterium in human serum (Serruto D. *et al*, 2010). The Arg-rich region appears to be extremely conserved among different NHBA peptides, underlying its importance in bacterial pathophysiology. Two proteases, the meningococcal NalP and the human lactoferrin are able to cleave the protein upstream and downstream of the Arg-rich region

respectively, releasing two different fragments (Serruto D. *et al*, 2010). NHBA has been associated to biofilm formation and NalP cleavage has been shown to negatively regulate this process (Arenas J. *et al* 2012). More recently, it was also demonstrated that the C-terminal fragment (C2), released upon NalP proteolysis, alters endothelial cell permeability by inducing the internalization of the adherens junction protein VE-cadherin, which is in turn responsible for the endothelial leakage. Thus, the NHBA-derived fragment C2 might contribute to the extensive vascular leakage typically associated with meningococcal sepsis (Casellato A. *et al* 2013).

9. NalP

NalP is an autotransporter with subtilisin-like serine protease activity that is involved in autoproteolytic processing, resulting in secretion of the NalP passenger domain into the bacterial supernatant (Turner DP *et al* 2002, Van Ulsen P. *et al* 2003). The expression of NalP is phase-variable because of slipped-strand mispairing of a poly-cytidine tract in the coding sequence (Saunders NJ. *et al* 2000). NalP contains a lipobox at the C-terminal end of the signal sequence; the lipid moiety permits anchorage in the outer membrane (Van Ulsen P. *et al* 2003). Lipidated NalP proteins are only temporarily retained at the cell surface; however, the lipid moiety retards the release of the NalP passenger domain from the bacterial surface and allows the partial or total cleavage of surface protein targets on bacterial surface (Roussel-Jazédé V. *et al* 2013), including IgA protease, adhesion and penetration protein App (Van Ulsen P, *et al* 2003), autotransporter serine protease AusI (Van Ulsen P. *et al* 2006), *Neisserial* heparin binding antigen NHBA (Serruto D. *et al* 2010), and lactoferrin binding protein LbpB (Roussel-Jazédé V. *et al* 2010). This activity modulates the expression of meningococcal proteins at bacterial surface and also has recently been implicated in the formation and regulation of Nm biofilm (Arenas J. *et al* 2013). Recently, it was demonstrated that it is able to cleave human C3, the central component of the complement cascade, facilitating C3b degradation and increasing the survival of Nm in human serum (Del Tordello E. *et al* 2014).

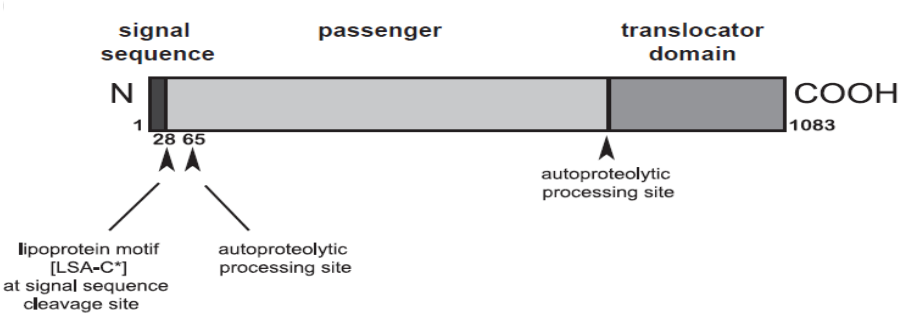


Fig.6: Schematic illustration of NalP protein sequence, divided in its main domains. The passenger domain contains the catalytic domain and at the C-term of the protein the translocator domain is shown.

Aims

In this study we aimed to evaluate the contribution of NHBA amino acid sequence variability to cross-protection and immunogenicity, and also to identify the immunogenic regions of this protein antigen.
Moreover we aimed at investigating other unknown biological functions of the NHBA protein, focusing on its hypothetical novel role as minor meningococcal adhesin.
Finally, for its possible immunogenic and functional implications, we wanted to analyse NHBA NalP-mediated cleavage in different NHBA peptides.

Materials and Methods

Bacterial strains and growth conditions

The strain 5/99 is a serogroup B clinical isolate, isolated in Norway in 1999. It belongs to cc8 (ST-1349) and has the following typing: B:2b:P1.5,2. The strain M10713 is a serogroup B clinical isolate, isolated in USA in 2003. It belongs to cc41/44 (ST-136) and has the following typing: B:P1.17, 16-3.

The strain UK013 is a serogroup B clinical isolate, isolated in United Kingdom in 1999. It belongs to cc11 (ST-269) and has the following typing: B:ND: P1.22,9.

The strain 8047 is a serogroup B clinical isolate, isolated in USA in 1978. It belongs to cc11 and has the following typing: B: 2: P1.2.

The strain NGH38 is a serogroup B clinical isolate, isolated in Norway in 1988. It belongs to ST-36 and has the following typing: B: ND: P1.3.

The strain M14933 is a serogroup B clinical isolate, isolated in USA in 2006. It belongs to cc32 and has the following typing: B:ND:P1.22-1,14.

Nm strains were routinely grown on GC agar (DifcoTM) or Mueller Hinton (MH) agar (DifcoTM) at 37◦C+5% CO2. When required, erythromycin, kanamycin and chloramphenicol were added to achieve a final concentration of 5 μg/ml, 100 μg/ml and 5 μg/ml, respectively.

Escherichia coli strain DH5α (Invitrogen) was cultured at 37°C in Luria Bertani (LB) agar or LB broth at 37°C and when required ampicillin, kanamycin, erythromycin and clormaphenicol were added at a final concentrations of 100 μg/ml, 25 μg/ml, 5 μg/ml, and 20 μg/ml respectively.

DNA manipulation and construction of plasmids

Constructs and recombinant strains used in this study are listed in Table 1S. Oligonucleotides used for DNA manipulation are reported in Table 2S. Forward and reverse primers to amplify the *nhba* genes for complementing strains were designed on the basis of a DNA alignment of *nhba* sub-variant genes. Forward primers include a AseI restriction site and reverse primers contain a NsiI restriction site. The sub-variants were amplified using genomic DNA from the Nm strains indicated in Table 1S as template. PCRs were performed on genomic DNA using Phusion High-Fidelity Taq DNA Polymerase (Finnzymes). Each PCR product was digested with AseI and NsiI enzymes, and cloned into the NdeI/NsiI sites of the pCom-PInd vector (Serruto *et al* 2010). Resulting plasmids were checked by sequencing.

For the generation of the chimeric *nhba* genes, several fusion PCRs were performed in order to generate each insert.

The insert for strain p2 Short was prepared by fusing 2 PCR products using *nhba* p2 as template and two primer pairs: p2A F with p2A R, and p2C F with p2C R.

The insert for strain p18 Long was prepared by fusing 3 PCR products using *nhba* p18 as template and three primer pairs: p18A F with p18A R; p18B F with p18B R, and p18C F with p18C R.

The insert for strain CH-G1 was prepared by fusing 2 PCR products: the first generated using primers p2A F and p2B R and *nhba* p2 as template; the second using primers p18C F and p18 R and *nhba* p18 gene as template.

The insert for strain CH-G3 was prepared by fusing 3 PCR products: the first generated using primers p18A F with p2-238 R and *nhba* p18 long as template; the second using primers p2-238 F and p2-301 R and *nhba* p2 gene as template, and the last one using primers p18-301 F with p18 C R and *nhba* p18 gene as template.

The insert for strain CH-G4 was prepared by fusing 3 PCR products: the first generated using primers p18A F with p18A R and *nhba* p18 as template; the second using primers p2-B F and p2-301 R and *nhba* p2 gene as template, and the last one using primers p18-301 F with p18 C R and *nhba* p18 gene as template.

The insert for strain CH-C1 was prepared by fusing 2 PCR products: the first generated using primers p18A F and p18A R and *nhba* p18 as template; the second using primers p2B F and p2C R and *nhba* p2 gene as template.

The insert for strain CH-C2 was prepared by fusing 2 PCR products: the first generated using primers p18A F and p18-301 R and *nhba* p18 long as template; the second using primers p2-301 F and p2C R and *nhba* p2 gene as template.

Also for the preparation of these chimeric inserts 5' Forward primers include a AseI restriction site and 3' reverse primers contain a NsiI restriction site to allow cloning into the NdeI/NsiI sites of the pCom-PInd vector.

Plasmid DNA to be used for Nm transformation was linearized using SpeI. The plasmid pBSUDgna2132erm was constructed to generate the *nhba* isogenic mutants (Δ *nhba* strains; Serruto *et al* 2010) and linearized with ApaI.

Constructions of Nm knock-out and complemented strains

For transformation by naturally competent Nm, ten to fifteen single colonies of a freshly grown overnight culture were suspended in 100 µl of PBS, spotted onto GC agar plates to which 5–10 µg of linearized plasmid DNA was added, allowed to dry, and incubated for 6–8 h at 37°C. Transformants were then selected on plates containing erythromycin (5µg/ml), kanamycin (100µg/ml), and/or chloramphenicol (5µg/ml), and single colonies were passaged on selective media for further analysis. Single colonies were resuspended in 100µl of PBS, placed in a boiling water bath for 10 min, and centrifuged in a bench top centrifuge for 5min at 8000×g. Two microliters of the sample was used as a template for PCR analysis in order to evaluate the correct double homologous recombination event resulting in the knock out of the gene or in the insertion of the complementation cassette.

The *nhba* gene to be complemented was inserted in the intergenic region between the nmb1428 and nmb1429 genes and the recombination event occurs between the upstream and downstream region of this locus, allowing the insertion of a chloramphenicol resistance cassette (used for the screening of the positive colonies) and the gene of interest. The primers used were designed to amplify the downstream region of the nmb1428 gene locus (PCR1) and the different *nhba* genes plus the upstream region of nmb1429 gene locus (PCR2).

Total cell extract preparation and Western blotting

Nm strains were grown overnight on chocolate agar plates at 37°C in 5% CO2. Colonies from each strain were collected and used to inoculate 6 ml Mueller-Hinton broth, containing 0.25% glucose to an initial OD_{600} of 0.05–0.06. For the complemented strains, 0.1mM IPTG was added to the culture to allow NHBA protein expression. The culture was incubated at 37°C with shaking until an OD600 0.5 was reached, and then centrifuged for 10 min at 8000×g. The supernatant was discarded and the pellet was resuspended in 50µl of PBS.

Proteins were then separated by SDS/PAGE electrophoresis using the NuPAGE Gel System (Invitrogen) and transferred onto nitrocellulose membranes for Western blotting analysis. Western blots were performed according to standard procedures. The different forms of NHBA were identified with polyclonal mouse antisera raised against the recombinant NHBA protein (diluted 1:1,000) and an anti-mouse antiserum conjugated to horseradish peroxidase (Dako) as secondary antibody. Bands were visualized with Super Signal West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's instructions.

Polyclonal antibodies used in this study

To prepare mice antisera, 20μg of individual antigens NadA, NHBA-GNA1030, GNA2091-fHbp or a combination of 20μg of each of antigen with or without 10μg of OMV derived from strain NZ98/254 were used to immunize 6-week-old CD1 female mice (Charles River). Five to ten mice per group were used. The antigens were administered intraperitoneally together with aluminium hydroxide (3 mg/ml) on days 0, 21 and 35.

FACS analysis

The ability of polyclonal sera raised against NHBA to bind to the surface of live meningococci was determined using FACS-Scan flow cytometer with 5/99 wild-type and all recombinant strains generated. Antibody binding was detected using a secondary antibody anti-mouse (whole molecule) FITC-conjugated (Sigma).

Serum bactericidal assay with human sera

SBAs were performed as described by Borrow *et al*. with minor modifications (Borrow R. *et al* 2005). Human plasma obtained from volunteer donors under informed consent was selected for use as complement source with a particular MenB strain only if it did not significantly reduce CFU of that strain relative to T0 when added to the assay at a final concentration of 50%. The final assay mixture contained 25% human plasma. The activity of plasma complement was reconstituted by the addition of divalent cations and heparin immediately before use.

Human serum samples

Healthy human volunteers were immunized under informed consent with the experimental serogroup B vaccine containing 50 μg each of GNA2091-fHbp, NHBA-GNA1030, and NadA, and plus or minus 25 μg of Outer Membrane Vesicles from the New Zealand strain, NZ98/254 and adsorbed to aluminum hydroxide. Sera samples before and after immunization were obtained from the following clinical trials:
Study 1 (V72P10) was a Phase 2b/3 clinical trial conducted in healthy adolescents evaluating safety, immunogenicity, and schedule finding. Two pooled sera were derived from 13 subjects before and after 2 doses of 4CMenB vaccine at 0 and 2 months.
Study 2 (V72P12) is Phase 2b clinical study evaluating the safety, tolerability and immunogenicity of 4CMenB administered to infants at 2, 4 and 6 months of age or and 3, 5 and 7 months of age.

Extensions of this clinical study (V72P12E1) investigated a fourth (booster) dose at 12, 18 or 24 months of age.

Two pooled sera were derived from 109 and 40 subjects who received the primary series of 3 doses of routine vaccine (V72P12) at 2 months of age and two pooled sera were derived from 69 and 40 infants who received the primary series of 3 doses of 4CMenB plus a booster in the second year of life.

Study 3 (V72P13) is Phase 3 clinical study evaluating the safety, tolerability and immunogenicity of 4CMenB administered to infants at 2, 4 and 6 months of age. Extensions of this clinical study (V72P13E1) investigated a fourth (booster) dose at 12 months of age.

Two pooled sera were derived from 180 and 40 subjects who received the primary series of 3 doses of routine vaccine (V72P13) at 2 months of age and 4 pooled sera were derived from 27, 20, 40 and 27 infants who received the primary series of 3 doses of 4CMenB plus a booster at 12 months of age.

Cell culture

HEC-1B (human endometrial cells, from ATCC® HTB113™) were maintained in Modified Eagle Medium (MEM, Invitrogen®) supplemented with 150 mM L-glutamine, non-essential amino acids, sodium-pyruvate, antibiotics and 10% heat-inactivated FBS (Gibco®).

CHO-K1 (*ATCC*® CCL-61) and CHO pgsA-745 (*ATCC*® CRL-2245) were maintained in 45% Dulbecco's Modified Eagle Medium (DMEM, Invitrogen®) and 45% F-12 medium (Invitrogen®) supplemented with antibiotics and 10% heat-inactivated FBS (Gibco®).

Cells were grown at 37°C with 5% CO2.

Binding with recombinant protein and immunofluorescence analyis

Hec-1B, CHO cells or CHO pgsA-745 epithelial cells were seeded on 8-well chamber slides coated with collagen I (BD BioCoat) and cultured for 2 days.

Cells were then incubated with 0.1 mg/ml of recombinant NHBA (diluted in MEM + 1%FBS) for 90 minutes at 37°C, or for 1 hour at 4°C. After washing to remove unbound protein, cells were fixed with 3.7% paraformaldehyde. Samples were washed extensively and incubated for 30 minutes with 3% BSA. Samples were then stained with anti-heparan sulfate antibody (H18 90, US biological, 1:100) and anti-NHBA polyclonal rabbit sera (1:100) for 1 h at room temperature. After two washes, samples were incubated for 30 minutes at room temperature with Alexa Fluor 568 goat anti-mouse IgG (1:500), Alexa Fluor 488 anti-rabbit (1:500) and Alexa Fluor 647- conjugated phalloidin (1:200) (Molecular Probes).

Glass coverslips were mounted with ProLong® Gold antifade reagent with DAPI and analysed with Zeiss LSM710 confocal microscope.

Heparinase and choindroitinase treatment

Hec-1B cells were seeded on 8-well chamber slides coated with collagen I (BD BioCoat) and cultured for 2 days. Cells were then incubated with either with heparinase III (Sigma) at 4 U/ml, or with 0.25 U/ml choindroitinase ABC (Sigma) dissolved in MEM + 1%FBS at 37 ◦C for 1.5 h and washed once in PBS, before proceeding with the binding of the recombinant protein. Choindroitin- sulfate was stained with anti-choindroitin sulfate mouse monoclonal antibody (clone CS-56, Sigma, 1:100).

Adhesion assay

Hec-1B cells (1,5 x 10^4) were seeded in a 24-well tissue culture plate (NUNC) and cultured for 48 hours in complete medium. Before the infection, cells were washed with MEM containing 1% (vol/vol) FCS (infection medium) and infected with a bacterial suspension at a multiplicity of infection (MOI) of approximately 1:100. After 3 h of incubation (at 37°C with 5% CO_2), the monolayers were washed gently 4 times with infection medium and incubated for 15 minutes at 37°C with 200 µl of 1% saponin (Sigma-Aldrich, United Kingdom). After incubation, viable counts of bacteria were made on GC agar plates.

