SYSTEMATIC ANALYSIS OF FHBP SEQUENCE COVERAGE BY ANTIBODIES AGAINST MENINGOCOCCAL GMMA FROM AFRICAN GROUP W STRAINS

Maria Grazia Aruta

Dottorato in Scienze Biotecnologiche – XXVIII ciclo
Indirizzo Biotecnologie Industriali e Molecolari
Università di Napoli Federico II

Dottorato in Scienze Biotecnologiche – XXVIII ciclo
Indirizzo Biotecnologie Industriali e Molecolari
Università di Napoli Federico II

SYSTEMATIC ANALYSIS OF FHBP SEQUENCE COVERAGE BY ANTIBODIES AGAINST MENINGOCOCCAL GMMA FROM AFRICAN GROUP W STRAINS

Maria Grazia Aruta

Dottorando:	Maria Grazia Aruta
Relatore:	Prof. Giovanni Sannia, Dr. Oliver Koeberling
Coordinatore:	Prof. Giovanni Sannia

Il miglior riconoscimento
per la fatica fatta
non è ciò che se ne ricava,
ma ciò che si diventa
grazie ad essa.

John Ruskin

Index

Summary

FHbp sequence diversity expressed by African isolates is limited among currently circulating strains in sub-Saharan Africa but could change in the future.
The primary aim of the project was to systematically investigate and dissect the breadth and nature of fHbp sequence coverage irrespective of the fHbp sequence prevalence. This was obtained by using a combination of bioinformatic tools and analysis of functional antibody raised against fHbp over-expressed in meningococcal GMMA from an African group W strain.
The work is divided into three parts:
1. Comparative structure/sequence analysis of the amino acids involved in factor H binding including more than 700 individual existing and published fHbp sequences. The results obtained show a differential degree of variability of the factor H binding site between fHbp belonging to variant 1 versus those belonging to variant 2 or 3 with greater sequence diversification of the factor H binding site in the v.1 group.
2. Systematic selection of fHbp sequences based on a gradually different number of differences in the factor H (fH) binding site compared to fHbp variant 1 ID9 expressed in the group W GMMA. Applying these criteria a panel of 11 African and non-African wild type strains (belonging to serogroup A, C, W, Y or X) was selected that expressed a subset of the identified phylogenetically diverse fHbp sequences. Use of fHbp selection criteria to generate a panel of eight genetically defined isogenic strains that were engineered to express seven existing diverse fHbp v.1 sequences and one naturally existing and published fHbp v.1/2 hybrid protein. The wild type and isogenic strains as test strains in serum bactericidal assay to measure functional activity of antibodies raised against GMMA from a group W strain with over-expressed fHbp v.1 ID 9.
The sera had bactericidal activity against 10 out of the 11 diverse wild type strains. However, against 8 of the susceptible strains also sera raised against GMMA that did not contain fHbp were bactericidal suggesting that other yet unknown target(s) contribute to the generation of bactericidal antibodies.The sera raised in mice against the group W GMMA with over-expressed fHbp v.1 also had bactericidal activity against the panel of eight isogenic strains engineered to express diverse fHbp sequences including the strain engineered to express the natural fHbp v.1/2 hybrid sequence. The results with the isogenic strains expressing diverse fHbp sequences suggest that differences in the fH binding site contribute to susceptibility of the isolate to killing by anti fHbp antibodies but also overall sequence diversity plays a role.
3. As proof of principle to broaden coverage of GMMA from a group W strain with over-expressed fHbp v.1 a mutant group W strain engineered to over-express the native fHbp v.2 sequence, which is predominant among African group W isolates was generated. GMMA from the mutant had approximately 10-fold higher amount of fHbp than in GMMA from wild type meningococcus; penta-acylation of lipidA after inactivation of lpxL1 was confirmed by MALDI-TOF analysis and Monocyte-Activation Test (MAT) assay showed an approximately 100-fold decreases ability of GMMA from the mutant to activate cytokine release from human blood cells. By HPLC-SEC MALLS integrity, size and impurities of the vesicles were characterized.

Riassunto

Introduzione

La meningite causata dal batterio *Neisseria meningitidis* si verifica in tutto il mondo sia in forma endemica che epidemica. Infetta soltanto esseri umani; non esiste un portatore animale. *N. meningitidis* appartenente ai gruppi capsulari A, C, W e X è responsabile delle epidemie di meningite nella "cintura meningococcica africana" (un'area che si estende dall'Etiopia al Senegal) e sono necessari vaccini a buon mercato che possano garantire una ampia protezione contro i ceppi epidemici africani.
I moduli generalizzati per gli antigeni di membrana (GMMA) da ceppi di *N. meningitidis* geneticamente ingegnerizzati sono in fase di studio come potenziali candidati vaccini. Le GMMA sono vescicole della membrane esterna, rilasciate da batteri geneticamente modificati, che possono essere usate come vettori di antigeni.
In topo, le GMMA meningococciche inducono prevalentemente una risposta anticorpale diretta versa antigeni proteici.
La proteina di legame al fattore H (fHbp) è un antigene chiave del meningococco.
Si tratta di una lipoproteina presente sulla superficie della membrane esterna e può essere divisa in tre varianti antigeniche. Ogni variante può essere ulteriormente divisa in sub-varianti che differiscono da pochi amminoacidi e finora più di 900 sub-varianti sono state identificate, distinguibili da un'unica sequenza definita ID. Una delle funzioni di fHbp è legare il fattore umano H, un regolatore negative della cascata del complemento. Ogni variante fHbp si lega al fattore H mediante una regione della proteina detta sito di legame al fattore H.
Gli anticorpi elicitati contro una particolare variante fHbp sono in generale funzionali contro i ceppi di *N. meningitidis* che esprimono la stessa variante di fHbp.
Ad ogni modo, differenze nella sequenza di fHbp entro ogni variante possono incidere sulla suscettibilità di un ceppo alla lisi mediata da anticorpi anti-fHbp.
E' stato osservato che GMMA derivanti da un ceppo di serogruppo W overesprimente fHbp v.1 inducevano in topi anticorpi contro fHbp. In saggi gli anticorpi avevano la capacità di uccidere un pannello di ceppi africani di serogruppo A, W e X esprimenti fHbp v.1. Il pannello includeva ceppi rappresentativi esprimenti sequenze di fHbp che sono prevalenti tra gli attuali ceppi africani epidemici. La diversità della sequenza di fHbp è limitata tra gli isolati africani invasivi attualmente circolanti e poche sub-varianti di fHbp sono espresse dalla maggior parte dei ceppi. L'epidemiologia è dinamica e I ceppi con diverse sequenze di fHbp potrebbero diventare predominanti in future nella cintura meningococcica Africana. Un'analisi sistematica può fornire indicazione sull'ampiezza della copertura delle sequenze di fHbp che può emergere in future nell'Africa sub-Sahariana.

Scopo

Lo scopo primario del progetto è investigare ed esaminare sistematicamente l'ampiezza e la natura della copertura delle sequenza di fHbp mediata dagli anticorpi contro fHbp. Ciò è ottenuto usando una combinazione di strumenti bioinformatici come base per

analizzare l'attività funzionale degli anticorpi. L'analisi può supportare la selezione di sequenze di fHbp da includere in vaccini basati su fHbp per l'Africa sub-Sahariana.

Metodi

Ho messo a punto un'analisi comparativa di struttura/sequenza di più di 700 sequenze fHbp ID esistenti e pubblicate, appartenti alla variante 1 (N=383), variante 2 (N=151) e 3 (N=149). In particolare, ho comparato il livello di diversità degli amminoacidi in contatto col fattore H tra varianti diverse e entro la stessa variante nelle diverse sequenze di fHbp. Essendo accessibili in quanto di superficie ci si aspetta che tali amminoacidi siano sotto pressione immunitaria. Usando fHbp variante 1 come modello ho usato i risultati ottenuti mediante analisi bioinformatica per applicare nuovi criteri per la selezione di un pannello di diverse sequenze di fHbp a esprimere in *N. meningitidis* con l'obiettivo finale di studiare la copertura della sequenza di fHbp. Le sequenze di fHbp sono state selezionate sulla base di un livello di differenze nel sito di legame al fattore H gradualmente differente. Entro ogni livello una sequenza è stata selezionata sulla base della percentuale di identità più bassa comparata con l'fHbp inclusa nel vaccine.

1. Applicando questi criteri, ho selezionato un pannello di ceppi wild type (appartenenti ai sierogruppi A, C, W, Y e X) che esprimevano un sottoinsieme delle sequenze di fHbp selezionate.
2. Ho applicato i criteri di selezione per generare, partendo da un ceppo parente, un pannello di 8 ceppi isogenici geneticamente definiti che sono stati ingegnerizzati per esprimere 7 sequenze fHbp v.1 diverse esistenti e un ibrido fHbp v.1/v.2. I mutanti sono stati generati usando un ceppo parente resistente all'uccisione mediante siero contro GMMA senza fHbp. Le sequenze di fHbp selezionate sono state integrate nel genoma ed espresse sotto il controllo di un promotore inducibile da IPTG. Il vantaggio di questo pannello è che esso ha permesso di investigare in modo specifico l'attività funzionale di anticorpi contro fHbp dato che i mutanti differivano soltanto per la sequenza di fHbp che essi esprimevano. Ho usato i ceppi come ceppi test negli SBA (saggi per investigare la battericidia dei sieri) per misurare l'attività funzionale di anticorpi elicitati contro GMMA derivanti da un ceppo di sierogruppo W con fHbp v.1 overespressa in presenza di complemento umano e di coniglio.

Come prova di principio, ho generato un ceppo mutante di sierogruppo W (basato sullo stesso ceppo parente wild type che è stato usato per generare il mutante overesprimente fHbp v.1) e ingegnerizzato per overesprimere fHbp v.2 nativa, che è predominante tra gli isolati africani di sierogruppo W. Le GMMA dai mutanti generati sono stati caratterizzati dal punto di vista della forma tramite microscopia elettronica a colorazione negativa, dal punto di vista dell'espressione di fHbp tramite western blotting, dal punto di vista della reattogenicità tramite analisi del lipide A per MALDI-TOF e test di attivazione dei monociti (MAT) e dal punto di vista della dimensione, del contenuto di acido nucleico e di proteine solubili tramite HPLC-SEC.

Risultati

Dal confronto tra le sequenze del sito di legame al fattore H è risultato che per la variante 1 tra i 54 amminoacidi in contatto con il fattore H, 29 (55%) delle posizioni sono variabili. Al contrario, entro i gruppi relativi alla variante 2 e 3 rispettivamente soltanto 13 e 10 dei 54 residui in contatto con il fattore H sono variabili (24% e 18% rispettivamente). Un confronto a coppie del numero dei differenti residui tra ogni sequenza ID ha rivelato che entro il gruppo variante 1, il sito di legame al fattore H tra le sequenze individuali può differire in più di 13 posizioni. Al contrario, il massimo numero di differenze in amminoacidi osservato nel sito di legame al fattore H tra coppie di peptidi ID appartenenti alla variante 2 è stato 5 (osservato per 4 paia di sequenze) e 6 per la variante 3. Quando abbiamo comparato i residui in contatto con il fattore H tra variante 2 e 3, 125 peptidi IDs differivano per 5 o 6 amminoacidi, mentre all'interno di entrambe le varianti da 96 a 107 sequenze hanno lo stesso sito di legame al fattore H.
Le sequenze diverse di fHbp v.1 selezionate per essere espresse nei ceppi usati negli SBA avevano tra 0 e un massimo di 10 di differenze in amminoacidi nel sito di legame a fattore H comparati all'fHbp usata nel vaccino un'identità di sequenza complessiva tra 89.6% e 94.9%. Tramite SBA usando complemento di coniglio contro il pannello di ceppi wildtype esprimenti le diverse sequenze di fHbp, 10 su 11 ceppi si sono rivelati sensibili all'uccisione mediata da siero elicitato contro GMMA con fHbp v.1 over-espressa (media geometrica dei titoli osservati > 1000 contro 8 ceppi). Ad ogni modo, contro 8 su 11 ceppi anche i topi immunizzati con le GMMA controllo (senza fHbp) hanno sviluppato una risposta anticorpale battericida indicando la presenza di anticorpi contro target ancora sconosciuti. Tramite FACS, è stato osservato che l'espressione superficiale di fHbp è stata similare negli 8 diversi ceppi isogenici. Quando pool di sieri elicitati contro GMMA con fHbp over-espressa sono stati testati in presenza di complemento di coniglio (ricordando che il fattore H di coniglio non lega fHbp) contro il pannello di ceppi isogenici i titoli battericidi sono stati alti indipendentemente dall'fHbp espressa dal ceppo. Quando gli stessi sieri e isolati sono stati testati in presenza di complemento è stato osservato un trend secondo cui titoli battericidi più alti erano ottenuti quando il numero di differenze nel sito di legame al fattore H di fHbp espresse nel ceppo test era basso, comparato con il sito di legame al fattore H di fHbp usata nel vaccino. I risultati suggeriscono che le differenze nel sito di legame al fattore H sono cruciali rispetto a differenze osservate sull'intera sequenza.
I risultati della caratterizzazione delle GMMA derivanti dal ceppo Men W OE nativa fHbp v.2 ID23 ΔcpsΔlpxL1Δgna33 hanno rivelato, tramite western blotting, più di 10 volte maggiore l'espressione di fHbp nelle GMMA dai ceppi mutanti rispetto a quella osservata nelle GMMA derivanti dal ceppo wild type e un pattern proteico nei mutanti similare a quello osservato in Men W wild type tramite SDS PAGE/Coomassie blue. Tramite MAT assay, è stato osservato che mentre le GMMA non detossificate iniziavano a indurre il rilascio di interleuchina 6 (IL-6) già a basse concentrazioni (10^{-2}ng/mL), quelle detossificate iniziavano a rilasciarla a concentrazioni 100 volte maggiori (10^{1}ng/mL). Dopodichè, i ceppi di sierogruppo W, wild type e mutanti, aventi una differente combinazione delle mutazioni descritte in precedenza, sono stati analizzati, come già anticipato, tramite HPLC-SEC per valutare la dimensione e la presenza di proteini solubili e acido nucleico. In tutti i campioni analizzati mediante il canale della fluorescenza non c'era presenza dei picchi corrispondenti a proteine solubili, in genere eluenti a tempi differenti rispetto alle GMMA. Tramite il canale UV, in base al rapporto

ABS260/ABS280, è stata valutata l'assenza di acidi nucleici indipendentemente dal trattamento con la benzonasi. Questi risultati suggeriscono che le GMMA sono state purificate bene mediante filtrazione attraverso filtro da 0.22 µm.

Conclusioni

L'approccio basato sull'utilizzo di una matrice per l'analisi comparative del sito di legame al fattore H nelle tre varianti di fHbp v.1, v.2 e v.3 ha rivelato un grado di variabillità differenziale di tale sito, in particolare con una maggiore diversificazione osservata relativamente alla variante 1, rispetto alle alter due varianti di fHbp analizzate. La matrice è stata utilzzata come nuovo approccio per selezionare in modo razionale 8 diverse sequenze di fHbp appartenenti alla variante 1 così da analizzare l'ampiezza della protezione e della copertura che la sequenza di fHbp potrebbe garantire, indotta da GMMA derivanti da meningococco di sierogruppo W contenente fHbp v.1. Ciò che è stato osservato è stato che MenW GMMA overesprimenti fHbp v.1 inducevano anticorpi battericidi (in presenza di complemento umano) contro i ceppi isogenici test esprimenti tutte le diverse sequenze di fHbp. L'attività battericida è risultata quindi dipendente dall'espressione di fHbp nelle GMMA. I risultati hanno indicato che gli anticorpi indotti da MenW GMMA con over-espressa fHbp v.1 potrebbero garantire protezione contro un'ampia varietà di sequenze di fHbp.

II Introduction

2.1 Neglected diseases

The neglected tropical diseases (NTDs) are a group of chronic, disabling, and disfiguring conditions that occur most commonly in the setting of extreme poverty, especially among the rural poor and some disadvantaged urban populations (1, 2). Today, the world's greatest concentration of poverty occurs in sub-Saharan Africa (SSA). According to a recent World Bank analysis, 51% of the population of SSA lives on less than US$1.25 per day, and 73% of the population lives on less than US$2 per day (**Errore. L'origine riferimento non è stata trovata.**) (1, 3).

Table 1: World Bank data related to poverty in Sub-Saharan Africa.

Poverty in Sub-Saharan Africa	
Percentage of SSA population living on less than US$1.25 per day	51%
Total SSA population living on less than $1.25 per day	390.6 million
Percentage of world's population living on less than US$1.25 per day in SSA	28%
Percentage of SSA population living on less than US$2 per day	73%
Total SSA population living on less than $2 per day	556.7 million
Percentage of world's population living on less than US$2 per day in SSA	22%

Gram-negative bacteria, as invasive Salmonella, Shigella and Meningitis, are the major causes of neonatal sepsis in developing countries, causing a wide range of diarrheal, respiratory and invasive diseases (3). Together these diseases are responsible for well in excess of 160 million clinical cases per year with more than 1 million deaths.
Besides the progressive improvement of basic hygienic and sanitary conditions, immunization still represent the most cost effective and efficient way to control and possibly defeat infectious diseases. Unfortunately, vaccines are not yet available to prevent many major bacterial infections especially affecting developing countries.
An ideal vaccine should be easy to administer, i.e. not requiring high professional skills for being administered, well tolerated, formulated for protecting against multiple pathogens (both for reducing cost and the number of injections per child), easy to manufacture, low cost (ideally lower than 1 $ per dose) and able to induce long-term protection.

2.2 Meningitis

Meningitis is inflammation of the meninges. The meninges is the collective name for the three membranes that envelope the brain and spinal cord (central nervous system), called the dura mater, the arachnoid mater, and the pia mater (Figure 1). The meninges' main function, alongside the cerebrospinal fluid is to protect the central nervous system.

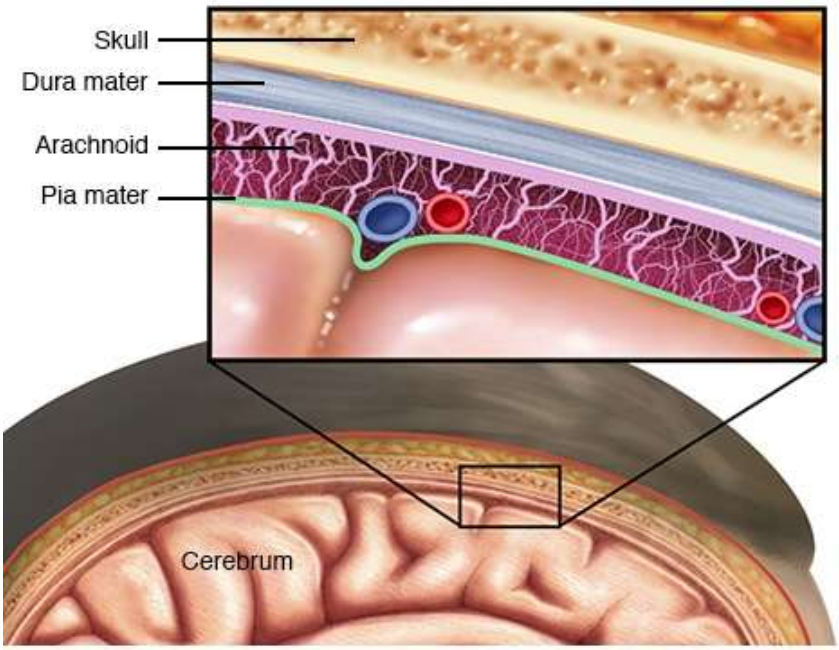


Figure 1: Meningitis is an infection and inflammation of the fluid and three membranes (meninges) protecting the brain and spinal cord. The tough outer membrane is called the dura mater, and the delicate inner layer is the pia mater. The middle layer is the arachnoid, a web-like structure containing the fluid and blood vessels covering the surface of the brain.

Meningitis is generally caused by infection of viruses, bacteria, fungi and parasites. Anatomical defects or weak immune systems may be linked to recurrent bacterial meningitis.

Although viral meningitis is the most common, it is rarely a serious infection. It can be caused by a number of different viruses, such as mosquito-borne viruses. There is no specific treatment for this type of meningitis. In the vast majority of cases the illness resolves itself within a week without any complications.

Bacterial meningitis is generally a serious infection. It is caused by four types of bacteria:

- ***Haemophilus influenzae* type b (Hib)** was once the leading cause of bacterial meningitis in children, but new Hib vaccines have greatly reduced the number of cases of this type of meningitis.
- ***Neisseria meningitidis* (meningococcus)** causes a blood and/or brain infection that affects mainly teenagers and young adults.
- ***Streptococcus pneumoniae* (pneumococcus)** is the most common cause of bacterial meningitis in infants, young children and adults in the United States (5).
- ***Listeria monocytogenes* (listeria)**, found in unpasteurized cheeses, hot dogs and luncheon meats, can cross the placental barrier, and infections in late pregnancy may be fatal to the baby.

2.2 Neisseria meningitidis

Neisseria meningitidis is an exclusively human, obligately aerobic, non sporulating, oxidase-positive, encapsulated or not, Gram negative pathogen, often diplococcal in form, about 0.6-1.0 µm in diameter (Figure 2) and recognized as the leading cause of meningitis and other forms of meningococcal disease such as meningococcemia, a life-threatening sepsis, in children and young adults.

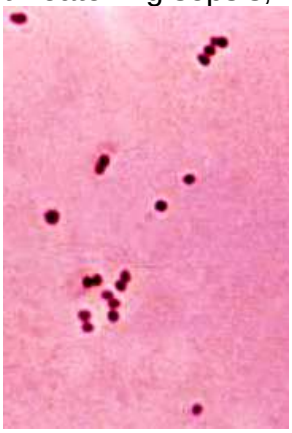


Figure 2: The micrograph depicts the presence of aerobic Gram-negative Neisseria meningitidis diplococcal bacteria; Mag. 1150X.

About 10% of adults are carriers of the bacteria in their nasopharynx (6, 7).
It causes the only form of bacterial meningitis known to occur epidemically (8), mainly in Africa and Asia.
N. meningitidis is spread through saliva and respiratory secretions for example during coughing, sneezing, kissing, and chewing on toys. It infects the cell by sticking to it with long thin extensions called pili and the surface-exposed proteins Opa and Opc and has several virulence factors.

2.2.1 Biology and microbiology

Neisseria meningitidis virulence depends on several factors as the iron sequestration mechanisms, the presence of endotoxin, the expression of surface adhesive proteins and of polysaccharide capsule, which is absent from *N. gonorrhoeae*, even though both organisms share about 90% homology at the nucleotide level.
N. meningitidis strains are primarily classified in serogroups based on capsule type that they express (11). Further classification is based on major outer-membrane porins into serotypes and serosubtypes as well as LPS (lipopolysaccharide) into immunotypes (12). Moreover, MLST (multi-locus sequence typing) classifies strains into STs (sequence types) based on variations among seven housekeeping genes (13).
Based on the sequencing of several genomes including those of strains MC58 (serogroup B, ST-32) (14), Z2491 (serogroup A, ST-4) (15), FAM18 (serogroup C, ST-11) and NMB-CDC (serogroup B, ST-8), it has been discovered that the chromosome is between 2.0 and 2.2 megabases in size and contains 2,000 genes (16). The core meningococcal genome, encoding for essential metabolic functions, represents about 70%

of the genome, while large genetic islands are predicted to encode hypothetical surface proteins and virulence factor.
N. meningitidis strains causing invasive disease are characterized almost always by the presence of a capsule, which is essential for the survival of the organism in the blood as it provides resistance to antibody/complement-mediated killing and inhibits phagocytosis (17).
Genes responsible for generation of the capsule are located in a single chromosomal locus *cps* that is divided in three regions A, B and C. Region A encodes enzymes for the biosynthesis and polymerization of the polysaccharide, and regions B and C carry the genes responsible for its translocation from the cytoplasm to the cell surface (18). "Capsule switching" can occur and is the result of horizontal gene exchange and recombination in the locus of serogroup specific capsule biosynthesis genes (19) and is one of the mechanism of escape from vaccine-induced or natural protective immunity and a virulence mechanism shown by other encapsulated bacterial pathogens (e.g., *Streptococcus pneumoniae*).
Although 13 meningococcal serogroups have been described (A, B, C, D, 29E, H, I, K, L, Y, W, X and Z), the majority of diseases is caused by organisms expressing one of six capsule types namely A, B, C, Y, W135 (described in **Errore. L'origine riferimento non è stata trovata.**) and X, differently distributed.

Table 2: Predominant meningococcal serogroups with respective capsular structures and operon compositions.

Serogroup	Capsule Structure			Operon
A	Non-sialic acid capsule	Homopolymers of N-acetyl-D-mannosamine-1-*P*	(α1→6)-linked-*N*-acetyl-D-mannosamine-1-phosphate	Gene operon: *mynA-mynD*
B	Sialic acid capsule (5-N-acetyl-neuraminic acid)	Homopolymers of sialic acid	(α2→8)-linked-*N*-acetyl-neuraminic acid *Mimics human structures – Poorly immunogenic*	Gene operon: *siaA-siaD*
C			(α2→9)-linked-*N*-acetyl-neuraminic acid *Highly immunogenic*	
W135		Heteropolymers of sialic acid-containing disaccharides	(α2→6)-linked-6-D-Gal(α1→4)-*N*-acetyl-neuraminic acid	
Y			(α2→6)-linked-6-D-Glc(α1→4)-*N*-acetyl-neuraminic acid	

As reported in the table above, the main meningococcal capsular polysaccharides associated with invasive disease contain sialic acid [NANA (5-N-acetylneuramic acid) and cps region A of these serogroups harbours a set of conserved genes *siaA, siaB* and

siaC, responsible for the synthesis of sialic acid in the form of CMP-NANA, required for incorporation into the capsular polysaccharide. The fourth gene in this region, *siaD*, encodes a serogroup-specific polysialyltransferase involved in capsule polymerization (20, 21). In serogroup A, the locus contains four genes named *mynA–D* (22) responsible of biosynthesis of repeating units of N-acetyl-mannosamine-1-phosphate. The incorporation of Neu5Ac into meningococcal capsules allows the meningococcus to become less visible to the host immune system (23, 24) because of molecular mimicry. The most striking example is observed in the serogroup B capsule (25), an α(2–8)-linked sialic acid homopolymer identical in structure to the human fetal neural cell-adhesion molecule (NCAM). Such identity is responsible for the particularly poor immune response generated against serogroup B capsule by humans (26).
The subcapsular cell envelope of *N. meningitidis* consists of an outer membrane (OM), a peptidoglycan layer, and a cytoplasmic or inner membrane (Figure 3).

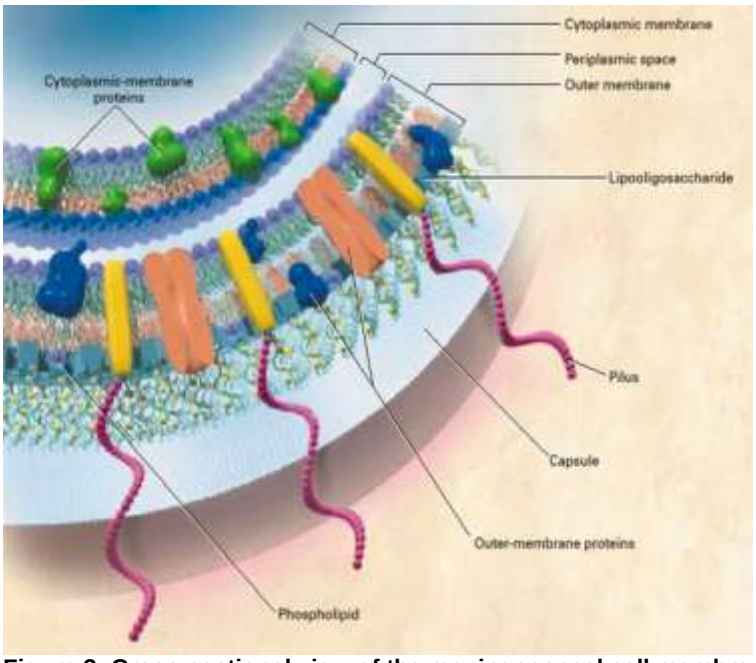


Figure 3: Cross-sectional view of the meningococcal cell membrane.

The OM has an outside layer primarily composed of lipopolysaccharide (LPS), proteins and an inside layer composed of phospholipids (27) that contains proteins primarily responsible for regulating the flow of nutrients and metabolic products. The structure of peptidoglycan, recognizable by components of the innate immune system (28), consists of two layers (29) with different variations in the degree of cross-linking and O-acetylation in the different strains of *N.meningitidis*.
Meningococcal LPS (also referred to as LOS, lipo-oligosaccharide) (30, 31) has a conserved region (the inner core) composed of heptose (Hep) and 2-keto-3-deoxy-**D**-*manno*-2-octulosonic acid (Kdo) attached to a lipid A moiety embedded in the outer membrane. Attached to Hep_2Kdo_2-Lipid A are variable sugar residues (the heterogeneous outer core), including the α-chain, which, in *N. meningitidis* and *N.gonorrhoeae*, is composed of glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), and *N*-acetylneuraminic acid (NeuNAc),

added by glycosyltransferases encoded by *lgt* genes (32, 34) (Figure 4). Meningococci have been classified into 12 immunotypes based on the expression of LOS antigens detected by solid-phase radioimmunoassay, passive hemaglutination methods (36-38), and mobility on SDS-PAGE gels (36, 39). The α-chains of neisserial LOS mimics many human glycosphingolipids (40-42). Of the 12 LOS immunotypes expressed by meningococci (43), eight contain the lacto-*N*-neotetraose structure (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), which is common to the human I erythrocyte antigen.

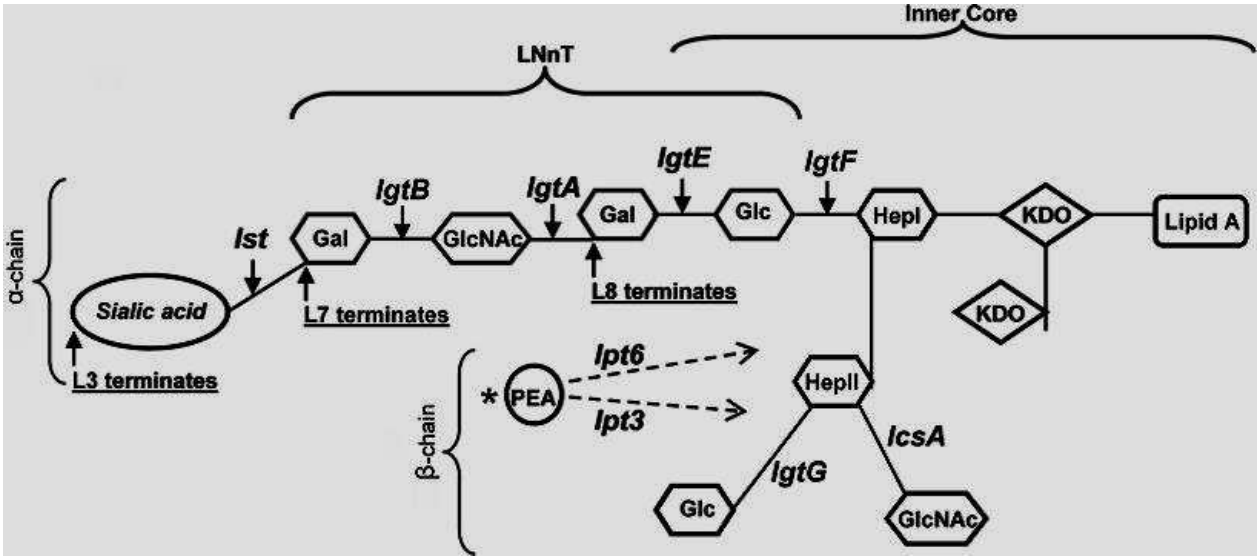


Figure 4: Schematic diagram showing structural organization of N. meningitidis LPS and some important determinants of immunotypes. The membrane-located part of meningococcal LPS comprises lipid A bound to two KDO and two heptose (HepI and HepII) moieties. Extended from the membrane are the structurally variable α- and β-chains of LPS. The different immunotypes of LPS are determined by variations both in the HepI α-chain extensions (Glc, Gal, GlcNAc, Gal and sialic acid) and the HepII β-chain extensions (GlcNAc, PEA and Glc). These arise as a result of phase variation in a number of genes shown and account for the antigenic variation of meningococcal LPS (34). Phosphatidylethanolamine (PEA) may be added at position 3 or 6 on HepII by two distinct enzymes encoded by lpt3 and lpt6 (35). The α-chain structures of the L3, L7 and L8 immunotypes are indicated. LNnT and sialylation: the immunotypes L7/L9, L2/L4/L5 contain identical α-chains terminating in LNnT (Gal-GlcNAc-Gal-Glc structure bound to HepI) which can be sialylated. The L7 immunotype, found in serogroup B and C isolates, refers to the immunotype lacking sialic acid, whereas L3 is sialylated. Phase variation of the lgtA gene gives rise to the immunotype L8, which cannot be sialylated (34).

Meningococcal LOS plays a role in the adherence of the meningococcus (32) and in activation of the innate immune system (33). It binds to a series of host transfer molecules and receptors on monocytic and dendritic cells of the innate immune system, including LPS-binding protein (LBP), CD14, and myeloid differentiation protein 2 (MD2), part of the Toll-like receptor 4 (TLR4) (44, 45) complex. This triggers the secretion of various cytokines (46) (including IL-6 and TNF-α). There is a direct correlation between LPS levels and severity of meningococcal disease (47, 48). In addition, LOS also plays an important role in resistance to other host defenses. Meningococci are resistant to cationic antimicrobial peptides (CAMPs) due to the lipid A phosphoethanolamine structures present on lipid A head groups. CAMPs, present in macrophages and neutrophils, are key components of the innate immune responses through their nonoxidative killing action and their signaling functions (49).

N. meningitidis strains express a number of surface and secreted proteins that bind to human molecules. The adhesive properties of capsulate *N. meningitidis* are mediated by major and minor adhesins. The first group is characterized by pili and opacity proteins, Opa and Opc. Meningococcal pili (50), composed of two major pilin families, undergo both phase and antigenic variation and extend several thousand nm beyond the capsule initiating the binding to epithelial cells (51). Opc is only expressed by *N. meningitidis* and encoded by a single gene. Opa proteins, expressed by both meningococci and gonococci, encoded by multiple genes and interact with multiple members of the CEACAM (carcinoembryonic antigen-related cell-adhesion molecule) family (52) that, during inflammation, are expressed at high level, facilitating Opa interactions and therefore cellular attachment and invasion.
Minor adhesion are molecules expressed at low levels in vitro, but that may be upregulated in vivo but their potential roles in pathogenesis remain to be fully defined: NadA (neisserial adhesinA), NhhA (Neisseria hia homologue A), App (adhesion and penetration protein), and MspA (meningococcal serine protease A) (53).
Iron-binding proteins (HmbR (hemoglobin), TbpA and TbpB (transferrin), HbpA and HbpB (lactoferrin), HpnA and HpnB (hemoglobin-haptoglobin complex)) enable meningococci to acquire iron, a crucial growth factor during colonization and disease (54, 55).
Neisserial porins, PorA and PorB, although not considered adhesins, interact with numerous human cells and proteins. Through them small hydrophilic nutrients diffuse into the bacterium via cation or anion selection. PorB is a major OM porin that inserts in membranes, induces Ca2+ influx and activates TLR2 and cell apoptosis (56). PorA is a major component of OM vesicle-based vaccines and a target for bactericidal antibodies (57).

2.2.2 Epidemiology

The global incidence of meningococcal disease changes in relation to geographical areas; worldwide, 500,000-1,200,000 invasive meningococcal diseases (IMD) occur each year, with 50,000-135,000 deaths (58, 59). Low socioeconomic status and immune deficiencies contribute to meningococcal infection, especially in infants and adolescents, particularly vulnerable because of the disappearance of maternal antibodies early in life and the high rate of nasopharyngeal colonization (60, 61). The serogroups causing IMD vary geographically for differences in population immunity and environmental factors (Figure 5). Before introduction of the MenA conjugate vaccine (2010) meningococcus serogroup A (MenA) occurred in Africa with incidence ranges between 10 and 1000 cases per 100,000 inhabitants and year during epidemics, whereas serogroups B (MenB), C (MenC) and Y (MenY) are predominant in the other continents, including Europe, North America and Australia (60, 62) with incidence ranges between 0.3 and 3 cases per 100,000 inhabitants/year (61).

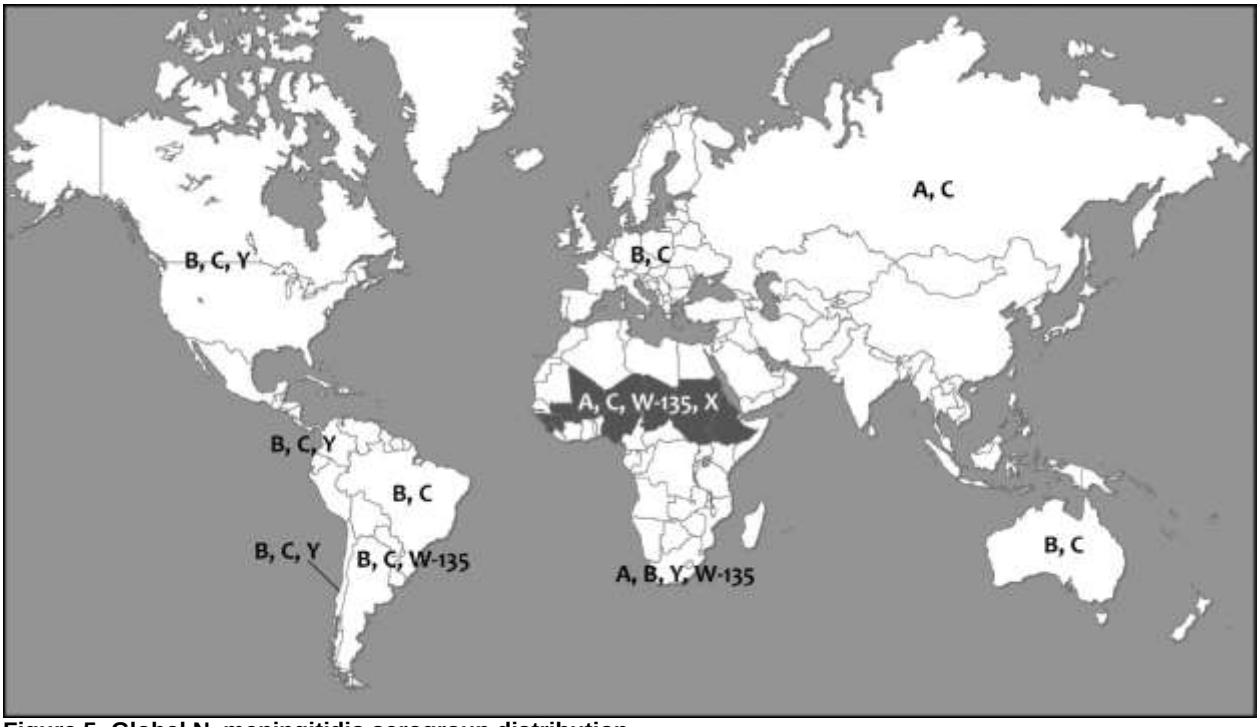


Figure 5: Global N. meningitidis serogroup distribution.

N. meningitidis is unique among the major bacterial agents of meningitis in that it causes epidemic as well as endemic (sporadic) disease. The area which extends from Ethiopia to Senegal, described by Lapeyssonnie as the African “meningitis belt” (63) (Figure 6) continues to have major outbreaks of meningococcal disease (200, 201). During African epidemics, attack rates can reach 1 000 cases/100 000 population (202). In the largest meningococcal epidemic outbreak recorded, over 300 000 cases and 30 000 deaths occurred in sub-Saharan Africa in 1996–97 due to serogroup A *N. meningitidis* (200). MenA was responsible for the majority of cases until the introduction of the group A conjugate vaccine in 2010, while meningococcus serogroup W (MenW) predominated between 2010 and 2014 (62). Although cases caused by MenC were rare in Africa, in 2013 and 2014 two outbreaks due to a novel strain of MenC were reported in Nigeria and Kebbi, respectively and it was identified among the majority of disease causing isolates in 2015 (64). Moreover, during 2006-2010 outbreaks of MenX were described in Niger, Togo, Kenya, Uganda, and Burkina Faso (65).

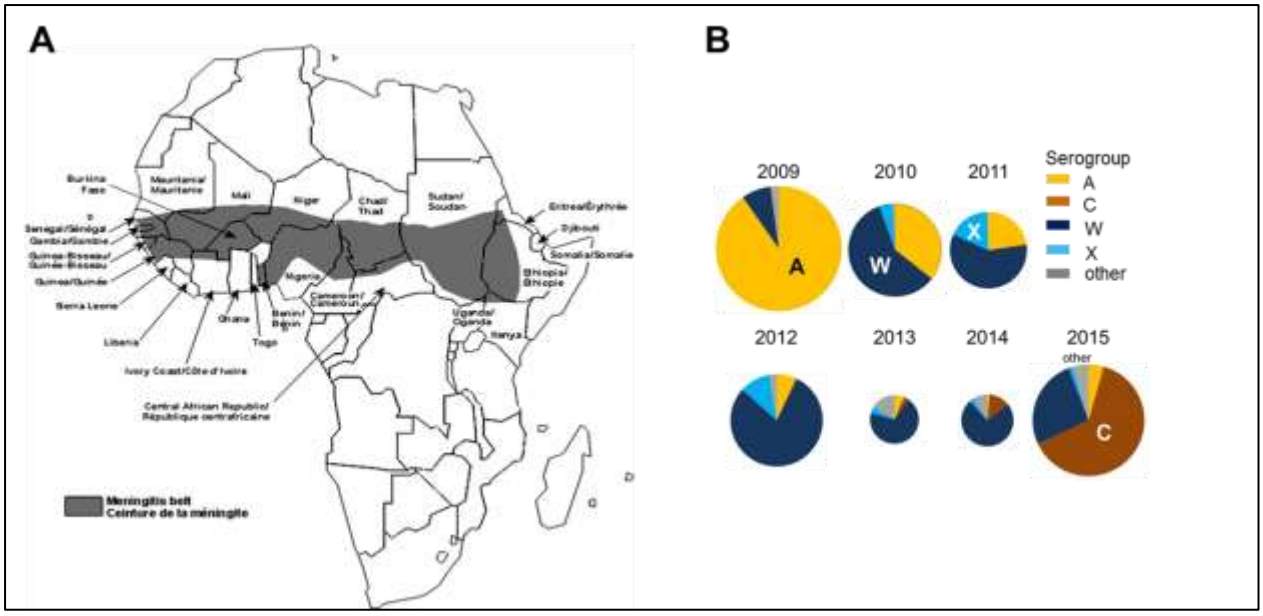


Figure 6: A. The sub-Saharan African meningitis belt. Source: CDC website, NCID, Travelers' health. B. The sub-Saharan serogroup distribution (circle diameter is proportional to the number of cases identified). Source WHO, weekly meningitis bulletin.

In Europe, MenB is the main cause of IMD, followed by MenC and MenY (62). In countries with established MenC vaccination programs, the incidence of MenC disease has significantly declined (60, 62). In comparison with the US, IMD caused by MenY is rare in Europe. However, an increase in this serogroup has been reported in recent years, particularly in the Nordic European countries (66).

The dynamic nature of meningococcal epidemiology is due to a variety of factors: age, with the highest rates occurring in infants (67); lack of serum bactericidal antibodies (SBA) (68, 69); low socioeconomic status (67,70–72); asplenia, genetic polymorphisms or deficiencies in components of the innate immunesystem, and HIV infection (73–81); environmental and behavioral risk factors such as active and passive smoking, bar and pub patronage, intimate kissing, and university dormitory attendance (82-91); recent or concomitant upper respiratory infection, including influenza (92-97); climactic conditions (98) and crowding (99).

Other factors involved in dynamics of meningococcal epidemiology are related to the antigenic variability that occurs mainly through horizontal gene transfer, which allows the organism to acquire large DNA sequences. The meningococcus also uses gene conversion, which is autologous recombination and does not require the acquisition of DNA from another strain (100, 101). *N. meningitidis* is also capable of varying its antigenic profile through variable gene expression and phase variation, which can occur through slipped-strand mispairing of variable number tandem repeats, variation in poly-C tracts, and use of insertion sequences (102-106). Capsular switching is the mechanism by which *N. meningitidis* can change its capsular phenotype. It occurs through horizontal gene transfer and is detected by identifying strains that are related genetically as defined by, for example, MLST (multi-locus sequence typing) but express different capsular polysaccharide. Capsular switching is presumed to occur during co-colonization of the pharynx with at two or more meningococcal strains (203, 204).

Meningococcal outbreaks can be started or sustained by capsular switching, which is believed to allow immunologic escape from the original serogroup (107-110). Capsular switching is believed to have been at least in part responsible for a serogroup W outbreak that occurred in 2000 in Mecca, Saudi Arabia, with subsequent global spread of the epidemic strain (111).

2.2.3 Pathogenesis

Meningococci that are transmitted by aerosol or contact with secretions colonize upper respiratory mucosal surfaces (e.g., the nasopharynx), may spread to adjacent mucosal surfaces (e.g., lower respiratory tract), and may invade epithelial surfaces and gain access to the bloodstream to produce systemic and focal infections (112). Acquisition of meningococci can lead to colonization (carriage), and under certain circumstances result in invasive disease (113-118).
Carriage is an immunising event leading to protective immunity against the organism (119). *N. meningitidis* colonises 8–25% of healthy individuals; the duration of meningococcal carriage can vary from days to many months (115-117). Adhesion to mucosal surface is an essential step in the establishment of a carrier state. Once meningococci penetrate the mucus barrier of the upper respiratory tract and attach to human epithelial cells, a series of interactions take place that result in a microcolony formation (114) (Figure 7).

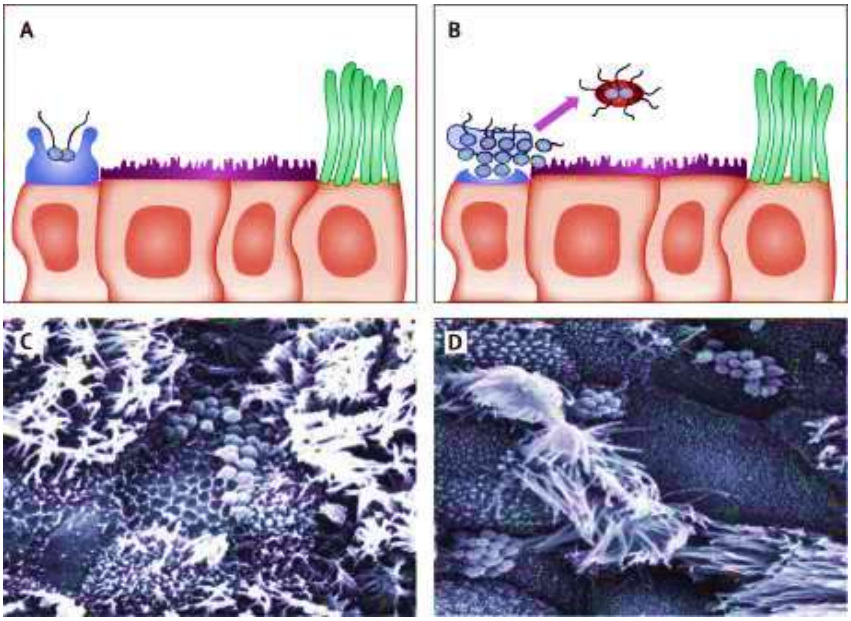


Figure 7: Events in acquisition and carriage of N meningitidis (A), N meningitidis attachment (B), microcolony formation (C), and colonisation (D) at nasopharyngeal mucosal surfaces.

Adhesion to epithelial cells of the nasopharynx is mediated by Type IV pili (120). They are multimeric proteins, with PilE and PilV that are involved in adhesion to host cells and recently shown to activate β2-adrenergic receptor (β2-AR), promoting the endothelial signaling events enabling *Neisseria meningitidis* translocation through the brain endothelium (121, 122). The mechanism of Type IV pili adhesion has been further

elucidated by Bernard et al. who identified CD147, a member of the immunoglobulin (Ig) superfamily, as receptor for PilE and PilV-mediated adhesion to human brain or peripheral endothelial cells and shown the role of CD147 in vascular colonization by meningococci (123).
The second step in meningococcal colonization is mediated by the opacity proteins, Opa, and Opc, with a typical integral membrane protein structure, which bind to carcinoembryonic antigen cell adhesion molecule (CEACAMs) receptor and extracellular matrix components, respectively. Additional antigens having a role in adhesion are NhhA (Neisseria hia/hsf homologue), a trimeric autotransporter with homology to Hia and Hsf adhesins of Haemophilus influenzae, App (Adhesion and penetration protein, homologous to Haemophilus Hap) and NadA (Neisseria adhesin A), a trimeric autotransporter belonging to the Oca family (Figure 8).

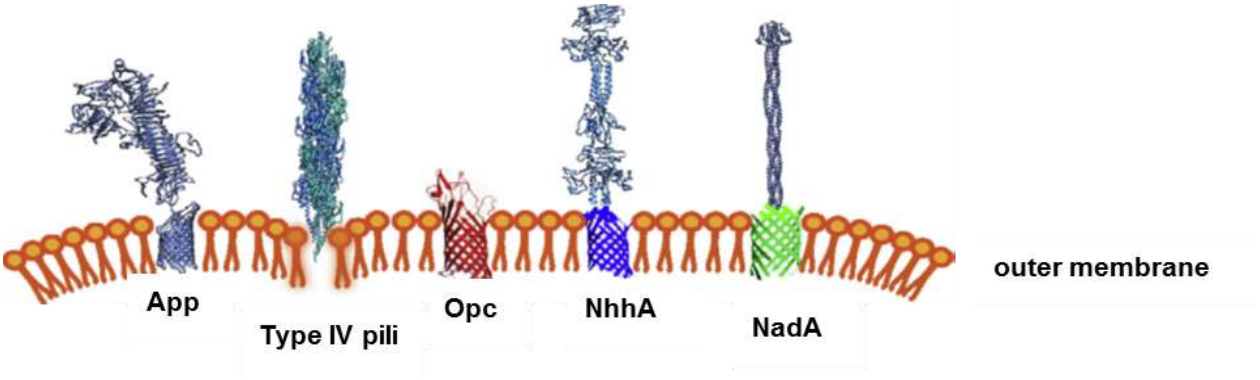


Figure 8: Neisseria meningitidis outer membrane proteins involved in colonization. App (adhesion and penetration protein); Type IV pili; Opc (opacity protein C); NhhA (Neisseria hia/hsf homologue); NadA (Neisseria adhesin A).

Meningococci can cross mucosal surfaces, enter the bloodstream and produce a systemic infection. Once access to the bloodstream is obtained, meningococci may multiply rapidly to high levels. Meningococci may also translocate across the blood-meningeal barrier, proliferate in the CNS and cause meningitis (124).
Major meningococcal contributors that influence the ability to cause invasive meningococcal disease include: capsular polysaccharide, other surface structures [pili, OMPs (e.g. PorA, PorB, Opa, Opc), lipooligosaccharide (LOS)] and the genotype. Resistance to complement-mediated lysis and phagocytosis is determined by the expression of the capsule and lipooligosaccharide (125). Meningococcal endotoxin released in outer membrane blebs also plays a major role in the inflammatory events of meningococcemia and meningococcal meningitis (126).

2.2.4 Neisseria interaction with complement components

Within the blood stream, meningococci produce a strong inflammatory response and activate the complement and the coagulation cascades (Figure 9).

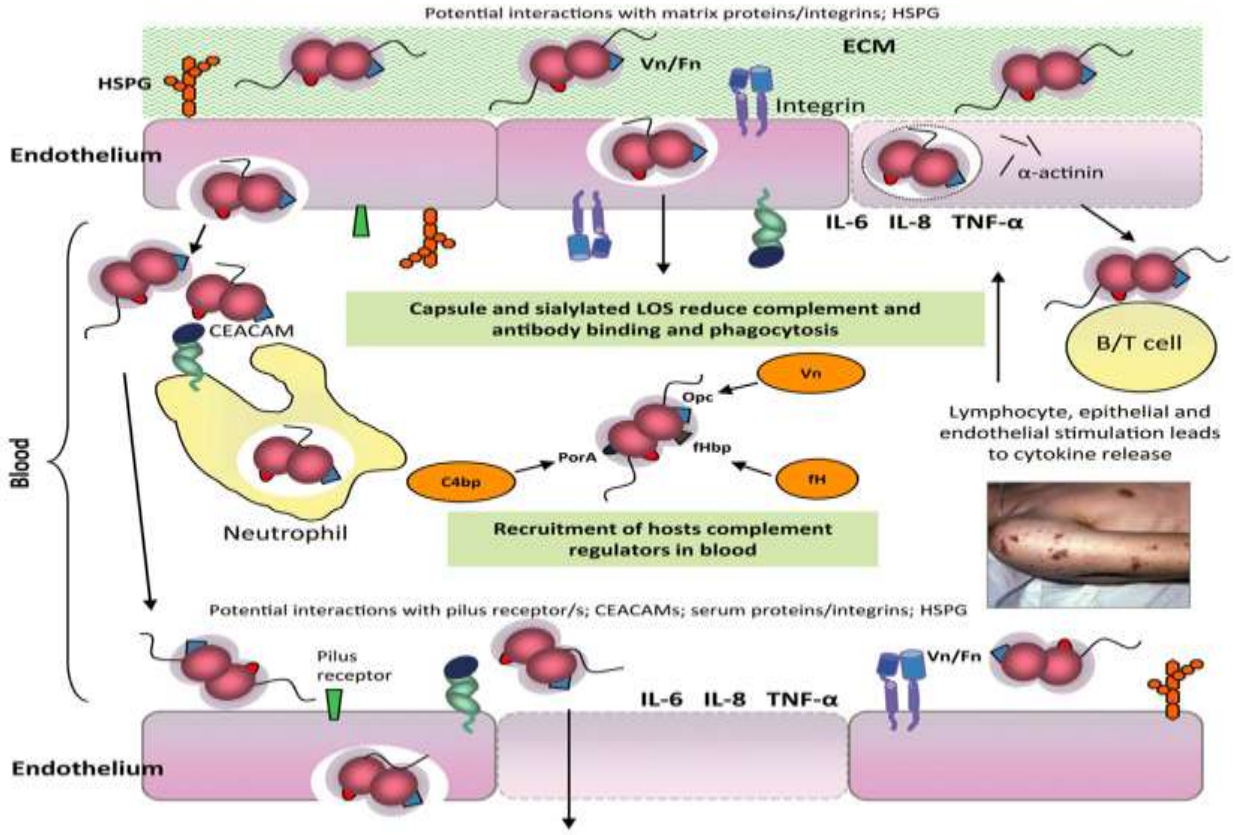


Figure 9: Meningococcal entry into and survival within the vasculature
Capillaries in close proximity to mucosal epithelial tissues are a possible point of entry into the blood for N. *meningitidis*. *In vivo*, meningococci initially encounter the basolateral surface of endothelial cells and need to traverse in a basal to apical direction to enter the vasculature. The *in vitro* studies, however, do not allow easy examination of basal interactions as cultured cells present their apical surfaces to the media. Both integrins and HSPGs are known to be expressed on the basolateral surface of endothelial cells and, hence, are likely targets for vascular penetration. However, it should be noted that these receptors are also expressed apically and are also probably involved in the exit from the bloodstream. Once in the blood, only capsulate meningococci appear to survive; whether acapsulate bacteria arising naturally can survive in any microenvironment is not known. In addition, meningococci are able to bind to a number of negative regulators of complement such as C4bp, factor H and vitronectin. Acquisition of such factors could lead to decreased complement-mediated killing *in vivo*. Interactions with vascular cells via protein adhesins and their cognate receptors and via LPS–TLR4 provoke an inflammatory response leading to cytokine release and cellular damage. This could increase further cell barrier penetration and leakage, which accounts for the damage and clinical symptoms observed during meningococcal sepsis, typified in latter stages by a petechial rash. LPS has also been shown to be toxic for human endothelial cells *in vitro* (145).

The lipid A moiety of LPS is the active component responsible for eliciting the inflammatory response associated with meningococcal sepsis. As already described, LPS induces the release of several cytokines, including IL-6 and TNF-α (tumour necrosis factor-α), as well as chemokines, ROS (reactive oxygen species) and NO, acting in part through TLR (Toll-like receptor) 4 (127, 128). Natural LPS variants lacking

a single acyl chain engage less well with TLR4, yet can cause clinical disease and so may be better placed to evade the innate immune system (129).
In the blood, *N. meningitidis* encounters numerous host killing mechanisms, including antibody/complement-mediated lysis, as well as opsonophagocytic killing. Disruption of genes associated with capsule and LPS synthesis results in an increase in meningococcal sensitivity to serum killing, indicating the importance of these polysaccharides for survival in the blood (130).
Negative regulators of complement can be recruited by meningococci to promote their survival. Factor H is recruited by fHbp (factor H-binding protein; also named GNA1870), a 27 kDa lipoprotein which is expressed by all meningococcal strains and which promotes serum resistance (131). The porin PorA of meningococci can also bind a complement regulator, C4bp (C4-binding protein), and influence serum resistance.
However, capsule may inhibit C4bp binding to PorA (132). It has been suggested that both PorA and PorB may be involved in bacterial uptake via re-arrangement of the cytoskeleton (133). Porins may also act via TLR2 as an adjuvant leading to the stimulation of B-cells (134). It has also been demonstrated that PorB has an antiapoptotic effect on epithelial cells, by localizing in the mitochondrial compartment, enhancing survival of the cell upon apoptotic stimuli (135). Porins therefore appear to have multiple roles in meningococci from aiding not only colonization, but also survival in the blood.
Besides facilitating entry into the vasculature, some bacterial adhesins may also function directly in resisting complement-mediated killing. Vitronectin inhibits the formation and insertion of a MAC (membrane attack complex) into bacterial membranes. In binding directly to vitronectin, Opc-expressing bacteria are able to resist serum-mediated killing (136). Thus meningococci have the means to interact with several regulators of the complement pathways which could lead to increased bacterial survival in the blood.
N. meningitidis can bind to and influence cells within the vasculature. Peripheral blood mononuclear cells from individuals immunized with a range of outer membrane proteins of *N. meningitidis* have a higher proliferative response to Opa than to other neisserial proteins (137).
Another study demonstrated a suppressive effect on T-cell activation and proliferation in response to Opa-containing meningococcal OMV preparations (138). However, no such deleterious effects of Opa containing OMVs used as vaccines have been reported (139). Recent studies have also shown Opa-independent proliferation of T-cells in the presence of *N. meningitidis* (140).Thus the influence of Opa proteins on immune cells is unclear and whether the Opa receptor CEACAM1, which is expressed on stimulated T-cells is involved, remains to be clarified.
Engagement of CEACAM3 by Opa-expressing *N. gonorrhoeae* has been postulated to result in increased cell death of neutrophils during infection (141). Such interactions of *N. meningitidis* could also lead to evasion of killing by promotion of neutrophil cell death, but this remains to be investigated.
Of the minor adhesins, NadA-expressing *Escherichia coli* adhere to and activate human monocytes and macrophages.
In addition, purified NadA induced high levels of TNF-α and IL-8 production by these cells (142). Recent work has demonstrated that OMVs containing NadA possessed

enhanced immune stimulation compared with controls, suggesting an additional role for this adhesin in septic shock (143).
In conclusion, it is widely believed that the key players in meningococcal survival in the blood include capsule and LPS. In addition, proteinaceous adhesins also play important roles in entry to and exit from the vasculature and may also modulate immune responses.

2.3 Meningococcal vaccines

Meningococcal infection continues to be reported worldwide, especially in the poorest countries, where universal vaccine coverage is absent and antibiotic resistance increasingly more common (9), (10).
Meningococcal disease has the highest incidence in infants and children aged <4 years and adolescents (126). 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.

2.3.1 State of the art in developed countries

Capsular polysaccharide vaccines to decrease A, C, Y, and W meningococcal disease were introduced in the 1970s and 1980s on the basis of Gotschlich, Gold, Goldschneider, and Artenstein's classic studies (205-207). These vaccines are safe with mild local adverse events, are effective (>85%) in children (older than 2 years) and adults, but are less immunogenic (C less than A) in children younger than 24 months, do not induce immunological memory and have little or no effect on nasopharyngeal carriage.
A major advance in the prevention of meningococcal disease has been the development of meningococcal polysaccharide and protein conjugate vaccines and their introduction into the UK, other parts of Europe, Canada, and the USA (208-213). They have been shown to be very effective in preventing the disease in all age groups (144). These vaccines have been proven to be safe and effective, with infrequent and mild side effects, and immunogenic, particularly for children under 2 years of age whereas polysaccharide vaccines are not. Moreover they induce immunological memory, and decrease nasopharyngeal carriage and hence transmission of meningococci. In the UK, introduction of the C conjugate meningococcal vaccines in 2000 to all children and young adults greatly reduced the rate of serogroup C disease (90% vaccine eff ectiveness at 3 years for patients aged 11–18 years) (208). A major protective effect of the C conjugate vaccine is mediated through herd immunity (209, 155).
The development of vaccines for serogroup B *Neisseria meningitidis* has been a challenge (145) because it is identical to the polysialic acid present in many human glycoproteins including the fetal neural cell adhesion molecule. As a consequence the serogroup B capsular polysaccharide is poorly immunogenic and safety concerns exist because it may induce the production of autoantibodies (146) therefore different

approaches based on protein antigens were used for the development of a vaccine against serogroup B disease. The first protein based MenB vaccine contained outer membrane vesicles (OMVs) which are obtained by detergent extraction of the bacterial biomass. They have been shown to be efficacious predominantly against the homologous strain that was used to generate the OMV. OMV protective ability resides mainly in the PorA antigen, an integral membrane protein with β-barrel structure and protruding loops made by variable sequences which constitute the main epitopes and which are the driving cause for the OMV vaccine strain specificity (147). This antigenic diversity of the meningococcal surface proteins has been the main limitation in the design of broadly protective meningococcal vaccines. The challenge of the antigen diversity has been addressed by two different approaches.

The first one has been based on multiple antigens identified by 'reverse vaccinology'. The Reverse Vaccinology (RV) approach is one of the most powerful examples of biotechnology applied to the field of vaccinology for identifying new protein-based vaccines. RV combines the availability of huge amounts of genomic data, the analyzing capabilities of new bioinformatic tools, the application of high throughput expression and purification systems combined with serological screening assays, for a coordinated screening process of the entire genomic repertoire of bacterial pathogens, viruses or parasites, for selecting the best vaccine antigens (4). In particular, *in silico* methods are used for selecting surface-exposed or secreted proteins. After antigen selection, great effort needs to be applied in amplifying the genes, cloning them in suitable vectors for protein expression, purifying the proteins and testing them in vitro for functionality, or in vivo for their ability to raise protective antibodies. It is possible to select the best antigens on the basis of their ability to induce antibodies by serum bactericidal assay (SBA) (150). SBA is an assay for measuring functional quality of antibody (ability to kill a strain) and for *Neisseria meningitidis* it is a predictor for protection against invasive disease: specifically when measured with human sera and human complement a bactericidal titer of >4 predicts protection against invasive disease

The application of RV to *Neisseria meningitidis* serogroup B represents the first success of this novel approach and resulted in the development of the first MenB vaccine licensed so far in Europe, Australia, Canada and Chile (4CMenB or Bexsero) (148, 149). It is a multi-component meningococcal B vaccine containing three surface-exposed recombinant proteins (fHbp, NadA and NHBA) and New Zealand strain detergent extracted outer membrane vesicles (NZ OMV) with PorA 1.4 antigenicity. The second approach has been based on the combination of two variants of the lipidated recombinant fHbp, (151).

2.3.2 State of the art in Africa

Quadrivalent conjugate vaccines based on the capsular polysaccharide are available against strains with capsular groups A, C, W and Y but are unlikely to be affordable for use in Africa.

The use of unconjugate A, C or A, C, W polysaccharide vaccine has been adopted for many years in response to epidemics in the African meningitis belt, but they are not effective in children under the age of 2 years and they do not induce immunologic memory, so they have not prevented the continuous occurrence of epidemics.

In 2010, in Niger, Burkina Faso and Mali, a polysaccharide conjugate vaccine (MenAfriVac) has been first introduced for preventive mass immunization in the meningitis belt against serogroup A. Where the vaccine has been implemented, the number of reported cases caused by serogroup A strains has decreased dramatically, but group W and X strains remain a persistent problem because the protection is serogroup- specific. This observation underlines the need for an affordable vaccine that can provide broad protection against all three main serogroups causing meningitis in Africa and potentially against new disease-causing serogroups that may emerge in the region in the future (152).
Vaccines based on GMMA (Generalized Modules for Membrane Antigens) from genetically engineered strains are under investigation (153).

2.3.3 Native outer membrane vesicles (NOMV) and Generalized Modules for Membrane Antigens (GMMA)

Gram-negative bacteria naturally release blebs from the outer membrane that were described as native or spontaneous Outer Membrane Vesicles (nOMV, sOMV) (153) and are attractive vaccine candidates as they present surface antigens in their natural context.
While outer membrane vesicles (OMV) are obtained by detergent-extraction of homogenized bacteria, NOMV are obtained from culture supernatant and not treated with detergent (154).
NOMV are derived from genetically engineered strains that contain modifications to increase immunogenicity by over-expression of antigens, like fHbp, and to increase safety, by deletion of *lpxL1* in case of *N. meningitidis*. In detail, NOMV contain immunostimulatory components, especially lipopolysaccharide (LPS), so, for vaccine use, they need to be genetically detoxified reducing their reactogenicity by modifying lipid A, the endotoxic part of LPS, through deletion of late acyltransferase and generating a lipid A with a different acylation pattern (a penta-acylated lipid A in place of esa-acylated lipid A).
In contrast, OMV are “detoxified” by the use of the detergent which removes the LOS.
By avoiding the use of detergent protein, antigens like lipoproteins (as fHbp) that are detergent soluble, are maintained in NOMV, while they are also extracted by the detergent used to obtain the OMV.
These NOMV are small spherical structures (Figure 10), 30-250 nm in diameter, which protrude from the outer membrane and then are released into the environment (156) and have a different composition than OMV (154).

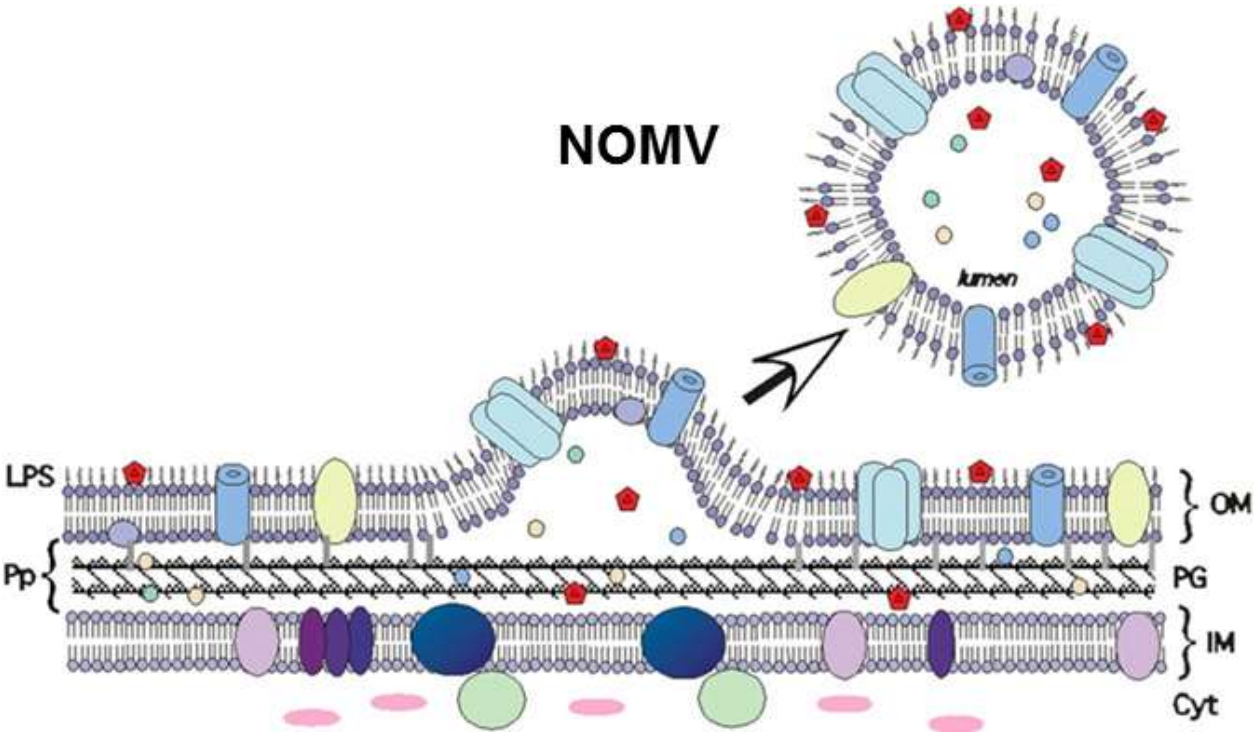


Figure 10: Native Outer Membrane Vesicles (NOMV) generation.