For inhibition experiments, 10^7 bacteria were pre-incubated with serial dilutions of anti-NHBA mouse polyclonal antibodies, or pre-immune sera as negative control, for 1h at 37°C in an Eppendorf tube. Bacteria were then washed with PBS, and added to the cells as in a normal adhesion experiment.

SUPPLEMENTARY MATERIAL

Table S1: Strains and plasmids used in this study

Name	Relevant characteristics	Reference or source
Strains		
Neisseria meningitidis		
5/99ΔnadAΔ2132	nadA, gna2132 null mutant of 5/99, EryR, KanR	Serruto et al 2010
5/99ΔnadAΔ2132C2132 p1	Complemented gna2132 mutant expressing the gna2132 gene from M11822 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p2	Complemented gna2132 mutant expressing the gna2132 gene from M10837 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p3	Complemented gna2132 mutant expressing the gna2132 gene from M14933 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p5	Complemented gna2132 mutant expressing the gna2132 gene from M2934 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p10	Complemented gna2132 mutant expressing the gna2132 gene from M10713 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p17	Complemented gna2132 mutant expressing the gna2132 gene from UK013 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p18	Complemented gna2132 mutant expressing the gna2132 gene from UK355 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p20	Complemented gna2132 mutant expressing the gna2132 gene from 961-5945 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p21	Complemented gna2132 mutant expressing the gna2132 gene from NM117 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p29	Complemented gna2132 mutant expressing the gna2132 gene from M16686 strain, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 CH-G1	Complemented gna2132 mutant expressing the engineered chimeric NHBA gene, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 CH-G3	Complemented gna2132 mutant expressing the engineered chimeric NHBA gene, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 CH-G4	Complemented gna2132 mutant expressing the engineered chimeric NHBA gene, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 CH-C1	Complemented gna2132 mutant expressing the engineered chimeric NHBA gene, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 CH-C2	Complemented gna2132 mutant expressing the engineered chimeric NHBA gene, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p2 short	Complemented gna2132 mutant expressing the engineered chimeric NHBA gene, EryR, KanR CmR	This study
5/99ΔnadAΔ2132C2132 p2 long	Complemented gna2132 mutant expressing the engineered chimeric NHBA gene, EryR, KanR CmR	This study
MC58 Δ2132	gna2132 null mutant of MC58, EryR	Serruto et al 2010
Δ2132 c2132 p18	Complemented gna2132 mutant expressing the gna2132 gene from UK355 strain, EryR, KanR CmR	This study
MC58 Δ2132 c2132 p18 L	Complemented gna2132 mutant expressing the gna2132 gene from UK355 strain, EryR, KanR CmR	This study
58 Δ2132 c2132	Complemented gna2132 mutant expressing the gna2132 gene from MC58strain, EryR, KanR CmR	This study
M10713 Δ2132	gna2132 null mutant of M10713, EryR	This study
UK013 Δ2132	gna2132 null mutant of UK013, EryR	This study
NGH38 Δ2132	gna2132 null mutant of NGH38, EryR	This study
8047 Δ2132	gna2132 null mutant of 8047, EryR	This study
Escherichia coli		
DH5-α	supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Invitrogen
BL21(DE3)	hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)	Invitrogen
Plasmids		
pBluescript (pBS)		Stratagene
pCOMP-pind	Derivative of pSLComCmr plasmid	Stephens DS (2007)
pBS-UDgna2132erm	Construct for generating knockout of the gna2132 gene, with a Ery cassette	Serruto et al. 2010
pBS-UDgna2132kan	Construct for generating knockout of the gna2132 gene, with a Kan cassette	Serruto et al. 2010
pBS-gna2132	Construct for generating complementing strains of the gna2132 gene of MC58 strains, with a CmR cassette	This study

Table S2: Oligonucleotides used in this study

Name	Sequence	Restriction site
NHBA F1	gagaATTAATATGTTTAAACGCAGCGTAATC	Ase I
NHBA F2	gagaATTAATATGTTTAAACGCAGTGTG	Ase I
NHBA F2b	gagaATTAATATGTTTAAACGCAGTGTGATT	Ase I
NHBA F3	gagaATTAATATGTTTGAACGCAGTGTG	Ase I
NHBA R	tgcATGCATTCAATCCTGCTCTTTTTTG	Nsi I
p2A-F	gagaATTAATATGTTTAAACGCAGCGTAATC	Ase I
p2A-R	ACCTTGGGCAGCATTTTGCGGCATATCATTT	
p2C-F	GATATGCCGCAAAATGCTGCCCAAGGTACAAATCA	
p2C-R	tgcATGCATTCAATCCTGCTCTTTTTTG	Nsi I
p18A-F	gagaATTAATATGTTTGAACGCAGTGTG	Ase I
p18A-R	TGTATCGGCGGCATTTTGCGGCATATCATTTTG	
p2B-F	GATATGCCGCAAAATGCCGCCGATACAGATAGTTT	
p2B-R	GGATTCGGCGGACGTATTGCCGGCATTTTCC	
p18C-F	GCCGGCAATACGTCCGCCGAATCCGCAAATCA	
p18C-R	tgcATGCATTCAATCCTGCTCTTTTTTG	Nsi I
p18-G2-F	TTCTGTTGTGATTGATGGGCCGTCGCAAAA	
p2-G2-R	CGGCCCATCAAT CACAACAGAATTGCCCAC	
p2-238R	TATTGCCACTACAAGAATCGCCTTTACAGT	
p2-238F	GCGATTCTTGTAGTGGCAATAATTTCTTGGA	
p2-301R	CAGAACGCCTAAATCGCGCAAATGAAGTGG	
p18-301F	TGCGCGATTTAGGCGTTCTGCACGGTCGAG	
p2 301 F	TGCGCGAGTTAGGCGTTCTGCACGGTCGAG	
p18 301 R	CAGAACGCCTAACTCGCGCAGATGAAGTGG	

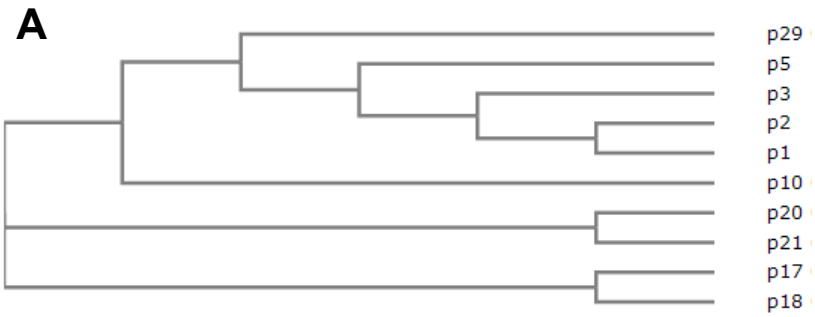
Results

3) Immunogenicity of NHBA

1.1 Sequence variability of different NHBA peptides

Analysis of gene sequences revealed the existence of more than 400 peptides in group B meningococcal strains and the distribution of these diverse peptide sequences has some association with clonal complexes and sequence types (Bambini *et al* 2009, Muzzi *et al* 2013). To evaluate the contribution of NHBA amino acid sequence variability to cross-protection and immunogenicity, we selected 10 divergent NHBA sequences (highlighted with asterisks in Fig. 5B) that represent well amino-acidic diversity and are epidemiologically representative of the ones found in European and American clinical isolates. For this analysis, the peptides p1, p2, p3, p5, p10, p17, p18, p20, p21 and p29 were selected.

Generation of a phylogenetic tree and multiple sequence alignment in Fig.7 demonstrate the genetic distance existing among these different peptides. Peptide 2 is the one present in the formulation of the 4CMenB vaccine. The multiple amino acid alignment indicates that as expected the C-terminus is highly conserved and variable segments of NHBA are present in the N-terminal and central regions of the primary amino acid sequence (Fig. 7B). These regions have been nominated A and B respectively (Fig. 5A).



B

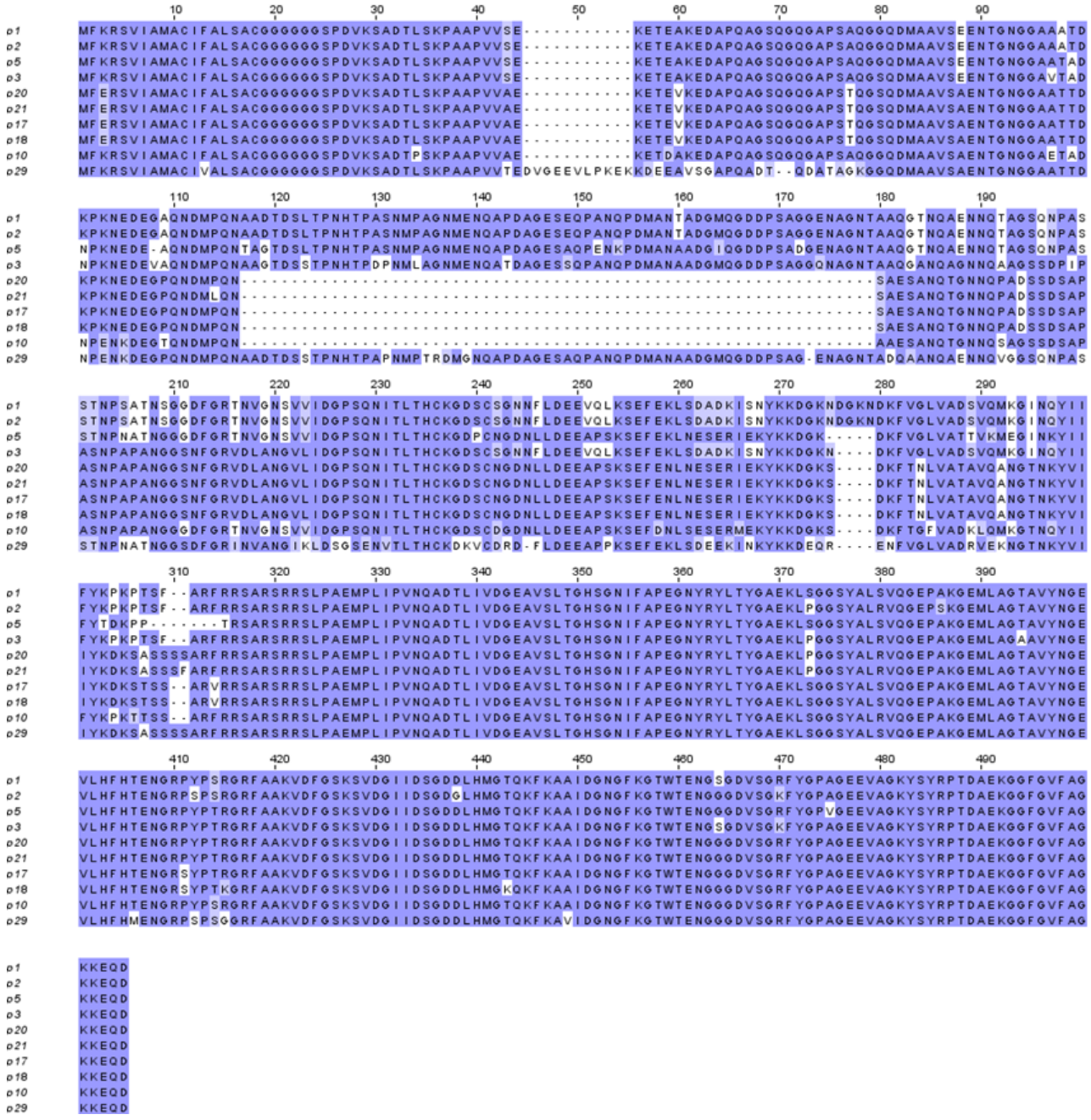


Fig.7 Sequence variability of different NHBA peptides. A) Phylogenetic tree of the 10 NHBA peptides selected for this analysis. B) Multiple alignment of the selected NHBA peptides (numbered on the left), where p2 indicates the peptide present in the vaccine formulation.

1.2 Generation of Nm isogenic strains expressing different NHBA peptides

To dissect and evaluate specifically the contribution of amino acid sequence variability to NHBA immunogenicity, we built a series of identical recombinant strains susceptible to bactericidal killing only by anti-NHBA antibodies and differing only in the amino acid sequence of the NHBA protein expressed (Fig. 8). To generate these isogenic recombinant strains we selected strain 5/99 (B:2b:P1.5,2), which is a well characterized strain expressing high levels of NadA, very low levels of NHBA and very low levels of fHbp variant 2.8. In the SBA assay the natural strain is killed only by anti-NadA antibodies and it is not killed by antibodies against NHBA, by antibodies against the NZ OMV or by antibodies against fHbp variant 1.1 or variant 2.8 (Serruto *et al* 2010; Brunelli *et al* 2011). To create a strain susceptible only to NHBA, the *NadA* and *nhba* genes were deleted by insertion of erm and kan resistance cassettes, respectively (Serruto *et al* 2010). The endogenous fHbp (sub-variant 2.8) was not deleted because serological characterization of the double mutant with antibodies raised against fHbp 1.1 showed that they do not interfere with the SBA analysis (Brunelli *et al* 2011).

The resulting double mutant strain, named 5/99 Δ*NadA* Δ*nhba* (5/99ΔΔ), was then engineered to express different NHBA peptides under the control of an IPTG inducible Ptac promoter in order to achieve similar levels of expression for each peptide (Fig. 8A). For this analysis we decided to express the peptides p1, p2, p3, p5, p10, p17, p18, p20, p21 and p29 that well represent amino-acidic diversity of NHBA peptides. Coding gene sequences were amplified from representative Nm strains, cloned in the pComPind vector and subsequently transformed into 5/99ΔΔ, generating ten different recombinant strains (Fig. 8A).

To evaluate the expression and the surface exposure of NHBA in the recombinant 5/99 complemented strains, we performed Western Blotting and FACS analysis. Testing mouse anti-sera raised against the different NHBA peptides revealed that the detection is variant-specific and a stronger recognition was observed against the homologous variant (data not shown). We selected sera from an immunization scheme which resulted reactive towards the conserved C-terminus of the protein and therefore a good reagent for assessing levels of diverse peptide sequences for testing in Western blot. Western Blotting analysis with this sera confirmed that the recombinant strains were able to express a comparable amount of the different peptides (Fig. 8B). FACS analysis was performed using different mouse polyclonal anti-NHBA sera, raised against each homologous variant and it showed a comparable level of surface expression of the different NHBA peptides across all the recombinant strains (Fig. 8C). The 5/99ΔΔ strain used as negative control showed no significant staining. These data suggest that in the

recombinant strains, the diverse NHBA peptides are expressed to comparable levels exposed on the surface of the bacterium.

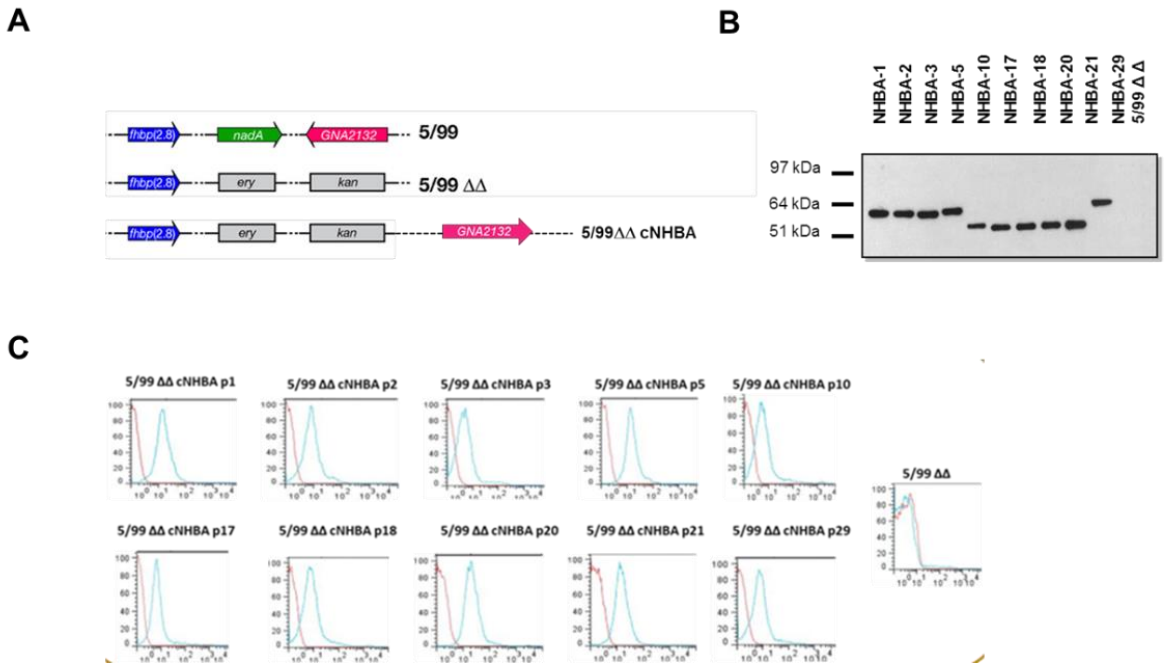


Fig. 8: Generation and analysis of recombinant Nm strains expressing different NHBA peptides

A) *N. meningitidis* strain 5/99 was engineered to express different NHBA variants under the control of an IPTG inducible Ptac promoter. *NadA* and *nhba* genes were respectively replaced by *erm* and *kan* resistance cassettes. The *nhba* gene to be expressed was inserted in the intergenic region between the open reading frames nmb1428 and nmb1429. B) Western Blot analysis of total cell extracts prepared from the recombinant strains. NHBA expression was induced with 0.1 mM IPTG and detected using a specific polyclonal mouse anti-sera raised against the recombinant NHBA p21 antigen. C) FACS analysis of Nm 5/99 recombinant strains using polyclonal mice sera against NHBA. Blue profiles represent the binding of polyclonal antibodies to the different homologous NHBA peptides. Red profiles represent the reaction with secondary antibody (negative control).