NOMV, in general, maintain the structure, composition, and orientation of the outer membrane, including the glycerophospholipids, outer membrane proteins and also contain soluble periplasmic proteins entrapped in the lumen (157, 158).
Gram-negative bacteria, both pathogenic and non-pathogenic, secrete NOMV during constitutive growth, including *E. coli*, *N. meningitidis*, *V. cholerae*, *Pseudomonas aeruginosa*, *Brucella melitensis*, and *Helicobacter pylori* especially during the end of log phase growth and at sites of cell division (159).
For the most part, NOMV are devoid of inner membrane and cytosolic constituents.
NOMV have been discussed to have different biological roles, e.g. as delivery vehicles for active components to host cells, as nucleators in the formation of bacterial communities (biofilms) and as contributors to bacterial survival and virulence (158).
As NOMV represent the envelope of Gram-negative bacteria and, thus, components on the NOMV that are recognized by the immune system resemble components on the surface of the bacterium (160), they have been tested for use as vaccines. Also, NOMV combine both adjuvant and carrier activity, increasing the low immunogenic properties of some protein antigens (161). It was also demonstrated that proteins from a foreign source can be localized in the outer membrane and periplasm of the parent cell and successively internalized into OMVs (162).
NOMV have been shown to induce protection in mice against multiple pathogens, including *Salmonella enterica* serovar Typhimurium (163), *Helicobacter pylori* (164), *Vibrio cholera* (165, 166) and *Shigella bodyii* (167) or to elicit antibodies with in vitro bactericidal activity in mice and humans, e.g. for *Neisseria meningitidis* (168, 169). In addition, NOMV have been tested as veterinary vaccine candidate, e.g. against *Edwardisiella tarda* implicated in the death of see water fish (170).

The yield of NOMV is usually too low for a practical production. The application of NOMV as vaccines requires high production and efficient purification. Various methods have been employed to induce and increase blebbing of the outer membrane of Gram-negative microbes. These techniques include inhibiting protein synthesis, autolytic cell wall degradation by lysine starvation, increasing growth temperature (even to 55^{o}C), and the addition of sub-lethal doses of antibiotics (158, 161). However, all of these conditions alter the composition of OMVs so that they are no longer “native” and have not resulted in true high yield production.
As mentioned above, NOMV preferentially form where the link between the inner and outer membrane is weakened. In the case of *Shigella* and *Salmonella*, this linkage can be genetically disregulated by deletion of *tolR*, a gene of the Tol-Pal pathway (153, 171). The Tol-Pal system is present in most of Gram-negative bacteria and is required to maintain outer membrane integrity. Thus, a mutation in any of *tol-pal* genes, and especially on *tolR*, confers a defect in other membrane integrity, resulting in a major release of outer membrane particles (171). In the case of *N. meningitidis*, which do not have a homologue of *tolR*, deletion of *gna33*, encoding a membrane bound lipoprotein with murein hydrolase activity, has been shown to impair the integrity of the cell envelope and increase the release (154). Vesicles derived from bacteria with a genetically upregulated outer membrane shedding have been termed generalized modules for membrane antigens or “GMMA” (153, 171).
Widely used methods at research scale is the separation of NOMV from the bacterial cells by low speed centrifugation, in which the outer membrane particles remain in the culture supernatant, followed by collection of the particles by using ultrafiltration or ultracentrifugation (172). Also alternative methods have been tested, e.g. purification over a sucrose density gradient or gel filtration chromatography, resulting in vesicles of high purity and being relatively homogeneous. Those systems were based on differential diffusion and pore size exclusion, but the use of ammonium sulfate in the precipitation of OMVs before density gradient centrifugation can lead to artifacts (158).
A method to collect GMMA from bacterial suspensions has been developed at large scale, by using two consecutive Tangential Flow Filtration steps: the first one with 0.22 µm to separate the biomass from the GMMA, and the second one with a smaller cut-off for purifying and concentrating GMMA (153), thus resulting in an high yield process.

2.3.3 Factor H binding protein

One of the main protective meningococcal protein antigens is factor H binding protein (fHbp) (173).
It was identified as a putative surface-exposed lipoprotein during the screening of the genome of serogroup B strain MC58, and then referred to as GNA1870 and, once purified as recombinant protein, recognized as very effective in inducing bactericidal antibodies (174, 175).
FHbp is a major component of licensed meningococcal vaccines and vaccines under development (176-179). As mentioned above, a multicomponent vaccine containing fHbp (4CMenB or Bexsero) was approved by the European Medicines Agency (180).

FHbp is a 28 kDa surface-anchored lipoprotein that consists of two *β*-barrels connected by a short linker (181), a flexible peptide chain connects these domains to the outer membrane, to which it is anchored by an N-terminal lapidated cysteine (182-184).
It is expressed by almost all *N. meningitidis* strains and all serogroups, even though the level of expression can significantly vary between different strains (175).
Based on amino acid alignments, fHbp can be divided into three variant groups (v.1, 2 and 3) (175) or, into 2 sub-families, with subfamily A corresponding to v.2 and v.3, and B corresponding to v.1 (185) (Figure 11).

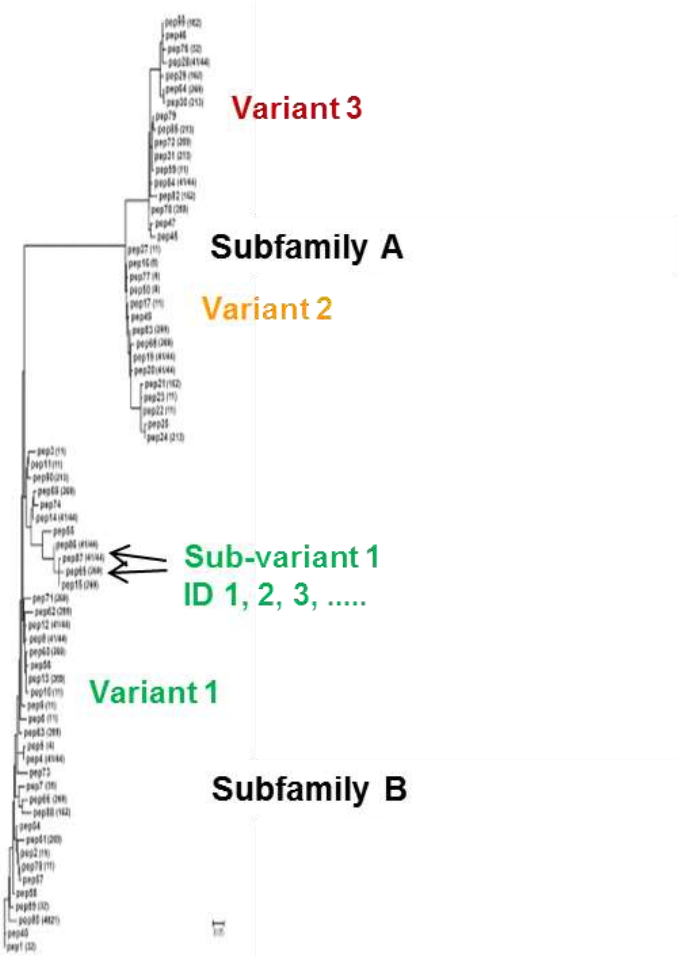


Figure 11: Phylogenetic tree showing the two different classifications of fHbp. Based on amino acid alignments, fHbp can be divided into three variant groups (v.1, 2 and 3) or, according to Pfizer nomenclature, into 2 sub-families, A corresponding to v.2 and v.3, and B corresponding to v.1.

Variant 1 fHbps are expressed by approximately 60% of invasive meningococcal serogroup B isolates in North America and Europe, while variant 2 and variant 3 fHbps are present in around 30% and 10% of the isolates, respectively (186-188). Immunological cross-reactivity between these variant groups is limited, although cross-reactivity is observed between v.2 and v.3 fHbps (175, 189).
The % identity within each variant is between 89% and 100% for variant 1, and between variants like v.1 versus v.2 is around 60%, whereas the sequence identity between v.2 and v.3 is around 80 %.

Each variant can be further divided into sub-variants, with small differences in their aminoacid sequence; each sub-variant is identified by a specific ID number. As reported by the public fHbp allel and peptide database on http://pubmlst.org/neisseria/fHbp/, the subvariant identified to date are 535 for variant 1, 196 for variant 2 and 187 for variant 3. Immunogenicity studies in mice conducted with recombinant fHbp showed that even within the same variant, different fHbp subvariants induce antibodies that have highest serum bactericidal activity against strains expressing the same subvariant (190). Furthermore, fHbp binds the negative human complement regulator factor H (fH) at high affinity, with a dissociation constant (*Kd*) for v.1, 2 and 3 fHbps with fH in the nanomolar range (181). Factor H is an abundant complement component (191) and the main negative regulator of the alternative complement pathway by acting as a cofactor for Factor I cleavage of C3b, and by accelerating the decay of the alternative pathway C3 convertase, C3bBb (192). Recruitment of fH to the surface of *N. meningitidis* reduces complement activation and promotes immune evasion by the bacterium (193, 194). Antibodies raised against fHbp have two modes of action: first, they block factor H binding, so that the bacterium is no longer able to escape complement-mediated killing; second, antibodies bound to the surface of meningococcus activate the classical complement pathway (193). Factor H consists of 20 complement control protein domains (CCPs), and it has been shown that CCPs 6 and 7 (fH6-7) are sufficient for high affinity interactions between fHbp and factor H (181). The interaction between fHbp and factor H could have consequences for fHbp-based vaccines and affect their efficacy and immunogenicity. As factor H engages a large area of fHbp, immunogenic epitopes on fHbp might be hidden. Indeed, several bactericidal mAbs raised against fHbp recognise epitopes that include the factor H binding site (195-197), suggesting that some important epitopes might be concealed when factor H is bound to fHbp. For this reason, current vaccine candidates under investigation are fHbp mutant proteins that do not bind factor H.

Also, down-regulation of complement activation by locally recruited factor H could impair antibody production and hence reduced immunogenicity.

African strains currently express a limited number of fHbp peptides: a study analyzing 106 invasive isolates from sub-Saharan Africa has shown that 100% of serogroup A isolates express fHbp v.1, peptide ID 4 or 5 that differ by one amino acid; the majority of X strains express fHbp v.1, and among them 63% express ID 74; fHbp v.1 ID9 is expressed by 20 % of serogroup W strains (190). However, among serogroup W strains the distribution of fHbp variants is quite heterogeneous (34% for v.1; 58% for v.2 and 8% for v.3); on the other hand, almost all W strains isolated in Africa express PorA subtype P1.5,2 (190). PorA is one of the main outer membrane porins in *N. meningitidis*. It is able to induce a strong and protective antibody response in the host but antibody responses are specific for the PorA subtype (Figure 12).

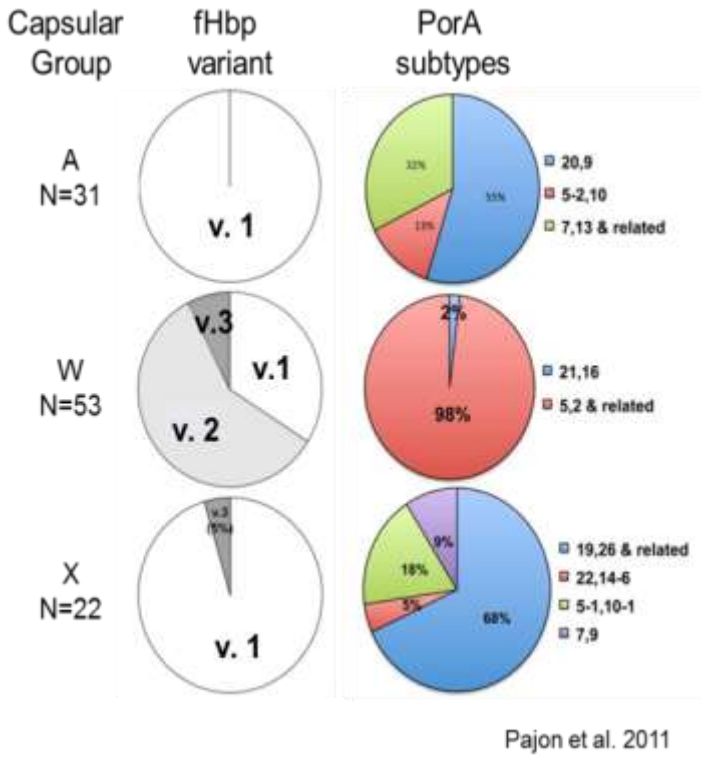


Figure 12: FHbp and PorA distribution in African strains.

A single GMMA vaccine has been investigated for use against African strains from an African group W strain with PorA P1.5,2, engineered to over-express fHbp v.1. To increase safety of the vaccine strain, capsule biosynthesis genes have been deleted. In order to increase the tolerability of the vaccine, the genetic modification of Lipooligosaccharide (LOS) is necessary. The lipid A, portion of the LOS is responsible for the endotoxin activity of LOS. Inactivation of *lpxL1* gene that encodes for late-functioning acyltransferases of lipid A biosynthesis results in a penta-acylated lipid A, instead of the wild type hexa-acylated lipid A with lower toxicity, but retaining the adjuvant activity (198). To increase blebbing *gna33* has been deleted (199). In mice, GMMA from this mutant elicited bactericidal antibodies against the homologous W strain and also cross protective antibodies against a panel of genetically diverse African A and X strains expressing fHbp v.1 sequence IDs that are predominant among current African isolates.

This vaccine approach is promising and has the potential to provide protection against the majority of current invasive A, W and X strains circulating in sub-Saharan Africa, but, such a vaccine, it would not be expected to cover strains with a heterologous PorA compared to that included in the vaccine and expressing fHbp v. 2 or 3.

The dynamic nature of meningococcal epidemiology in Africa and the resulting goal to expand protection have been the rational for test, as proof of concept, of over-expression of a fHbp v.2 in serogroup W strain. Despite a relatively high conservation and small number of fHbp sequences expressed by current invasive strains from Africa, the distribution and prevalence may change in the future. Therefore an analysis of sequence coverage and of functionality of anti-fHbp antibodies induced by a GMMA

containing fHbp v.1, against strains expressing a broad panel of highly diverse fHbp sequences IDs, independent of their prevalence among existing isolates was performed. In order to reach this aim we first performed a comparative sequence/structure analysis of fHbp v.1, 2 and 3 analyzing the factor H binding site. Being a surface exposed portion of the molecule, the factor H binding site is subject to a balance between immunological selection and functional activity. Variations in the amino acids characterizing the factor H binding site could have a greater impact on the ability of antibodies to kill a particular strain than variations of amino acids that are “hidden” in the molecule and not accessible to antibodies Moreover, we selected as model a panel of diverse fHbp sequences based on diversity in the factor H binding site and overall diversity. On the basis of the first sequences selection we chose a panel of wild type strains (NOT serogroup B) that expressed a subset of the selected sequences and a panel of isogenic strains. To generate the last panel we selected one parent strain and generated eight mutants that we engineered to express eight highly diverse fHbp sequences. The advantage of this panel is that it allowed us to specifically investigate functional activity of antibodies against fHbp because the mutants only differed by the fHbp sequence that they expressed. The work demonstrate that fHbp expression level, fHbp sequence diversity and intrinsic susceptibility of killing play a key role contributing to susceptibility of strain killing by anti fHbp antibodies.

III Aim of thesis

Current african A, W and X strains causing diseases are relatively conserved in terms of the fHbp variant and fHbp sequence that they express. Meningococcal epidemiology is dynamic and fHbp sequences that are expressed by African isolates could change in the future. The primary aim of the project is to systematically investigate and dissect the breadth and nature of fHbp sequence coverage by antibodies against fHbp. This is obtained by using a combination of bioinformatic tools as the basis to analyze functional antibody activity. The analysis can support the selection of fHbp sequences to be included in fHbp based vaccines for sub Saharan Africa.
In detail, in order to reach this objective, by a comparative structure/sequence analysis of >700 existing and published individual fHbp IDs belonging to three variants, we selected fHbp sequences based on a gradually different level of differences in the factor H binding site. Applying these criteria, we selected a panel of wild type strains (belonging to serogroup A, C, W, Y or X) that expressed a subset of the selected fHbp sequences. Moreover, we applied the selection criteria to generate, starting from one parent strain, a panel of eight genetically defined isogenic strains that were engineered to express seven existing diverse fHbp v.1 sequences and one fHbp v.1/2 hybrid protein. The selected fHbp sequences were integrated into the genome and expressed under control of an IPTG inducible promoter. The advantage of this panel is that it allowed to specifically investigate functional activity of antibodies against fHbp because the mutant only differed by the fHbp sequence that they expressed. We used the strains as test strains in serum bactericidal assay to measure functional activity of antibodies raised against GMMA from a group W strain with over-expressed fHbp v.1 in the presence of baby rabbit or human complement. As proof of principle, We generated a mutant group W strain (based on the same parent wild type strain that was used for the mutant to over-express fHbp v.1) and engineered it to over-express the native fHbp v.2, which is predominant among African group W isolates, with deletion of capsule biosynthesis gene to increase safety of the vaccine strain, with deletion of lpxL1 to obtain strains with a reduced endotoxin activity and with deletion of *gna33* to increase the release of GMMA. The vesicles obtained were then characterized based on the fHbp expression by western blotting, on reactogenicity by MALDI-TOF analysis of LipidA and Monocyte-Activation Test (MAT) assay, and on size, nucleic acid and soluble proteins content by HPLC-SEC MALLS.

IV Material and methods

4.1 Bacterial growth conditions

4.1.1 Escherichia Coli

Escherichia Coli DH5α competent cells (Invitrogen) were used for plasmid cloning. Bacteria were grown at 30 or 37°C in Luria-Bertani Broth (LB) or LB agar plates with the addition of required antibiotics. Antibiotics used for selection were: ampicillin (100 µg/mL); chloramphenicol (20 µg/mL); kanamycin (20 µg/mL); erythromycin (20 µg/mL); spectinomycin (50 µg/mL).

4.1.2 Neisseria Meningitidis

Parent wild type strain used for generating *Neisseria meningitidis* mutants is a serogroup W meningococcus, "NVGH 0385", an ST11 strain isolated in 2004 in Ghana; the native fHbp expressed belongs to variant 2, and PorA subtype is P1.5,2.
Neisseria meningitidis strains were grown at 37°C on GC agar plates or in liquid medium containing yeast extract, casaminoacids, and lactic acid, in a humid atmosphere containing 5% CO2.
When required, antibiotics were added to plates and liquid medium at concentration of 5 µg/mL for chloramphenicol, 80 µg/mL for kanamycin, 5 µg/mL for erythromycin, 60 µg/mL for spectinomycin.

4.2 Molecular biological DNA techniques

4.2.1 Polymerase chain reaction (PCR)

The Polymerase Chain Reaction (PCR) is used to amplify a gene or DNA fragment of interest. For subsequent cloning of the amplified fragment, restriction sites were added to the 5' end of both PCR primers. In addition, 6 bases were added at the 5' end of the primer. These additional bases provide an overhang for the restriction enzyme to bind the recognition site and cut efficiently. When selecting a restriction site(s) to add to the primers, it is important to determine which site(s) will be compatible with the selected vector, whether directional cloning is desired and, confirm that the recognition site(s) does not occur within the gene or DNA fragment. Primer sequences of 18 to 22 nucleotides were created on the base of the template sequence, taking note of the melting temperatures. The optimal annealing temperature was initially determined by using a gradient between 55°C and 65°C for the annealing step. Lyophilized primers were obtained from Sigma Aldrich and dissolved with sterilediH_2O to a final stock concentration of 100 µM. For use in a PCR a primer stock of 10 µM diluted in diH_2O was used. The polymerases used were TAQ, Deep Vent and Pfu obtained from New England Biolabs.

Taq DNA Polymerase (New England BioLabs) is a thermostable DNA polymerase that possesses a 5´→3´ polymerase activity and a 5´ flap endonuclease activity.

The conditions for PCR amplification with Taq DNA Polymerase are shown in Table 3.

Table 3: Conditions for PCR amplification with Taq DNA Polymerase.

Component	25 µl reaction	50 µl reaction	Final Concentration
10X ThermoPol Reaction Buffer	2.5 µl	5µl	1X
10 mM dNTPs (NEB)	0.5 µl	1µl	200µM
10 µM Forward Primer	0.5 µl	1 µl	0.2 µM (0.05–1 µM)
10 µM Reverse Primer	0.5 µl	1 µl	0.2 µM (0.05–1 µM)
Template DNA	variable	variable	<1 µg
Taq DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl
Nuclease-free water	to 25 µl	to 50 µl	

The thermal cycling conditions adopted for Taq DNA Polymerase-mediated PCR amplification are reported in Table 4.

Table 4: Thermal cycling conditions for Taq DNA polymerase-mediated PCR.

Step	Temperature	Time	Number of Cycles
Initial Denaturation	95°C	30 seconds	1 cycle
Denaturation	95°C	15-30 seconds	30 cycles
Annealing	45-68°C	15-60 seconds	
Extension	68°C	1 minute per kb	
Final Extension	68°C	5 minutes	1 cycle
Hold	4-10°C	Indefinite	1 cycle

Deep VentR DNA Polymerase (New England BioLabs) is a high-fidelity thermophilic DNA polymerase. The fidelity of Deep VentR DNA Polymerase is derived in part from an integral 3´→ 5´ proofreading exonuclease activity. Deep VentR is even more stable than VentR at temperatures of 95 to 100°C.

Both Taq and Deep Vent polymerase were supplied with 10X ThermoPol Reaction Buffer, which contains a nonionic detergent to increase enzyme stability during longer incubations.

The conditions for PCR amplification with Deep Vent DNA Polymerase are shown in Table 5.

Table 5: Conditions for PCR amplification with Deep Vent DNA Polymerase.

Component	Volume(µl)	Final Concentration
ThermoPol Reaction Buffer (10X)	5µl	1X
Deoxynucleotide (dNTP) (NEB) Solution Mix (10 mM)	1µl	200µM
Upstream Primer (10 µM stock)	0.5-2.5 µl	0.1-0.5 µM
Downstream Primer (10 µM stock)	0.5-2.5 µl	0.1-0.5 µM
DNA template	variable	<1 µg
Deep Vent DNA Polymerase	0.25-0.5 µl	0.5-1 unit
Nuclease-Free Water to final volume of	50µl	

The thermal cycling conditions adopted for Deep Vent DNA Polymerase-mediated PCR amplification were reported in Table 6.

Table 6: Thermal cycling conditions for Deep Vent DNA polymerase-mediated PCR.

Step	Temperature	Time	Number of Cycles

Initial Denaturation	95°C	2-5 minutes	1 cycle
Denaturation	95°C	15-30 seconds	20–30 cycles
Annealing	55-65°C	15-30 seconds	
Extension	72°C	1 minute per kb	
Final Extension	72°C	5 minutes	1 cycle
Hold	4-10°C	Indefinite	1 cycle

Pfu DNA Polymerase (Promega) is a thermostable enzyme that replicates DNA at 75°C. It catalyzes the polymerization of nucleotides into duplex DNA in the 5´→3´ direction in the presence of magnesium. The enzyme has a molecular weight of approximately 90,000 daltons as estimated from the predicted amino acid sequence and exhibits 3´→5´ exonuclease (proofreading) activity. Pfu DNA Polymerase is recommended for use in PCR and primer extension reactions that require high fidelity and it was used for self-priming PCR (Figure 13). In this reaction two DNA fragments were used as template that overlap for 30 bp. This allows the generation of a full-length DNA fragment by a self-priming PCR followed by a PCR with primers annealing at the ends of the two fragments.

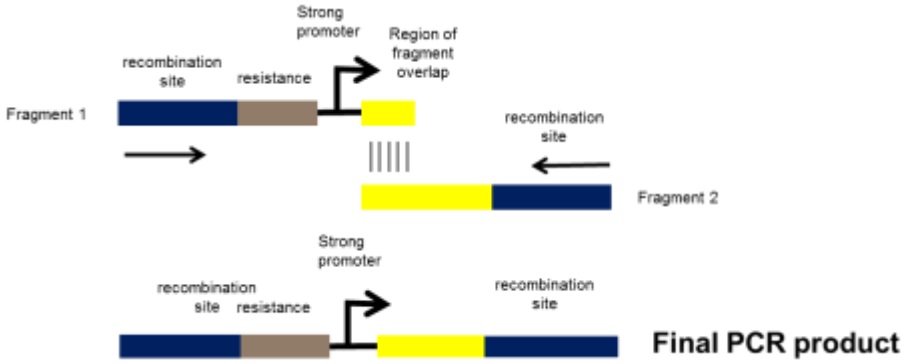


Figure 13: Example of a SELF-PRIMING PCR. The two DNA pieces used as template in the PCR reaction overlap for 30 bp of N-term fHbp. This allows the generation of a full-length DNA fragment by a self-priming PCR followed by a PCR with primers (schematically represented by the black arrows) annealing at the external extremities of the DNA fragments

The conditions for PCR amplification with Pfu DNA Polymerase are shown in Table 7.

Table 7: Conditions for PCR amplification with PFU DNA Polymerase.

Component	Volume(µl)	Final Concentration
Pfu DNA Polymerase 10X Buffer with MgSO4	5µl	1X
dNTP mix (NEB),10mM each	1µl	200µM each
Upstream primer	5–50pmol	0.1–1.0µM
Downstream primer	5–50pmol	0.1–1.0µM
DNA template	variable	<0.5µg/50µl
Pfu DNA Polymerase (2–3u/µl)	variable	1.25u/50µl
Nuclease-Free Water to final volume of	50µl	

The thermal cycling conditions adopted for Pfu DNA Polymerase-mediated PCR amplification were reported in
Table 8.

Table 8: Thermal cycling conditions for PFU DNA polymerase-mediated PCR.

Step	Temperature	Time	Number of Cycles

Initial Denaturation	95°C	1–2 minutes	1 cycle
Denaturation	95°C	0.5–1 minute	25–35 cycles
Annealing	42–65°C	30 seconds	
Extension	72–74°C	2–4 minutes	
Final Extension	72–74°C	5 minutes	1 cycle
Soak	4°C	Indefinite	1 cycle

4.2.2 Colony PCR

The colony PCR (for screening of *E. coli* colonies after transformation for uptake of plasmid with the correct insert(s) was performed using the same protocol as described for Deep VentR DNA Polymerase (New England BioLabs). A tiny part of the colony was picked using a pipette tip and place in the PCR tube. In PCR block to heat to 92°C for 1 minute helps break open the cells and kills DNAses. The PCR reactions were separated by agarose gel electrophoresis.

4.2.3 Agarose gel electrophoresis

Gel electrophoresis is the standard lab procedure for separating DNA by size (e.g. length in base pairs) for visualization and purification. Electrophoresis uses an electrical field to move the negatively charged DNA toward a positive electrode through an agarose gel matrix. The gel matrix allows shorter DNA fragments to migrate more quickly than larger ones. Thus, it is possible accurately determine the length of a DNA segment by running it on an agarose gel alongside a DNA ladder (a collection of DNA fragments of known lengths). For a standard 1.2% Agarose Gel, 1.2 g of agarose were measured and agarose powder was poured into microwavable flask along with 100mL of 1X Tris Acetate EDTA buffer (TAE) and heated for 1-3min (until the agarose is completely dissolved). Agarose solution was let cool down for 5min. 10 µL of 10,000X SYBR® Safe stain concentrate was added to 100 mL of agarose solution and mixed well. SYBR® Safe stain has been specifically developed for reduced mutagenicity, making it safer than ethidium bromide for staining DNA in agarose or acrylamide gels and the detection sensitivity with SYBR® Safe stain is comparable to that obtained with ethidium bromide.
The gel was placed into a gel tray with the well comb in place and let solidify for 30 minutes at roome temperature or at 4°C for 10-15 minutes. The gel and staining solution were covered from light by covering it with aluminum foil or by placing it in the dark. The gel was run using a running buffer appropriate to the SYBR® Safe gel stain formulation. No post-staining or destaining is required. GelPilot Loading Dye was added to each sample.
It contains three tracking dyes (xylene cyanol, bromophenol blue, and orange G) for two purposes: 1) it provides a visible dye that helps with gel loading and will also allows to gauge how far the gel has run while you are running your gel; and 2) it contains a high % glycerol, so after adding it your sample is heavier than water and will settle to the bottom of the gel well, instead of diffusing in the buffer.
Once solidified, the agarose gel was placed into the gel box (electrophoresis unit) and gel box was filled with 1xTAE until the gel was covered. A molecular weight ladder was loaded into the first lane of the gel. Samples were loaded into the additional wells of the

gel. The gel was run at 80-150V until the dye line was approximately 75-80% of the way down the gel (a typical run time is about 1-1.5 hours, depending on the gel concentration and voltage). DNA bands stained with SYBR® Safe DNA gel stain can be detected using a standard UV transilluminator, a visible-light transilluminator such as the Safe Imager™ blue-light transilluminator from Molecular Probes (S37102), or a laser-based scanner. Bound to nucleic acids, SYBR® Safe stain has fluorescence excitation maxima at 280 and 502 nm, and an emission maximum at 530 nm.
In case of purification of DNA away from the agarose gel by Gel extraction kit (Qiagen) for later use, wavelength UV exposure should be for as short a time as possible to minimize damage to the DNA.
For further manipulation, such as molecular cloning, the PCR reactions were purified using the PCR purification kit (Qiagen).

4.2.4 DNA purification and quantitation

The QIAquick PCR Purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, and other impurities from DNA samples. Using a microcentrifuge or vacuum manifold, DNA ranging from 100 bp to 10 kb is purified.
DNA fragments purified with the QIAquick system are ready for direct use in all applications, including sequencing, microarray analysis, ligation and transformation, restriction digestion, labeling, microinjection, PCR, and in vitro transcription.
In detail, 5 volumes of PB (Binding Buffer: 5 M Gu-HCl and 30% isopropanol) were added to 1 volume of the PCR reaction and mix.
A QIAquick column was placed in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 30–60 s. Flow-through was discarded and the QIAquick column was placed back in the same tube. To wash, 0.75 ml PE (Washing Buffer: 10 mM Tris-HCl pH 7.5 and 80% ethanol) were added to the QIAquick column and centrifuged for 30–60 s. The flow-through was discarded and the QIAquick column was placed back in the same tube. The QIAquick column was centrifuged once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer. Each QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) were added to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, 30 µl elution buffer were added to the center of the QIAquick membrane, the column is let stood for 1 min, and then centrifuged.
The NanoDrop ND-1000 spectrophotometer uses a patented sample retention system that holds 1 µl of sample without the need for traditional containment devices such as cuvettes and capillaries. Using fiber optic technology and surface tension, the sample is held in place between two optical surfaces that define the pathlength in a vertical orientation. Direct coupling of the sample to the optics of the spectrophotometer removes interference caused by incident light and transmitted light passing through containment walls of traditional cuvettes, microcell cuvettes, and capillaries. Total measurement cycle time, including preparation and removal of the sample, is ~ 30 sec.
The measurement and subsequent concentration calculation is volume independent. Once the instrument lever arm is lowered, the upper optical surface engages with the

sample, forming a liquid column with the path length defined by the gap between the two optical surfaces. During each measurement, the sample is assessed at both a 1-mm and 0.2-mm path, providing a large dynamic range of nucleic acid detection. The software automatically calculates the nucleic acid concentration. All measurements are automatically normalized to 340 nm.

4.2.5 Enzymatic restriction of DNA

Preparation of DNA for cloning is dependent upon restriction enzyme digestion to generate compatible ends. DNA templates used for restriction were plasmids or PCR fragments. In all cases, one or more restriction enzymes are used to digest the DNA resulting in either non-directional or directional insertion into the target vector.
Enzymatic digestions were performed for 2 hours or overnight at 37°C using 1 µl enzyme/500-1000 ng DNA. Enzymes from New England Biolabs, were used at conditions reported in Table 9.

Table 9: NEBuffer Activity/Performance Chart with Restriction Enzymes.

Enzyme	Recognition Sequence	Supplied NEBuffer	% Activity in NEBuffer	Heat Inactivation	Incubation Temperature
XmaI	C/CCGGG	CutSmart® Buffer	100	65°C	37°C
XbaI	T/CTAGA	CutSmart® Buffer	100	65°C	37°C
NsiI	ATGCA/T	NEBuffer 3.1	100	65°C	37°C
NdeI	CA/TATG	CutSmart® Buffer	100	65°C	37°C
SpeI	A/CTAGT	CutSmart® Buffer	100	80°C	37°C
BamHI	G/GATCC	NEBuffer 3.1 CutSmart® Buffer	100	No	37°C

After restriction of the DNA, the fragments were purified using the PCR purification kit (Qiagen) or fragmehts were separated by Agarose gel electrophoresis and purified from the gel using the gel extraction kit (Qiagen).

4.2.6 Gel extraction of DNA

The QIAquick Gel Extraction Kit enables removal of nucleotides, enzymes, salts, agarose, ethidium bromide, and other impurities from samples, ensuring up to 80% recovery of DNA. Using a microcentrifuge or vacuum manifold, DNA ranging from 70 bp to 10 kb is purified from 1–24 samples. Purified DNA can be used, for example, in sequencing.
DNA fragments purified with the QIAquick system are ready for direct use in all applications, including sequencing, ligation and transformation, restriction digestion, labeling, microinjection, PCR, and in vitro transcription.
The DNA fragment was excised from the agarose gel with a clean, sharp scalpel.
The gel slice was weighed in a colorless tube. 3 volumes Buffer QG (5.5 M guanidine thiocyanate (GuSCN) and 20 mM Tris HCl pH 6.6) were added to 1 volume gel (100 mg gel ~ 100 µl). The maximum amount of gel per spin column is 400 mg. For >2% agarose gels, 6 volumes Buffer QG were added and incubate at 50°C for 10 min (or until the gel

slice has completely dissolved). The tube was vortexed every 2–3 min to help dissolve gel.
1 gel volume isopropanol was added to the sample and mixed.
A QIAquick spin column was placed in a provided 2 ml collection tube or into a vacuum manifold. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 1 min. The flow-through was discarded and the QIAquick column was placed back into the same tube.
If DNA will subsequently be used for sequencing, in vitro transcription, or microinjection, 500 µl Buffer QG were added to the QIAquick column and centrifuged for 1 min.
The flow-through was discarded and the QIAquick column was placed back into the same tube.
To wash, 750 µl Buffer PE (Washing Buffer: 10 mM Tris-HCl pH 7.5 and 80% ethanol) were added to QIAquick column and centrifuged for 1 min.
The flow-through was discarded and the QIAquick column was placed back into the same tube.
If the DNA will be used for salt-sensitive applications (e.g., sequencing, bluntended ligation), the column is let stand 2–5 min after addition of Buffer PE.
The QIAquick column was centrifuged in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube.
To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water were added to the center of the QIAquick membrane and the column was centrifuged for 1 min. For increased DNA concentration, 30 µl Buffer EB were added to the center of the QIAquick membrane, the column was let stand for 1 min, and then centrifuged for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.

4.2.7 Ligation of DNA fragments

For ligation reactions T4 DNA Ligase (Invitrogen) was used to ligate DNA fragments.
T4 DNA Ligase catalyzes the formation of phosphodiester bonds in the presence of ATP between double-stranded DNAs with 3´ hydroxyl and 5´ phosphate termini. The unique T4 DNA Ligase buffer optimizes ligation, which can be performed in 5 minutes. The conditions adopted for cloning are reported in Table 10.

Table 10: Conditions adopted for the reaction of ligation.

Component	Blunt ends
5X Ligase Reaction Buffer	4 µl
Insert: Vector Molar Ratio	3:1
Vector Ends	15-60 fmol
Insert Ends	45-180 fmol
Total DNA	0.1-1.0 µg
Autoclaved distilled water	to 20 µl
Temperature	14°C
Time	16-24 h

4.2.8 Neisseria genomic DNA prep

Sigma's GenElute Bacterial Genomic Kit is used as a simple and convenient technique to isolate high quality DNA from Gram negative bacteria, as *Neisseria meningitidis*.
The GenElute kit combines the advantages of a silica-based system with a microspin format and eliminates the need for expensive resins, alcohol precipitation, and hazardous organic compounds such as phenol and chloroform. Bacteria are first incubated with the appropriate enzymes to ensure efficient cell lysis and DNA release from the cells. The bacteria are lysed in a chaotropic salt-containing solution to ensure the thorough denaturation of macromolecules. The addition of ethanol causes the DNA to bind when the lysate is spun through a silica membrane into a microcentrifuge tube. After washing to remove the contaminants, the DNA is eluted in 200 µL of a Tris-EDTA solution. The expected yield of genomic DNA will vary depending on the cell density of the bacterial culture and the bacterial species and strain used.
DNA purified with the GenElute kit has an A260/A280 ratio between 1.6 and 1.9 and can be up to 50 kb in length. An absorbance of 1.0 at 260 nm corresponds to approximately 50 mg/mL of double-stranded DNA.
This DNA purified in this way is ready for downstream applications such as restriction endonuclease digestions, PCR, and Southern blots.
In detail, 1.5 mL of *Neisseria meningitidis* cells pellet of an overnight bacterial broth culture was harvested by centrifuging for 2 minutes at 12,000–16,000 × g. The culture medium was completely removed and discarded. The pellet was resuspended thoroughly in 180 µL of Lysis Solution T.
If RNA-free genomic DNA was required, 20 µL of RNase A Solution were added, mixed and incubated for 2 minutes at room temperature.
20 µL of the Proteinase K solution were added to the sample, mixed and incubated for 30 minutes at 55 °C. 200 µL of Lysis Solution C were added and vortexed thoroughly (about 15 seconds), and incubated at 55 °C for 10 minutes.
A homogeneous mixture is essential for efficient lysis.
500 µL of the Column Preparation Solution were added to each pre-assembled GenElute Miniprep Binding Column seated in a 2 mL collection tube and centrifuged at 12,000 × g for 1 minute. The eluate was discarded. 200 µL of ethanol (95–100%) were added to the lysate and mixed thoroughly by vortexing for 5–10 seconds. A homogeneous mixture is essential. The entire contents of the tube was transferred into the binding column and centrifuged at ≥ 6500 × g for 1 minute. Discard the collection tube containing the eluate was discarded and the column was placed in a new 2 mL collection tube. 500 µL of Wash Solution 1 were added to the column and centrifuged for 1 minute at ≥ 6500 × g. The collection tube containing the eluate was discarded and the column was placed in a new 2 mL collection tube. 500 µL of Wash Solution were added to the column and centrifuged for 3 minutes at maximum speed (12,000–16,000 × g) to dry the column. The column must be free of ethanol before eluting the DNA. The column was centrifuged for an additional 1 minute at maximum speed if residual ethanol is seen. Finally, the collection tube containing the eluate was discarded and the column was placed in a new 2 mL collection tube.

200 µL of the Elution Solution were pipetted directly into the center of the column and centrifuged for 1 minute at ≥ 6500 × g to elute the DNA. To increase the elution efficiency, the sample was incubated for 5 minutes at room temperature after adding the Elution Solution and then centrifuged.
The yield can be improved by 20–50% when performing a second elution.
The eluate contains pure genomic DNA. For short-term storage of the DNA, 2–8 °C is recommended. For longer-term storage, –20 °C is recommended. Avoid freezing and thawing, which causes breaks in the DNA strand. The Elution Solution will help stabilize the DNA at these temperatures.

4.2.9 Plasmidic Miniprep

The protocol of Plasmid DNA Purification using the QIAprep Spin Miniprep Kit and a Microcentrifuge is designed for purification of up to 20 µg of high-copy plasmid DNA from 1–5 ml overnight cultures of E. coli in LB medium.
The pelleted bacterial cells were resuspended in 250 µl Buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 100 µg/ml RNaseA) and transferred to a microcentrifuge tube. 250 µl Buffer P2 (200 mM NaOH and 1% SDS) were added and the tube was mixed thoroughly by inverting 4–6 times. 350 µl Buffer N3 (4.2 M Gu-HCl and 0.9 M potassium acetate pH 4.8) were added and the tube was mixed immediately and thoroughly by inverting 4–6 times. The samples were centrifuged for 10 min at 13,000 rpm (~17,900 x *g*) in a table-top microcentrifuge. 800 µl of the supernatant were added to the QIAprep 2.0 spin column by pipetting. The samples were centrifuged for 30–60 s and the flow-through was discarded. The QIAprep 2.0 spin column was washed by adding 0.5 ml Buffer PB (Binding Buffer: 5 M Gu-HCl and 30% isopropanol) and centrifuged for 30–60 s. The flow-through was discarded. The QIAprep 2.0 spin column was washed by adding 0.75 ml Buffer PE (Washing Buffer: 10 mM Tris-HCl pH 7.5 and 80% ethanol) and centrifuging for 30–60 s. The flow-through was discarded and the sample was centrifuged at full speed for an additional 1 min to remove residual wash buffer. The QIAprep 2.0 column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water were added to the center of each QIAprep 2.0 spin column, let stand for 1 min, and centrifuged for 1 min. Plasmid DNA was stored at -20°C for long term or 4°C for short term.

4.2.10 *Escherichia Coli* transformation

The transformation of *E. coli* is performed using chemically competent cells (subcloning efficient *E.coli* DH5α, Invitrogen). The appropriate amount of competent cells was thawed on ice. 50 µl aliquots of cells were put into pre-chilled tubes. 5-10 µl of a ligation reaction mix or 5 ng of pure plasmid DNA were added to each tube and mixed gently. The tubes were incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 42°C. The tubes were placed immediately on ice for 2 min. 950 µl of LB medium were added to each tube and incubate for 1 hour at 37°C.The cultures were transferred to 1.5 ml microcentrifuge tubes and centrifuged for 1 min at 6000 rpm. 950 µl of the supernatant were removed and the pellet was resuspended. The suspension was

suspended on a LB agar plate containing the appropriate antibiotic. The plates were incubated overnight at 37°C.

4.2.11 *Neisseria Meningitidis* transformation

The meningococcus strain to be transformed was plated on a GC agar plate from a glycerol stock kept at -80°C. After ON growth at 37°C, 5% CO2, 5 to 10 single colonies were transferred onto a fresh GC agar plate in a single spot. 1-3 µg of DNA in a maximum volume of 20 µL used to transform meningococcus was directly dropped onto bacteria, and then mixed with a 1µL loop. The plate was incubated at 37°C, 5% CO2 for 5-6 hours. After incubation bacteria were collected, resuspended in 100 µL of PBS 1X, plated onto a fresh GC agar plate containing the appropriate antibiotics to select the colonies that have taken up the DNA and express the appropriate resistance. Plates were incubated at 37°C, 5% CO2 for 18-36 hours, or until transformants appeared big enough to be subcultures. Single colonies were subcultured on fresh GC agar plates with antibiotics and incubated at at 37°C, 5% CO2 for 18-36 hours. Glycerol stocks of the colonies were prepared.

4.2.12 Preparation of bacterial glycerol stocks for long term storage

For a Glycerol stock preparation, sterile pipet tips and sterile microfuge tubes were used. Few colonies of the clone of interest were picked off the plate and added to a sterile 1.5 ml microfuge tube containing 0.5 ml of sterile liquid meningococcal growth mediun with 20% (v/v) glycerol solution. The solution was mixed by inversion and freezed on dry ice or directly placed into the -80°C freezer. The tube was labeled with clone informations.
To recover the bacteria the frozen surface of the culture was scraped with a sterile inoculating needle, and then the bacteria that adhere to the needle were immediately streaked onto the surface of the plate. The glycerol stocks should not freeze/thaw too many times. Placing the glycerol stock on dry ice while streaking onto the plate will prevent it from thawing completely and will improve the shelf life.

4.3 Generation of *Neisseria Meningitidis* mutants

4.3.1 Overview of definitions and objectives of mutations in Neisseria meningitidis genome

Gene to delete	Aim of mutation	Reference
gna33	To improve the production of outer membrane vescicules production, interfering with biosynthesis of cell wall	Adu Bobie et al. Infect. Immun. 2004, 72(4); 1914-1919 (214)
lpxL	To remove an enzyme responsible of last step of lipopolysaccaride synthesis, reducing its tossicity	Koeberling et al. 2008. J Infect Dis 19:262-70 (215)
Genes responsible of	To make the strain sensitive to serum and human blood and so non-virulent	Kahler et al. 1998; Mackinnon et al. 1993

capsule biosynthesis (*syn* or *ctr*)		(216) Vogel et al. 1996, 1997 (217)
Gene to overexpress		
fHbp	To increase fHbp expression on outer membrane in order to improve the immunogenicity of outer membrane vescicules vaccine	Koeberling et al., CVI 2011(218)

Inserts and plasmids used to generate modifications in the host

Inserts	**Plasmids**
Spectinomycin resistance (*spec*)	pSL1190
Erythromycin resistance (*erm*)	pBluescript
Kanamycin resistance (*kan*)	pUC18 or pUC19
Chloramphenicol resistance (*cat*)	pSL1190 or pBluescript
Factor H binding protein (fHbp)	pSL1190 or pBluescript

4.3.2 Plasmids and primers

Table 11: List of plasmids used in this study.

Plasmid name	**Resistance**	**Description**	**Reference**
psynX	Chloramphenicol and ampicillin	This plasmid is an expression vector that contains an upstream flanking region that allows homologous recombination with genes of the capsule biosynthesis locus and a portion of fHbp gene that allows recombination with fHbp in *Neisseria* genome under the control of a strong synthetic promoter designed at GSK Vaccines Siena	This study
psynX_nmb1869	Chloramphenicol and ampicillin	psynX containing an upstream flanking region that allows recombination with the gene nmb1869, located upstream of fHbp in the genome of	This study

		Neisseria Meningitidis serougroup B	
Plasmid used for deletion of the group W capsule biosynthesis genes (without replacement with fHbp)	Spectinomycin and ampicillin	This plasmid is an expression vector that contains flanking regions that allow the capsule biosynthesis gene deletion in place of spectinomycin resistance cassette by allelic exchange in the genome of *Neisseria Meningitidis*	Personel communication
pLpxL1kan	Kanamycin and chloramphenicol	This plasmid is an expression vector that contains flanking regions that allow *lpxL1* gene deletion in place of kanamycin resistance cassette by allelic exchange in the genome of *Neisseria Meningitidis*	Koeberling et al. 2008. J Infect Dis 19:262-70 (215)
pBSUDGNA33ERM	Erythromycin and ampicillin	This plasmid is an expression vector that contains flanking regions that allow *gna33* gene deletion in place of erythromycin resistance cassette by allelic exchange in the genome of *Neisseria Meningitidis*.	Adu Bobie et al. Infect. Immun. 2004, 72(4); 1914-1919 (214)
pComPind	Chloramphenicol and ampicillin	This plasmid is an expression vector that allows the integration of a target gene into the Neisseria chromosome into a non coding region between nmb1428 and nmb1429. Expression of the inserted gene is under control of an IPTG inducible promoter. This plasmid was used as the parent plasmid to generate a	Iewa et al., J Bacteriology 2005(219)

		set of derivative constructs that contain different full length fHbp IDs under IPTG inducible control	
pCompind fHbp ID5	Chloramphenicol and ampicillin	pComPind containg the gene encoding the full length fHbp ID 5	This study
pCompind fHbp ID15	Chloramphenicol and ampicillin	pComPind containg the gene encoding the full length fHbp ID 15	This study
pComPind fHbp ID55	Chloramphenicol and ampicillin	pComPind containg the gene encoding the full length fHbp ID 55	This study
pComPind fHbp ID359	Chloramphenicol and ampicillin	pComPind containg the gene encoding the full length fHbp ID 359	This study
pComPind fHbp ID283	Chloramphenicol and ampicillin	pComPind containg the gene encoding the full length fHbp ID 283	This study
pComPind fHbp ID473	Chloramphenicol and ampicillin	pComPind containg the gene encoding the full length fHbp ID 473	This study
pComPind fHbp ID306	Chloramphenicol and ampicillin	pComPind containg the gene encoding the full length fHbp ID 306	This study
pComPind fHbp ID419	Chloramphenicol and ampicillin	pComPind containg the gene encoding the full length fHbp ID 419	This study
pBSUDgna1870ERM	Erythromycin and ampicillin	This plasmid is an expression vector that contains flanking regions that allow fHbp v.1 gene deletion in place of erythromycin resistance cassette by allelic exchange in the genome of *Neisseria Meningitidis*	This study

Table 12: List of primers used in this study. Fw means forward and rev means reverse.

Primer name	**Primer sequence 5'>3'**	**Primer use**
nmb1869 fw	CCCCCCGGGGCCTGTAAACAAAATGCCG	Amplification of the portion of the gene nmb1869 located upstream of fHbp in the genome of Neisseria Meningitidis serogroup B

		and used as flanking region
nmb 1869 rev	GCTCTAGAGCATACCGCATCAATGAGG	Amplification of the portion of the gene nmb1869 located upstream of fHbp in the genome of Neisseria Meningitidis serougroup B and used as flanking region
nmb1869 fw2	CCCCCCGGGCCCGCGCAAATACCTGAGC	Amplification of the portion of the gene nmb1869 located upstream of fHbp in the genome of Neisseria Meningitidis serougroup B and used as flanking region
CpsUp-fw	TCCCCCCGGGTATCGCCAACAAACGGCACAG	Amplification of a fragment of the group W capsule biosynthesis locus used as upstream recombination site in plasmid that allows the capsule biosynthesis gene deletion in place of spectinomycin resistance
CpsDo-rev	GGACTAGTGTTTGTTTGCCGCATGCTAATGCC	Amplification of a fragment of the group W capsule biosynthesis locus used as downstream recombination site in plasmid that allows the capsule biosynthesis gene deletion in place of spectinomycin resistance
REVcpsUP_rev	GCTCTAGATTTCGATTAAGTGCTATAATTAGGCC	Amplification of a fragment of the group W capsule biosynthesis locus used as upstream recombination site in plasmid that allows the capsule biosynthesis gene deletion in place of spectinomycin resistance
FWcpsDO_fw	GCCAATGCATCAATATGCTGCCATTACTCC	Amplification of a fragment of the group W capsule biosynthesis locus used as downstream recombination site in plasmid that allows the capsule biosynthesis gene deletion in place of spectinomycin resistance
fHbpID5_fw	CGCGGATCCCATATGAATCGAACTGCCTTCTGCTGCC	Amplification of the full length fHbp gene ID 5 from genomic DNA of *Neisseria meningitidis* serogroup A as template

fHbpID5_rev	AACTGCAGAACCAATGCATTGGCTGCTTGGCGGCAAGAC	Amplification of the full length fHbp gene ID 5 from genomic DNA of *Neisseria meningitidis* serogroup A as template
fHbpID9_fw	CGCGGATCCCATATGAACCGAACTACCTTTTTCTGCC	Amplification of the full length fHbp gene ID 9 from genomic DNA of *Neisseria meningitidis* serogroup W as template
fHbpID9_rev	AACTGCAGAACCAATGCATTGGCTGCTTGGCGGCAAGAC	Amplification of the full length fHbp gene ID 9 from genomic DNA of *Neisseria meningitidis* serogroup W as template
fHbp-f41_fw	GGATCAGTCCGTCAGTAAAAACGAGAAAC	Insertion of the mutation R41S by site directed mutagenesis (using the Q5® Site-Directed Mutagenesis Kit, NEB) in the plasmids where the different fHbp genes were added
fHbp-r41_rev	GTTTCTCGTTTTTACTGACGGACTGATCC	Insertion of the mutation R41S by site directed mutagenesis (using the Q5® Site-Directed Mutagenesis Kit, NEB) in the plasmids where the different fHbp genes were added
lpxL1up-fw	GCATTTGTATTTTGCCGTCTG	Amplification of a fragment of *lpxL1* gene used as upstream recombination site in plasmid that allows the *lpxL1* gene deletion in place of kanamycin resistance
lpxL1do-fw	ATGCATGCCCCGATTTTTACTGACTACG	Amplification of a fragment of *lpxL1* gene used as downstream recombination site in plasmid that allows the *lpxL1* gene deletion in place of kanamycin resistance
lpxl1do-rev	CGCCATTTTCTACGCTTTGCCAAG	Amplification of a fragment of *lpxL1* gene used as downstream recombination site in plasmid that allows the *lpxL1* gene deletion in

		place of kanamycin resistance
lpxL1up-rev	GCTCTAGAGCCGTCTGAACGTAGTCAGTAAAAATCGGGGC	Amplification of a fragment of *lpxL1* gene used as upstream recombination site in plasmid that allows the *lpxL1* gene deletion in place of kanamycin resistance
GNA33_R1	CGGGAAAGAGCTGTATGTCGAACG	Amplification of a fragment of *gna33* used as downstream recombination site in pBsudGNA33ERM that allows the *gna33* gene deletion in place of kanamycin resistance
GNA33_R2	GGCGGTTTGAAGCCGAGTTTGACG	Amplification of a fragment of *gna33* used as downstream recombination site in pBsudGNA33ERM that allows the *gna33* gene deletion in place of kanamycin resistance
GNA33_R3	GCACAGGCATACCGCGACGCGCG	Amplification of a fragment of *gna33* used as downstream recombination site in pBsudGNA33ERM that allows the *gna33* gene deletion in place of kanamycin resistance
ERM_fw1	CTAGTCTAGATTTGTGTATAATAGG	Amplification of the full length *erm* gene from the full length *erm* synthesized and present in pBsudGNA33ERM
ERM_rev1	CGCGGATCCTTATTTCCTCCCG	Amplification of the full length erm gene from the full length erm synthesized and present in pBsudGNA33ERM
ERM_fw2	CTAGTCTAGAGAGTGTGTTGATAGTGCAG	Amplification of the full length erm gene from the full length erm synthesized and present in pBsudGNA33ERM
ERM_rev2	CGCGGATCCCTTTAGTAACGTGTAACTTTCC	Amplification of the full length erm gene from the full length erm synthesized and present in pBsudGNA33ERM

fHbp f1	CGCGGATCCCATATGAATCGAACTGCCTTCTGCTGCC	Amplification of the full length fHbp gene ID5, 15, 55 and 306 from genomic DNA of *Neisseria meningitidis* serogroup C as template or the full length fHbp gene ID283, 359, 473 and 419 synthesized (Invitrogen)
fHbp r1	GCCAATGCATGCGGTTGGCCTGAAAATAGGG	Amplification of the full length fHbp gene ID5, 15, 55 and 306 from genomic DNA of *Neisseria meningitidis* serogroup C as template or the full length fHbp gene ID283, 359, 473 and 419 synthesized (Invitrogen)

4.4 Determination of protein concentration by Lowry assay

Standard curve for the assays were prepared with bovine serum albumin (Pierce) in the range 4-50 µg. Detergent Compatible (DC) Protein assay (Bio-Rad) is used following the producers specifications. Different quantities of GMMA were adjusted to a final volume of 25 µL with water. 125 µL of Reagent A' (prepared adding 20 µL of reagent S to every mL of reagent A) were added and samples were vortexed. 1 mL of reagent B was added and absorbances were read at 750 nm after 15 minutes incubation.

4.5 SDS-Polyacrylamid gel electrophoresis of proteins

Whole cell lysates samples were prepared by centrifuging 1 mL of bacteria liquid culture (OD 1) at 13000 rpm for 10 minutes, and resuspending the pellet in 200 µL sample buffer 2X (NuPAGE® Sample Reducing Agent (10X) plus NuPAGE® LDS Sample Buffer (4X) to final concentration 2X); samples were then boiled for 10 minutes.
GMMA samples were prepared by mixing equal volumes of GMMA and sample buffer 2X (NuPAGE® Sample Reducing Agent (10X) plus NuPAGE® LDS Sample Buffer (4X) to final concentration 2X); samples were then boiled for 10 minutes. Both whole cell lysates and GMMA samples were separated by SDS-PAGE using NuPAGE® Novex® 12% Bis-Tris Protein Gels; gels were run in NuPAGE® MOPS SDS Running Buffer, at 200V for 1 hour.

4.6 Western Blotting

The term "blotting" refers to the transfer of biological samples from a gel to a membrane and their subsequent detection on the surface of the membrane. Western blotting (also called immunoblotting because an antibody is used to specifically detect its antigen) was introduced by Towbin, et al. in 1979 and is now a routine technique for protein analysis.

The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture. Western blotting can produce qualitative and semiquantitative data about that protein.
It was performed using the iBlot system (Invitrogen). Protein transfer on nitrocellulose membrane was performed as per instruction of the manufacturer. The membrane was blocked in PBS 3% milk for 2 hours at room temperature, washed in PBS-Tween 0.1%, and incubated with primary antibody. For fHbp detection, primary antibody was a mouse anti-fHbp v.1 (or anti-fHbp v.2) polyclonal (or monoclonal used at 1 µg/mL final concentration) antibody, diluted 1:2000 in PBS-Tween 0.1%; secondary antibody was an anti-mouse IgG conjugated with horseradish peroxidase (Sheep anti-mouse IgG – HRP, GE Healthcare, diluted 1:10000 in PBS-Tween 0.1%). After 1 hour incubation with secondary antibody, membranes were washed with PBS-Tween 0.1%. 4 mL of ECL (Pierce ECL Western Blotting Substrate, Thermo Scientific) were added to the membranes for 1 minute; membranes were removed from the substrates and placed in plastic sheet protectors, exposed to Amersham Hyperfilm ECL film (GE Healthcare) and developed.

4.7 Dot blotting

Dot blotting is a technique for detecting, analyzing, and identifying proteins, similar to the western blot technique but differing in that protein samples are not separated electrophoretically but were spotted directly onto the membraneusing a dot blot devide that allowed spotting of 96 samples.
20 to 50 uL of samples were spotted onto the nitrocellulose membrane. Vacuum was applied to spot the sample on the membrane.. The membrane was removed from the blotting device and let dry for a few minutes. Non-specific sites were blocked by soaking in PBS 3% milk for 2 hours at room temperature, washed in PBS-Tween 0.1%, and incubated with primary antibody. For fHbp detection, primary antibody was a mouse anti-fHbp v.1 (or anti-fHbp v.2) polyclonal (or monoclonal used at 1 µg/mL final concentration) antibody, diluted 1:2000 in PBS-Tween 0.1%; secondary antibody was an anti-mouse IgG conjugated with horseradish peroxidase (Sheep anti-mouse IgG – HRP, GE Healthcare, diluted 1:10000 in PBS-Tween 0.1%). After 1 hour incubation with secondary antibody, membranes were washed with PBS-Tween 0.1%. 4 mL of ECL (Pierce ECL Western Blotting Substrate, Thermo Scientific) were added to the membranes for 1 minute; membranes were removed from the substrates and placed in plastic sheet protectors, exposed to Amersham Hyperfilm ECL film (GE Healthcare) and developed.

4.8 Coomassie blue stain of proteins

For Comassie staining, after run gels were washed with distilled H2O and stained with Coomassie Blue Stain (Sigma-Aldrich) following the data sheet’s procedures for proteins.

4.9 FACS analysis of fHbp surface expression on live bacteria

Surface expression of fHbp in strains that were engineered to express fHbp under the control of IPTG-inducible promoter was measured on live bacteria by FACS-Scan flow cytometer. Antibody binding was detected using a secondary antibody anti-mouse (whole molecule) FITC-conjugated (Sigma).
Neisseria Meningitidis strain to test in the assay was subculture from glycerol stock on GC agarplate and incubate O/N at 37°C, 5% CO2.
7 ml of Mueller-Hinton Broth (MHB) (DIFCO, #275730) were inoculated with single cells to OD600 = 0.12 to 0.15. Bacteria were incubated on a shaker at 37%, 5% CO2 until OD600 reached 0,6 - 0,65. 1 mL of culture were then transferred into 6 mL warm MHB + Isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentrations adopted in this work (0.05 mM or 0.5 mM). Bacteria were incubated at 37%, 5% CO2. When the culture reached the target OD 600 = 0.6 to 0.65, it was transferred into 50 mL Falcon tube, and washed with PBS + 1%BSA, centrifuged for 10 minutes at 3500 rpm, RT. After another washing in PBS + 1%BSA, the supernatant was discarded and pellet resuspended in DPBS + BSA to OD600 = 0.6 to 0.7. 80 µl of bacterial suspension (OD 0.6) were incubated with 20 µl of polyclonal sera (1:100 final dilution) at room temperature for 60 minutes. After a centrifugation at 13,400 RCF for 3 minutes at room temperature, the bacterial pellets were washed once with 1 mL PBS + 1%BSA and after a second centrifugation step the pellets were resuspended in 100 µl PBS + 1%BSA containing 1:300 dilution anti-mouse-FITC and incubated for 30 minutes RT. After a centrifugation at 13,400 RCF for 3 minutes, the pellet of bacteria were washed as described above and resuspended in 1 ml filter sterilized freshly made 0.5% formaldehyde in 1X PBS. Samples (10-20 µl) were transferred to FACS tubes, adding 500 µl of sterile PBS. Fluorescence was measured using a FACS machine.

4.10 Preparation and purification of GMMA

Strains were subcultured on GC agar plates with antibiotics if required and incubate for 18 to 24 hours at 37°C, 5% CO2. 25 mL of complete medium (Table 13) was inoculated with single colonies to O.D. = 0.1 and incubated on shaker at 37°C, 5% CO2 150 rpm for 12 +/- 2 hours.

Table 13: Complete medium. The medium was adjusted to pH 7.2 with NaOH and filter sterilized.

Composition of complete medium (g/L)	
L-glutamic acid	1
L-cystein hydrochloride	0.03
$Na_2HPO4 \times 7\ H_2O$	3.25
KCl	0.09
$MgSO_4 \times 7\ H_2O$	0.6
Yeast extract	5
Casamino acids	10
Lactic acid	7.5

200 ml of medium (in 1 L baffled flask, vented) was inoculated with over-night culture to O.D. = 0.15 and incubate for 8 to 10 hours at 37°C, 150 rpm. O.D. was measured every two hours to generate growth curve (end O.D. should be around 7 to 8). The cultures were centrifuged and the supernatants were sterilized by filtration through a 0.22 µm filter. To collect the GMMA the sterile culture supernatants were centrifuged (186,000 g, 2 hours, 4°C), the membrane pellets were washed once with PBS and after a second centrifugation step the GMMA pellets were resuspended in 1X PBS and were sterilized by filtration through a 0.22 µm filter.

4.11 Characterization and quality control of GMMA

4.11.1 Negative Staining Trasmission Electron Microscopy

A drop of 5 µL of GMMA suspension prediluted 100 µg/mL was placed onto 300 mesh copper formvar/carbon-coated grids and adsorbed for 5 min. Grids were then washed with few drops of distilled water and blotted with a Whatman filter paper. For negative staining, grids were treated with 2% uranyl acetate in ddH_2O for 1 min, blotted with Whatman filter paper and air-dried and observeded with a Tecnai 2 Spirit transmission electron microscope (FEI, Eindoven, the Netherlands) operating at 80 kV. Electron micrographs were recorded at a nominal magnification of 87,000 – 105,000 X.

4.11.2 Monocyte Activation Test (MAT)

PBMC isolation and freezing

Buffy coat from different donors were used to isolate PBMC by Ficoll (Amersham Pharmacia Biotec, cat N° 17-1440-03) density centrifugation and following protocol reported in (220, 221).
PBMC were frozen resuspending in freezing medium (heat-inactivated FBS (Fetal Bovine Serum), 10%DMSO) at a concentration of $5x10^7$ cells/cryovial. Cryovials were prepared dispensing 1 mL aliquots of cell suspension in pre-cooled 2 mL cryovials marked with subject's code and were immediately transferred into the Cell Freezing Box (Nalgene) and stored at –80°C for 24-48h and then transfered in Liquid Nitrogen tank.

PBMC thawing and stimulation

For thawing, each cryovial conserved in liquid nitrogen was warmed at 37°C in water bath gently shaking until a small, pea-sized pellet of ice remained. 1 mL of thawing medium (PBS without Ca^{2+} and Mg^{2+}, with 2.5 mM EDTA and 20 µg/mL DNAse (Sigma, 20 mg/mL stock) warmed at 37°C was added directly into each cryovial; thus, the cell suspension was added to a 40 mL of warm thawing medium. Each cryovial was rinsed with 1 mL of fresh thawing medium to collect residual cells and samples were centrifuged at 320 g for 10 minutes at room temperature. Supernatants were discarded

by gently inverting the tube and the pellet of cells were washed with 40 mL of pre-warmed thawing medium and centrifugation and discarding of supernatants were repeated. Pellet containing PBMCs was diluted in adequate volume of medium RPMI-1640 complete (with 25 mM HEPES, glutamine, 10% Fetal Bovine Serum (FBS) + 1% Antibiotics Penicilline-Streptomicine (InvitroGen)).
For the stimulation PBMC were cultured at a density of 2 x 10^5 cells/well with 180 µL of medium RPMI-1640 complemented with 25 mM HEPES, 2 mM glutamine, 10% FBS, 1% Pen-Strep (InvitroGen) in 96-well round bottom plates. After incubation at 37°C for 30 min, 20 µL of 10-fold serial dilutions of in PBS (0.0001-1,000 ng/mL final concentration in the assay) were added.

Cytokine analysis by ELISA

For IL-6 detection by ELISA Nunc MaxiSorp 96-well plates were coated overnight at 4°C with 2 µg/mL human IL-6 capture antibody (eBioscience 14-7069) in PBS, subsequently washed with PBS with 0.05% Tween 20 (PBS-T), blocked for 1 h with PBS with 1% BSA at room temperature (RT) and washed 3-times with PBS-T. 50 µL of supernatants from PBMC experiments, diluted 1:4 with PBS, were incubated for 2 h at RT. A two-fold dilution series of recombinant human IL-6 (eBioscience 39-8069) of 31.24 pg/mL to 4,000 pg/mL in RPMI with 10% FBS was included as standard curve on each plate (in duplicate). Plates were washed with PBS-T. Bound IL-6 was detected using 2 µg/mL of biotin-conjugated anti-human IL-6 (e-Bioscience 13-7068) in PBST with 0.1% BSA for 2 h at RT, followed by 3 washes with PBS-T, 20 min incubation at RT with streptavidin-horse radish peroxidase (R&D Systems, DY998) diluted 1:200 in PBS-T with 0.1% BSA, 3 washes with PBS-T, and a color reaction with 100 µL/well of substrate (R&D Systems, DY999) for 8 min at RT in the dark. The reaction was stopped by adding 50 µL/well of 12.5% sulfuric acid. The plates were read at 450 nm and 630 nm and the $OD_{450nm-630nm}$ was determined. IL-6 concentrations in the samples were calculated in comparison to the IL-6 standard using a four parameter logistic curve and plotted against the amount of GMMA in the sample. Results below the detection limit were assigned half of the detection limit. The average of the IL-6 release at the lowest concentration of GMMA that did not trigger activation was defined as background level. For comparison of the stimulatory activity of GMMA, the amounts of GMMA needed to obtain a 10-fold increase of IL-6 release over background were determined from the generated curves.

4.11.3 MALDI-TOF and MS/MS analysis on lipid A

Lipid A was precipitated from GMMA using mild acid hydrolysis with 1 % acetic acid for 2 h at 100°C (222). Samples were centrifuged at 14,000 g for 15 min, the pellets resuspended in water, and washed twice with water. The pellets were dried overnight using a speedvac and resuspended in chloroform-methanol 4:1 and mixed with an equal volume of Super DHB solution (Sigma-Aldrich). 2 µL of the mixture were loaded to the target plate (MTP 384 target plate ground steel BC, Bruker Daltonics) and analyzed by Ultraflex MALDI-TOF (Bruker Daltonics) in reflectron ion-negative mode. A Peptide Calibration Standard (Bruker Daltonics), mixed with the Super DHB solution, was

included in each analysis. For MS/MS analysis of lipid A, main peaks from the linear mode analysis were selected for Collision Induced Dissociation (CID) and resulting fragments were detected by MALDI-TOF-TOF in ion negative mode. For each sample, spectra represent the integration of the analysis of 20 different areas of the spot by 50 single laser shots. The *m/z* rations were determined by Flex Analysis software in comparison to the Peptide Standard.