1.3 Sera from mice immunized with NHBA p2 kill most of the recombinant isogenic strains tested with different efficiency

The recombinant strains differing only in the amino acid sequence of the expressed NHBA peptide were analyzed for their susceptibility to killing by antibodies from mice immunized with either the recombinant NHBA-GNA1030 antigen, or with GNA2091-fHbp, NadA, OMV-NZ or with the full

4CMenB vaccine formulation. Pooled mouse sera and rabbit complement were used in the SBA assay and the 5/99ΔΔ was used as a negative control. The results reported in Table 1 show that, the 5/99ΔΔ strain was not killed by sera raised against the 4CMenB vaccine or any of the recombinant antigens and showed negative titers. All the NHBA-expressing strains were resistant to killing by sera raised against the GNA2091-fHbp, NadA or OMV-NZ, but showed significant susceptibility to sera derived from mice immunized with both the recombinant NHBA-GNA1030 or with the 4CMenB vaccine. Moreover, the bactericidal titer from sera of mice immunized with NHBA-GNA1030 against the isogenic strains varied from 64 to 8192, while those of mice immunized with 4CMenB were in general higher and varied from 256 to 16384. Highest titers were obtained against the recombinant strain expressing the peptide p2, homologous to the vaccine antigen, or against peptides which present the highest similarity at amino-acid sequence level (p1, p3, p5). Titres with the NHBA-GNA1030 immunized sera exhibit an inverse correlation to the degree of genetic variance of the amino-acid sequence of NHBA to that of the p2 variant. The only exception was the relatively high titres (1024) exhibited against the strain expressing p21, which is genetically more distant from p2. Instead SBA activity of sera of mice immunized with the full 4CMenB formulation split the recombinant strains into 2 general groups, those with high titres (p1, p2 , p3 and p5), and those with medium titres (p10, p29, p17, p20 and p21), with lowest titres for the p18 expressing strain. In conclusion, we found that sera from mice immunized with recombinant NHBA-GNA1030 or with the 4CMenB vaccine are able to kill strains expressing all the selected peptides; however their titers can significantly vary and in general are affected by the amino-acid sequence of NHBA.

	5/99 ΔNadA ΔNHBA cNHBA										
	p1	**p2**	**p3**	**p5**	**p10**	**p17**	**p18**	**p20**	**p21**	**p29**	**//**
NHBA-GNA1030	8192	8192	8192	1024	512	256	64	512	1024	256	<16
GNA2091-fHbp	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16
NadA	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16
OMV-NZ	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16	<16
4CMenB	16384	16384	16384	8192	1024	2048	256	2048	2048	1024	<16

Table 1: SBA titers using mice sera and rabbit complement. The 5/99 ΔΔ recombinant isogenic strains expressing the different NHBA were grown in presence of 0.1mM IPTG and analyzed for their susceptibility to killing by antibodies from mice vaccinated with the recombinant NHBA-GNA1030 antigens, with the 4CMenB vaccine or with the other components of the vaccine (GNA2091-fHbp, NadA, OMV-NZ).

1.4 Cross protective ability of sera from vaccinated adolescents and infants

To investigate the cross-protective capabilities of NHBA in humans, sera from adults, adolescents and infants immunized with the 4CMenB vaccine were tested for their ability to kill 5/99ΔΔ complemented strains in hSBA. In order to study purely NHBA-mediated responses, pre-screening of individual vaccinee sera against the 5/99ΔΔ strain was performed to exclude human vaccinees that showed responsive titres to other antigens in the vaccine. For the adult and adolescent sera, those individual sera giving positive titres to the 5/99ΔΔ strain (and thereby having antibodies to antigens other than the 4CMenB antigens) were excluded and individual sera that gave negative titres against the 5/99ΔΔ recombinant strain were selected and pooled. Instead a pool of 40 infant sera without pre-screening was used (due to limitation in serum availability). The recombinant strain expressing the homologous NHBA peptide 2 (5/99ΔΔ p2) was killed with adult, adolescent and infant sera pools with titres of 32, 32 and 64, respectively. Strains expressing NHBA peptides p1, p3, p5, were efficiently killed (titres of 32 or 64) with pooled sera from each age group, whereas strains expressing NHBA peptides p17, p18, p20 and p21 were resistant to killing by each pooled sera and showed negative titers comparable between the pre-immune and the immune sera (Table 2). Strains expressing either p10 or p29 exhibited negative or low titres (either 1:4 titres with infant sera, 1:8 titres by pooled adolescent sera, and 1:8 and 1:4 titres respectively with pooled adult sera). In conclusion, pooled sera from vaccinees showed different SBA titers against the recombinant isogenic strains expressing the different NHBA peptides and appeared to be greatly affected by NHBA amino-acidic sequence variability. The antibodies elicited to the p2 antigen in the vaccine by vaccinees from all age groups exhibited an effective bactericidal response to the recombinant strain expressing those NHBA variants (p1, p3 and p5) genetically most similar to p2 and had intermediate responses or failed to mount an effective bactericidal response to the more distant (p10, p29) or most distant peptide variants (p17, p18, p20, p21) respectively. Therefore with human sera, an inverse relationship to bactericidal titres and genetic distance is evident. Furthermore, we elucidated that under the conditions of the recombinant background, two main groups of NHBA sequences with distinct SBA outcomes are observed: p1, p2, p3, p5, p10 and p29, which were covered by anti-NHBA antibodies in the vaccine and p17, p18, p20 and p21, which were not.

		5/99 ΔNadA ΔNHBA complemented NHBA									
		p1	**p2**	**p3**	**p5**	**p29**	**p10**	**p17**	**p18**	**p20**	**p21**
ADULTS	Pre	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	Post 3	64	32	32	32	8	4	<4	<4	<4	<4
ADOLESCENTS	Pre	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	Post 3	16	32	64	32	8	8	<4	<4	<4	<4
INFANTS	Pre	<2	<2	<2	<2	<2	<2	<2	<2	<2	<2
	Post 4	64	64	32	64	4	4	<2	<2	<2	<2

Table 2: SBA titers from adults, adolescents and infants receiving the 4CMenB vaccine.

The 5/99 ΔΔ recombinant isogenic strains expressing the different NHBA peptides were grown in presence of 0.1mM IPTG and analyzed for their susceptibility to killing by antibodies from adults, adolescents and infants vaccinated with the 4CmenB vaccine. Pre: sera collected before vaccination. Post 4: sera collected after the 4th vaccine dose. A dilution which results in 50 % killing of the test strain is considered a positive bactericidal titre. Grey boxes: negative titers, <4 adolescents and <2 in infants. Green boxes: positive titers, >= 8 or when there is an increase of at least four fold between Pre and Post immunization.

1.5 Generation of Nm recombinant strains expressing NHBA chimaeras

The previous hSBA analysis resulted in a clear segregation of the NHBA sequences into those resulting in positive (SBA+) or negative (SBA-) hSBA titres on the basis of the ability of pooled vaccinee sera to kill the recombinant strain which expresses them. In order to identify crucial regions for immunogenicity and functional protective responses the amino-acidic sequence of the NHBA peptides previously tested in hSBA were compared using a Pam40 matrix. This analysis identifies indels and SNPS which associate with the different hSBA outcomes. The analysis revealed the presence of a 60 amino-acid insertion sequence, specifically present in the NHBA peptides p1, p2, p3, p5 and p29, which were expressed by strains positive in hSBA. Interestingly this indel segregates NHBA sequences into long and short variants (Fig 5).Moreover, several polymorphisms, which gathered in three main regions of the protein located in the A and B N-terminal regions of the protein (Fig. 5A), were also highlighted and named group1, group2 and group 3.

To understand the specific contribution of these polymorphic regions we further engineered the recombinant isogenic strains to express chimeric NHBA peptides, and then tested them by hSBA. The inserted region corresponding to residues 106-168 of the *nhba* gene encoding for peptide p2, was deleted from the NHBA p2 scaffold (positive in hSBA) and it was inserted in the NHBA p18 scaffold (negative in hSBA). The resulting strains were named p2 short and p18 long, respectively. Bactericidal

responses appeared not to be dependent on the insertion, since positive titers were maintained in the strain expressing NHBA p2 short, while negative titers were observed in the one expressing NHBA p18 long.

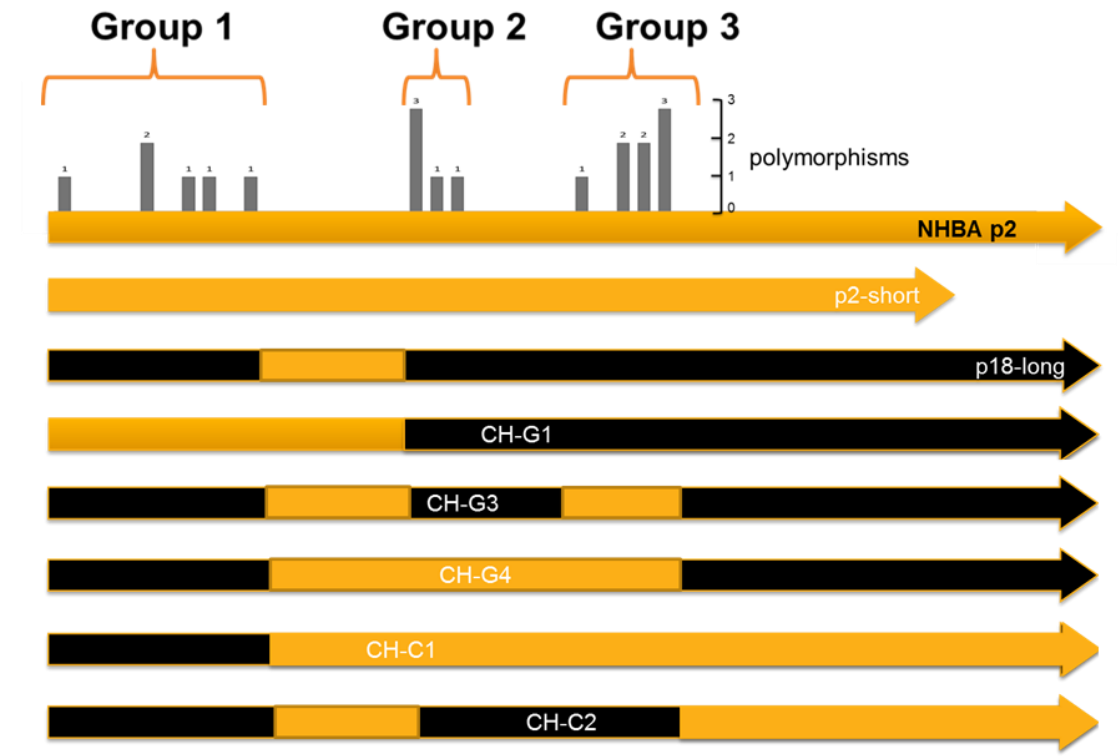
Moreover, four recombinant strains that express chimeric proteins carrying different regions from NHBA p2, chosen as positive reference, were generated in the p18-long background (negative in hSBA).

FACS analysis with 2 different sera raised against either the C-terminal portion of p2 or against the full length p21 protein, resulted in positive FACS signals for each of the chimera-expressing strains. Slightly different degrees of fluorescence were observed depending on the antibodies used suggesting that these chimeras are recognized differently by distinct sera and making absolute quantification of the proteins on the surface elusive (data not shown). The positivity of the FACS results, however, suggests that none of the chimera results in an unstable protein and all are expressed on the surface of the recombinant strains.

The recombinant strain expressing the engineered chimeric peptide CH-C2 resulted resistant to killing by all sera tested, demonstrating that under the conditions of the experiment and in this background the conserved C-terminus on its own does not determine bactericidal activity. Also the recombinant strain expressing the chimeric peptide CH-G3, in which only group 3 polymorphisms were substituted, was resistant to killing. The recombinant strain expressing the chimeric NHBA peptides CH-G1 was killed by adult and adolescent sera, and strains expressing CH-G4 or CH-C1 exhibited positive titres either with only adolescent or with both adult and adolescent sera respectively. However, these titres were bacteriostatic rather than fully bactericidal (killing at the lowest serum dilution was not 100%, but there was a survival of 30-40%). None of the chimaera was bactericidal for the pooled infant sera.

In conclusion, group 1 polymorphisms present at the distal N-terminus, and groups 2 and 3 together carry functional bactericidal epitopes for anti-NHBA antibodies elicited in adolescent and adults. Functional epitopes present along all the protein sequence are likely to be required for full bactericidal activity testing pooled infant sera.

A



B

	Adults		Adolescents		Infants	
	Pre	Post-3	Pre	Post-3	Pre	Post-4
p2 short	<4	64	<4	128	<2	64
p18 long	<4	4	<4	<4	<2	<2
CH-G1	<4	32	<4	32	<2	<2
CH-G3	<4	<4	<4	<4	<2	<2
CH-G4	<4	4	<4	32*	<2	<2
CH-C1	<4	16*	<4	16*	<2	<2
CH-C2	<4	<4	<4	<4	<2	<2

Fig. 9: Generation of Nm recombinant strains expressing NHBA chimaeras

A) Schematic representation of the *N. meningitidis* recombinant strains. Region corresponding to residues 106-168 of the *nhba* gene was deleted from the NHBA p2, while it was inserted in the NHBA p18 (p2 short, p18 long respectively). Moreover, four recombinant strains that express chimeric proteins carrying different regions from p2 were generated in the p18-long background. **B)** hSBA analysis of recombinant isogenic strains expressing

chimeric NHBA peptides using adults, infants and adolescents pooled sera. The asterisks (*) indicate a bacteriostatic effect of the serum. Grey boxes: negative titers, <4 adolescents and <2 in infants. Green boxes: positive titers, >= 8 or when there is an increase of at least four fold between Pre and Post immunization.

1.6 Coverage and cross-protection in mismatched natural strains

Having observed that bactericidal titers were affected by NHBA amino acid sequence variability in the model system of the recombinant isogenic strain where factors such as the level of NHBA expression, the synergistic effects between NHBA and other antigens, or the inherent strain resistance to complement-mediated killing are avoided, we next wanted to analyse the contribution of anti-NHBA antibodies elicited by 4CMenB in vaccinees to killing of natural clinical isolates expressing the same NHBA peptides analysed in the recombinant background.

Furthermore, in order to investigate predominantly the NHBA contribution we selected a panel of test strains that were mismatched for the other vaccine antigens (fHbp var.2 or 3, no NadA and PorA different from 1.4). Strains expressing NHBA peptide 3 could not be considered for this analysis, due to the lack of mismatched strains within our strain collection.

Pooled sera from infants or adolescents vaccinated with the 4CMenB vaccine were able to kill between 25 and 100% of natural strains harboring all different NHBA peptides (considering only those for which the number of strains tested was higher than 1) and the efficacy did not specifically correlate with the sequence of the peptide expressed by each of them (Fig 10). The pooled adolescent sera resulted in bactericidal activity against a significantly larger proportion of these strains. These results showed antibodies raised against 4CMenB are able to kill natural strains harboring the different p20, p21, p29, p1, p5, p18, p10 and p17 NHBA peptides and as these strains do not express other subtypes or variants of the antigens in the vaccine, it is likely that the antibodies elicited to the NHBA p2 antigen are cross-protective towards these strains.