4.11.4 HPLC-SEC MALLS analysis of GMMA samples

The GMMA have a size range that allows to enter in the pore of the Size Exclusion High Pressure Liquid Chromatography (HPLC-SEC) eluting as entire vesicles. The procedure was used to analyze GMMA samples and evaluate their soluble protein content, the radii populations and the presence of a relevant amount of nucleic acids.
In particular the detectors adopted were:

- UV, 280/260 ratio to detect DNA presence and its MW
- Fluorimeter, ex280/em336 to detect protein related to GMMA particles and free soluble proteins
- Multi Angle Laser Light Scattering (static scattering) for particle sizing

GMMA ultracentrifuged pellets, resuspended in 1X PBS and sterilized by filtration through a 0.22 µm filter as described in the paragraph 4.10, were diluted in PBS 1X to a final concentration 150 µg/mL in a final volume for injection of 80 µL.
To clarify how MALLS works in Figure 14 is reported the chromatogram related to an injection of 80 µL of sample. The HPLC system used is SEPAX 2000A+1000A column in series while the eluent used is PBS 0.5 ml/min.

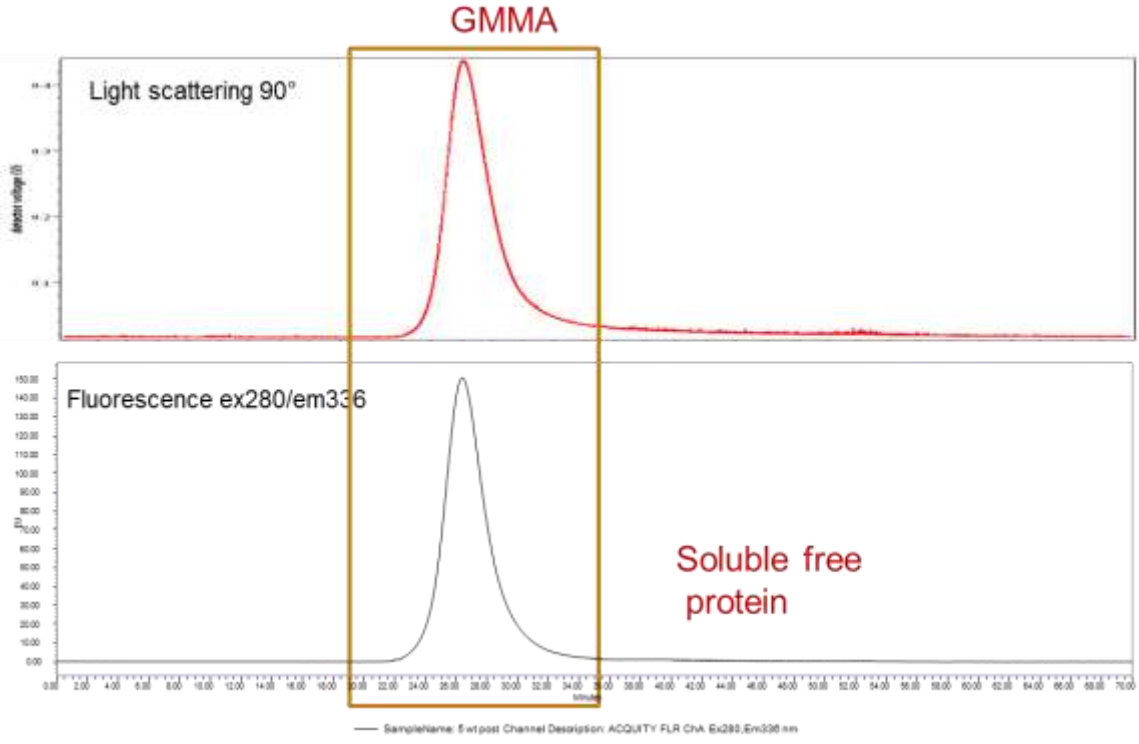


Figure 14: Chromatogram related to an injection of 80 µL of GMMA sample (150 µg/mL). The HPLC system used is SEPAX 2000A+1000A column in series while the eluent used is PBS 0.5 ml/min for a 70 min run time.

In the chromatogram reported above is possible to check the peak related to the GMMA and the absence of the peak related to the soluble free proteins, generally located on the right in the chromatogram because of their smaller size that delayed their time of elution in respect to the bigger GMMA samples.
The size determination by MALLS was performed by the following detector flow cell scheme (Figure 15).

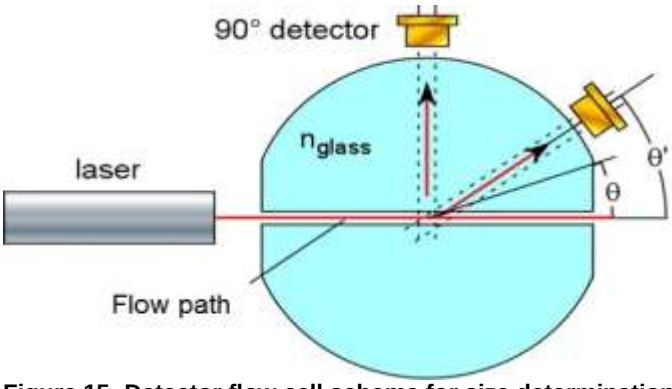


Figure 15: Detector flow cell scheme for size determination in MALLS analysis.

Then a multiple chromatogram was acquired (18, one for each angle) as reported in Figure 16. The analysis performed was in function of Light Scattering intensity = f (angle, MW, Particle size).

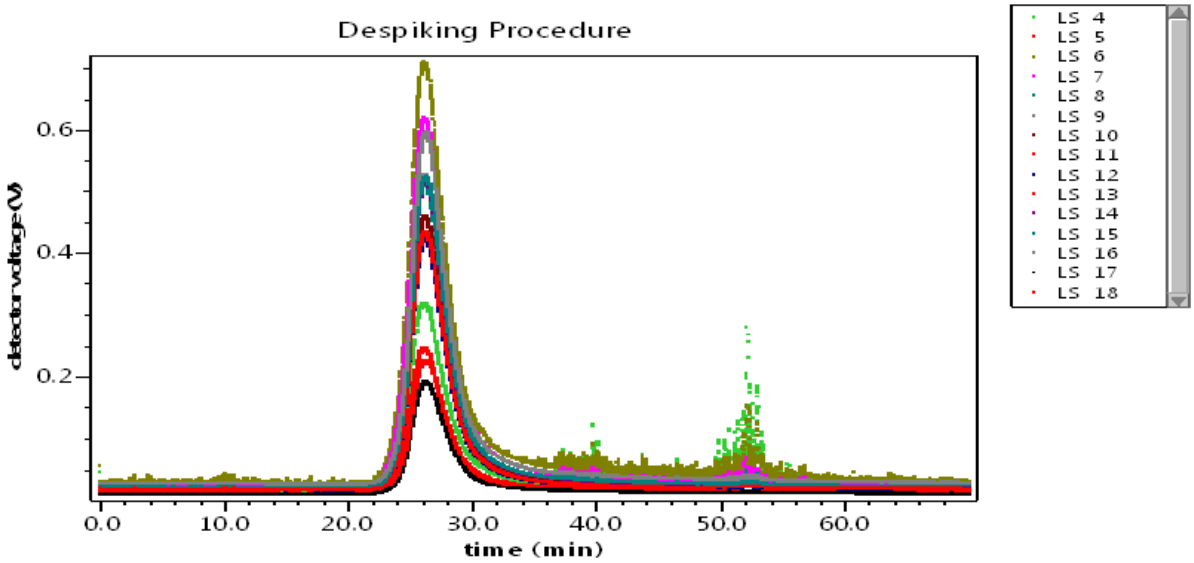


Figure 16: Multiple chromatogram in function of Light Scattering intensity related to one of the GMMA sample analyzed in this work (GMMA deriving from *Neisseria Meningitidis* serogroup W wild type strain).

Successively, it was chosen the best fitting model and the fit degree for the data to achieve, from LS equations, the particles radius. Each model, in theory, works better with a different range of MW / radius, but it depends also on the product to characterize. For this reason they have to be tried on each peak.
In Figure 17 it is reported, as example, trials on first LS peak.
In this case the model/fit degree was applied on data obtained from:

- retention time (RT) corresponding to the top of the peak
- averaging for the whole peak

The parameters considered were:

- RMS (root mean square radius)
- Peak R_n (average radius)
- Peak R_w (mass weighted average radius)
- Peak R_z (mass^2 weighted average radius)

The criteria to evaluate the method were based on:

- R^2 for fitting on RT
- Errors on the RMS of the whole peak

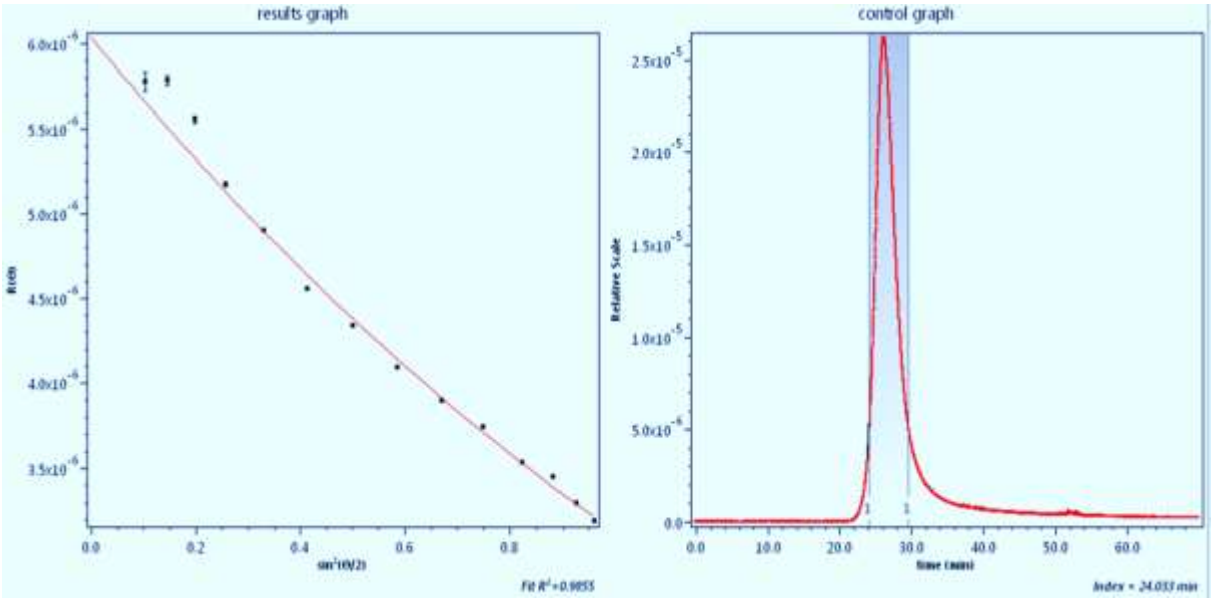


Figure 17: Analysis of first LS peak reported in Figure 16.

The configuration of MALLS is reported in Figure 18.

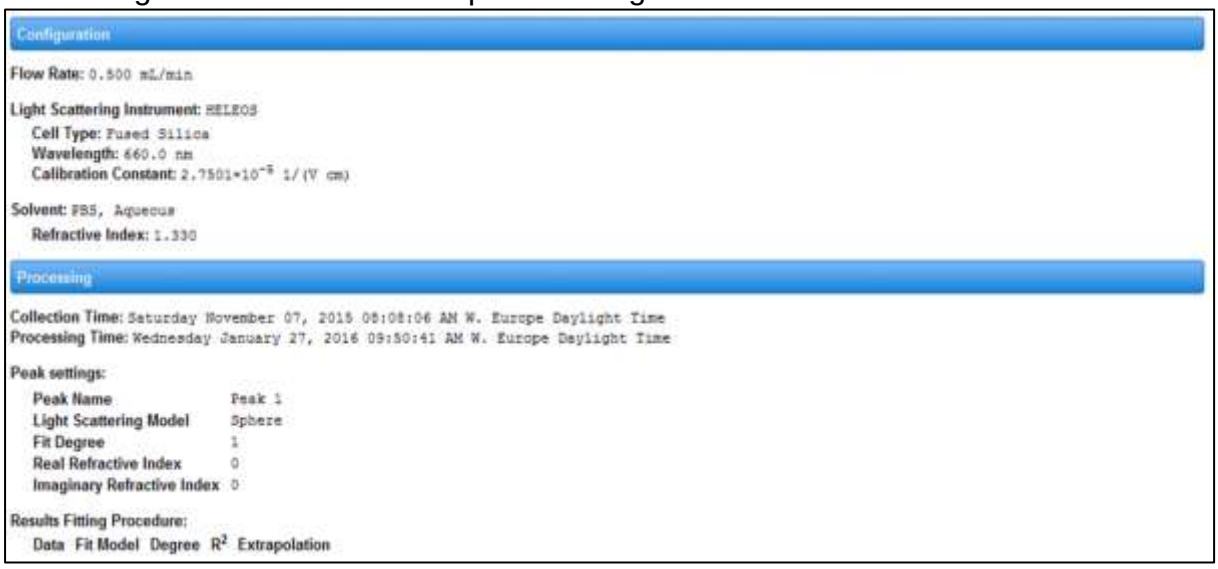


Figure 18: Typical MALLS configuration.

4.12 Mice immunization

In order to obtain serum samples for analysis, mouse immunization studied were carried out previously at the Toscana Life Science animal facility, according to formal approved GVGH protocols. Female CD-1 mice (4 to 6 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA).
Eight mice per group were immunized intraperitoneally two times with 4 weeks intervals. Serum samples were obtained 2 weeks after the second dose. Mice were immunized with the following antigens: 0.5 or 2.5 µg "native outer membrane vescicles" (nOMV) from a serogroup W mutant with deleted capsule, deleted lpxL1 and over-expressed fHbp ID5; 1 or 5 µg GMMA from a serogroup W mutant with deleted capsule, deleted *lpxL1*, deleted *gna33* and over-expressed fHbp ID9 R41S A control vaccine consisted of 5 µg GMMA from a serogroup W mutant with deleted capsule, deleted *lpxL1*, deleted *gna33.* All vaccines were adsorbed on 3 mg/mL Aluminium hydroxide in a 200 µL formulation containing 10 mM Histidine and 0.9 mg/mL NaCl. Sera were stored at −80 °C until use. All animal work was approved by the Italian Animal Ethics Committee.

4.13 Characterization of immune responses by Serum Bactericidal Assay (SBA)

Neisseria Meningitidis strain to test in the assay was subculture from glycerol stock on GC agarplate and incubate O/N at 37°C, 5% CO2.
7 ml of Mueller-Hinton Broth (MHB) (DIFCO, #275730) supplemented with 0.25% glucose were inoculated with single cells to OD600 = 0.12 to 0.15. Bacteria were incubated on a shaker at 37%, 5% CO2 until OD600 reached 0,6 - 0,65. 1 mL of culture were then transferred into 6 mL warm MHB (+ 0.25% glucose and 0.02 mM CMP-NANA and Isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentrations adopted in this work (0.05 mM or 0.5 mM)). Bacteria were incubated at 37%, 5% CO2 until OD 600 = 0.6 to 0.65. Culture was then transferred into 50 mL Falcon tube, and washed with Dulbecco's saline phosphate buffer (Sigma #D8662) supplemented with BSA 1% (DPBS+BSA), centrifuged for 10 minutes at 3500 rpm, RT. The supernatant was discarded and pellet resuspend in DPBS + BSA to OD600 = 0.6 to 0.7. Bacteria were diluted in DPBS+BSA to a final working dilution of 1:12,500.
The assay was done in sterile 96 well plates in a final volume of 40 µl per well. In a separate plate ("dilution plate"), sera dilutions were performed, starting from a 1:10 dilution, followed by 7 serial dilutions 1:4. 10 µL of serum sample were added from the dilution plate into the assay plate. In control wells were added wither DPBS + BSA only, or active complement, or heat-inactivated complement without serum. These controls were used to calculate the average of colonies at time zero (T0). 10 µL of bacteria working dilution were added to each well of the assay plate containing buffer only or diluted sera. Active and inactivated complement were added to control wells. 12 µL of controls were plated on square GC agar plate, loading 12 uL on top of the plate, and letting the drops run down to bottom of plate. Active complement was added to all the assay wells. Plate was sealed and incubated (37°C, 5% CO2) for 60 min. After 60 minute (T60) 12 µL of each well were plated on GC agarplate (as before) and incubated

O/N at 37°C, 5% CO2. 1. Colonies were counted on T0 plates and T60 plates. The bactericidal titer is defined as the serum dilution that results in 50% decrease of CFU at T60, compared to the average number of CFU at T0 in the six control wells that did not contain the test serum.

4.14 FHbp sequence diversity analysis by pairwise amino acid comparison

A matrix approach was adopted in order to analyze sequence diversity in the factor H binding sites in an existing and published panel of fHbp v.1, 2 and 3 sequence IDs.. Starting from the crystal structure of fHbp variant 1 and 3 from NCBI's Entrez Structure database, the amino acids that are in contact with factor H has been identified. Selection criteria were amino acid residues in fHbp that are in a maximum distance of 5Å to factor H. Factor H binding site sequence used for the analysis was characterized by 48 out 255 amino acids (full length fHbp v.1 ID1, published crystal structure) and 54 out 262 amino acids (full length fHbp v.3 ID28, published crystal structure). The factor H binding site for variant 2 has been obtained by alignment of sequence fHbp ID 28 (published crystal structure of the v.3 protein, as reported previously) and fHbp ID 16 (variant 2). Factor H binding sites in the other published fHbp IDs belonging to fHbp v.1 (N= 382), 2 (N= 150) and 3 (N= 148) have been identified by alignment with the reference sequence for fHbp variant 1 (ID1), variant 2 (ID16) and variant 3 (ID28). A snapshot of the pairwise comparison of the fH binding site sequences using the matrix shown in Figure 19.

	16	17	18	19	20	21	22	23	24	25	26	27	32	33	34	41	42	43
16	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
17	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
18	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
19	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
20	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
21	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
22	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
23	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
24	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0
25	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0
26	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0
27	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
32	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
33	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
34	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	2	1	1
41	2	2	2	2	2	2	2	2	1	1	1	2	2	2	2	0	1	1
42	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0
43	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0

Figure 19: Screenshot of matrix used to compare Factor H binding sites in fHbp v.2. Each variant can be divided into sub-variants (each one identified by a specific ID number) differing in their amino acid sequence. The numbers on the left and top correspond to the numbers of ID compared. Numbers in matrix from 0 to 2, in this case, correspond to the differences in aminoacid sequence between each ID pair.

It is possible to observe on the left and top the numbers of IDs compared, while the numbers within the matrix correspond to the numbers of differences in terms of aminoacid residues between the factor H binding sites of each ID. The darker colour is proportional to the increase of number of differences.
By matrix it was possible to calculate the number of positions of a sequence that were different from those of another sequence, as reported in the following equation.

Number of differences = **n° ij**= number of aminoacids differences comparing sequence **i** and sequence **j**

V – Results

5.1 Generation and characterization of GMMA from a serogroup W strain with over-expressed fHbp v.2

The majority of current African invasive group A and X isolates express fHbp variant 1. Current African serogroup W strains are more heterogeneous and strain have been identified that express fHbp v.1, 2 or 3. However, the majority of group W strains express a single subtype of the porin protein PorA. Antibodies against PorA provide subtype specific protection. Therefore, GMMA from a group W strain expressing the PorA subtype common among W strains and over-expressed fHbp variant 1 have the potential to provide protection against the majority of the current invasive African strains. However they are not expected to cover strains with a different PorA subtype and expressing fHbp variant 2 or 3. As a proof of principle I generated and characterized a GMMA from an African group W strain engineered to over-express fHbp v.2 (Figure 20).
The approach was to use a group W wildtype strain that naturally expresses fHbp v.2 (ID23) and upregulate the native fHbp v.2 expression by replacing the native promoter with a strong promoter and leaving the fHbp in its native site.
Additional mutations that were introduced in the strain were:

- Deletion of capsule biosynthesis gene to increase safety of the vaccine strain. In fact, strains without capsule were not infectious (223) and allowed to present better antigens like fHbp known to be protective and avoided potential generation of serogroup specific antibodies
- Deletion of *lpxL1* (224) to obtain strains with a reduced endotoxin activity
- Deletion of *gna33* (225) to increase the release of GMMA (Figure 20).

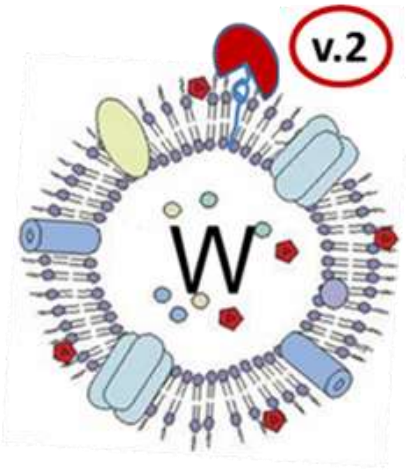
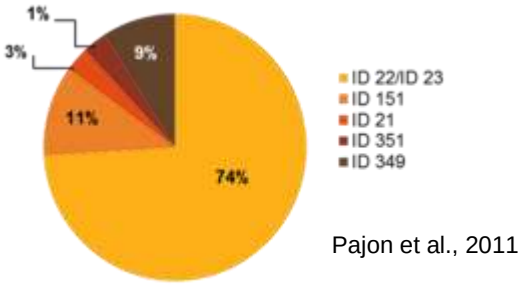


Figure 20: One GMMA from a serogroup W strain with over-expressed native fHbp v.2.

The initial selection of ID belonging to v.2 chosen to overexpress was based on current epidemiology and, as shown in the chart (Figure 21), among the invasive MenW strains that express fHbp v.2 or 3, ID22 and 23, differing by only one aminoacid (Figure 22), are represented in 74% of W strains.



Pajon et al., 2011

Figure 21: FHbp variant 2 (ID22, ID23, ID151, ID21) and 3 (ID351, ID349) distribution among the invasive MenW strains.

```
CLUSTAL O(1.2.1) multiple sequence alignment


ID22      CSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNG
ID23      CSSGGGGVAADIGAGLADALTAPLDHKDKGLQSLTLDQSVRKNEKLKLAAQGAEKTYGNG
          ****************************.*****************************

ID22      DSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKI
ID23      DSLNTGKLKNDKVSRFDFIRQIEVDGQLITLESGEFQIYKQDHSAVVALQIEKINNPDKI
          ************************************************************

ID22      DSLINQRSFLVSGLGGEHTAFNQLPSGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRI
ID23      DSLINQRSFLVSGLGGEHTAFNQLPSGKAEYHGKAFSSDDPNGRLHYSIDFTKKQGYGRI
          ************************************************************

ID22      EHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVK
ID23      EHLKTPEQNVELASAELKADEKSHAVILGDTRYGGEEKGTYHLALFGDRAQEIAGSATVK
          ************************************************************

ID22      IREKVHEIGIAGKQ
ID23      IREKVHEIGIAGKQ
          **************
```

Figure 22: FHbp variant 2 ID22 and ID23 (most prevalent among the invasive MenW strains) differ by only one aminoacid as checked by ClustalW Bioinformatics software.

5.1.1 Over-expression of native fHbp variant 2 in MenW

The plasmid used to generate *N. meningitidis* mutants with over-expressed native fHbp (Figure 23) contains the upstream recombination site that allows homologous recombination with genes of the capsule biosynthesis locus (capsW up), a chloramphenicol resistance gene (cm), a portion of fHbp that allows recombination with fHbp (fHbp Nterm) and a strong synthetic promoter. By homologous recombination in *Neisseria* the native fHbp promoter will be replaced with the strong synthetic promoter.

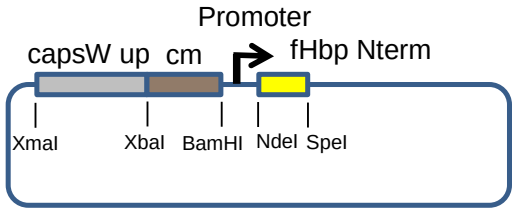


Figure 23: Schematic representation of the plasmid psynX containing the cps upstream flanking region, the chloramphenicol resistance cassette, a portion of fHbp gene downstream a strong synthetic promoter.

The first step has been the replacement (by enzymatic digestion and ligation) of the upstream recombination site (capsW up) with a flanking region that allows recombination with the gene nmb1869, located upstream of fHbp in the genome of

Neisseria Meningitidis. To do this, a portion of the gene *nmb1869* was amplified by PCR from the genome of *Neisseria meningitidis* group W.

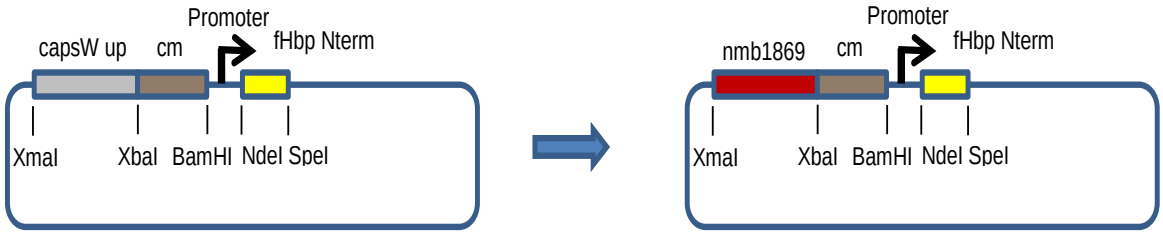


Figure 24: Substitution of the recombination site in the plasmid used for over-expression of fHbp v.2 in native site of MenW strain.

The recombination site the chloramphenicol resistance gene, the strong promoter and the downstream recombination site fHbp ID16 N-terminal was amplified by PCR (Figure 25).

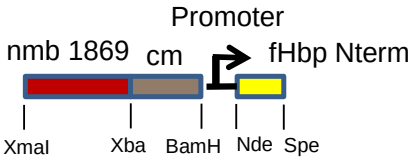


Figure 25: PCR product used for *Neisseria Meningitidis* transformation.

The primers used allowed the amplification of the portion of the gene located upstream of fHbp in the genome of Neisseria Meningitidis serougroup B and used as flanking region and were designed to add two restriction sites for XmaI upstream and XbaI downstream of the gene of interest (nmb1869). The PCR product was used for transformation of *Neisseria Meningitidis*. Homologous recombination of the PCR fragment with the chromosome is represented in
Figure **26**. Chloramphenicol resistant clones were selected.

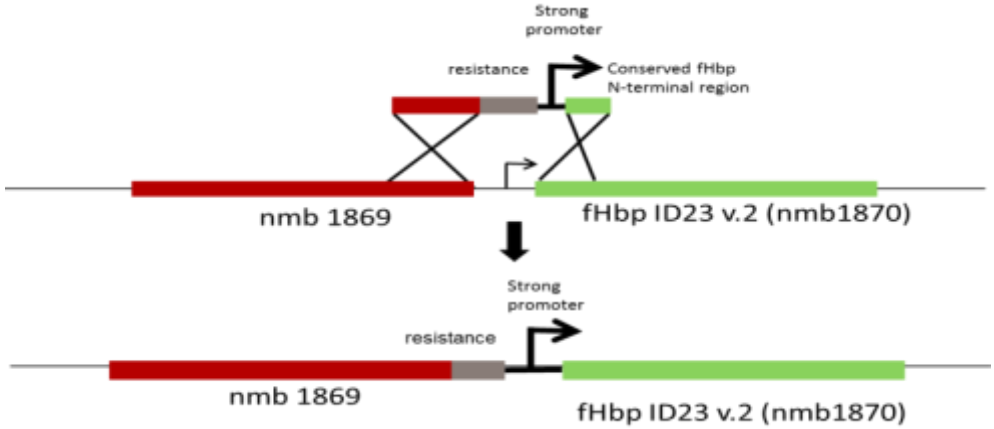


Figure 26: Homologous recombination of the PCR fragment with the chromosome.

Neisseria meningitidis serogroup W was transformed with the PCR product previously described resulting in 40 chloramphenicol resistant colonies. Whole cell lysates was prepared and analyzed by SDS-PAGE and western blotting detecting with an antibody

specific for fHbp variant 2. Seven colonies out of 40 colonies screened showed over-expression of fHbp v.2 ID23 (Figure 27) which was approximately 10-fold above native fHbp expression in this strain.

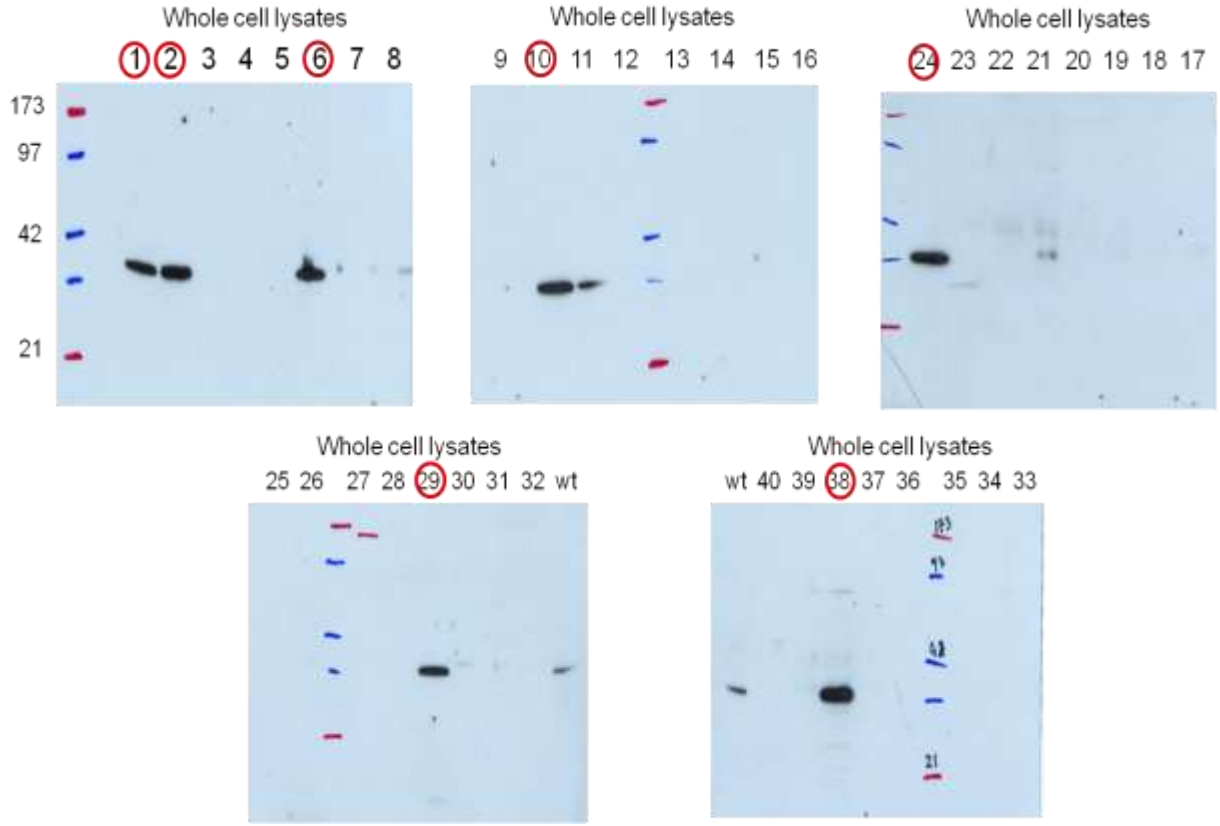


Figure 27: FHbp v.2 expression in whole cell lysates from 40 colonies obtained after transformation with the PCR fragment containing th strong promoter. Number of cells loaded was normalized based on optical density of the cell suspension. Wt = expression of fHbp in the respective wildtype strain.

5.1.2 Deletion of capsule in MenW strains over-expressing fHbp v.2,

The primers used for amplification of the fragment containing the recombination site allow the capsule biosynthesis gene deletion by replacement with a spectinomycin resistance and were designed to add two restriction sites for each recombination site: XmaI upstream and XbaI downstream of the gene of interest (cps up); NsiI upstream and SpeI downstream of the gene of interest (cps down).
The mutation generated was the deletion of capsule biosynthesis genes in group W strain over-expressing fHbp v.2. The analysis of colonies obtained after transformation with the DNA fragment was carried out by dot blot with whole cell lysates and using a monoclonal antibody against group W capsule polysaccharide. The deletion of capsule was observed in all 3 colonies selected on spectinomycin. Control wild type gave a strong signal while mutants capsule KO were negative (Figure 28).

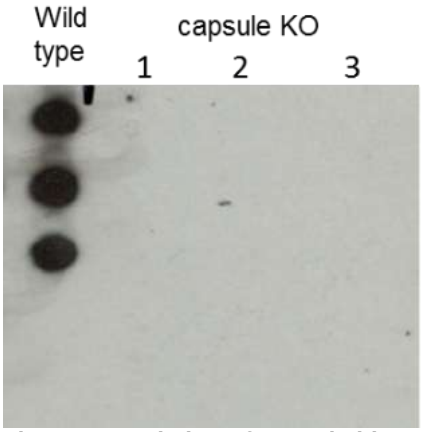


Figure 28: Deletion of capsule biosynthesis genes in group W strain over-expressing fHbp v.2 as determined by dot blot with heat killed bacteria and using a groupW monoclonal antibody for detection. Wild type = parent group W wild type strain expressing the capsule and used to generate the mutants. Capsule KO = Three out of 3 clones obtained after transformation with the plasmid used to delete the capsule.

5.1.3 Deletion of lpxL1

The lipid A, portion of the LOS is responsible for the endotoxin activity of LOS. Inactivation of lpxL1 gene that encodes for a late-functioning acyltransferases of lipid A biosynthesis results in a penta-acylated lipid A, instead of the wild type hexa-acylated lipid A with lower toxicity, but retaining the adjuvant activity (226).
A plasmid containing kanamycin resistance cassette and the flanking regions of *lpxL1* gene was used (Figure 29). The deletion of *lpxL1* gene was obtained by allelic exchange with kanamycin resistance cassette.

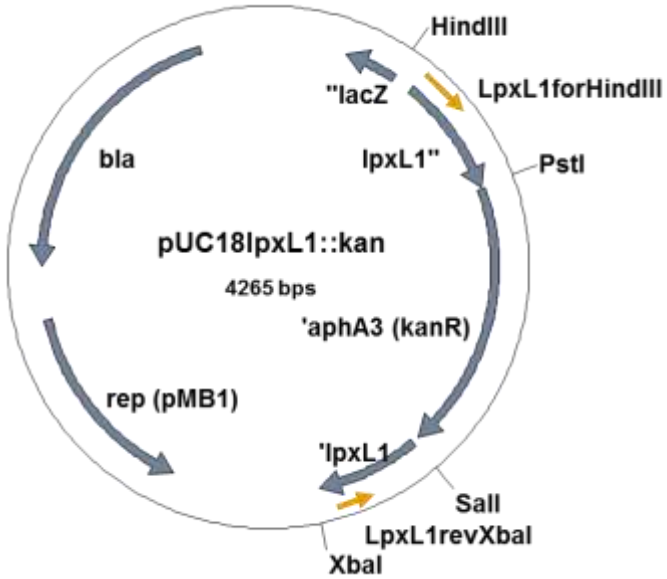


Figure 29: Plasmid pLpxL1kan used to delete *lpxL1* gene.

The primers used for amplification of *lpxL1* recombination sites were designed to add two restriction sites for each recombination site: XmaI upstream and XbaI downstream

of the gene of interest (*lpxL1* up); NsiI upstream and SpeI downstream of the gene of interest (*lpxL1* down).
The insertion of kanamycin resistance gene and so the deletion of gene *lpxL1* in capsule KO mutants, over-expressing fHbp v.2 was verified by PCR. The positive result occurred in all 7 colonies selected previously on kanamycin (Figure 30).

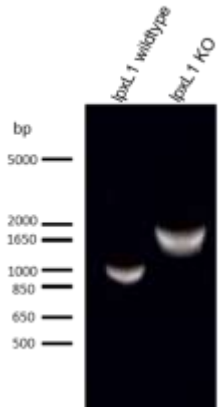


Figure 30: Deletion of lpxL1 in capsule KO mutant, over-expressing fHbp v.2. LpxL1 wild type = parent group W wild type strain used to generate the mutants. LpxL1 KO = one out of 7 clones obtained after the transformation with the plasmid used to delete gene lpxL1 by the insertion of kanamycine resistance gene. Transformats were tested by PCR using genomic DNA as template and primers LpxL1forHindIII and LpxL1revXba. The higher band in the mutant results from integration of the kanamycin gene.

5.1.4 Deletion of gna33

Mutants obtained so far were further mutated for gene gna33 deletion To delete the gene *gna33* a plasmid containing erythromycin cassette and the flanking region of *gna33* gene was used in order to obtain in removing *gna33* and integrating in place of it the erythromycin cassette resistance by homologous recombination. The insertion of erythromycin resistance gene and the deletion of gene *gna33* in capsule KO, *lpxL1* KO mutants, over-expressing fHbp v.2 was confirmed by PCR. The positive result occurred in 7 out of 16 colonies screened on erythromycin (Figure 31).

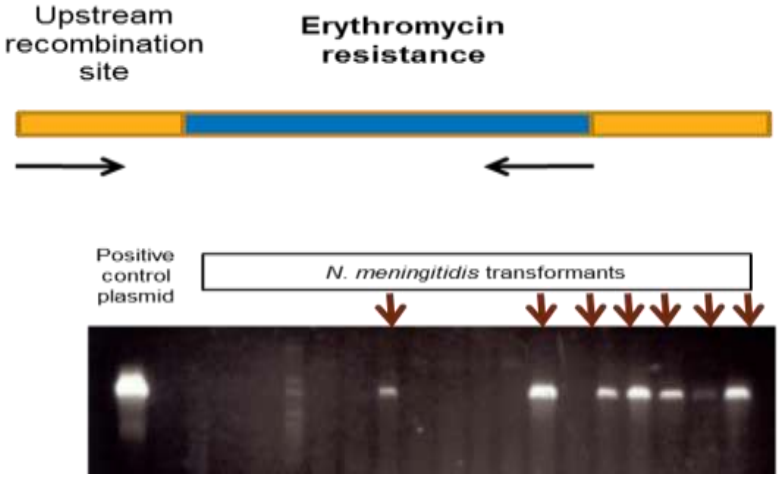


Figure 31: Deletion of gene gna33 in capsule and lpxL1 KO mutants, over-expressing fHbp v.2. Plasmid used for deletion of gna33. transformation is reported as positive control.Transformats having the gna33 deletion

were verified by PCR with chromosomal DN Aas template and primers annealing in the recombination site and the inserted erythromycin gene.

5.2 Comparative characterization of Group W GMMA over-expressing two different variants of fHbp

GMMA were generated from Men W strains ΔcpsΔlpxL1Δgna33 over-expressing the native fHbp variant 2, described in the previous paragraph.
In this section it is report a comparative characterization between GMMA obtained from Men W strains ΔcpsΔlpxL1Δgna33 over-expressing native fHbp v.2 ID23.
GMMA from Men W strains ΔcpsΔlpxL1Δgna33 over-expressing native fHbp v.2 were obtained by ultracentrifugation (186,000 g, 2 hours, 4°C) of the sterile culture supernatants (200 mL supernatant/strain), a washing with 1X PBS, a second ultracentrifugation step (186,000 g, 1 hours, 4°C) and resuspension in 1X PBS (~ 5 mL/strain) and sterilization by filtration through a 0.22 µm filter and quantitation by Lowry assay.
The GMMA characterization was based on:

- Total protein composition by separation with SDS PAGE and staining with coomassie blue
- FHbp expression by western blotting
- Lipid A analysis by MALDI-TOF in order to detect the effect of *lpxL1* knock out
- Endotoxin activity evaluation by MAT assay
- DNA, protein related to GMMA particles and free soluble proteins presence and particle sizing by HPLC-SEC MALLS, before and after benzonase treatment

5.2.1 Detection of total protein and fHbp in GMMA by SDS PAGE, Comassie blue and western blotting

In Figure 32 is shown the total protein composition of vesicle preparations from the group W wild type, from a mutant derived from the wild type over-expressing the native fHbp v.2 and from a mutant with over-expressed fHbp v.2 and deleted *cps*, *lpxL1* and *gna33* genes (3 different clones) after separation by SDS PAGE and staining with coomassie blue.
As shown, the protein pattern of mutants is similar to that of Men W wild type and Men W overexpressing fHbp v.2, which was used as parent strain for the subsequent genetic modifications. Bands show the typical pattern of major proteins present in meningococcal outer membrane preparations including Opa (ca. 27 kDa), RmpM (ca. 34 kDa), PorB (ca. 38 kDa) and PorA (ca. 42 kDa). -Fhbp cannot be separately visualized by 1D gelelectrophoresis and coomassie stain because the band is at the same level as the Opa protein.

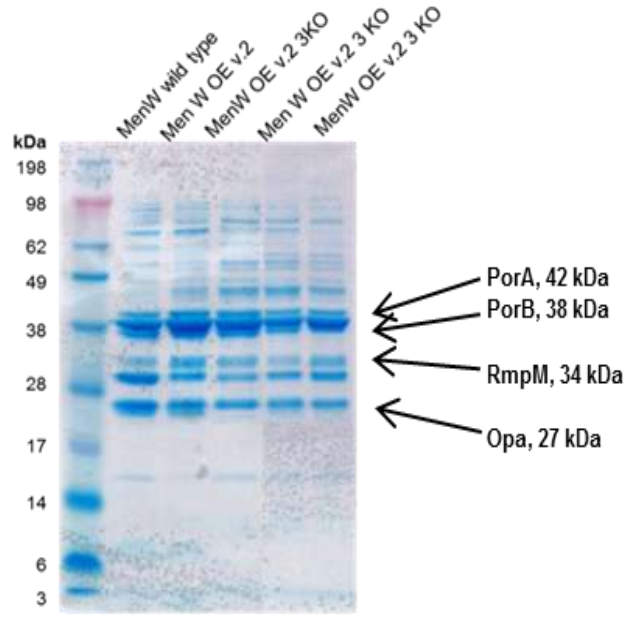


Figure 32: Total protein content of GMMA from MenW ΔcpsΔlpxL1Δgna33 over-expressing native fHbp as detected by SDS PAGE and coomassie blue stain. This protein pattern was compared with that deriving from MenW wild type and from MenW overexpressing fHbp v.2, strain used then for the deletion of the capsule biosynthesis genes, *lpxL1* and *gna33*. 5 ug GMMA based on total protein were loaded per lane.

In Figure 33 it is reported the detection of fHbp in GMMA prepared from the same strains analyzed after separation by SDS PAGE and staining with coomassie blue (group W wild type; a mutant derived from the wild type over-expressing the native fHbp v.2; 3 clones of a mutant with over-expressed native fHbp v.2 ID23 and deleted *cps*, *lpxL1* and *gna33* genes). The over-expression of native fHbp v.2 ID23 was detected by western blotting using a monoclonal antibody raised against fHbp v.2. It was approximately 10-fold above native fHbp expression in the mutants with over-expressed fHbp and capsule, *lpxL1* and *gna33* KO. Expression of fHbp in these mutants was similar to the amount of fHbp in GMMA from OE v.2.

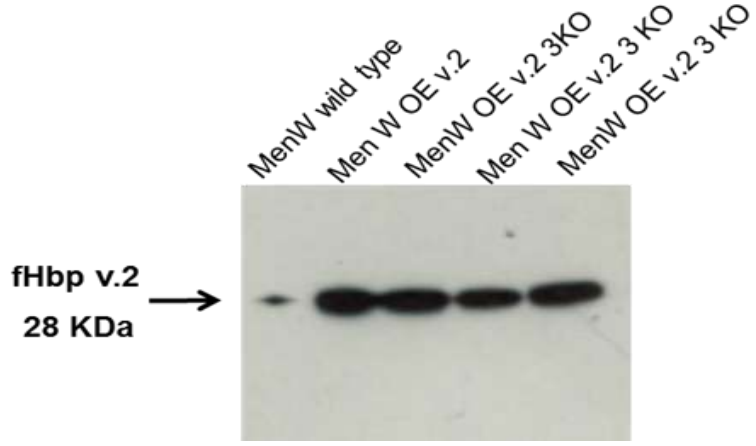


Figure 33: Detection of fHbp in GMMA from MenW ΔcpsΔlpxL1Δgna33 over-expressing the native fHbp v.2 ID23 using a monoclonal antibody raised against fHbp v.2.

5.2.2 MALDI-TOF analysis of lipid A from N. meningitidis GMMA lpxL1 knock out

The lipid A, portion of the LOS is responsible for the endotoxin activity of LOS. Inactivation of *lpxL1* gene that encodes for an enzyme responsible of last step of lipopolysaccaride synthesis (late-functioning acyltransferases) results in a penta-acylated lipid A, instead of the wild type hexa-acylated lipid A with lower toxicity, but retaining the adjuvant activity. To prove the correct deletion of the fatty acyl chain lipid A from GMMA with *lpxL1* KO, lipid A from the various mutants has been extracted from GMMA, analyzed by MALDI-TOF and compared with GMMA from the wild type strain.
The obtained spectra and *Neisseria* lipid A species corresponding to the observed m/z (227) are reported in Figure 34.
The main peaks in the mass spectra obtained by MALDI-TOF in ion-negative reflectron mode from GMMA purified from lipid A unmodified strains of *Neisseria*, present two main peaks; the first one has an *m/z* correspondent to the theoretical mass (1838 Da, 1837 *m/z*) of an hexa-acylated lipid A that present a phosphoethanolamine attached on the phosphate 1' due to the action of transferases encoded by *eptA,* instead the second ones is correspondent to an hexa-acylated lipid A without the phosphoetanolamine (1715 Da, 1714 *m/z*). It was possible observe that the two peaks corresponded to the same two lipid A molecules described before but that had lost a phosphate in 4' position, most likely during the analysis.

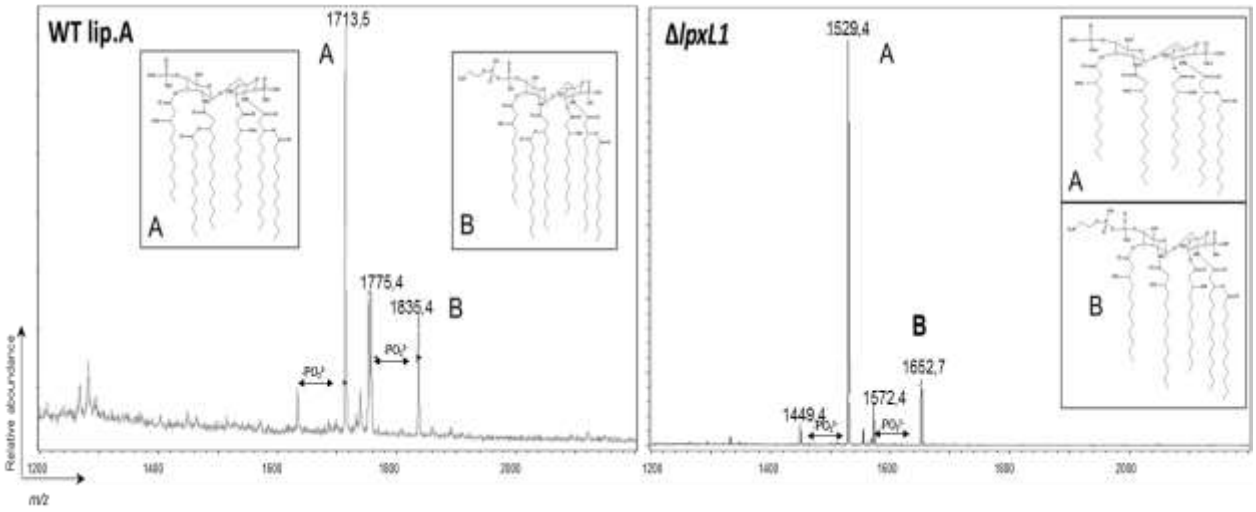


Figure 34: MALDI-TOF spectra obtained analyzing lipid A extracted from *N. meningitidis* GMMA *lpxL1* knock out and wild type. Spectra were acquired in reflectron ion-negative mode.

In the lipid A purified from GMMA from *lpxL*1 mutant strains two populations were observed, with and without phosphoetanolamine, with a shift of exactly 182 Dalton corresponding to the lack of a lauroyl (-C_{12} fatty acid chain), from the two lipid A species observed in GMMA purified from lipid A WT strain, consisting in a *lpxL1* knock out. The discrepancy between the theoretical *m/z* and the observed ones is only 1 Dalton and it was due to calibration (the discrepancy was the same for all the peaks observed). In

fact, the *m/z* shift was perfect at Dalton level in chemical shift due to the lack of fatty acid chains and/or phosphoethanolamine.

5.2.3 Evaluation of endotoxin activity of Neisseria GMMA after genetic manipulation

In order to directly detect the reduction of reactogenicity after lipid A modifications in *N. meningitidis*, GMMA from strains with wild type (Men W wt; Men W OE native fHbp v.2 ID23; Men W OE fHbp v.1 ID5, fHbp v.2 and Δcps) and modified lipid A (Men W OE native fHbp v.2 ID23, ΔcpsΔlpxL1Δgna33; Men W OE fHbp v.1 ID5, ΔfHbpΔcpsΔlpxL1; Men W OE fHbp v.1 ID5 ΔcpsΔlpxL1Δgna33) were tested by MAT to measure the ability of the preparations to induce the release of IL-6 from human peripheral blood mononuclear cells.
As shown in Figure 35, the concentration of GMMA with wildtype lipidA required to induce a 10 fold increase of IL6 was 10-1 ng/mL for GMMA from the wild type and a mutant over-expressing fHbp v.1 (ID5). GMMA from a mutant with wild type lipid A and overexpressed fHbp v.2 had a 10 fold lower ability to induce IL-6 release. GMMA from mutants containing the lpxL1 KO mutation had an approximately 100 -fold decreased ability to stimulate Il-6 release from human PBMC and the relative decrease was similar for GMMA from strains that over-express fHbp v.1 or v.2.

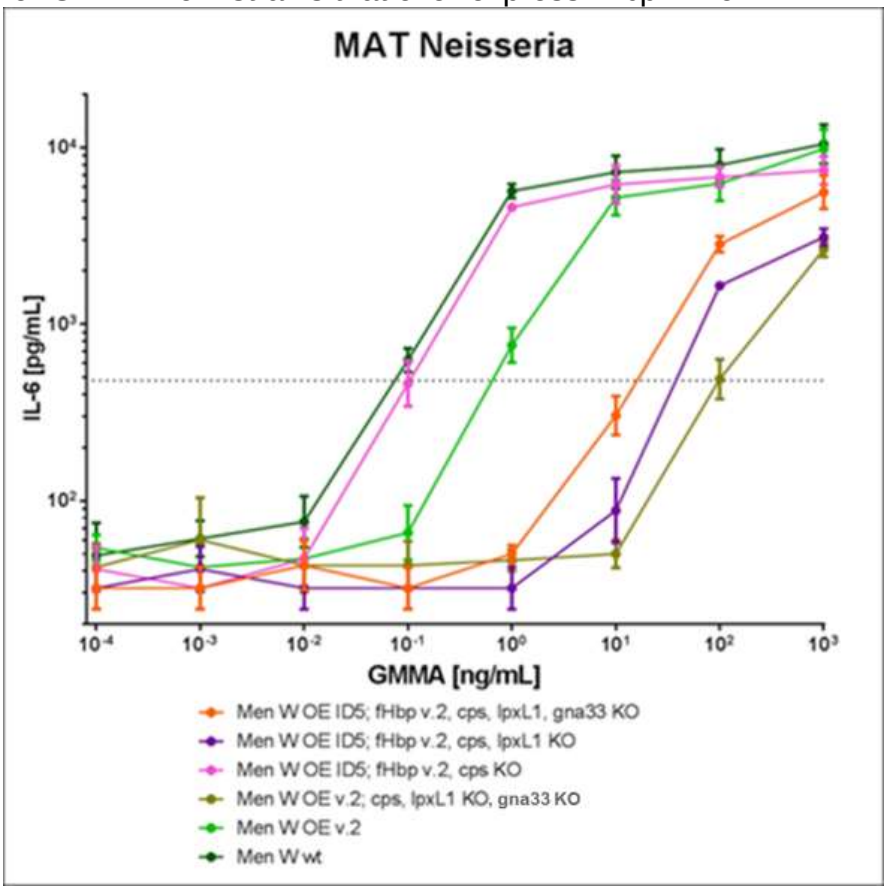


Figure 35: Evaluation of endotoxin activity in Neisseria GMMA by measuring IL-6 release by human PBMC: PBMC were stimulated for 4 hours with different concentration of GMMA (0,001-1000 ng/mL, 10-fold steps dilutions).

5.2.4 Analysis of GMMA samples from group W mutants with over-expressed fHbp fHbp v.2 by HPLC-SEC MALLS

Outer membrane preparations from serogroup W wild type and mutants having a different combination of mutations resulting in deletion of the capsule biosynthesis genes, *lpxL1*, *gna33* and over-expressed fHbp were analyzed by HPLC-SEC in order to evaluate their soluble protein content, the size and the presence of nucleic acids before and after benzonase treatment (2 µL benzonase/2 mL sample diluted in buffer 20 mM Tris-HCl, 2 mM MgCl2). Benzonase is a nuclease that effectively remove nucleic acids. Soluble proteins presence is assessed using the fluorescence channel (336 nm) (Figure 36). In all samples analyzed except for MenW GMMA OE native fHbp v.2 cps KO, *lpxL1* KO, *gna33* KO. It is possible to observe for all the samples detected one distinct peak at 27 min corresponding to the GMMA. Moreover there was a loss of proteins after benzonase treatment, as shown in the graph below, more evident in GMMA from wildtype strains.

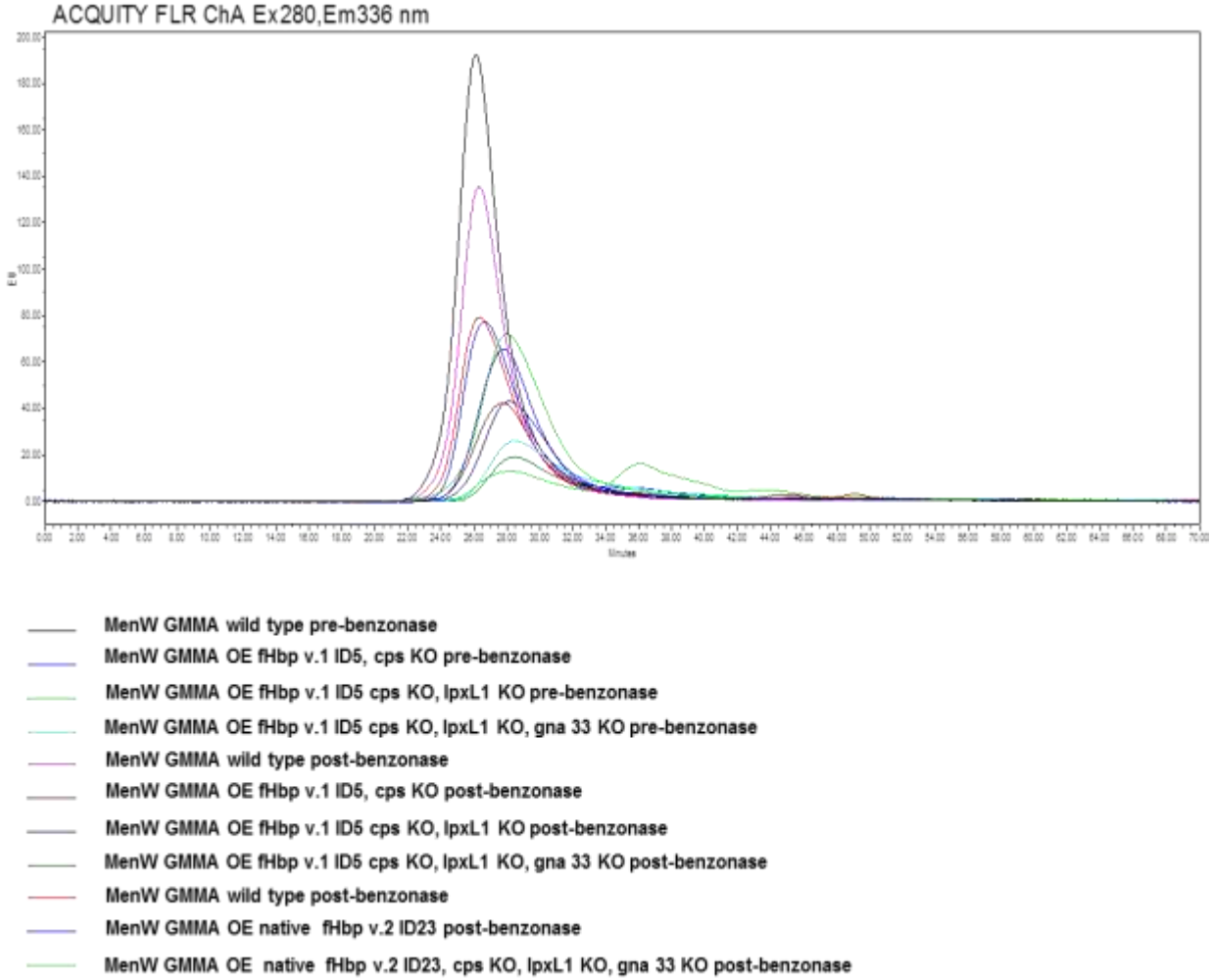


Figure 36: Evaluation of outer membrane preparations from different serogroupW mutants by HPLC-SEC.

GMMA radii were evaluated by MALLS). In particular, the parameters adopted were the following:

- Peak Rn (average radius): $(\Sigma n_i * R_i)/R_i$
- Peak Rw (mass weighted average radius): $(\Sigma n_i * R_i * R_i)/R_i$
- Peak Rz (mass^2 weighted average radius): $(\Sigma n_i * R_i^2 * R_i)/R_i$

R_i is the radius calculated while n_i is the number of particles detected.

The values of Rn, Rw and Rz reported in the Table 14 allow to calculate the index of polydispersivity (Rw/Rn). If the values of the three radii are similar and if the index of polydispersivity is ~ 1, so if the value of Rw is similar to the value of Rn, it means that there aren't polydisperse populations as resulted in the case of samples indicated by the arrows (GMMA deriving from Men W wild type before and after benzonase treatment; GMMA deriving from Men W cps KO, *lpxL1* KO and *gna33* KO OE heterologous fHbp v.1 ID5 before and after benzonase treatment; GMMA deriving from Men W OE native fHbp v.2 ID23 and GMMA deriving from Men W cps KO, *lpxL1* KO and *gna33* KO OE native fHbp v.2 ID23, after benzonase treatment). These populations of GMMA were therefore homogeneus and monodisperse in respect to GMMA deriving from Men W cps KO, before and after benzonase treatment and in respect to GMMA deriving from Men W cps KO and *lpxL1* KO both OE heterologous fHbp v.1 ID5, before and after benzonase treatment.

The results indicate that the monodispersivity of vesicles is particularly evident in wild type strains and in strains characterized by all the mutations described previously. It is wortwhile to characterize and to detect the index of polydispersivity in GMMA deriving from different Men W strains, anyone with a single mutation in order to understand if any mutation is crucial affecting the GMMA radii or if it is the combination of all mutations, in a synergistic way, to play a role in the dispersivity observed.

Table 14: Evaluation of GMMA radii and relative uncertainty by MALLS detection.

SAMPLES DETECTED	Rn geo (nm)	Uncertainty	Rw geo (nm)	Uncertainty	Rz geo (nm)	Uncertainty	
MenW GMMA wild type pre-benzonase	50.2	2.00%	51.4	1.90%	53.2	1.70%	←
MenW GMMA OE fHbp v.1 ID5 cps KO pre-benzonase	47.7	2.60%	51.6	2.20%	57.6	1.80%	
MenW GMMA OE fHbp v.1 ID5 cps KO, lpxL1 KO pre-benzonase	46.6	2.10%	50.7	1.80%	58.5	1.40%	
MenW GMMA OE fHbp v.1 ID5 cps KO, lpxL1 KO, gna 33 KO pre-benzonase	54.2	1.80%	54.5	1.80%	54.8	1.80%	←
MenW GMMA wild type1 post-benzonase	49.4	2.70%	51.1	2.50%	53.8	2.30%	←
MenW GMMA OE fHbp v.1 ID5 cps KO post-benzonase	47.1	2.20%	51.7	1.90%	58.6	1.50%	
MenW GMMA OE fHbp v.1 ID5 cps KO, lpxL1 KO post-benzonase	44.6	2.30%	48.2	2.00%	55.1	1.50%	
MenW GMMA OE fHbp v.1 ID5 cps KO, lpxL1 KO, gna 33 KO post-benzonase	51.3	2.30%	51.6	2.30%	52	2.30%	←
MenW GMMA wild type2 post-benzonase	47.2	2.80%	48.4	2.60%	50.3	2.30%	←
MenW GMMA OE native fHbp v.2 ID23 post-benzonase	50.3	2.50%	50.5	2.40%	50.7	2.40%	←
MenW GMMA OE native fHbp v.2 ID23 cps KO, lpxL1 KO, gna 33 KO post-benzonase	50.1	2.60%	50.3	2.60%	50.5	2.60%	←

Nucleic acid presence in the preparations was assessed on the ABS260/ABS280 ratio on the UV channel (Figure 37). In general, whennucleic acids are present, soluble proteins and GMMA are not separated by ultracentrifugation.
In the first group of samples analyzed nucleic acid and soluble proteins weren't present more than GMMA proteins, independently of benzonase treatment, while the second group of samples showed DNA and soluble proteins, also after benzonase treatment.

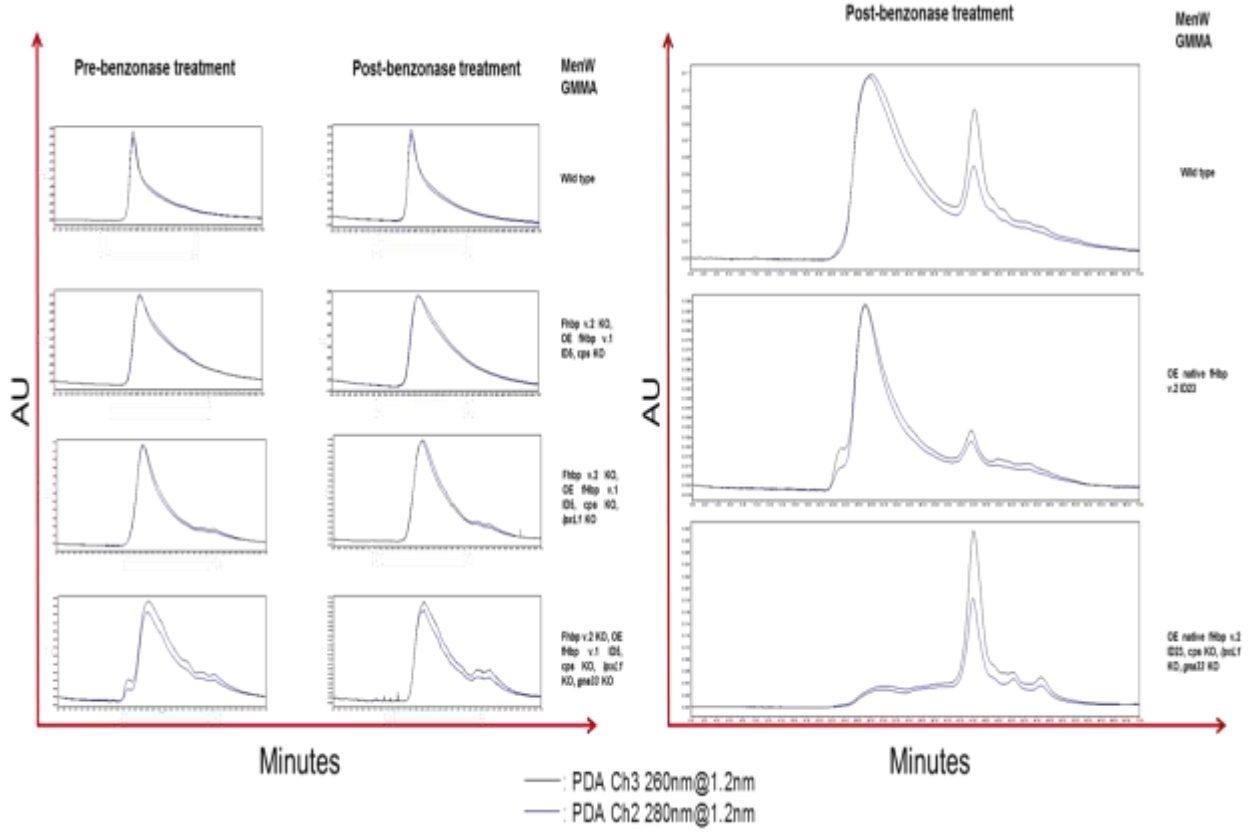


Figure 37: Evaluation of nucleic acid presence by UV channel in GMMA from different strains pre and post benzonase treatment.

5.3 Analysis of the breadth of fHbp sequence coverage by antibodies against fHbp in GMMA

As described in the introduction, fHbp can be divided into 3 variants. Each variant contains many subvariants that differ by few amino acids. Each subvariant sequence is identified by an individual identification number (sequence ID). A sequence analysis of fHbp in more than 100 invasive serogroup A, W and X isolates from 11 countries and collected between the 1980's and 2010 revealed a small number of predominant fHbp sequences expressed in these strains. As a consequence, the analysis of coverage by antibodies raised against fHbp containing vaccines was previously performed on invasive wild type strains expressing one of the predominant fHbp sequence IDs. In the case of strains expressing fHbp variant 1, serogroup A strains with ID 5 were selected (100% of strains expressed either ID4 or ID5 that differ be one amino acid). In the case of serogroup W strains, about one third expressed fHbp variant 1 and the majority had

ID 9. The serogroup X strain tested expressed one of the prevalent variant 1 ID 73 or 74. A recent serogroup C outbreak in Niger (group C outbreaks have not been observed since the 1980s) demonstrates that the epidemiology is dynamic and the possibility exists that fHbp sequences which are underrepresented or currently do not occur will be expressed by African isolates and become predominant in the future. Antibodies raised against a particular fHbp variant induce antibodies that in general are bactericidal against strains expressing the same variant. However, a systematic analysis of the breadth of fHbp sequence ID coverage independent of the predominance in current strains has not been performed.
I therefore performed a systematic fHbp structure sequence analysis of existing published sequence IDs belonging to variant 1, 2 and 3 taken from the Neisseria MLST database http://pubmlst.org/neisseria/. Using fHbp variant 1 as a model I used the bioinformatic data to rationally select a panel of phylogenetically highly diverse fHbp sequences. To test fHbp sequence coverage I tested serum bactericidal activity of sera made in mice against GMMA with over-expressed fHbp. Test strains was a panel of 11 invasive wildtype *N. meningitidis* strains expressing the selected diverse fHbp sequences. I also generated a panel of eight isogenic strains that were obtained by engineering a parent strain to express selected diverse fHbp sequences and were used as test strains to measure serum bactericidal antibody responses.

5.3.1 Structure and sequence analysis of fHbp v.1, v.2 and v.3

A structure/sequence analysis of 683 fHbp IDs belonging to fHbp v.1, 2 and 3 was carried out.
The amino acids in the different fHbp sequences that are in contact with factor H were analyzed. Being accessible for a soluble complement factor of the host these amino acids must be exposed on the surface of the bacterium and likely to be target of antibodies and under immune selection pressure. In fact, previous studies with anti fHbp monoclonal antibodies indicate that some of them have the ability to block binding of factor H to fHbp. Our hypothesis was that variations in these amino acids have a greater impact on the ability of antibodies to kill a particular strain than variations of amino acids that are “hidden” in the molecule and not accessible to antibodies.
Starting from molecular model of fHbp v.1 and v.3 in complex with factor H from NCBI’s Entrez Structure database, we identified the amino acids that are in contact with factor H. We included residues in fHbp that are at a calculated distance of $\leqslant$ 5Å to factor H indicating an interaction (Figure 38, yellow residues).