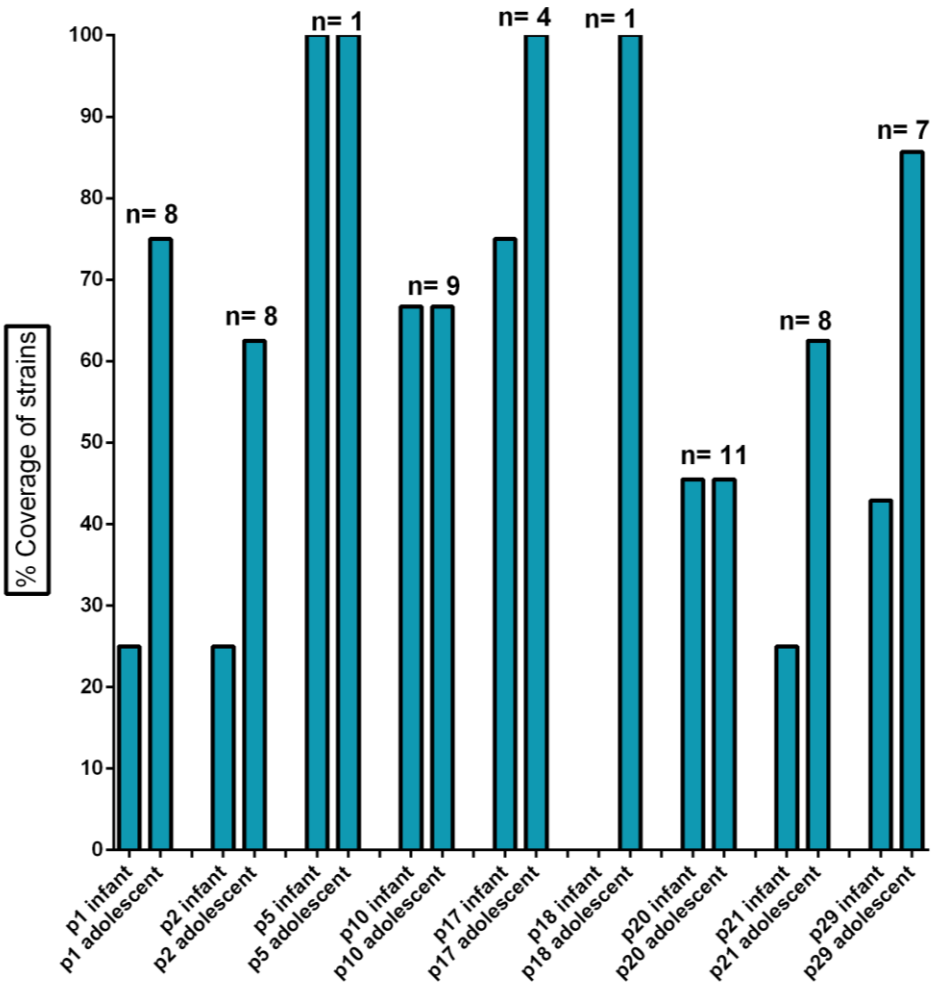


Fig. 10: Coverage in natural mismatched MenB strains

A panel of MenB mismatched clinical isolates harboring different NHBA peptides was tested in a SBA using human complement (hSBA) and pooled adolescent and infant sera receiving the 4CMenB vaccine. Results are expressed as percentage of coverage between the total natural strains expressing each selected peptide. "N" above each bar indicates the total number of strains analysed in hSBA.

1.7 Specificity of NHBA-mediated immune response

To confirm that the bactericidal responses against these strains are NHBA-mediated, the direct role of anti-NHBA antibodies in bacterial killing was evaluated with two different approaches: performing a competitive hSBA analysis using the NHBA recombinant antigen as competitor, and testing the hSBA activity of isogenic *nhba* deletion mutants in different *N. meningitidis* genetic backgrounds. In both approaches, human sera from different age groups vaccinated with either the 4CMenB or rMenB

vaccines (4CMenB lacking the OMV component) were used. Here, we reported results obtained in the M10713 strain, which expresses a p10 NHBA peptide variant. Competitive hSBA analysis using adult, and infant sera vaccinated with either the rMenB or the 4CMenB vaccine showed that positive bactericidal titres were abolished when the fusion antigen NHBA-GNA1030 was used as a competitor (Table 3). Specific competition with the other components had no significant effect on the titres, with the exception of competition with OMV-NZ for the pooled infant sera from trial V72P13. In this case, addition of OMV-NZ abolished the bactericidal titres against the M10173 strain, (Table 3). This suggests that in this pooled sera there may be synergy between non-PorA antibodies present in the OMV and NHBA.

Similar competitive SBA analyses were performed on selected mismatched strains, expressing p29, p5, and p188. These experiments suggest that in strains expressing diverse peptide variants, the bactericidal responses observed in pooled vaccinee sera are due to antibodies specifically recognizing NHBA (data not shown).

The M10713 WT and *nhba* deletion mutant strains were also tested in hSBA analysis using single subjects infant sera, pooled infant sera and pooled adult sera vaccinated with the rMenB or the 4CMenB vaccine, respectively. The analysis showed that positive bactericidal titers were either abolished or significantly reduced in the deletion mutant strain in 7/9 infant sera tested, demonstrating that killing of M10713 in the majority of infant individuals is mediated by bactericidal responses against the NHBA vaccine antigen. Pooled infants and adults sera were also able to efficiently and specifically kill only the M10713 WT strain (Table 4). In conclusion, bactericidal antibodies recognize specifically the NHBA antigen and are required for killing of M10713 in hSBA.

Furthermore, an additional nine NHBA KO strains were generated in strains expressing diverse NHBA peptides (including p1, p3, p5, p29, p10, p1, p144, p17) and tested with pooled vaccinee sera. In all strains we could demonstrate that positive bactericidal titres were reduced either significantly or completely on deletion of the *nhba* gene (data not shown). This confirmed the specificity of NHBA-mediated killing in natural strains expressing diverse peptides.

Sample	Vaccine	Bleed	Inhibitor				
			None	NadA	NHBA-GNA1030	GNA2091-fHBP	OMV NZ
V72P1	rMenB	Post-3rd	32	16	<4	64	32
V72P9	rMenB+ OMV NZ	Post-3rd	16	16	<4	64	16
V72P13	rMenB+ OMV NZ	Post-4th	32	16	<4	64	<4

Table 3: Competitive hSBA (adults and infants)

The ability of excess purified recombinant proteins, OMV, or buffer alone to inhibit killing of M10713 by vaccine-elicited serum and serum pools was tested. The V72P1 serum sample is from an adult who received 3 doses of the recombinant protein MenB vaccine (without OMV). The V72P9 serum pool consists of sera from 25 infants who received 3 doses (6, 8, 12 months) of rMenB + OMV NZ. The V72P13 serum pool consists of sera from 141 infants who received 4 doses of rMenB + OMV NZ (2, 4, 6, 12 months).

Subject	SBA Titer	M10713	M10713 (ΔNHBA)
4-001	Pre-immune	<4	<4
	Post 4th	32	<4
4-002	Pre-immune	16	<4
	Post 4th	128	16
4-003	Pre-immune	<4	<4
	post 4th	32	<4
4-004	Pre-immune	32	<4
	Post 4th	128	8
4-005	Pre-immune	32	<4
	Post 4th	128	4
4-006	Pre-immune	<4	<4
	Post 4th	8	<4
4-007	Pre-immune	<4	<4
	Post 4th	64	8
4-008	Pre-immune	<4	<4
	post 4th	32	<4
4-009	Pre-immune	<4	<4
	Post 4th	64	8
V72P12	Pre-immune	4	<4
	Post 4th	16	<4
V72P1	Pre-immune	8	<4
	Post 3rd	32	4

Table 4: hSBA on M10713 WT and deletion mutant strain

The ability of infant immune sera collected from nine 13-month old infants who received 4 doses of rMenB + OMV NZ to kill the wild type and *nhba* knockout M10713 strains was tested in hSBA . The V72P12 serum was obtained from infants vaccinated with 4 doses of 4CMenB vaccine. The V72P1 serum was obtained from an adult immunized with 3 doses of the rMenB (no OMV) vaccine.

2) Characterization of the functional properties of NHBA

To evaluate the biological significance of the binding to heparin observed biochemically *in vitro* (Serruto *et al* 2010), we decided to explore the possible interaction of this protein with epithelial cells, and then its relevance for meningococcal pathogenesis, focusing in particular on the first step of bacterial adhesion and colonization.

2.1 rNHBA binds to human epithelial cells through GAGs, and specifically to HSPGs

The ability of NHBA to interact with epithelial cells was evaluated by adding the recombinant protein (rNHBA) to epithelial cells and analyzing the results of the binding by immunofluorescence. To investigate the potential contribution of glycosaminoglycans (GAGs) present in the extra cellular matrix (ECM) or on the cellular surface in mediating the binding to epithelial cells, we analysed and compared NHBA binding to wild-type CHO-K1 and CHO mutant pgsA-745 epithelial cells, which are defective for GAGs biosynthesis.

As shown in figure 11, confocal microscopy analysis of CHO cells incubated with the recombinant protein revealed that NHBA was able to bind only to wild-type cells, while no binding was observed in CHO mutant cells, devoid of GAGs expression.

Moreover, when the heparan sulfate is stained using a monoclonal anti-heparan sulfate antibody (10E4 epitope), a spatial co-localization of the protein was observed with heparan sulfate, suggesting that the interaction with GAGs was mediated specifically through the binding to heparan-sulfate.

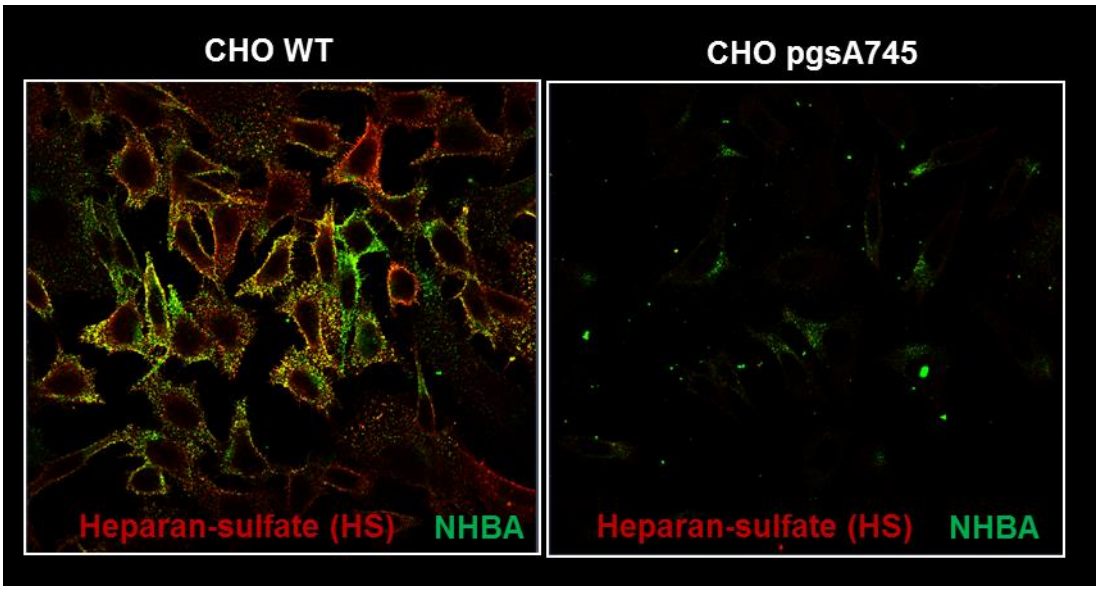
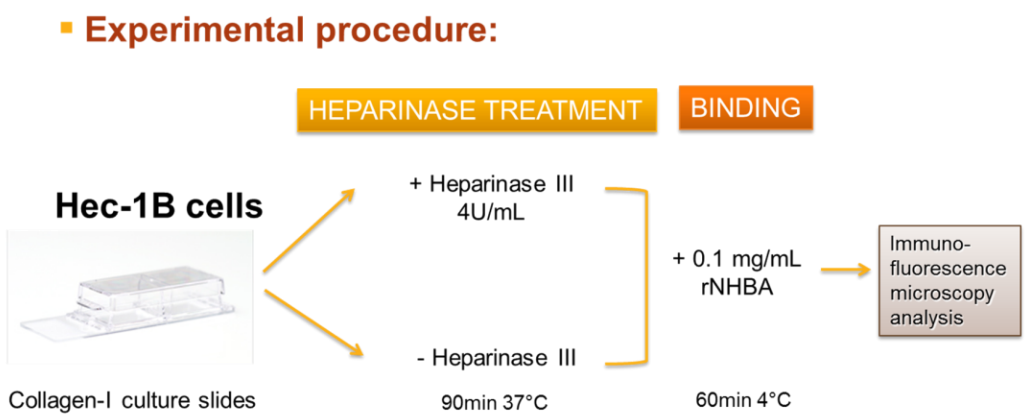


Fig. 11: Binding of the recombinant NHBA protein to wild type CHO-K1 and CHO pgsA-745 epithelial cell monolayers and colocalization with heparan-sulfate. NHBA was detected with a primary polyclonal mouse anti-NHBA serum (green staining). Heparan sulfate was stained using a mouse monoclonal anti-heparan sulfate antibody (10E4 epitope, red staining).

Recombinant purified NHBA protein was tested in *in vitro* binding studies also in Hec1B human epithelial cells. To understand the specific contribution of heparan-sulfate proteoglycans in mediating protein binding, cells were pre-treated with heparinase III that specifically removes heparan sulfate, and compared to untreated cells by confocal microscopy. Recombinant NHBA protein was able to bind to Hec-1B human epithelial cells and binding was abolished after heparinase III treatment. Also in this epithelial cell line, a partial colocalization of the protein with heparan sulphate was observed (Fig.12).

A



B

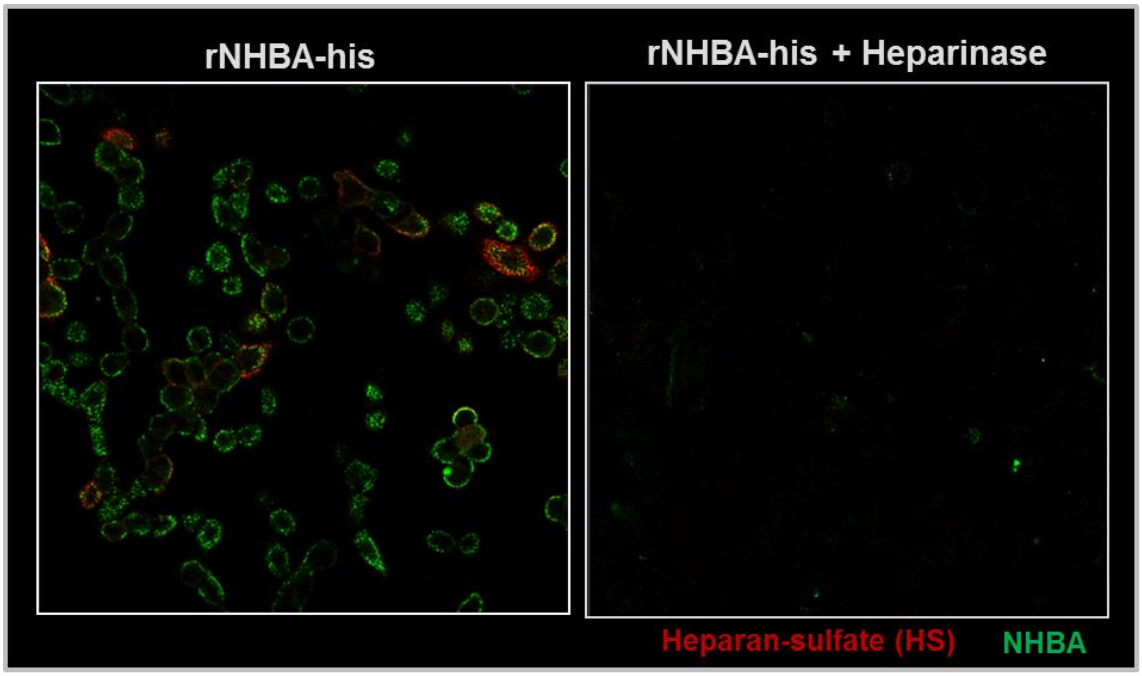


Fig. 12: Bindig of the recombinant NHBA protein to Hec-1B human epithelial cells and after treatment heparinase III treatment A) Schematic representation of the experimental procedure. **B)** Binding of the recombinant NHBA protein to Hec-1B cell monolayers and colocalization with heparan-sulfate. NHBA was detected with a primary polyclonal mouse anti-NHBA serum (green staining). Heparan sulfate was stained using a mouse monoclonal anti-heparan sulfate antibody (10E4 epitope, red staining) in not treated and treated Hec-1B cells, demonstrating the efficient removal of heparan sulfate from epithelial cells after heparinase III treatment.

To further confirm the specificity of the enzymatic treatment and of heparan sulphate proteoglycans in mediating protein binding to epithelial cells, we used a similar approach and treated Hec-1B epithelial cells with Choindroitinase ABC, which is known to effectively remove choindroitin-sulfate residues which are other GAGs commonly found in the ECM.