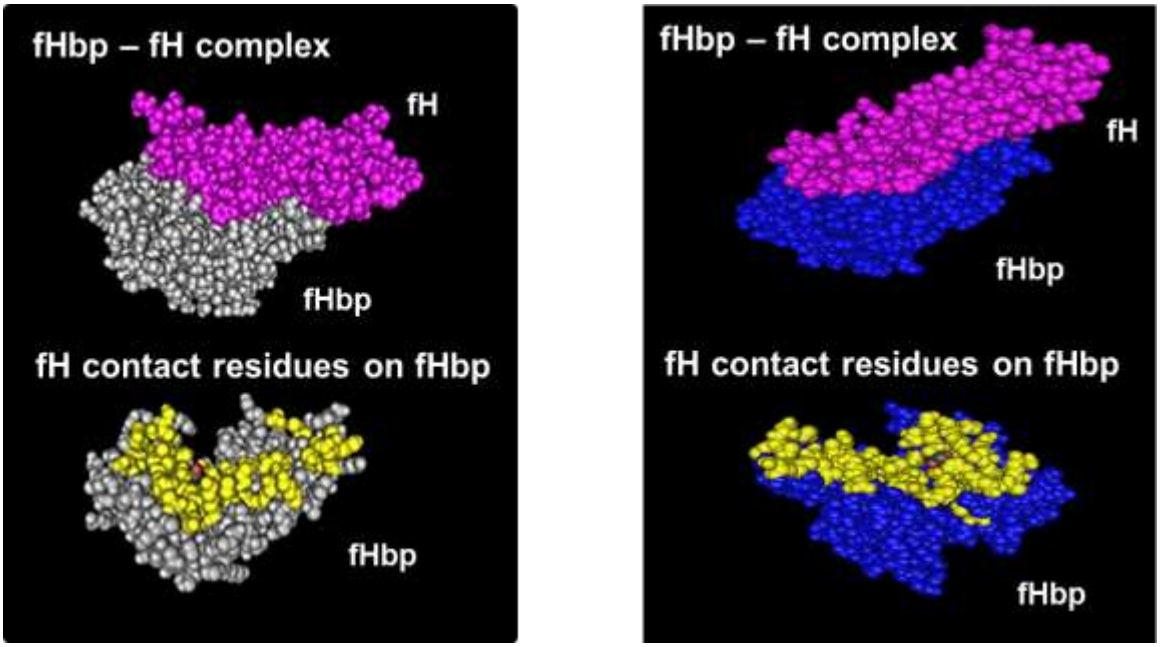


Figure 38: Molecular model of fHbp in complex with factor H. Left panel: fHbp v.1 (grey); right panel fHbp v.3 (blue). The model of the portion of factor H that binds to fHbp is shown in purple. The structures were generated using Cn3D from the published model on NCBI's Entrez Structure database. Amino acids on fHbp that are at a distance of ≤ 5Å to factor H presumably being involved in factor H binding are highlighted in yellow.

There is no publically available structure of fHbp v.2 in complex with factor H. However, because fHbp v.2 and 3 are closely related (around 80% sequence identity), I used the fHbp v.3 sequence to identify the factor H binding site in fHbp v.2 using ID16 as reference sequence. Successively, by sequence alignment, I identified the amino acids in contact with factor H in the published fHbp sequences belonging to v.1, 2 and 3.
I have systematically analyzed the amino acid diversity involved in factor H binding in 683 published fHbp peptide sequences belonging to variant 1 (N=383), 2 (N=151) and 3 (N=149).
The analysis of polymorphic sites in factor H binding site using ID9 as reference sequence for variant 1 and ID28 for variant 2 and 3 revealed how many variable positions are present in terms of chemical-physical features and how many of these variable positions are conservative or not conservative substitutions (Figure 39 and Figure 40).

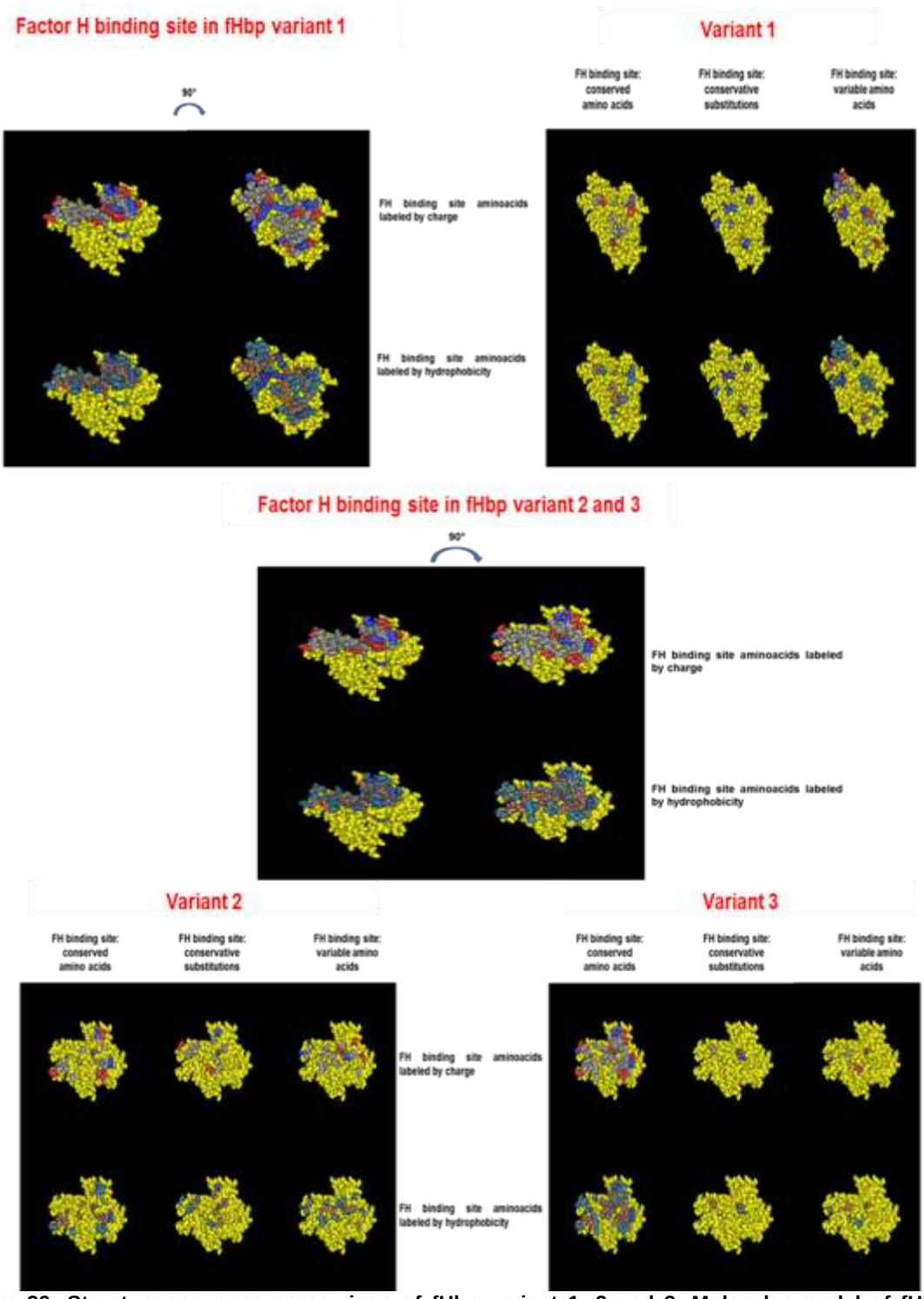


Figure 39: Structure-sequence comparison of fHbp variant 1, 2 and 3. Molecular model of fHbp showing physico chemical features of amino acids included in the site that is involved in factor H binding. Factor H binding site amino acids are coloured in relation to their charge (positively charged residues in blue, negative in red and neutral in gray) and to their hydrophobicity (standard hydrophobicity scale to draw the most hydrophobic residues in red and the least hydrophobic (most hydrophilic) in blue). Chemical properties of the amino acid residues in the factor H binding site of fHbp variant 2 are shown on the model publically available for fHbp v.3.

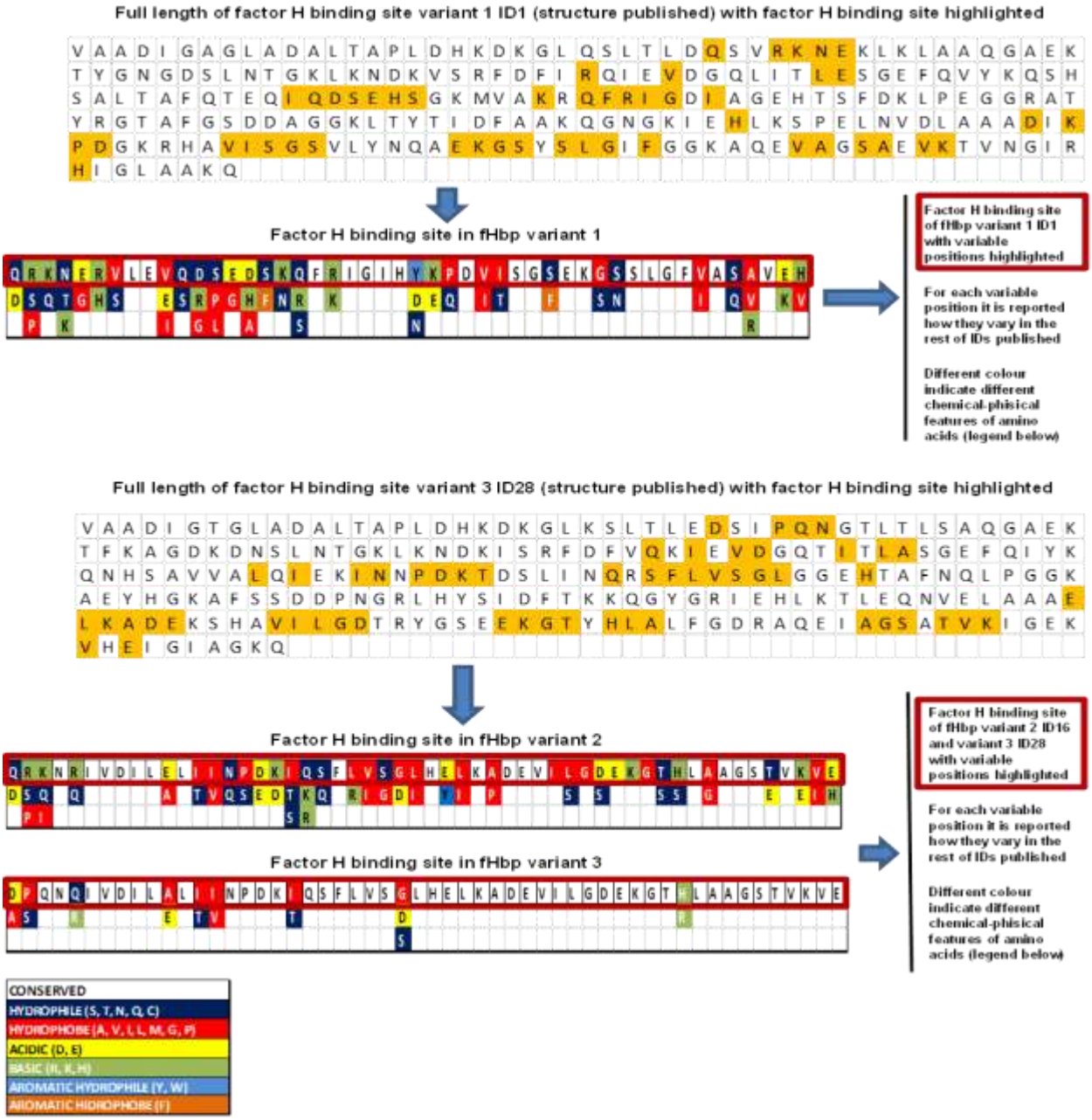


Figure 40: Structure-sequence comparison of fHbp variant 1, 2 and 3. Sequence and polymorphic sites of the amino acids of fHbp variant 1, 2 and 3 involved in binding of factor H. Starting from the molecular model the amino acid residues that are involved in factor H binding were mapped and extracted from the linear sequence of the full length fHbp. Factor H binding site amino acids are coloured in relation to their chemical-physical features (hydrophile in blue black, hydrophobe in red, acidic in yellow, basic in green, aromatic hydrophile in blue, aromatic hydrophobe in orange) and to their level of conservation ((*) identical residues, (:) highly conserved, (.) weakly conserved).

A summary of the structure-sequence comparison of fHbp variant 1, 2 and 3 looking at variable positions in the factor H binding site is reported in
Table **15**.
For variant 1, 48 amino acids out of 255 (full length of the reference sequence ID1) were in contact with factor H, characterizing the factor H binding site. Among them 30

positions out of 48 (length of factor H binding site of the reference sequence ID1) were variable. Among the 30 variable positions 21 were not conservative substitutions. For variant 2, 54 amino acids out of 254 (full length of the reference sequence ID16) were in contact with factor H, characterizing the factor H binding site, and among them 31 positions out of 54 (length of factor H binding site of the reference sequence ID16) were variable and among them 19 out of 31 were not conservative substitutions. For variant 3, 54 amino acids out of 262 (full length of the reference sequence ID28) were in contact with factor H, characterizing the factor H binding site, and among them 9 positions out of 54 (length of factor H binding site of the reference sequence ID28) were variable and among them 19 out of 31 were not conservative substitutions.

Table 15: Analysis of the factor H binding site of fHbp variant 1, 2 and 3. Reference sequences for the analysis were ID1 for variant 1, ID16 for variant 2 and ID28 for variant 3.

fHbp	Number of amino acids involved in factor H binding (% of full length fHbp)	Number (%) of variable positions in the factor H binding site of fHbp	Number (%) of not conservative substitutions in the variable positions of the factor H binding site
Variant 1	48 (18.8%)	30 (62.5%)	21 (43.8%)
Variant 2	54 (21.2%)	31 (57.4%)	19 (35.2%)
Variant 3	54 (20.6%)	9 (16.6%)	6 (11.1%)

A matrix approach previously performed on fHbp v.1 was adopted in order to analyze sequence diversity in the factor H binding sites in an existing and published panel of, fHbp 2 and 3 sequence IDs and compared with fHbp v.1. A snapshot of the pairwise comparison of the factor H binding site sequences using the matrix is shown in Figure 41.

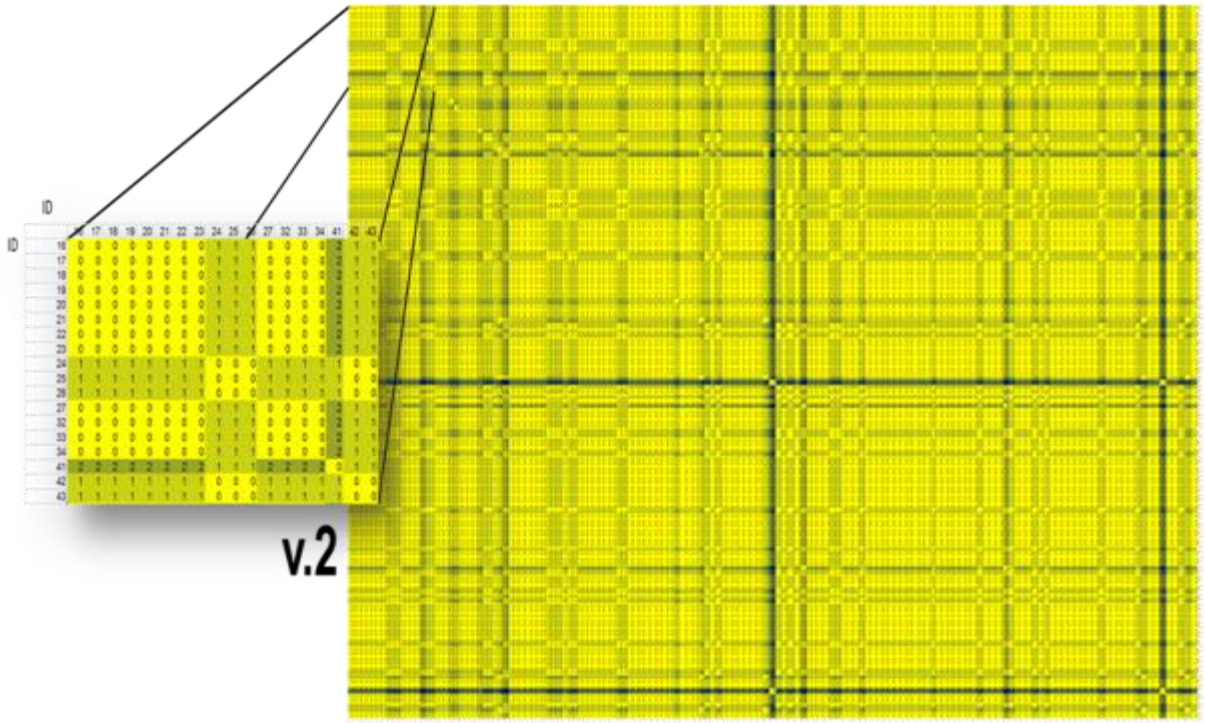


Figure 41: Matrix used to compare factor H binding sites in fHbp v.2. Each variant can be divided into sub-variants (each one identified by a specific ID number) differing in their amino acid sequence. The numbers on

the left and top correspond to the numbers of ID compared. Numbers in matrix from 0 to 2, in this case, correspond to the differences in aminoacid sequence.

As shown in the magnified section of the matrix each row and column corresponds to the individual ID compared. The numbers in the matrix correspond to the numberof differences in terms of aminoacid residues between the factor H binding sites between each ID. A darker colour reflects an increase of number of differences.

In Figure 42 is shown the pairwise comparison of factor H binding sites in fHbp variant 1, 2 and 3. In this format, the fHbp IDs were sorted by increasing number of differences in order to better appreciate the difference in conservation between v1 and v.2/3. In the figure it is also shown the comparison of factor H binding sites in fHbp v.2 and v.3 together, in order to demonstrate the presence of two branches, one related to v.2 and one other related to v.3.

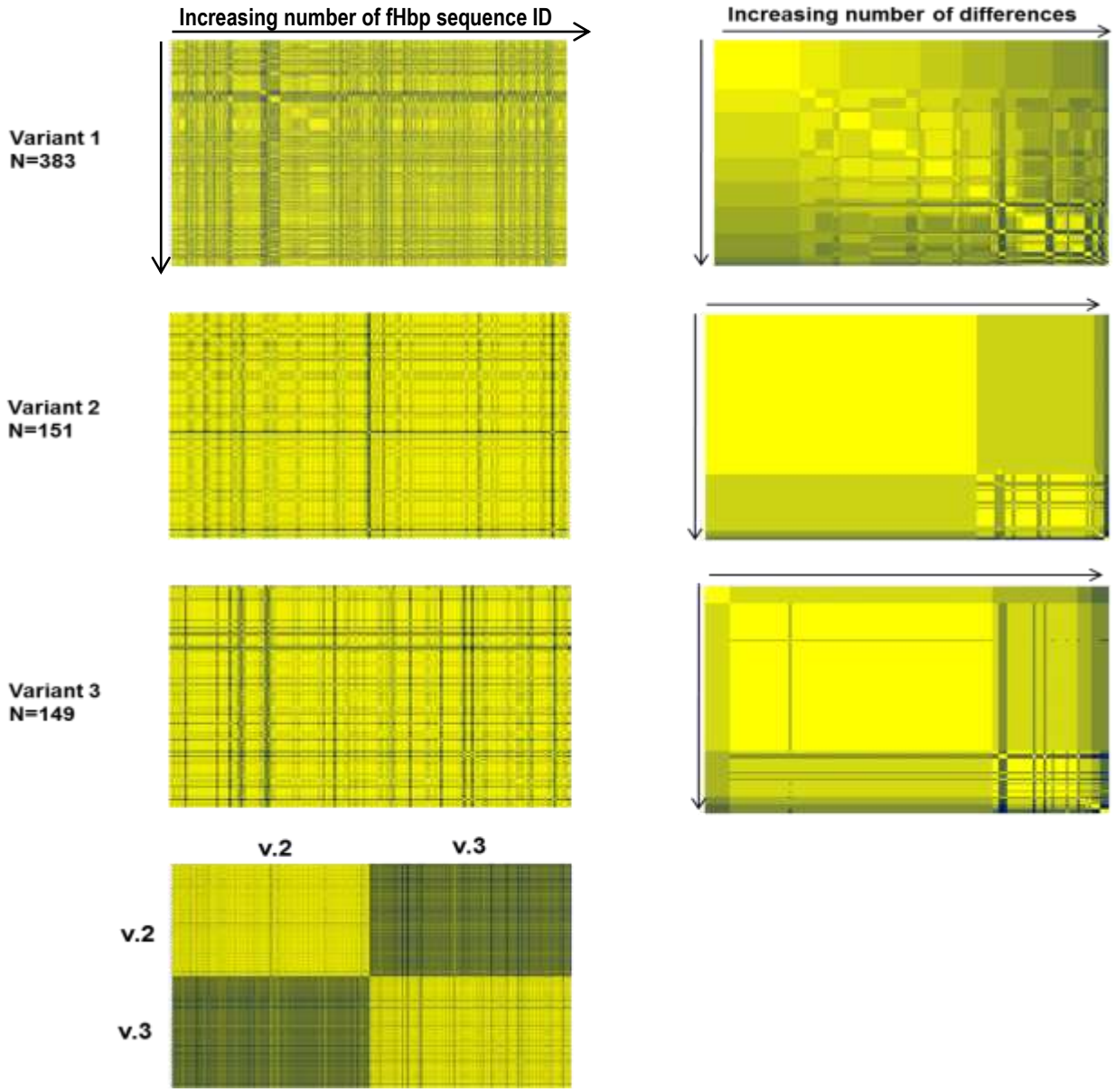


Figure 42: Pairwise comparison of factor H binding site using a matrix approach. Each matrix obtained was characterized on the left and top by the number of fHbp ID compared, while the numbers in the matrix

corresponded to the numbers of differences in terms of amino acid residues between each ID. The differences were shown also in terms of colour: the darker colour was proportional to the increase of number of differences. IDs in the matrices in the left are sorted by the increasing ID sequence number. IDs in the matrices on the right are the same ID but sorted by number of differences.

In detail, for variant 1, the factor H binding sites in 383 different fHbp IDs were compared. The highest number of IDs that share the same sequence of the factor H binding site is 81 (21%).
while the maximum number of amino acids differences observed between pairs of peptide IDs is 11 observed for 31 pairs of IDs.
For variant 2, the factor H binding sites in 151 different fHbp IDs were compared. The highest number of IDs that share the same sequence of the factor H binding site is 106 (69%) while the maximum number of amino acids differences observed between pairs of peptide IDs is 5 observed for 4 pairs of IDs.
For variant 3, the factor H binding sites in 149 different fHbp IDs were compared. The highest number of IDs that share the same sequence of the factor H binding site is 99 (66%) while the maximum number of amino acids differences observed between pairs of peptide IDs is 6 observed for 8 pairs of IDs.
When we compared in the matrix factor H binding sites deriving from the two variants, v.2 and v.3, together, 300 different fHbp IDs were compared. We observed the conserved clusters represented by a clear colour, one regard v.2 comparison, the second one regard v.3 (within the same variants) In contrast we could observe lower conservation and bigger differences for most of IDs analyzed between the variants, as reflected by the darker color. The highest number of IDs that share the same sequence of the factor H binding site is 107 while the maximum number of amino acids differences observed between pairs of peptide IDs is 6 observed for 125 IDs. The results observed by the pairwise comparison by matrix approach are reported in Table 16.

Table 16: Highest number of IDs sharing the same sequence of factor H binding site and maximum number of amino acids differences observed between IDs in the factor H binding site of the three variants of fHbp detected by matrix approach.

fHbp	Highest number (%) of IDs sharing the same sequence of factor H binding site	Maximum number of amino acids differences observed between IDs or pairs of peptide IDs in the factor H binding site
Variant 1	81 (21%)	11 for 31 pairs of IDs
Variant 2	106 (69%)	5 for 4 pairs of IDs
Variant 3	99 (66%)	6 for 8 pairs of IDs
Variant 2 + 3	107 (36%)	6 for 125 IDs

In conclusion we can assert that has been observed a similarity in factor H binding site between v.1 and v.2 in terms of number of variable positions, not observed between v.1 and v.3, while the matrix showed that there is lower variability overall in factor H binding site v.2 and v.3, compared with v.1. A possible explanation of it is that few sequences in v.2 analysis are characterized by this variability. When we compared the factor H binding site sequences between fHbp v.2 and 3, most of the pairs of IDs differed by 5 or 6 amino acids as reflected by the darker color. However, there were 4 IDs, 67, 175, 339, 381 that had from 0 to 3 differences compared to 236 out of 300 IDs including v.2 and 3. These results suggest that these IDs could be phylogenetically located between the two

variants. To further test it we compared v.2 and v.3 factor H binding site by a phylogenetic analysis (Figure 43). The tree obtained revealed the results obtained by matrix because we could observe the presence of two branches, one related to v.2 and one other related to v.3. Moreover the 4 IDs described previously are effectively located at the conjunction of the two branches.

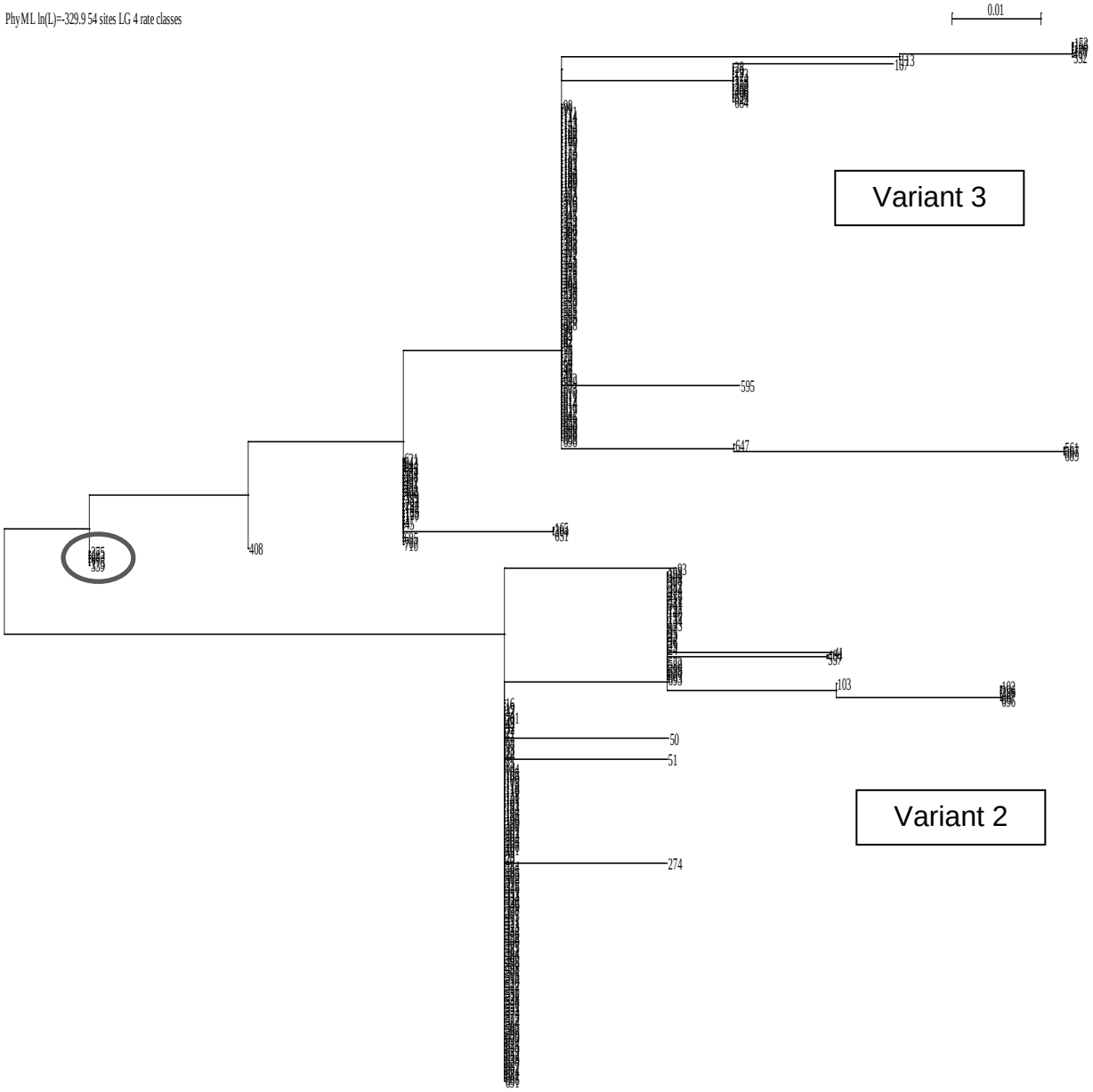


Figure 43: Phylogenetic analysis of factor H binding site of IDs belonging to v.2 or 3. The red circle indicate IDs located at the conjunction of the two branches. The tree was generated by seaview that drives the PhyML v3.1 program to compute maximum likelihood phylogenetic trees.

We also looked at full sequences of fHbp v.2 and v.3 and the tree obtained revealed the results obtained by matrix and by the phylogenetic analysis of factor H binding site of IDs belonging to v.2 or 3. Also in this case we could observe the presence of two

branches, one related to v.2 and one other related to v.3. Moreover the 4 IDs described previously (67, 408, 339 and 381, in red circle) were phylogenetically located at the point of separation of the two branches (Figure 44).

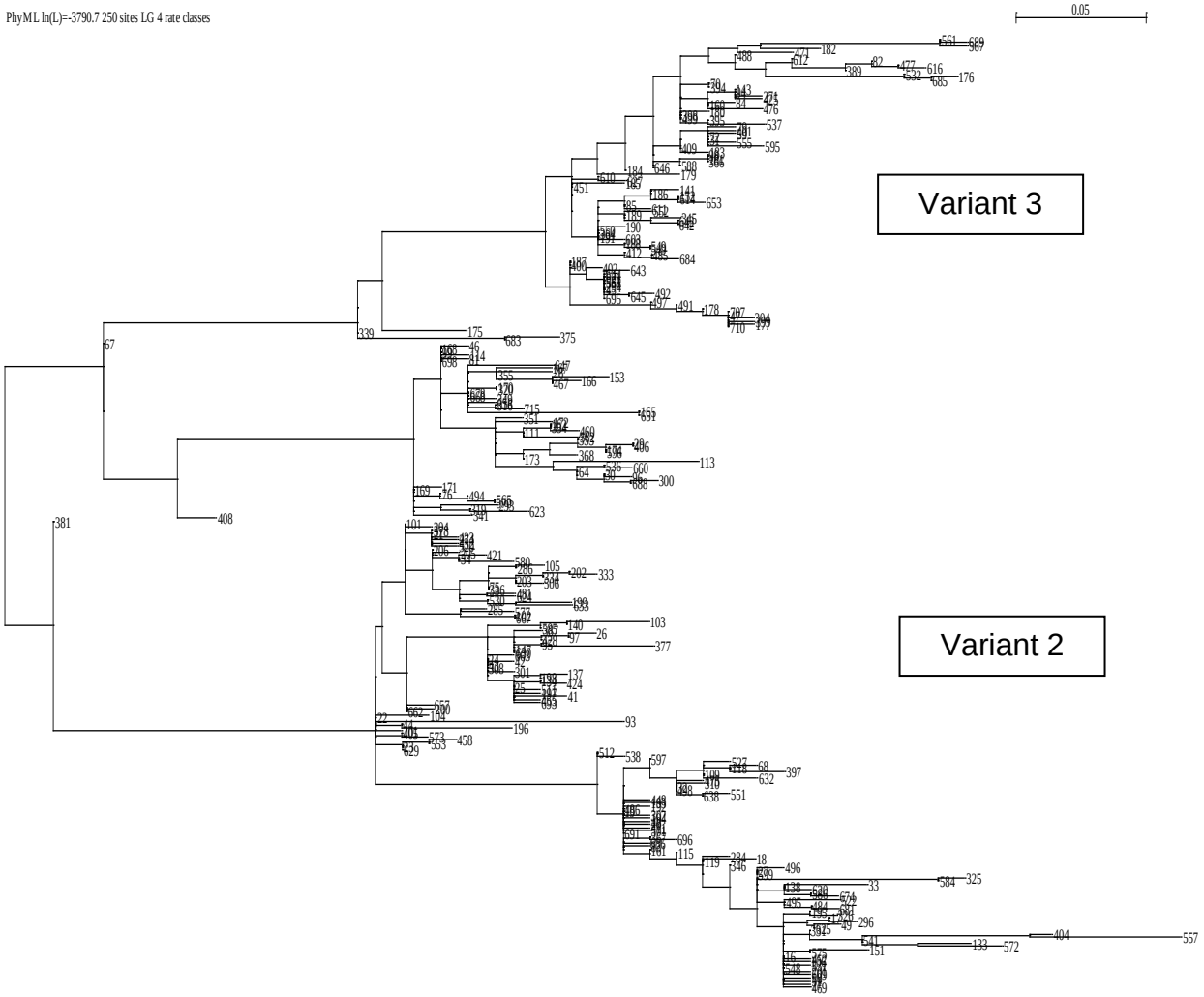


Figure 44: Phylogenetic analysis of full length fHbp IDs belonging to v.2 or 3. The tree was generated by seaview that drives the PhyML v3.1 program to compute maximum likelihood phylogenetic trees.

5.3.2 Investigation of functional activity of antibodies against fHbp on a strain panel expressing highly diverse fHbp sequences

Using fHbp variant 1 as model, the results from the bioinformatic analysis of the fHbp sequence IDs was used to rationally select diverse fHbp sequences for the analysis of functional activity of antibodies. The criteria for fHbp sequence selection were:

1. Different levels of differences in the factor H binding site of the fHbp ID to be tested in comparison with the fHbp expressed in the GMMA vaccine
2. Within each level of difference in the factor H binding site select an ID with a low % identity of the whole sequence compared with the fHbp expressed in the GMMA
3. Broad fHbp distribution in the phylogenetic tree

Test *N. meningitis* for the functional analysis of antibody activity by complement mediated serum bactericidal assay was:

1. A panel of wild type strains belonging to serogroup A, C, W, Y and X that expressed a subset of the selected sequences
2. A panel of isogenic strains based on one parent strain that was used to generate eight mutants engineered to express eight highly diverse selected fHbp sequences

The advantage of the latter panel was that it allowed to specifically investigate functional activity of antibodies against fHbp because the mutants only differed by the fHbp sequence that they expressed and the parent strain was resistant to killing by antibodies raised against GMMA without fHbp.