As shown in Fig. 13, while the treatment was able to effectively remove choindroitin-sulfate, protein binding was not affected.

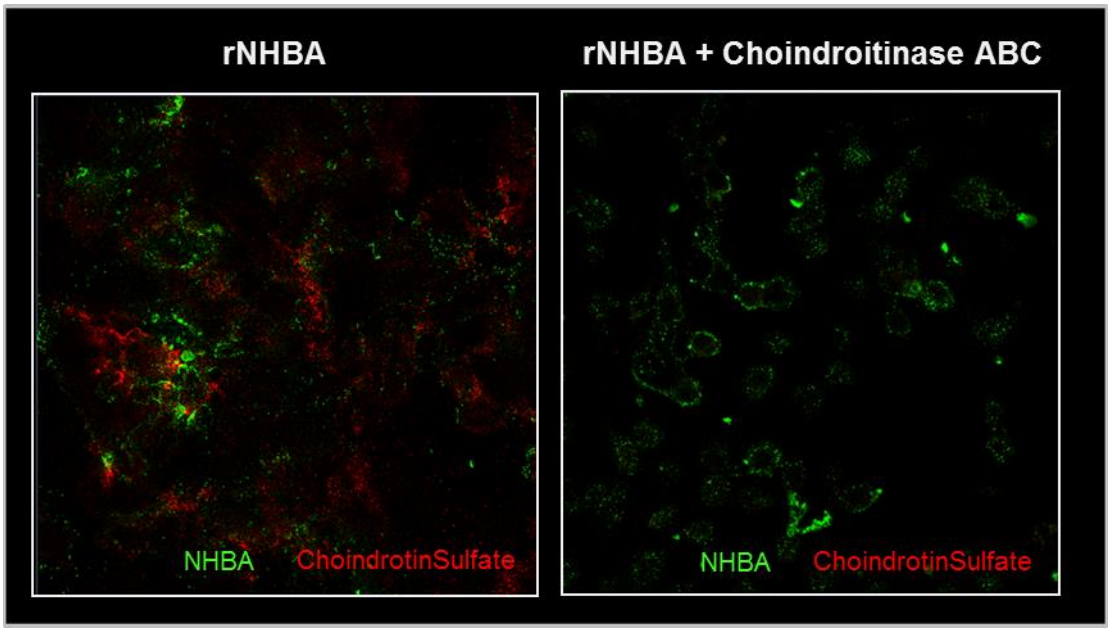


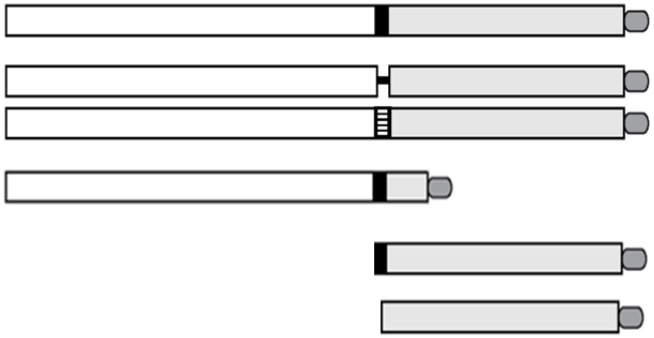
Fig. 13: Binding of the recombinant NHBA protein to Hec-1B cell monolayers and choindroitinase ABC treatment. NHBA was detected with a primary polyclonal mouse anti-NHBA serum (green staining). Choindroitin-sulfate was stained using a mouse monoclonal anti-choindroitin sulfate antibody (CS-56 clone, red staining) in not treated and treated Hec-1B cells, demonstrating the efficient removal of choindroitin sulfate from epithelial cells after choindroitinase treatment.

2.2 rNHBA binding to epithelial cells occurs through the Arg-rich region

To investigate the role of NHBA Arg-rich region in mediating the binding to CHO-K1 epithelial cells, the ability of binding of the WT recombinant protein and of different mutants in the Arg-rich region was compared *in vitro*. Both the recombinant mutant full-length proteins ΔRR-his, in which the Arg-rich region is completely deleted, and the mRR-his mutant, in which all the Arg residues were substituted with Gly, did not efficiently bind to CHO and Hec-1B cells, compared to the WT protein.

Moreover, the analysis showed that only fragments containing the Arg-rich region (AB3 and C2-his) were able to bind to epithelial cells, confirming the key role of this region in mediating the interaction (Fig.14).

A



Construct	Arg-rich region
rNHBA	+
dRR	-
mRR	-
AB3	+
C2	+
C1	-

B

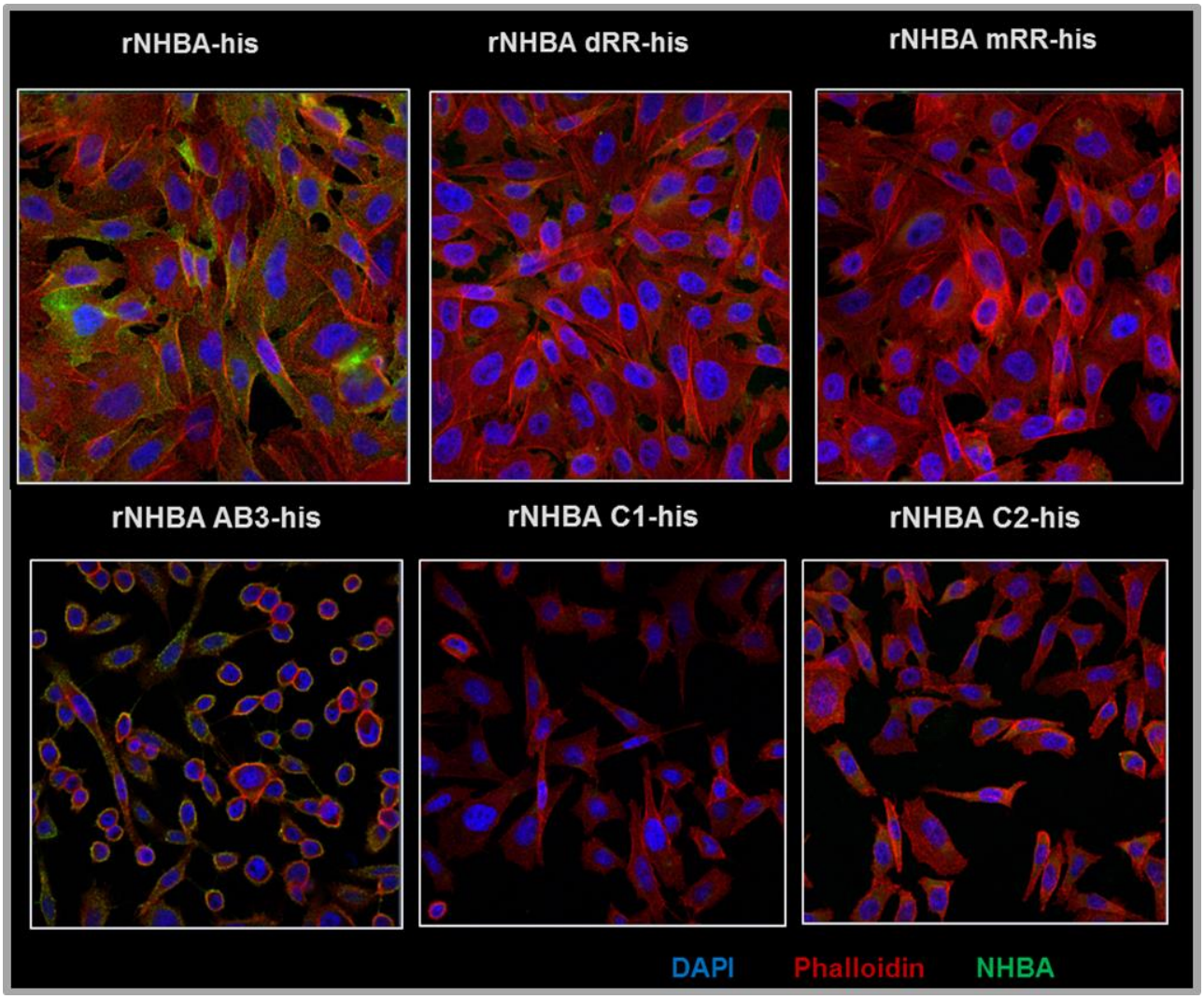


Fig. 14: rNHBA mutants for the Arg-rich region, or purified protein fragments devoided of that region do not bind to epithelial cells

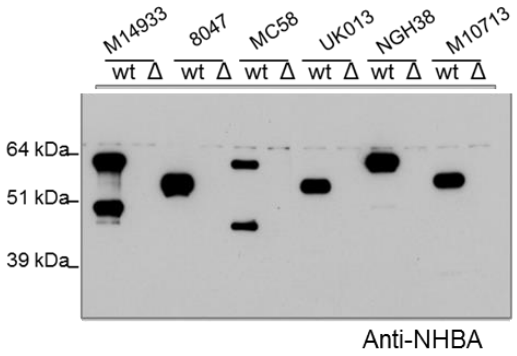
A) Schematic representation of the NHBA recombinant proteins used for the binding assay. ΔRR-his is a deletion mutant of the Arg-rich region, mRR-his is a mutant where all Arg residues were substituted with a Gly, the AB3 fragment is truncated at the level of the C-term after the Arg-rich region, the C2 fragment is represented by the C-term with the Arg-rich region, while the C1 fragment starts just after that region and does not contain the Arg-rich domain.

B) Binding of the recombinant purified NHBA protein to CHO epithelial cells compared to binding of the purified mutant proteins dRR-his, mRR-his or fragments AB3, C1 and C2. NHBA was detected with a primary mouse polyclonal anti-NHBA serum (green staining). Actin was stained with Phalloidin-568 dye (red) and nuclei with DAPI staining (blue).

2.3 Deletion of NHBA decreases the ability of meningococcal strains to bind epithelial cells

To investigate the role of NHBA in contributing to meningococcal binding to Hec-1B epithelial cells, the adhesion capability of different WT and *nhba* deletion mutant strains were compared in an *in vitro* adhesion assay. The natural wild-type strains tested showed a different adhesive capability, however, in all genetic backgrounds the deletion of *nhba* resulted in a reduction of adhesion with respect to each isogenic wild type strain (Fig. 15). Interestingly, this phenotype did not appear to be dependent on the different NHBA peptide expressed by each strain and by NalP cleavage, which was observed only for strains MC58 and M14933, both expressing p3 peptide variants.

A



Strain	Clonal Complex	NHBA
M14933	32	3
8047	11	20
MC58	32	3
UK013	269	17
NGH38	n.a.	2
M10713	41/44	10

B

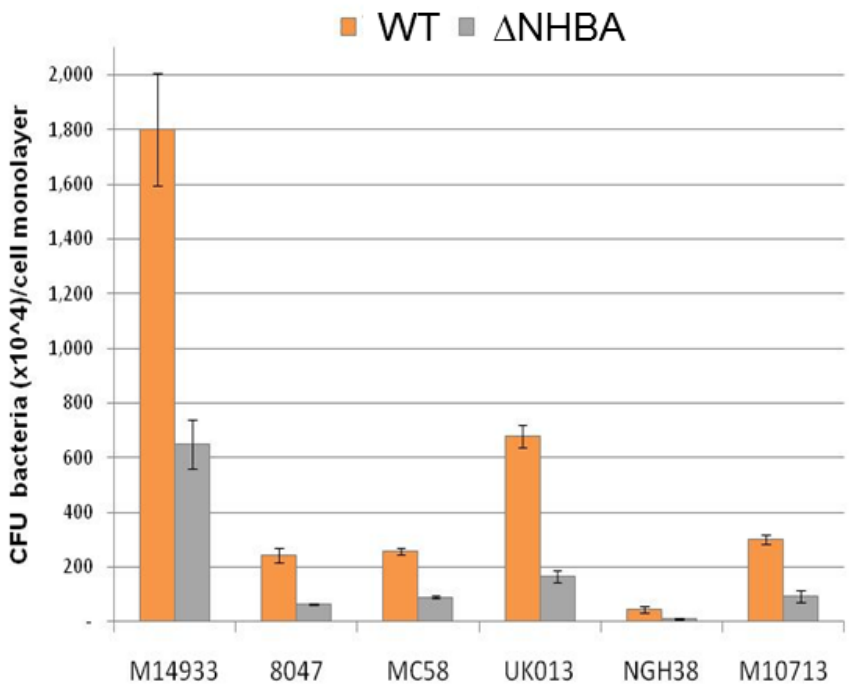


Fig. 15: NHBA deletion mutant adhere less than WT strains A) Western blot analysis of different natural strains expressing NHBA and of *nhba* isogenic deletion mutants. The table on the right shows the clonal complex and the NHBA peptide expressed by each of the strain tested in the adhesion assay. **B)** Adhesion assay of a panel of WT and *nhba* deletion mutant strains to Hec-1B human epithelial cells. Cell monolayers were infected at a MOI of 1:100 for 3 hours with a panel of different WT and *nhba* deletion mutant strains. After 3 hours, samples were treated with saponin and adherent bacteria were plated for CFU counting. The graph reports the number of CFU counted at the end of the assay.

Despite we generated and tested also a complementation mutant in MC58, able to successfully express NHBA also on the bacterial surface (as demonstrated by FACS analysis), we never observed any restoration of the impaired adhesion phenotype observed in the deletion mutant strain (Fig.16).

We generated also an alternative mutant, by complementing the protein *in trans* and inducing protein expression by IPTG addition, however, also this strategy did not allow to restore the adhesion capability of the mutant strain to the wild-type level (data not shown).

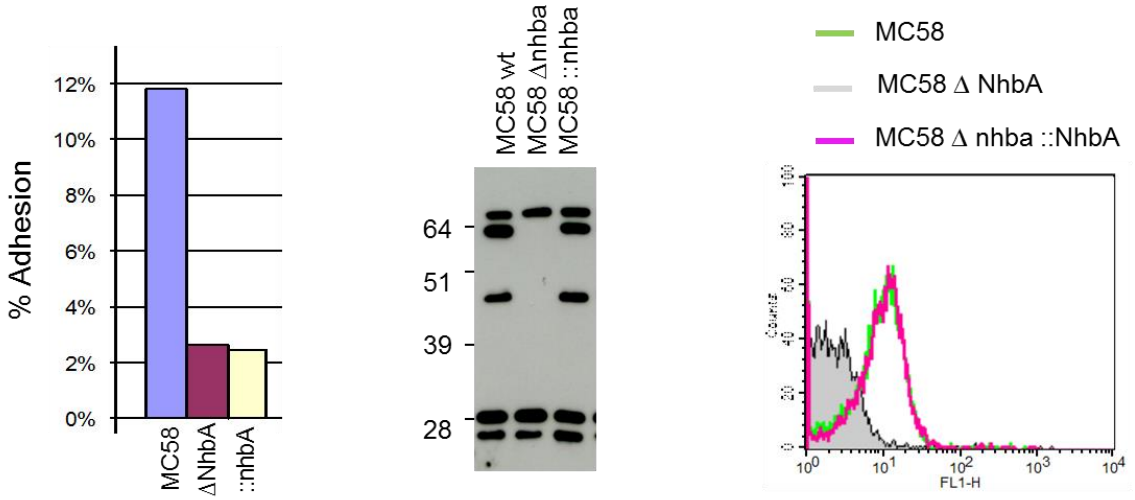


Fig. 17: Lack of complementation of the adhesion phenotype A) Adhesion assay of a panel of MC58 WT, *nhba* deletion mutant and complementing strain to Hec-1B human epithelial cells. After 3 hours, samples were treated with saponin and adherent bacteria were plated for CFU counting. The results are reported as percentage of CFU counts after 3 hours of infection/ CFU at time zero (starting inocolum). B) Western blot analysis of MC58 WT, *nhba* deletion mutant and complementing strain on total cell lysates, using polyclonal mice sera against NHBA. C) FACS analysis of MC58 WT, *nhba* deletion mutant and complementing strain using polyclonal mice sera against NHBA. Green profile represents the binding of polyclonal antibodies to the WT strain, while the pink the one of the complementing strain. The gray profile represents the binding of the anti-NHBA antibodies to the *nhba* deletion mutant strain (negative control).

2.4 Antibodies against NHBA are able to inhibit meningococcal adhesion

To further confirm the specificity of the role of NHBA in mediating adhesion to epithelial cells, bacteria were pre-incubated with anti-NHBA sera before the infection. All anti-NHBA sera were able to effectively inhibit meningococcal adhesion in the UK013 strain tested in a dose-dependent manner, compared to the negative control with no serum or in which bacteria were incubated with pre-immune sera (Fig.17).

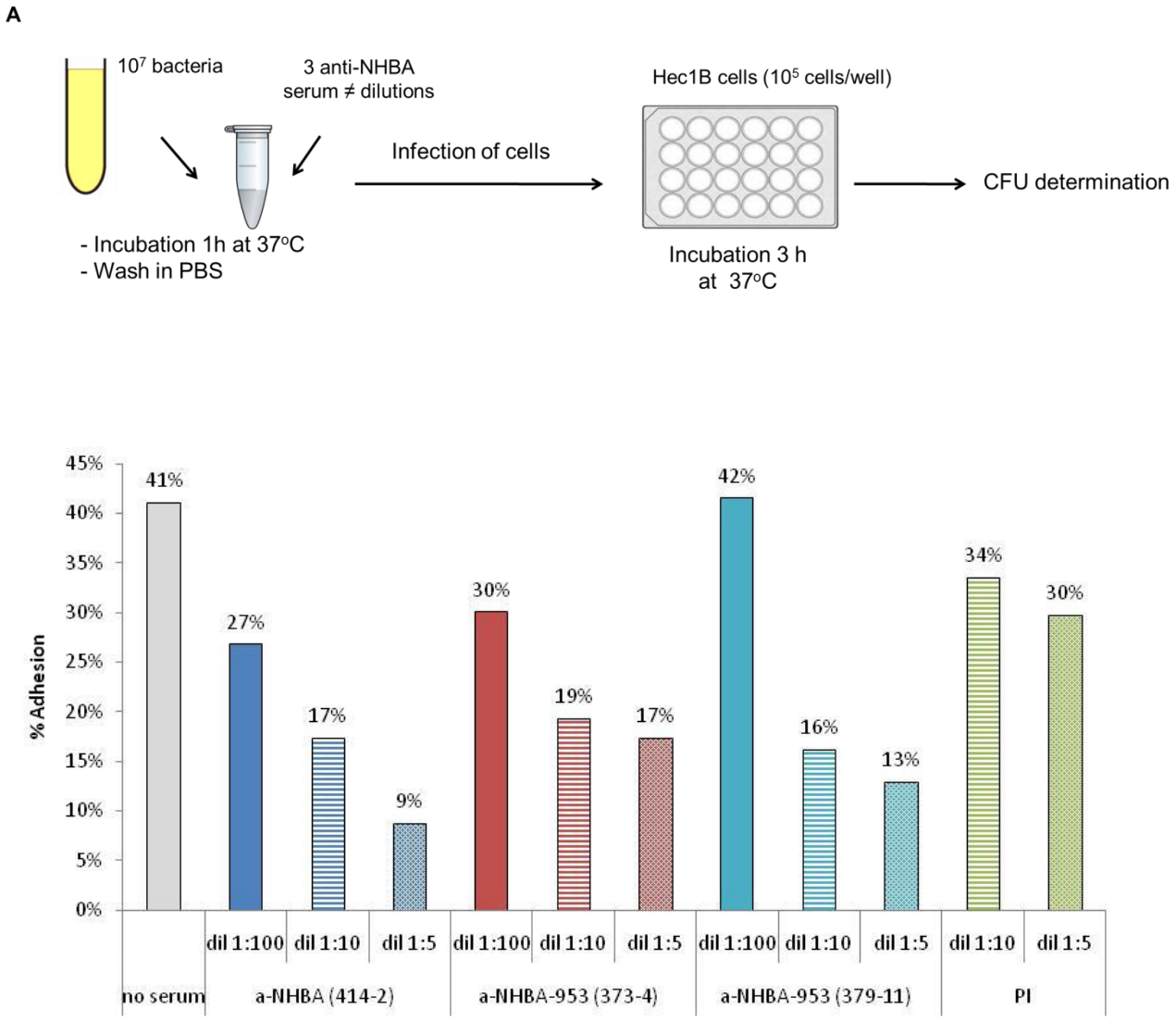


Fig. 17: Inhibition of meningococcal adhesion using anti-NHBA polyclonal sera A) Schematic representation of the experimental procedure. B) Anti-NHBA polyclonal sera were able to reduce bacterial adhesion to Hec-1B epithelial cells. Bacteria were pre-incubated with increasing concentrations of three different mouse polyclonal sera raised against NHBA protein for 1 hour at 37°C. Hec-1B cell monolayers were then infected at a MOI of 1:100 for 3 hours with UK013 WT strain. After 3 hours, samples were treated with saponin and adherent bacteria were plated for CFU counting. As a control, bacteria were incubated with the same dilution of pre-immune sera, or without any sera.

The results are reported as percentage of CFU counts after 3 hours of infection/ CFU at time zero (starting inocolum).

3) NHBA NalP-mediated cleavage

In order to understand if NalP mediates cleavage of all NHBA peptides we analysed a panel of natural and recombinant strains expressing different NHBA peptides.

Through a bioinformatics approach it was possible to identify at least eleven different NalP target domains (Serruto D. *et al* 2010, Muzzi A. *et al* 2013 and personal communication), six of which were represented in the NHBA peptides analysed in the first part of this study (Fig. 17).

NHBA peptides p1, p2 and p3 possess the same target sequence (-TSFA); while peptides p18 and p17 were characterized by a polymorphic sequence (-TSSA). Peptide p10 shows the same target sequence, but different flanking residues, which are part of the NalP target domain. NHBA peptide p21 possesses a longer target sequence, rich in Ser residues (-SASSSFA), more similar to the one present in peptide p20 and p29 (-SASSSSA). A peculiar situation was observed for NHBA peptide p5, which seems to lack the residues which are usually recognized by NalP.

Western blot analysis on natural strains expressing these different NHBA peptides revealed that NHBA peptides p10, p17, p20, p5 and p29 were never cleaved. Strains expressing NHBA p1, p2 and p3 were cleaved when NalP was expressed (data not shown).

NalP target domain name	NalP target domain sequence	Example of NHBA
NalP_td-1	YKPKP--TSFARFRRSA	p1 (L)
		p2 (L)
		p3 (L)
NalP_td-2	YKDKS--TSSAQFRRSA	p17 (S)
		p18 (S)
NalP_td-5	YKDKSASSSFARFRRSA	p21 (S)
NalP_td-6	YKDKSASSSSARFRRSA	p20 (S)
		p29 (L)
NalP_td-8	YKPKT--TSSARFRRSA	p10 (S)
NalP_td-11	YTDKP-------PTRSA	p5 (L)

Fig. 17: Analysis of NalP target domain sequences. Six different classes of NalP target domains were observed for NHBA peptides p1, p2, p3, p5, p10, p17, p18, p20, p21, p29. These classes are indicated in the first column of the table. The last column shows an example of NHBA peptides associated to the different NalP target domains. “S” or “L” indicates whether the NHBA peptide has a longer or shorter aminoacidic sequence.

To confirm what was observed in natural strains and to focus the analysis specifically on the NHBA sequence, we analysed NHBA expression in the 5/99 Δ*NadA* Δ*nhba* recombinant isogenic strains, complemented with the ten different NHAB peptides p1, p2, p3, p5, p10, p17, p18, p20, p21 and p29. Interestingly, only NHBA peptides which have a longer amino acidic sequence and present the "-TSFA" NalP target domain sequence were cleaved. Moreover, even among the longer peptides, peptide p5 and p29 were not cleaved (Fig.18). This well correlates with what we observed in natural strains and with the peculiarity of their NalP target domain sequence.

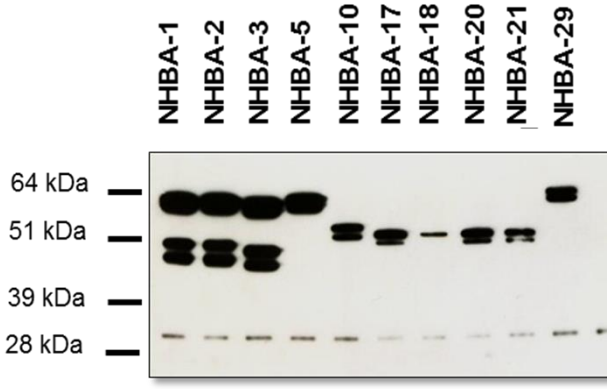


Fig. 18: Analysis of NalP cleavage on different NHBA peptides. Western blot analysis on total protein extracts using anti-NHBA polyclonal sera shows a different cleavage pattern between the NHBA peptides expressed by the recombinant 5/99 isogenic strains. Protein expression was induced with 0.1mM IPTG.

The differences observed between the different NHBA peptides could be either due to NalP specificity in the recognition of the target sequence or to a different conformation of the longer NHBA peptides which could be responsible of a better exposure of the NalP cleavage site, or to a combination of both factors.

To discriminate among these two factors we expressed in the MC58 background the wild-type NHBA peptide p18, and a p18 engineered peptide in which it was inserted the 60 aminoacid sequence present in the longer peptides. MC58 is a high NalP expresser strain and its endogenous NHBA peptide p3 presents a polymorphic NalP target domain sequence (NalP target domain 1), with respect to the one found in p18 (NalP target domain 2).

Western blot analysis showed that in MC58, NalP was able to cleave only the endogenous NHBA (p3), while it did not cleave the NHBA short WT p18 peptide. Interestingly, cleavage was observed in the

engineered p18 peptide, containing the insertion sequence (Fig. 19).

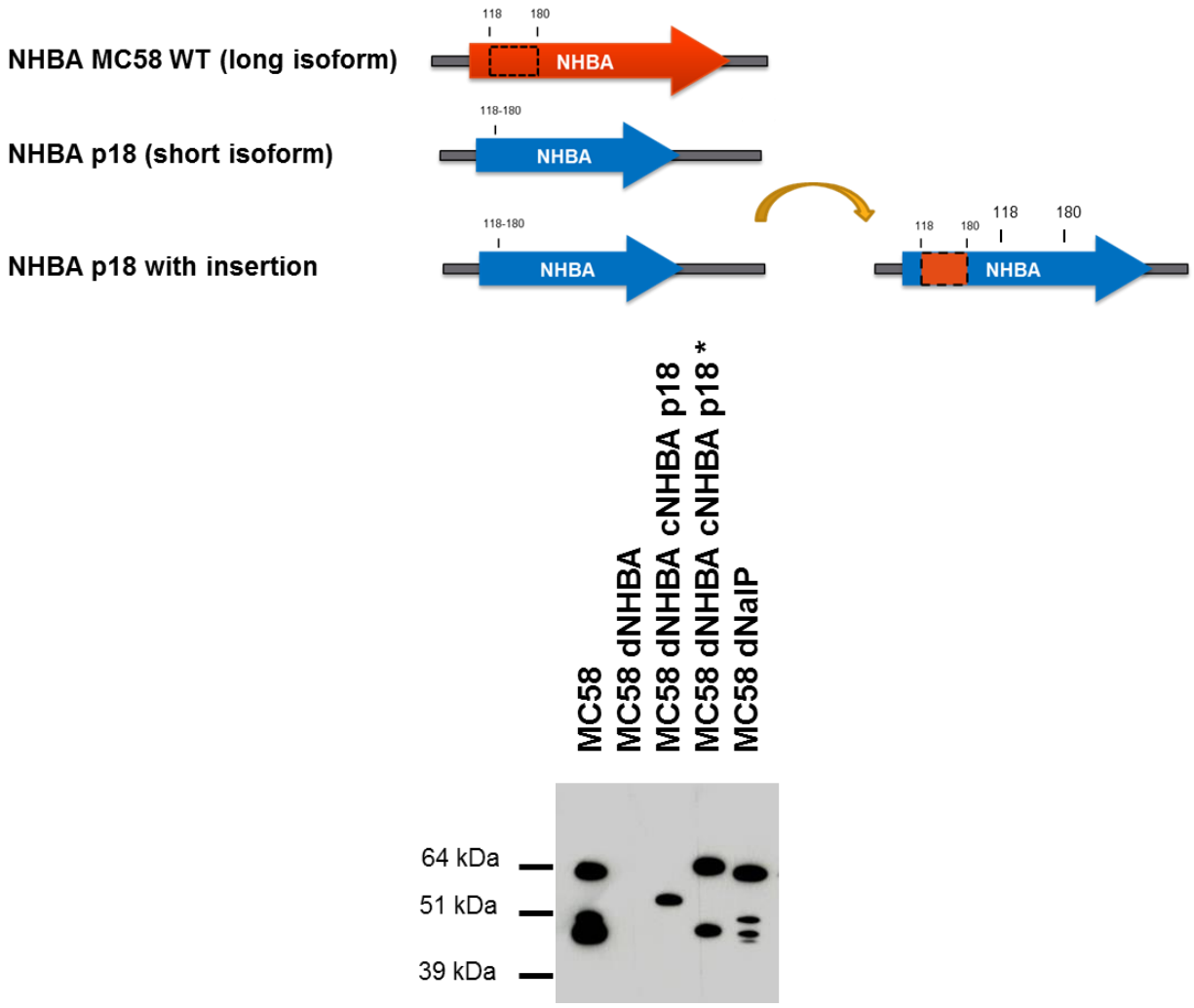


Fig. 19: Analysis of NHBA cleavage in MC58 recombinant strains. A) Schematic representation of the engineering of NHBA peptide p18, expressed in the MC58 genetic background. In red is represented the endogenous NHBA peptide p3, in blue NHBA peptide p18. The region corresponding to residues 118-180 of the *nhba* gene (highlighted in red) from the NHBA peptide 3, was inserted in the NHBA peptide 18. **B)** Western blot analysis on total protein extracts of MC58 wild-type, MC58 Δnhba and complemented with NHBA p18 wild-type and with the insertion. MC58 ΔNalP was used as a control for NHBA cleavage. Protein expression was induced with 0.1mM IPTG.

Discussion

1) Immunogenicity of NHBA

NHBA is one of the main antigens of the Novartis 4CMenB vaccine against serogoup B meningococcus. The study of the immunogenicity and contribution of this antigen to vaccine coverage in the multicomponent vaccine is challenging and many factors have to be taken into consideration. By vaccine coverage, we mean the ability of vaccine antigen-elicited antibodies to kill and protect against circulating strains, and the serum bactericidal antibody (SBA) assay is considered the "gold standard" for measuring serological protection against meningococcal strains (Frasch *et al*, 2009). However, the SBA reflects the cumulative effect of all of the antibodies present in a sample which may interact with a bacterial target strain. As such, NHBA-mediated killing of MenB strains with human sera, may depend on factors such as amino acid sequence variability and antigen level of expression in the different strains as well as the influence of antibodies directed against other antigens and inherent strain dependent resistance to complement mediated killing. Variability of NHBA sequences in circulated strains has been already reported (Muzzi A. *et al* 2013, Bambini S. *et al* 2009), however unlike the other main antigens in 4CMenB (fHBP, NadA and PorA) it has not been classified into distinct sequence variants, alleles or subtypes, since no specific groups of proteins can be easily identified. More than 400 distinct amino acid sequences are currently reported in the databases, and different levels of expression are common in the different natural strains (data not shown).

We constructed a panel of isogenic recombinant Nm strains that are only susceptible to killing by anti-NHBA antibodies and are able to express the same level of ten different NHBA peptides that included the most frequent NHBA peptide variants identified by molecular epidemiology surveys performed on different panels of strains (Bambini S. *et al*, 2009, Wang *et al*, 2011,). We used this panel to study the influence of amino acid variability in the induction of protective immunity. These were tested in SBA using mouse and human pooled sera.

Sera from mice vaccinated with NHBA-GNA1030 or with the full vaccine formulation were able to kill strains expressing all selected peptides, although bactericidal titres appeared to be affected significantly by NHBA amino acid sequence variability. We observed higher titers for the strain that expressed NHBA peptide p2 (which corresponds to the one present in the vaccine formulation), or for those strains expressing peptides which are most closely related to its amino acid sequence, such as p1, p3

and p5, and generally lower titres against the strains expressing the more distal peptides. However a complete correlation to sequence divergence and bactericidal titres is not evident, for instance, significantly different titres were observed between p17- and p18- expressing strains, and p20- and p21- expressing strains, while these paired peptides are very genetically related. From the human vaccinees responses to the NHBA-expressing recombinant strains, we can segregate the NHBA peptides into cross-protective (p1, p2, p3, p5) and not-cross protective (p17, 18, 20 and 21) with p10 and p29 showing an intermediate result giving titres of 1:4 or higher for all age groups. This reflects the genetic relatedness of these peptide sequences as highlighted in Fig 7, and confirms that aminoacid sequence diversity plays an important role in cross-bactericidal activity of NHBA-antibodies in the recombinant background where other factors are not in play. Interestingly there was no great age-related influence in cross-bactericidal anti-NHBA responses.

In a similar analysis carried out to study fHbp cross protection (Brunelli B. *et al* 2011), sera from vaccinated adults had a behavior similar to vaccinated mice, (6/8 subvariants showing positive or high titres, respectively), however, while the vaccinated infants showed cross-protection only to the most highly similar subvariant, adult sera could cross-protect against six out of eight suggesting that the breadth of cross-protection of fHbp decreases in infants compared to adults. In contrast, for NHBA in the 'clean' recombinant background, vaccinees responses showed good cross-protection for genetically close peptides, irrespective of the age group of the vaccinees.

Aminoacid sequence analysis revealed the presence of several polymorphisms along the molecule, which gathered in three main regions of the protein (Group 1, Group 2 and Group3), mainly located in the N-term and in the central portion of the molecule. Engineering of the recombinant isogenic strains to express different chimeric NHBA proteins allowed the mapping of important bactericidal epitopes in the N-term and central region of the protein which also show highest antigenic variation. The analysis in the chimera-expressing recombinant strains showed that the highly conserved C-terminus on its own is not sufficient to provide cross-protective bactericidal activity to the more divergent NHBA sequences. Group 1 polymorphisms present at the distal N-term and groups 2 and 3 all carry functional bactericidal epitopes. This explains the cross-protective limit of the most divergent sequences in the recombinant strains.

Moreover, the results suggest that functional epitopes present along all the protein sequence are required for full bactericidal activity when testing pooled infant sera, as chimeras containing either the N-term or central region of the p2 sequence were not killed efficiently in contrast to results with adult or adolescent sera where positive but lower titres were observed. The respective levels of bactericidal

antibodies that are present in the sera pools may be different, and possibly less in the infants sera below a specific threshold, which requires multiple NHBA epitopes, in the N-term, central and possibly C-term regions to be co-expressed before the test strain can be efficiently killed. The results obtained in the Nm recombinant strain background suggests that NHBA sequence variability indeed influences the bactericidal activity of the NHBA-antibodies in vaccinee sera, when looking uniquely at the NHBA contribution separately from all other vaccine responses and possible strain influences.

Interestingly, the data from the mismatched natural strains suggest that human antibodies raised against NHBA p2 present in the 4CMenB vaccine were able to efficiently kill strains expressing genetically different NHBA peptides, including the more genetically distant ones. Indeed, some of the killed natural strains expressed NHBA peptides which did not mediate killing when tested in the recombinant strain background (i.e. p17, p18, p20, p21). We analysed and compared in hSBA WT and *nhba* deletion mutants and performed a competitive hSBA analysis, using the recombinant purified NHBA protein. Both methods, allowed us to conclude that on the model strain M10713 as well as a number of others expressing different NHBA peptides (including a p17 expressor), bactericidal antibodies specifically recognizing the NHBA antigen mediated killing. We speculate that the killing in these natural strains may be either due to a different protein expression level, or to the generic characteristics of the strains analysed (for instance complement susceptibility), or to contributions from antibodies elicited to the other components of the vaccine. In fact, due to the presence of multiple antigens in the vaccine formulation (fHbp, NadA, NHBA and OMV-NZ), immune sera may contain antibodies against each of these antigens and the SBA reflects the cumulative effect of all of the antibodies present which may cause complement-mediated killing of a target strain. Moreover, a cooperative serum bactericidal activity was already demonstrated between human antibodies against fHbp and NHBA (Vu DM *et al* 2011) and contributions of non-PorA OMV antibodies have been implicated in NBA-mediated killing (Giuliani et al., vaccine 2010). The data presented in this thesis also suggest that the NHBA-GNA1030 antigen of the vaccine can elicit functional bactericidal antibodies against diverse peptide-expressing strains and the addition of OMV to the vaccine evokes a bactericidal response against components of OMV that complements the response to NHBA.

The fact that NHBA alleles show some association to clonal complexes and STs (Bambini S. *et al* 2009), might suggest that the NHBA-mediated antibody responses and SBA outcomes could reflect the molecular epidemiology or variability of the genetic characteristics of strains belonging to the same clonal complexes. However, as already reported recently (Vogel *et al* 2013), neither clonal complex alone nor antigen genotype alone is sufficient to predict which strains would be killed in the serum

bactericidal assay. Therefore the meningococcal antigen typing system (MATS) whose readout takes into account both expression levels and genetic distance of the allele is appropriate to predict NHBA-mediated killing of circulating strains due to vaccine responses.

Interestingly we observed a higher cross-protective ability from adolescent sera with respect to infant sera for natural strains, while from the analysis of the recombinant strains no age-related influence in cross-bactericidal anti-NHBA responses was observed. Therefore these age-related differences are seen predominantly on the cumulative effects of the vaccine responses and we speculate that this may be due to priming of the older age groups by natural bacterial carriage.

In conclusion, we show that the anti-NHBA antibodies are more efficient at killing strains expressing genetically close NHBA variants in an isogenic background, in the absence of any other effectors or negative strain-related factors. Moreover, the presence in the vaccine of the other antigens (including non-PorA OMV antigens) may compensate for the differential response to NHBA peptide variants and can induce broadly protective immune responses. These data underscore the importance of using a multicomponent vaccine to induce broadly protective immunity in infants and adolescents.

2) NHBA functional characterization

NHBA was shown to bind heparin *in vitro* through an Arg-rich region and upon binding, unencapsulated bacteria showed increased survival in human serum (Serruto D. *et al* 2010). Heparin binding is a common feature of several virulence factors and is usually associated with an increased serum resistance or with the facilitation of bacterial adhesion to host tissues by binding to heparin-like molecules. Heparan sulfate is a close structural relative of heparin, found in the extracellular matrix and on the surface of most animal cells and several bacterial adhesins are reported to bind heparin and heparan sulfate including the *N. meningitidis* Opc adhesin (F.P. de Vries, R. *et al* 1998); *Neisseria gonorrhoeae* Opa proteins (T. Chen, et al, 1995), heparin-binding hemagglutinin adhesin of *Mycobacterium tuberculosis* (Menozzi, F.D. *et al* 1996) and the filamentous hemagglutinin of *Bordetella pertussis* (Hannah, J.H. *et al* 1994). It was already hypothesized that *in vivo* NHBA was likely to bind glycoaminoglycans (such as heparan sulfate) that are present in mucosal secretions and in this context could contribute to the interaction of meningococcus with the host cells (Serruto D. *et al* 2010).

Confocal microscopy analysis experiments showed a direct binding of the recombinant NHBA protein to Hec1B and CHO epithelial cells. The binding appeared to be mediated specifically by heparan sulfate glycosaminoglycans, since the protein did not bind to CHO cells genetically deficient for glycosaminoglycans biosynthesis and showed a colocalization with heparan sulfate found in the ECM and on cell surface. Furthermore, digestion of Hec-1B cells with heparinase III but not choindroitinase specifically abolished cell binding.

Moreover, in accordance to what was previously reported for the binding to heparin *in vitro* (Serruto D. *et al* 2010), only protein fragments possessing the Arg-rich region were able to interact with epithelial cells, confirming the importance of this region in specifically mediating binding to the negatively-charged heparan sulfate proteoglycans. Significantly, the Arg-rich region is extremely conserved among different NHBA peptides, underlying its importance in bacterial pathophysiology.

Binding to a heparan sulfate proteoglycan on the target cell could be part of the mechanism used by meningococcus to adhere and then enter host cells, as it was already observed for other viral or parasite heparin-binding proteins.

In order to evaluate the role of NHBA as a novel meningococcal adhesin we studied the protein in the bacterial background. Analyzing a panel of *N. meningitidis* strains, we observed that the deletion of *nhba* resulted in a reduction of adhesion with respect to each isogenic wild type strain. Furthermore, the adhesion of the wild-type strain was prevented by using anti-NHBA polyclonal sera, demonstrating the specificity of the interaction. Despite the different strategies attempted to generate a fully functional complementation mutant in the MC58 background, we never obtained the complementation of the adhesion phenotype to the same levels of the wild-type strain. Since FACS analysis proved that the protein was successfully expressed on the bacterial surface, our hypothesis is that we are either inducing polar or secondary mutations by generating the *nhba* deletion mutant. It is more likely that a polar affect which alters some other gene within or near the *nhba* locus is contributing partially or fully to the phenotype of the knockout mutant as generation of the mutant in six different backgrounds faithfully resulted in the significant adhesion phenotype. To test these hypotheses we are now generating a revertant strain, to analyse if the adhesion capability of the wild-type strain can be in such a way restored. Furthermore the significant adhesion phenotype that we observed in the knockout would be uncharacteristic of a minor adhesin, as apart from Opc, Opa and pili which have an essential contribution to initial contact and adhesion in meningococcus, there is a large redundancy of minor adhesins, and knockout mutants of any of these demonstrate less evident adhesion phenotypes, in

particular, often only in acapsulate or non-piliated strains (Capecchi *et al*., 2005; Serruto *et al*., 2003, Hung MC *et al*, 2013, Scarselli M *et al* 2006).

NalP expression is known to mediate NHBA cleavage and to negatively influence biofilm formation (Arenas J. *et al* 2012), however its presence or absence in the panel of strains tested in this study, did not seem to influence NHBA-dependent adhesion to epithelial cells.

This correlates well with the fact that NalP was found to be non-functional in commensals and its expression seems to be mostly important in the context of proliferation and adaptation in human blood, when causing invasive disease. In accordance to this, the NHBA C2 fragment, generated by NalP cleavage was shown to increase permeability of endothelial cell monolayers (Casellato A. *et al* 2013).

These results suggest that NHBA could have distinct roles in different steps of meningococcal pathogenesis. NHBA has already been reported to influence bacterial survival in blood through the binding to heparin and in contributing to vascular leakage by altering endothelial permeability (Serruto D. *et al* 2010, Casellato A. *et al* 2013). Here we showed that it also contributes to the adhesion to the human epithelium and to host-cell interaction via the ability to bind to heparan sulfate proteoglycans.

3) NHBA NalP-mediated cleavage

It has been demonstrated that NHBA is a target of the meningococcal protease NalP and that this cleavage event results in the release of a 22-kDa fragment, also named C2, into the extracellular milieu. This process has been reported in the MC58 strain, which generally expresses high levels of NalP and NHBA peptide 3. Also by complementing *in trans* an heterologous NHBA peptide from the NZ98/254 strain (peptide 2), the same cleavage pattern was observed (Serruto D. *et al* 2010).

Here, we analysed the putative NalP cleavage site of the different NHBA peptides and observed at least eleven divergent target sequences. By Western blot analysis on natural strains and on the recombinant 5/99 isogenic strains we found that among the NHBA peptides analysed, only peptide p1, p2 and p3, were cleaved. These peptides all present the same target sequence (TSFA) and have a longer aminoacid sequence due to the 60 aminoacid insertion that segregates the NHBA proteins into long and short families (Fig 5A) .

This result may be explained either by polymorphisms at the level of the cleavage site or by a different conformation of the protein which masks the same site and does not make it accessible to NalP.

Interestingly, while no cleavage was observed when p18 was expressed in the MC58 background, when we expressed an engineered NHBA p18 peptide, including the insertion sequence found in the peptide p2, cleavage was observed. This result would suggest that not only the target aminoacid sequence is important for cleavage, but also the final protein conformation and exposure of the cleavage site, which is probably different in the shorter NHBA peptides. However, partial cleavage of the p3 peptide is also observed when expressed in a NalP knockout of MC58, suggesting that the long peptide conformation is generally more susceptible to proteolytic cleavage.

The cleavage event has raised concerns that NalP expression may reduce susceptibility of MenB isolates to 4CMenB induced immune response, at least in those isolates where NalP is expressed (Oldfield *et al* 2013). However we do not have strong evidence for a correlation between the immunogenicity and NalP activity. Cross-bactericidal activity of NHBA antibodies in human vaccinee sera was observed against p1, p2, p3 and p5 giving similar titres, in the recombinant isogenic background, when p5 is not cleaved and p1, p2 and p3 are. It would be interesting to understand the biological role of NHBA in those strains where NalP is not expressed or it is not active on NHBA, and the C2 fragment is not generated. For this analysis, site directed mutations of the cleavage site are ongoing. Furthermore in light of the recent report where the NHBA C2 fragment, generated by NalP cleavage was shown to increase permeability of endothelial cell monolayers (Casellato A. *et al* 2013), this phenomenon may only be limited to certain peptide-expressing strains. More research is needed to evaluate the relationship between cleavage, immunogenicity and the virulence/colonization ability of the strains.

References

Achtman M. (1995) Epidemic spread and antigenic variability of Neisseria meningitidis. Trends Microbiol. May;3(5):186-92.

Aguilera J-FJ, Perrocheau AA, 5 (2002) Outbreak of serogroup W135 meningococcal disease after the Hajj pilgrimage, Europe, 2000. Emerging Infect Dis 8: 761–767.

Allison H. Bartlett and Pyong Woo (2011) Heparan Sulfate Proteoglycans in Infection, Glycans in Diseases and Therapeutics, Chapter 2, Biology of Extracellular Matrix, Springer-Verlag Berlin Heidelberg .

Anderson AS, Hao L, Jiang Q, Harris SL, Jones TR, Perez JL, York L, Eiden J, Jansen KU**. (**2012) Potential impact of the bivalent rLP2806 vaccine on Neisseria meningitidis carriage and invasive serogroup B disease.Hum Vaccin Immunother. Dec 18;9(3).

Anderson EL, Bowers T, Mink CM, Kennedy DJ, Belshe RB, Harakeh H, Pais L, Holder P, Carlone GM. (1994) Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults. Infect Immun. Aug;62(8):3391-5.

Arenas J., Nijland R., Rodriguez FJ, Bosma TN, Tommassen J. (2013) Involvement of three meningococcal surface-exposed proteins, the heparin-binding protein NHBA, the α-peptide of IgA

protease and the autotransporter protease NalP, in initiation of biofilm formation. Mol Microbiol 87(2):254–268.

Bambini S, Muzzi A, Olcen P, Rappuoli R, Pizza M, Comanducci M. (2009) Distribution and genetic variability of three vaccine components in a panel of strains representative of the diversity of serogroup B meningococcus. Vaccine. May 11;27(21):2794-803.

Boisier P, Nicolas P, Djibo S, Taha M-K, Jeanne I, et al. (2007) Meningococcal meningitis:unprecedented incidence of serogroup X-related cases in 2006 in Niger. Clin Infect Dis 44: 657–663.

Borrow R, Fox AJ, Richmond PC, Clark S, Sadler F, Findlow J, Morris R, Begg NT, Cartwright KA. (2000) Induction of immunological memory in UK infants by a meningococcal A/C conjugate vaccine. Epidemiol Infect. Jun;124(3):427-32.

Branham SE (1953) Serological relationships among meningococci. Bacteriol Rev 17: 175–188.

Brunelli B., Del Tordello E., Palumbo E., Biolchi A., Bambini S., Comanducci M., Muzzi A., Pizza M., Rappuoli R., Donnelly JJ, Giuliani MM, Serruto D. Influence of sequence variability on bactericidal activity sera induced by Factor H binding protein variant 1.1. Vaccine. 2011 Jan 29;29(5):1072-81.

Casellato A, Rossi Paccani S, Barrile R, Bossi F, Ciucchi L, Codolo G, Pizza M, Aricò B, de Bernard M. (2013) The C2 fragment from Neisseria meningitidis antigen NHBA increases endothelial permeability by destabilizing adherens junctions. Cell Microbiol. Dec 13.

Cecchini P, Tavano R, Polverino de Laureto P, Franzoso S, Mazzon C, Montanari P, Papini E. (2011) The soluble recombinant Neisseria meningitidis adhesin NadA(Δ351-405) stimulates human monocytes by binding to extracellular Hsp90. PLoS One.;6(9):e25089.

Capecchi B, Adu-Bobie J, Di Marcello F, Ciucchi L, Masignani V, Taddei A, Rappuoli R, Pizza M, Aricò B. (2005) Neisseria meningitidis NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells. Mol Microbiol. Feb;55(3):687-98.

Chen T., Swanson J., Wilson J., Belland R.J. (1995) Heparin protects Opa+ Neisseria gonorrhoeae from the bactericidal action of normal human serum. Infect Immun, 63 (May 5) pp. 1790–1795.

Costantino P, Viti S, Podda A, Velmonte MA, Nencioni L, Rappuoli R. (1992) Development and phase 1 clinical testing of a conjugate vaccine against meningococcus A and C. Vaccine;10(10):691-8.

De Vries F.P., Cole R., Dankert J., Frosch M., van Putten J.P. (1998), Neisseria meningitidis producing the Opc adhesin binds epithelial cell proteoglycan receptors Mol Microbiol, 27 (March 6) pp. 1203–1212.

Deghmane AE, Giorgini D, Larribe M, Alonso JM, Taha MK. (2002) Down-regulation of pili and capsule of Neisseria meningitidis upon contact with epithelial cells is mediated by CrgA regulatory protein. Mol Microbiol. Mar;43(6):1555-64.

Del Tordello E, Vacca I, Ram S, Rappuoli R, Serruto D (2014) Neisseria meningitidis NalP cleaves human complement C3, facilitating degradation of C3b and survival in human serum. Proc Natl Acad Sci U S A. Jan 7;111(1):427-32.

Eugène E, Hoffmann I, Pujol C, Couraud PO, Bourdoulous S, Nassif X. (2002) Microvilli-like structures are associated with the internalization of virulent capsulated Neisseria meningitidis into vascular endothelial cells. J Cell Sci. Mar 15;115(Pt 6):1231-41.

Fairley CK, Begg N, Borrow R, Fox AJ, Jones DM, Cartwright K. (1996) Conjugate meningococcal serogroup A and C vaccine: reactogenicity and immunogenicity in United Kingdom infants. J Infect Dis. Dec;174(6):1360-3.

Finne, J., D. Bitter-Suermann, et al. (1987). "An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues." J Immunol 138(12): 4402-7.

Giuliani MM, Adu-Bobie J, Comanducci M, Aricò B, Savino S, Santini L, Brunelli B, Bambini S, Biolchi A, Capecchi B, Cartocci E, Ciucchi L, Di Marcello F, Ferlicca F, Galli B, Luzzi E, Masignani V, Serruto D, Veggi D, Contorni M, Morandi M, Bartalesi A, Cinotti V, Mannucci D, Titta F, Ovidi E, Welsch JA, Granoff D, Rappuoli R, and Pizza M (2006) A universal vaccine for serogroup B meningococcus. Proc Natl Acad Sci USA.103:10834–9.

Giuliani MM, Biolchi A, Serruto D, Ferlicca F, Vienken K, Oster P, Rappuoli R, Pizza M, Donnelly J. (2010) Measuring antigen-specific bactericidal responses to a multicomponent vaccine against serogroup B meningococcus. Vaccine. 2010 Jul 12;28(31):5023-30.

Hammerschmidt S[1], Müller A, Sillmann H, Mühlenhoff M, Borrow R, Fox A, van Putten J, Zollinger WD, Gerardy-Schahn R, Frosch M. (1996) Capsule phase variation in Neisseria meningitidis serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol Microbiol. Jun;20(6):1211-20.

Hannah J.H., Menozzi F.D., Renauld G., Locht C., Brennan M.J. (1994) Sulfated glycoconjugate receptors for the Bordetella pertussis adhesin filamentous hemagglutinin (FHA) and mapping of the heparin-binding domain on FHA. Infect Immun, 62 (November 11) pp. 5010–5019.

Hayrinen, J., H. Jennings, et al. (1995). Antibodies to polysialic acid and its N-propylderivative: binding properties and interaction with human embryonal brain glycopeptides. J Infect Dis 171(6): 1481-90.

Hill DJ, Griffiths NJ, Borodina E, Virji M. (2010) Cellular and molecular biology of Neisseria meningitidis colonization and invasive disease. Clin Sci (Lond). Feb 9;118(9):547-64.

Hill DJ, Virji M. (2012) Meningococcal ligands and molecular targets of the host. Methods Mol Biol.;799:143-52

Hilse R, Hammerschmidt S, Bautsch W, Frosch M (1996) Site-specific insertion of IS1301 and distribution in Neisseria meningitidis strains. J Bacteriol 178: 2527–2532.

Holst J, Feiring B, Naess LM, Norheim G, Kristiansen P, et al. (2005) The concept of "tailormade," protein-based, outer membrane vesicle vaccines against meningococcal disease. Vaccine 23: 2202–2205.

Hung MC, Heckels JE, Christodoulides M. (2013) The adhesin complex protein (ACP) of Neisseria meningitidis is a new adhesin with vaccine potential. MBio. Feb 26;4(2).

Joshi VS, et al. (2009) Meningococcal polysaccharide vaccines: A review. Carbohydr Polym; 75:553 – 65.

Kahler CM, Martin LE, Stephens DS, 6 (1998) The (alpha2-->8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B Neisseria meningitidis to resist the bactericidal activity of normal human serum. Infect Immun 66: 5939–5947.

Lieberman JM, Chiu SS, Wong VK, Partidge S, Chang SJ, Chiu CY, Gheesling LL, Carlone GM, Ward JI. (1996) Safety and immunogenicity of a serogroups A/C Neisseria meningitidis oligosaccharide-protein conjugate vaccine in young children. A randomized controlled trial. JAMA. May 15;275(19):1499-503.

Lucidarme J, Comanducci M, Findlow J, Gray SJ, Kaczmarski EB, Guiver M, Kugelberg E, Vallely PJ, Oster P, Pizza M, Bambini S, Muzzi A, Tang CM, Borrow R. (2009) Characterization

of fHbp, nhba (gna2132), nadA, porA, sequence type (ST), and genomic presence of IS1301 in group B meningococcal ST269 clonal complex isolates from England and Wales.J Clin Microbiol. Nov;47(11):3577-85.

Lucidarme J, Comanducci M, Findlow J, Gray SJ, Kaczmarski EB, Guiver M, Vallely PJ, Oster P, Pizza M, Bambini S, Muzzi A, Borrow R. (2010) Characterization of fHbp, nhba (gna2132), nadA, porA, and sequence type in group B meningococcal case isolates collected in England and Wales during January 2008 and potential coverage of an investigational group B meningococcal vaccine. Clin Vaccine Immunol. Jun;17(6):919-29.

Maiden M., Bygraves J., Feil E., Morelli G., Russell J., et al. (1998) Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. P Natl Acad Sci Usa 95: 3140–3145.

McNeil G, Virji M, Moxon ER. Interactions of Neisseria meningitidis with human monocytes. Microb Pathog. 1994 Feb;16(2):153-63.

Menozzi F.D., Rouse J.H., Alavi M., Laude-Sharp M., Muller J., Bischoff R. et al. (1996) Identification of a heparin-binding hemagglutinin present in mycobacteria. J Exp Med, 184 (September 3), pp. 993–1001.

Miller E, Salisbury D, Ramsay M. (2001) Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story. Vaccine. Oct 15;20 Suppl 1:S58-67.

Muzzi A, Mora M, Pizza M, Rappuoli R, Donati C. (2013) Conservation of meningococcal antigens in the genus Neisseria. MBio. Jun 11;4(3):e00163-13.

Muzzi A, Mora M, Pizza M, Rappuoli R, Donati C. (2013) Conservation of meningococcal antigens in the genus Neisseria. MBio. Jun 11;4(3)

Nägele V, Heesemann J, Schielke S, Jiménez-Soto LF, Kurzai O, Ackermann N. (2011) Neisseria meningitidis adhesin NadA targets beta1 integrins: functional similarity to Yersinia invasin. J Biol Chem. Jun 10;286(23):20536-46.

Nassif X. (1999) Interactions between encapsulated Neisseria meningitidis and host cells. Int Microbiol. Sep;2(3):133-6. Review.

Nassif, X. Marceau M, Pujol C, Pron B, Beretti JL, Taha MK. (1997)Type-4 pili and meningococcal adhesiveness. Gene 192, 149–153.

Parkhill J, Achtman M, James KD, Bentley SD, Churcher C, et al. (2000) Complete DNA sequence of a serogroup A strain of Neisseria meningitidis Z2491. Nature 404: 502–506.

Parkhill J, Achtman M, James KD, Bentley SD, Churcher C, et al. (2000) Complete DNA sequence of a serogroup A strain of Neisseria meningitidis Z2491. Nature 404: 502–506.

Patti JM, Allen BL, McGavin MJ, Höök M. (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol.;48:585-617.

Plested JS, Welsch JA, Granoff DM. (2009) Ex vivo model of meningococcal bacteremia using human blood for measuring vaccine-induced serum passive protective activity. Clin Vaccine Immunol. Jun;16(6):785-91.

Plested JS, Granoff DM. (2008) Vaccine-induced opsonophagocytic immunity to Neisseria meningitidis group B. Clin Vaccine Immunol. May;15(5):799-804.

R. Borrow, I.S. Aaberge, G.F. Santos, T.L. Eudey, P. Oster and A. Glennie, *et al.* (2005) Interlaboratory standardization of the measurement of serum bactericidal activity by using human complement against meningococcal serogroup b, strain 44/76-SL, before and after vaccination with the Norwegian MenB vac outer membrane vesicle vaccine. *Clin Diagn Lab Immunol*, 12 8, pp. 970–976.

Ramsay ME, Andrews N, Kaczmarski EB, Miller E. (2001) Efficacy of meningococcal serogroup C conjugate vaccine in teenagers and toddlers in England. Lancet. Jan 20;357(9251):195-6.

Rosenstein NE, PERKINS BA, Stephens DS, POPOVIC T, Hughes JM (2001) Meningococcal Disease. N Engl J Med 344: 1378–1388.

Rosenstein NE, Fischer M, Tappero JW. **(**2001) Meningococcal vaccines. Infect Dis Clin North Am. Mar;15(1):155-69

Rostand KS, Esko JD. (1997) Microbial adherence to and invasion through proteoglycans. Infect Immun. Jan;65(1):1-8.

Roussel-Jazédé V, Grijpstra J, van Dam V, Tommassen J, van Ulsen P (2013) Lipidation of the autotransporter NalP of Neisseria meningitidis is required for its function in the release of cell-surface-exposed proteins. Microbiology 159(Pt 2):286–295.

Roussel-Jazédé V, Jongerius I, Bos MP, Tommassen J, van Ulsen P (2010) NalP mediated proteolytic release of lactoferrin-binding protein B from the meningococcal cell surface. Infect Immun 78(7):3083–3089.

Sa E Cunha C, Griffiths NJ, Virji M. Neisseria meningitidis Opc invasin binds to the sulphated tyrosines of activated vitronectin to attach to and invade human brain endothelial cells. PLoS Pathog. 2010 May 20;6(5):e1000911.

Sarrazin S, Lamanna WC, Esko JD. (2011) Heparan sulfate proteoglycans. Cold Spring Harb Perspect Biol. Jul 1;3(7).

Saunders NJ, Jeffries AC, Peden JF, Hood DW, Tettelin H, Rappuoli R, Moxon ER. (2000) Repeat-associated phase variable genes in the complete genome sequence of Neisseria meningitidis strain MC58. Mol Microbiol 37(1): 207–215.

Scarselli M, Serruto D, Montanari P, Capecchi B, Adu-Bobie J, Veggi D, Rappuoli R, Pizza M, Aricò B. (2006) Neisseria meningitidis NhhA is a multifunctional trimeric autotransporter adhesin. Mol Microbiol. Aug;61(3):631-44. Epub 2006 Jun 27.

Schoen C, Blom J, Claus H, Schramm-Glueck A, Brandt P, et al. (2008) Whole-genome comparison of disease and carriage strains provides insights into virulence evolution in Neisseria meningitidis. P Natl Acad Sci Usa 105: 3473–3478.

Scholten RJ, Bijlmer HA, Poolman JT, Kuipers B, Caugant DA, Van Alphen L, Dankert J, Valkenburg HA. (1993) Meningococcal disease in The Netherlands, 1958-1990: a steady increase in the incidence since 1982 partially caused by new serotypes and subtypes of Neisseria meningitidis. Clin Infect Dis. Feb;16(2):237-46.

Serruto D, Rappuoli R., Pizza M. (2004) Meningoccocus B: from Genome to Vaccine. Genomics, proteomics and vaccines. Handbook of Meningococcal Disease: Infection Biology, Vaccination, Clinical Management. Wiley-VCH.

Serruto D, Spadafina T, Ciucchi L, Lewis LA, Ram S, Tontini M, Santini L, Biolchi A, Seib KL, Giuliani MM, Donnelly JJ, Berti F, Savino S, Scarselli M, Costantino P, Kroll JS, O'Dwyer C, Qiu J, Plaut AG, Moxon R, Rappuoli R, Pizza M, Aricò B. (2010) Neisseria meningitidis GNA2132, a heparin-binding protein that induces protective immunity in humans. Proc Natl Acad Sci USA 107(8): 3770–3775.

Serruto D, Bottomley MJ, Ram S, Giuliani MM, Rappuoli R. (2012) The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens.Vaccine. May 30;30 Suppl 2:B87-97.

Serruto D., Adu-Bobie J, Scarselli M, Veggi D, Pizza M, Rappuoli R, Aricò B. (2003) Neisseria meningitidis App, a new adhesin with autocatalytic serine protease activity. Mol Microbiol. Apr;48(2):323-34.

Sharip A, Sorvillo F, Redelings MD, Mascola L, Wise M, et al. (2006) Population-based analysis of meningococcal disease mortality in the United States: 1990-2002. Pediatr Infect Dis J 25: 191–194.

Shepard CW, Rosenstein NE, Fischer M, Active Bacterial Core Surveillance Team (2003) Neonatal meningococcal disease in the United States, 1990 to 1999. Pediatr Infect Dis J 22: 418–422.

Sjölinder H, Eriksson J, Maudsdotter L, Aro H, Jonsson AB. (2008) Meningococcal outer membrane protein NhhA is essential for colonization and disease by preventing phagocytosis and complement attack. Infect Immun. Nov;76(11):5412-20.

Stephens DS (2009) Biology and pathogenesis of the evolutionarily successful, obligate human bacterium Neisseria meningitidis. Vaccine 27 Suppl 2: B71–7.

Stephens DS, Greenwood B, Brandtzaeg P (2007) Epidemic meningitis, meningococcaemia, and Neisseria meningitidis. Lancet 369: 2196–2210.

Takahashi H, Carlson RW, Muszynski A, Choudhury B, Kim KS, et al. (2008) Modification oflipooligosaccharide with phosphoethanolamine by LptA in Neisseria meningitidis enhances meningococcal adhesion to human endothelial and epithelial cells. Infect Immun 76: 5777–5789.

Tappero JW, Lagos R, Ballesteros AM, Plikaytis B, Williams D, Dykes J, Gheesling LL, Carlone GM, Høiby EA, Holst J, Nøkleby H, Rosenqvist E, Sierra G, Campa C, Sotolongo F, Vega J, Garcia J, Herrera P, Poolman JT, Perkins BA. Immunogenicity of 2 serogroup B outer-membrane protein meningococcal vaccines: a randomized controlled trial in Chile. JAMA. 1999 Apr 28;281(16):1520-7.

Tettelin H, Saunders NJ, Heidelberg J, Jeffries AC, Nelson KE, et al. (2000) Complete genome sequence of Neisseria meningitidis serogroup B strain MC58. Science 287: 1809-1815.

Turner DP, Wooldridge KG, Ala'Aldeen DA (2002) Autotransported serine protease A of Neisseria meningitidis: An immunogenic, surface-exposed outer membrane, and secreted protein. Infect Immun 70(8):4447–4461.

Van Ulsen P, et al. (2003) A Neisserial autotransporter NalP modulating the processing of other autotransporters. Mol Microbiol 50(3):1017–1030.

Van Ulsen P, et al. (2006) A novel phase-variable autotransporter serine protease, AusI, of Neisseria meningitidis. Microbes Infect 8(8):2088–2097.

Virji M, Makepeace K, Ferguson DJ, Achtman M, Moxon ER. (1993) Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. Mol Microbiol. Nov;10(3):499-510.

Virji M, Makepeace K, Ferguson DJ, Achtman M, Sarkari J, Moxon ER. (1992) Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by Neisseria meningitidis. Mol Microbiol. Oct;6(19):2785-95.

Virji M. (2009) Pathogenic neisseriae: surface modulation, pathogenesis and infection control. Nat Rev Microbiol. Apr;7(4):274-86.

Vogel U, Frosch M. (1999) Mechanisms of neisserial serum resistance. Mol Microbiol. Jun;32(6):1133-9.

Vogel U, Taha MK, Vazquez JA, Findlow J, Claus H, Stefanelli P, Caugant DA, Kriz P, Abad R, Bambini S, Carannante A, Deghmane AE, Fazio C, Frosch M,Frosi G, Gilchrist S, Giuliani MM, Hong E, Ledroit M, Lovaglio PG, Lucidarme J, Musilek M, Muzzi A, Oksnes J, Rigat F, Orlandi L, Stella M, Thompson D, Pizza M,Rappuoli R, Serruto D, Comanducci M, Boccadifuoco G, Donnelly JJ, Medini D, Borrow R. (2013) Predicted strain coverage of a meningococcal multicomponent vaccine (4CMenB) in Europe: a qualitative and quantitative assessment. Lancet Infect Dis. May;13(5):416-25.

Vu DM, Wong TT, Granoff DM. (2011) Cooperative serum bactericidal activity between human antibodies to meningococcal factor H binding protein and neisserial heparin binding antigen. Vaccine. Feb 24;29(10):1968-73.

Zahlanie YC, Hammadi MM, Ghanem ST, Dbaibo GS. (2014) Review of meningococcal vaccines with updates on immunization in adults. Hum Vaccin Immunother. Feb 5;10(4).