The test animal sera for the analysis were obtained from mice immunized with:

1. GMMA from a serogroup W mutant with deleted capsule, deleted lpxL1, deleted gna33 and over-expressed fHbp ID9 R41S
2. "Native OMV" from a serogroup W mutant with deleted capsule, deleted lpxL1 and over-expressed fHbp ID5

5.2.3.1 Selection of a panel of wild type strains with phylogenetically diverse fHbp sequences for the analysis of functional activity of antibodies raised against GMMA with over-expressed fHbp

FHbp sequences IDs of *N. meningitidis* non serogroup B strains present at GVGH and in the GSK Siena Molecular Epidemiology Database were analyzed and strains with 44 different fHbp v.1 IDs have been identified using fHbp variant 1 as reference in matrix from variant 1. As reported in Table 17, the selection of fHbp sequences was performed considering a maximum of 7 to 0 as number of differences in the factor H binding site and a maximum of 99% to 89% as range of highest overall sequence diversity. The fHbp sequences selected for the study were highlighted in yellow.

Table 17: % overall identity and number of differences in factor H binding site of fHbp sequences IDs of *N. meningitidis* A, C, W, Y and X strains analyzed and identified using fHbp variant 1 as reference in matrix from variant 1.

fHbp Sequence ID	% overall identity to ID 9	Number of differences in fH binding site compared to ID9
1	94.35	4
2	94.76	2
3	95.16	1
4	95.97	2
5	95.56	2
6	96.0	2
8	97.6	0
9	100.0	0
10	97.2	0
11	96.4	0
12	98.0	0
13	98.0	0
14	94.4	5

15	89.1	6
37	94.4	3
38	93.2	6
73	94.0	5
74	94.0	6
78	94.4	2
126	99.6	0
130	94.4	3
213	91.1	4
254	95.2	5
258	96.4	0
299	97.6	0
303	96.4	2
306	95.2	0
321	93.6	6
328	94.0	2
369	95.97	2
391	93.55	7
445	97.18	0
447	93.95	5
449	96.77	0
456	96.37	0
457	95.16	5
462	97.98	0
465	96.37	1
490	96.37	2
513	95.56	2
514	94.76	3
515	96.77	2
516	93.95	4
517	95.97	1

As reported in Figure 45 and as already described in Table 17, the selection of fHbp sequences expressed by the *N. meningitidis* wild type strains and used for testing in serum bactericidal assays (indicated in purple) was performed predominantly on the base of:

- Number of differences in the factor H binding site compared with ID9
- Differences in the overall % identity compared with ID9

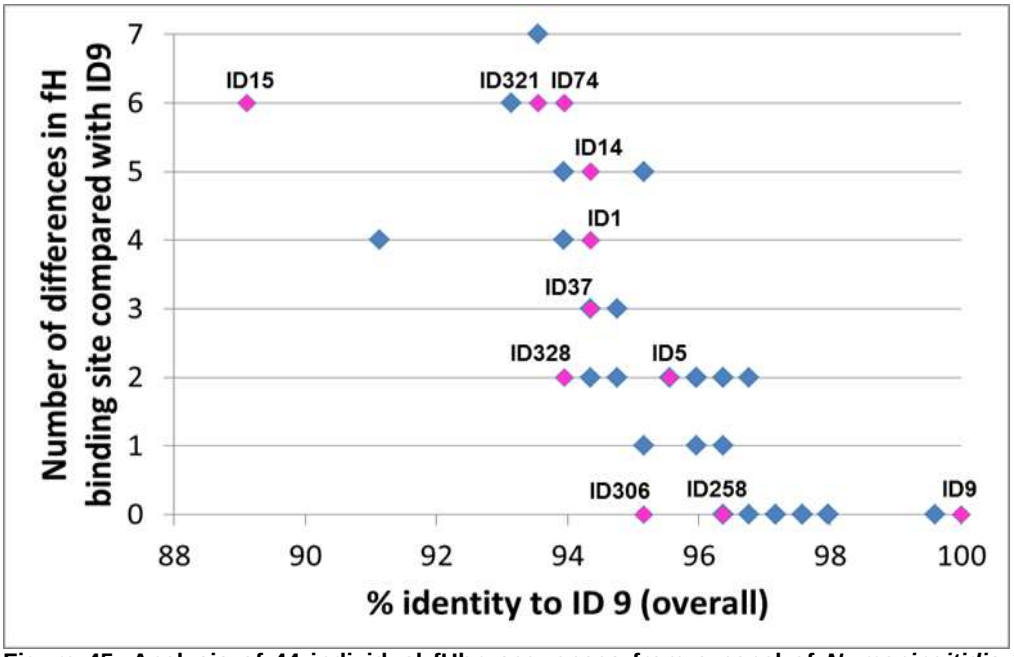


Figure 45: Analysis of 44 individual fHbp sequences from a panel of *N. meningitidis* A, C, W, Y, X strains available in the GVGH and GSK vaccines Siena strain collection. FHbp sequences identified for the selection of a panel of wild type strains. Each symbol represents an individual fHbp sequence ID belonging to variant 1.

Phylogenetic tree showing broad distribution of the identified sequences is shown in Figure 46.

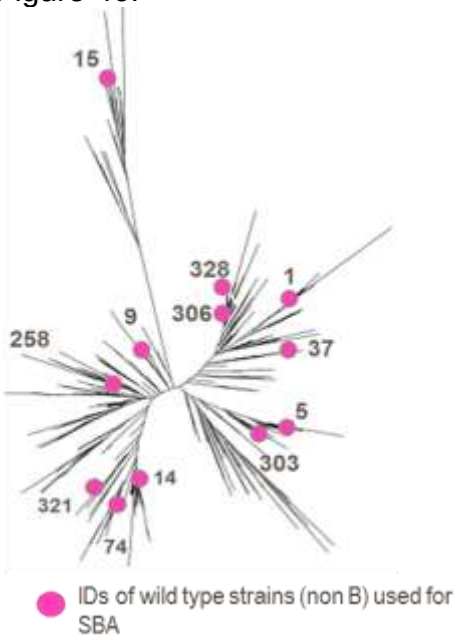


Figure 46: Phylogenetic tree of fHbp v.1 sequences. 380 existing fHbp sequences available on the Neisseria MLST database were included in the phylogenetic analysis. The tree was generated with with MAFFT version 7 The purple dots identify the phylogenetic distribution of the fHbp sequences that expressed by the N. meningitidis wild type strains used for testing in serum bactericidal assays.

In detail, we selected one or more fHbp sequence for each degree of difference in factor H binding site in respect to fHbp ID9 (reference sequence) as shown in Table 18.

Moreover the sequences chosen for the study were the most diverse in respect to fHbp ID9 (~95% overall identity) except for ID15 (89%).

Table 18: % overall identity and number of differences in factor H binding site of fHbp sequences IDs selected for testing in serum bactericidal assays.

Number of differences in factor H binding site compared to ID9	fHbp Sequence ID
0	306; 258
2	5; 328
3	37
4	1
5	14
6	15; 321; 74

The fHbp expression and the main features of *Neisseria meningitidis* wild type strains used for SBA analysis as serogroup, PorA subtype and sequence type, were reported respectively in Figure 47 and in Table 19.

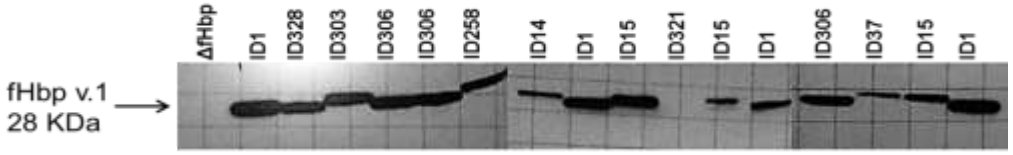


Figure 47: FHbp expression of *Neisseria meningitidis* wild type strains used for SBA analysis.

Table 19: Characteristics of *Neisseria meningitidis* wild type strains used for SBA analysis.

NVGH ID	Serogroup	fHbp variant	fHbp ID	PorA subtype	Sequence type
NVGH 2383	C	1	1	7-2,16	32
NVGH 2374	C	1	328	5-1,10-8	11
NVGH 2372	C	1	303	5-1,10-8	11
NVGH 2380	C	1	306	5-1,10-8	2994
NVGH 2378	C	1	306	5-1,10-8	655
NVGH 2394	Y	1	258	21,16	1466
NVGH 2381	C	1	14	17,16-4	3346
NVGH 2379	C	1	1	7, 16	2493
NVGH 2384	C	1	15	18-1,3	8339
NVGH 2390	W	1	321	5-2,10-1	6444
NVGH 2371	C	1	15	5-1,10-8	11
NVGH 2376	C	1	1	7-2,16	32
NVGH 2373	C	1	306	5-1,10-8	11
NVGH 2388	C	1	37	19,15	145
NVGH 2377	C	1	15	19-1, 15-11	467
NVGH 2382	C	1	1	5-2, 10-1	8299

5.2.3.2 Serum bactericidal antibody responses in sera from mice immunized with GMMA with over-expressed fHbp v.1

Serum bactericidal antibodies responses were measured using baby rabbit serum as complement source against the invasive wild type strains with diverse fHbp sequences and a heterologous PorA compared to that in the vaccine as described in Table 19.
As reported in Figure 48 high antibodies titers were observed in presence of sera against GMMA over-expressing fHbp v.1, except for the strain expressing ID306.
High antibodies titers that were not significantly different from those induced by GMMA with over-expressed fHbp were observed against 8 strains (expressing fHbp ID9, 328, 15, 258, 37, 321, 5, 1) also with sera against GMMA without fHbp. Two strains expressing ID74 and ID14 were resistant to killing by antibodies against GMMA without fHbp. While againt these two strains, antibodies against fHbp seem to be responsible for bactericidal activity, against the other test strains also antibodies againt other targets seem to contribute to bactericidal activity.

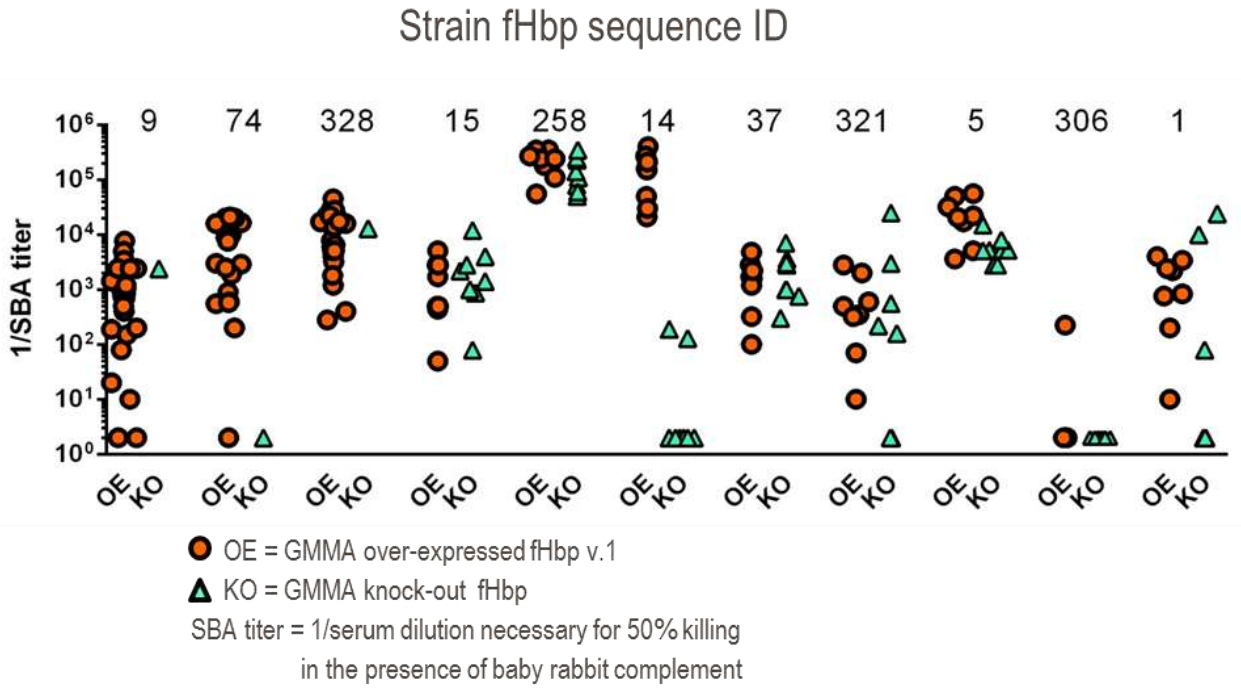


Figure 48: Serum bactericidal antibody responses against invasive Neisseria strains with different fHbp IDs. The numbers above the graph indicate the fHbp sequence ID expressed by the N. meningitidis wild type strain. "OE" indicate sera from mice immunized with 5 ug GMMA with over-expressed fHbp; "KO" indicate sera from mice immunized with 5 ug GMMA without fHbp. Each symbol represents the SBA titer measured in individual immunized mice. Mice were previously immunized twice four weeks apart with the vaccines over-expressing variant 1. Serum samples were obtained two weeks after the second dose. A control group of mice received GMMA without over-expressed fHbp. All vaccines were adsorbed on Aluminum hydroxide. Serum pools containing sera from eight mice immunized with GMMA without fHbp were tested against the strains expressing fHbp ID9, 74, 328, 15, 258, 14, 37, 321, 5, 306 and 1. Complement source was 20% baby rabbit serum screened to not have bactericidal activity against the test strain.

In addition, we applied the selection criteria adopted previously (described in paragraph 5.2.3.1), to generate, starting from one parent strain, a panel of eight genetically defined

isogenic strains that were engineered to express seven existing diverse fHbp v.1 sequences and one naturally existing fHbp v.1/2 hybrid protein. The isogenic strains were generated using a parent strain resistant to killing by sera against GMMA that did not conatin fHbp. After deletion of the native fHbp, the selected “heterologous” fHbp was stably integrated into the chromosome using derivatives of the published plasmid pComPind that allows controlled expression under control of IPTG.
As reported in Figure 49 the selection of fHbp sequences to express in isogenic strains and used for testing in serum bactericidal assays (coloured symbol) was performed on the base of:

- Number of differences in the surface exposed factor H binding site compared with ID9
- Highest and lowest % overall identity to ID 9 within each level of factor H binding site differences

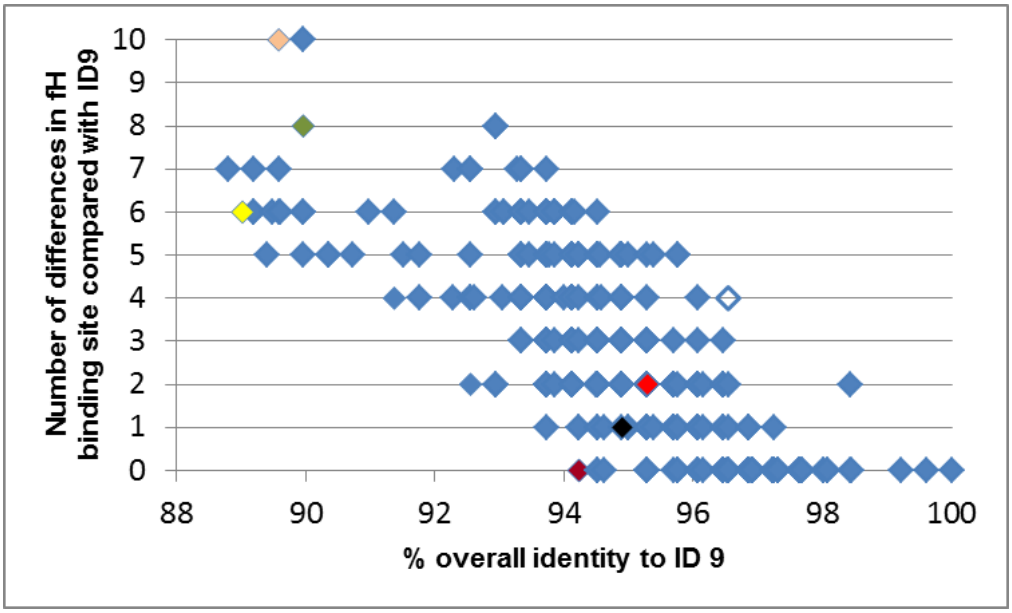


Figure 49: Analysis of fHbp v.1 sequences based on number of differences in fH binding site and % overall identity compared to ID 9. Eight candidates IDs were chosen out of 380 individual fHbp v.1 sequences analyzed. Among them, ID283 was chosen as hybrid v.1/v.2. Each symbol represents an individual fHbp sequence.

Phylogenetic tree showing broad distribution of the identified sequences is shown in Figure 50.

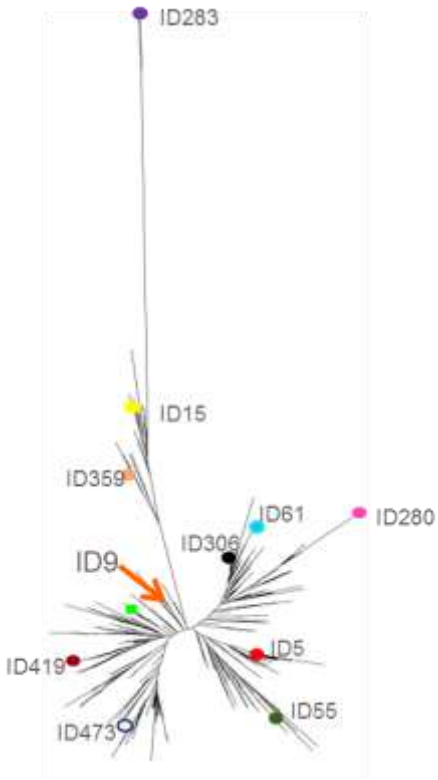


Figure 50: Phylogenetic tree of fHbp v.1 sequences. 380 existing fHbp sequences available on the Neisseria MLST database were included in the phylogenetic analysis. The tree was generated with with MAFFT version 7 The coloured dots identify the phylogenetic distribution of the fHbp sequences selected to express by isogenic strains.

Factor H binding proteins variant 1 selected to be expressed in isogenic strains are shown in Table 20 and sorted by the number of differences in factor H binding site compared with ID9. In detail, we selected one fHbp sequence for each degree of difference in factor H binding site in respect to fHbp ID9 (reference sequence). Moreover the sequences chosen for the study were the most diverse in respect to fHbp ID9 (89.6 to 95.3%overall identity) except for the hybrid v.1/v2 ID283 ID with 75.5 % overall identity. Moreover ID 306 was selected because the single wild type strain available with this ID was resistant and we wanted to test if this is because of the feature of this particular sequence ID or the strain.

Table 20: Number of differences in factor H binding site and % overall identity of candidate fHbp v.1 IDs compared with fHbp ID 9 and ID 5 used to over-express in the GMMA vaccine. fHbp ID 283 is a hybrid sequence between variant 1 and 2.

fHbp ID	Number of differences in factor H binding site compared with ID 9	% overall identity to ID 9	Number of differences in factor H binding site compared with ID 5	% overall identity to ID 5
419	0	94.2	2	90.6
306	1	94.9	3	94.9

5	2	95.3	0	100
473	4	94.5	4	92.6
15	6	89.6	4	89
55	8	90	8	87.1
359	10	90	10	87.1
283*		75.5		74.4

Parent strain selected to generate isogenic strains panel was isolate NVGH2381, which is resistant to killing by sera against GMMA without fHbp. The native fHbp v.1 was deleted by transformation with plasmid pBSUDgna1870ERM and homologous recombination with the erythromycin resistance gene present on the plasmid. Genes encoding for fHbp sub-variants (only ID 5, 55 15 and 306 because the other IDs (419, 473, 359 and 283) were synthesized (Invitrogen)) were amplified by polymerase chain reaction (PCR) from different *N. meningitidis* genomes (all from a C strain) and inserted in the genome in a non coding region between the genes nmb1428 and nmb1429 using a published plasmid (Figure 51) with a promoter that allows controlled expression of fHbp using IPTG.

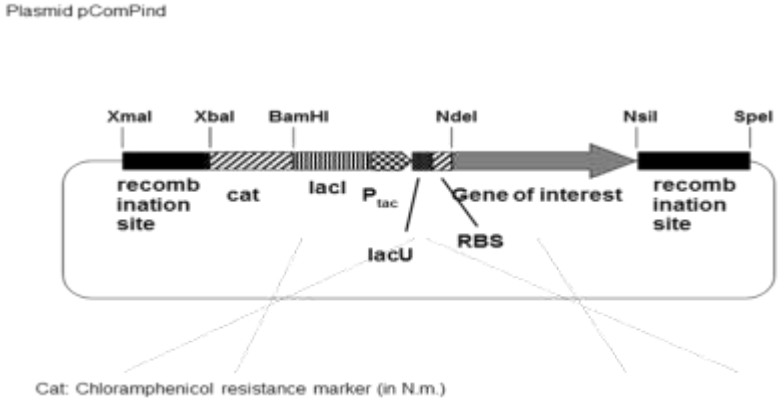


Figure 51: Plasmid pComPind used for isogenic strains generation.

Susceptibility to killing has been shown to also be dependent on the level of fHbp expression.

As mentioned previously, by using the isogenic strains we wanted to test susceptibility to killing under defined conditions having a combination of the different fHbp ID types and different levels of expression, independently of other additional strain specific factors that influence susceptibility to killing. The IPTG concentration useful to test the contribution of fHbp expression into analysis of functionality of antibodies by SBA was initially tested by growing a candidate mutant that was engineered to express fHBp ID 359 under different concentrations of IPTG and detection of fHbp in whole cell lysates. Figure 52. Under the conditions tested, with 0.01 mM IPTG no fHbp was detectable and the lowest concentration tested that induced fHbp expression was 0.05 mM. In addition, for following experiments also growth conditions with 0.5 m IPTG were selected which resulted in higher fHbp expression.

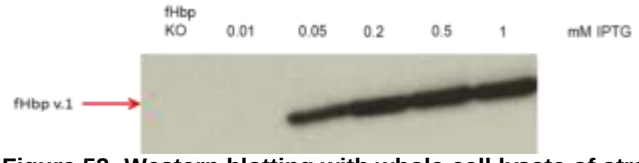


Figure 52: Western blotting with whole cell lysate of strain fHbp KO as negative control and of isogenic strain expressing ID359. FHbp expression was induced with different IPTG concentrations.

Screening of additional mutants for fHbp expression after transformation with plasmids containing fHbp ID 419, 5, 15, 55, 306, 473 and 61 was detected by colony blot. The growth conditions of cultures, then pelleted for dot blot, were the same adopted for Serum Bactericidal Assay, described in the next paragraph. 7 ml of Mueller-Hinton Broth (MHB) were inoculated with single cells to OD600 = 0.12 to 0.15. Bacteria were incubated on a shaker at 37%, 5% CO2 until OD600 reached 0,6 - 0,65. 1 mL of culture were then transferred into 6 mL warm MHB (+0.02 mM CMP-NANA and Isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentrations adopted in this work (0.05 mM or 0.5 mM)). Bacteria were incubated at 37%, 5% CO2 until OD 600 = 0.6 to 0.65. Culture was then transferred into 50 mL Falcon tube, and washed with Dulbecco's saline phosphate buffer supplemented with BSA 1% (DPBS+BSA), centrifuged for 10 minutes at 3500 rpm, RT. The supernatant was discarded and pellet resuspend in 1XPBS to OD600 = 0.6 to 0.7. 2 µl of samples were spotted onto the nitrocellulose membrane at the center of the grid and then serum anti fHbp v.1 was used to detect fHbp. As shown in Figure 53, at least one colony out of 4 transformed with the plasmids containing one of the fHbp IDs (except for fHbp ID61) expressed fHbp after 0.05 mM IPTG induction (except for fHbp ID61).

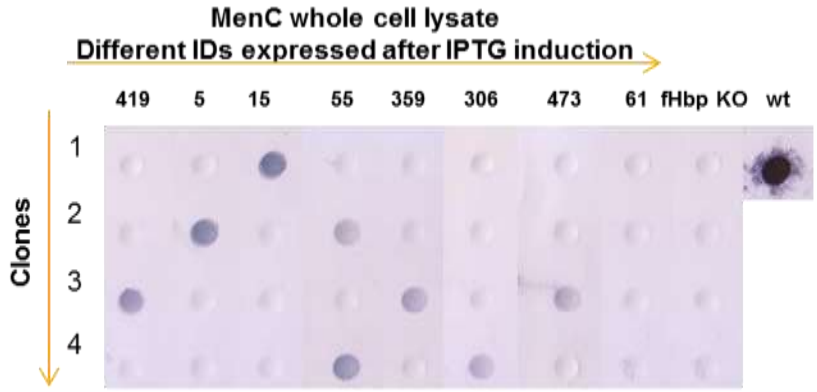


Figure 53: Colony blot to detect fHbp expression in isogenic strains using as detecting antibody a monoclonal antibody against fHbp v.1. The lysate of parent strain (Men C expressing ID14) was used as wild type control.

FHbp surface expression in isogenic strains was detected by FACS analysis using a polyclonal serum raised in mice against recombinant fHbp v.1 and v.2. The antibody was used at 2 dilutions 1:100 and 1:400. No differences in binding were observed between the two dilutions and thereforeonly data obtained at 1:100 dilution were shown (Figure 54).

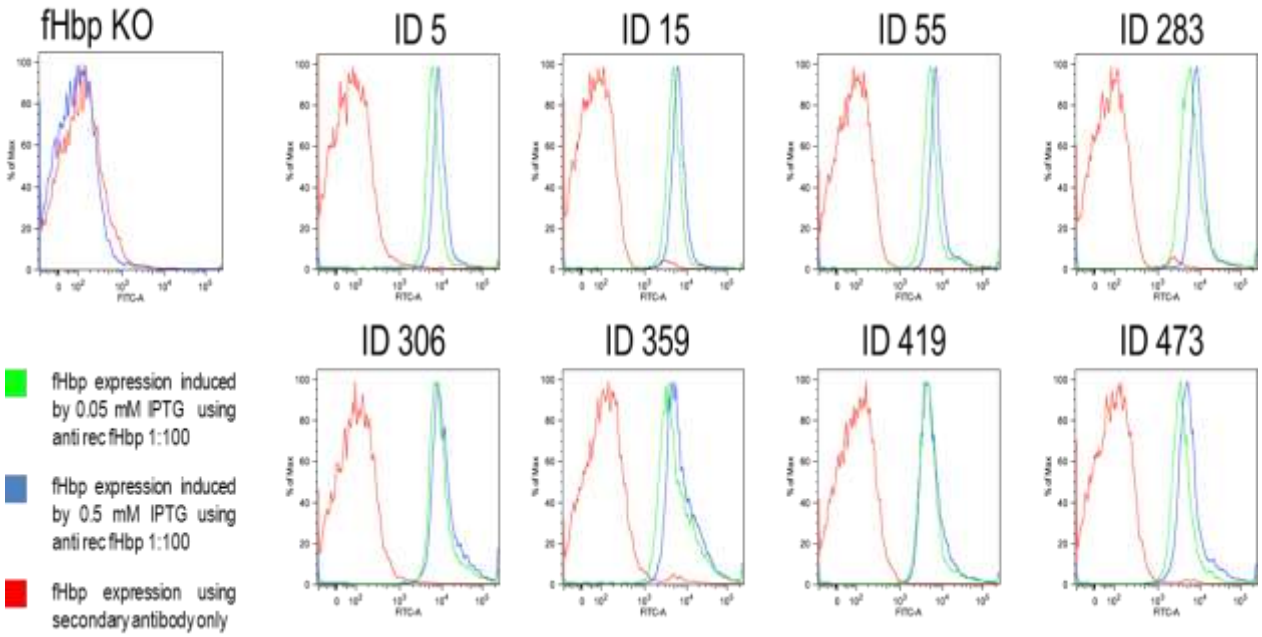


Figure 54: FHbp surface expression by FACS in isogenic *N. meningitidis* strains generated. FHbp expression was induced with 0.5 or 0.05 mM IPTG for 3 hours. Detecting antibody was a 1:100 dilution of a polyclonal serum raised against recombinant fHbp v.1 + v.2.

By FACS analysis FHbp is exposed at same levels on the surface of the mutants expressing the eight different fHbp IDs. Therefore, we can control fHbp expression and investigate killing of the different mutants depending purely on the fHbp sequence at a given IPTG concentration.
By FACS, it was not possible to appreciate big differences in the surface expression of fHbp related to the two different IPTG concentration so IPTG concentrations lower than 0.05 mM could be a way to regulate better fHbp expression.
For SBA analysis both rabbit and human complement were used to investigate potential breadth of coverage of antibodies induced by GMMA with over-expressed fHbp v.1 against the panel of the 8 isogenic strains generated and grown at 2 different IPTG concentrations. *N. meningitidis* only binds human complement factor H and not rabbit factor H. Testing both complement sources allows to investigate the potential contribution of competition of factor H and the antibody for binding to fHbp and resulting consequences for bactericidal activity.
I tested serum pools containing sera from eight mice from each group per pool. Figure 55 and Figure 56 shows the SBA titers measured with rabbit and human complement, respectively against the panel of isogenic strains. They are sorted by IDs with decreasing number of differences in factor H binding site compared with ID9 and by overall identity to ID9.
In presence of rabbit complement (Figure 55), no detectable bactericidal responses were obtained in case of control sera against GMMA without fHbp. In contrast, high SBA titers were obtained against all mutants expressing the different fHbp sequences indicating broad functional activity of anti fHbp antibodies against strains expressing the diverse fHbp sequences. Except for one strain (with ID 359 induced with 0.05 mM IPTG) titers ranged between 32,000 and 400,000.

In most cases SBA titers were similar independent of the IPTG concentration used to induce fHbp expression and the dose used for immunization (5 ug or 1.2 ug based on total protein). The first observation is in line with the similar fHbp expression levels observed by FACS in presence of 0.5 or 0.05 mM IPTG.

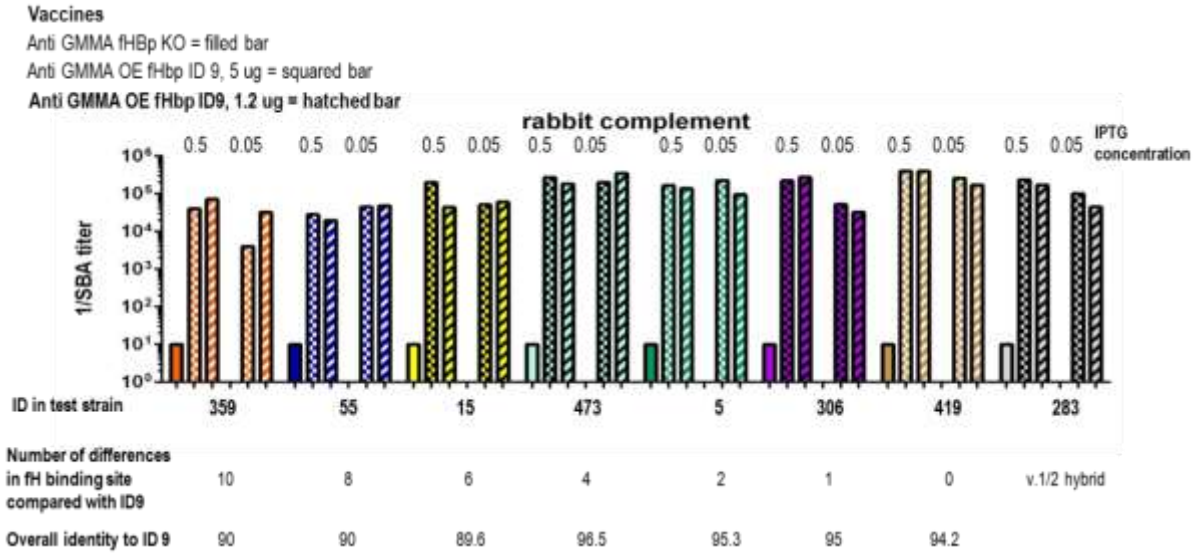


Figure 55: Serum bactericidal antibody responses observed against the isogenic strains generated, in presence of 20% rabbit complement. Mice were previously immunized twice four weeks apart with the vaccines over-expressing variant 1. Serum samples were obtained two weeks after the second dose. A control group of mice received GMMA without over-expressed fHbp. All vaccines were adsorbed on Aluminum hydroxide. Bars show titers obtained with serum pools for eight mice per group at two different concentrations of IPTG (0.5 and 0.05 mM) in order to investigate potential breadth of fHbp sequence coverage of antibodies induced by GMMA OE ID9 at 2 different quantities, 5 µg and 1.2 µg.

When bactericidal activity was measured with human complement (Figure 56), no detectable bactericidal activity was observed in case of sera against GMMA with deleted fHbp, while bactericidal activity was observed raised against GMMA with over-expressed fHbp ID9, indicating activity of anti-fHbp antibodies, dependent on fHbp expression in GMMA. Titers ranged between 150 (anti GMMA OE fHbp ID9 5µg against strain expressing ID359 grown in presence of 0.05 mM IPTG) and >327680 (for example with anti GMMA OE fHbp ID9 5 µg and 1.2 µg against strain expressing ID5 grown in presence of 0.05 mM IPTG).For practical reasons serum pools have been used and therefore no statistical comparison between the strains can be performed. However, with human complement the data indicate a trend that differences in titers are observed against strains expressing the different fHbp ID in particular with ID359<ID15<ID473<ID5. The number of differences in the fH binding site can in part contribute to susceptibility. For example fHbp IDs 359 and 15 have a similer overall identity to ID 9 in the vaccine (90% and 89.6 %, respectively). However, ID359 has 10 amino acid differences and ID 15 six amino acid differences in the factor H binding site comared to ID 9 and SBA titers measured against the strain engineered to express ID 15 are higher. Similar observation can be made against the strains expressing fHbp ID 473 and 5 (96.5 % and 95.3 % overall identity and 4 and 2 differences in the fH binding site, respectively). When expressed in the isogenic strain also the mutant with ID306 was susceptible to killing. This indicates that in the wild type strain expressing this ID

other mechanisms contribute to the resistance and not the fHbp sequence expressed. Interestingly, also the strain engineered to express the fHbp v.1/2 hybrid protein was susceptible to killing.

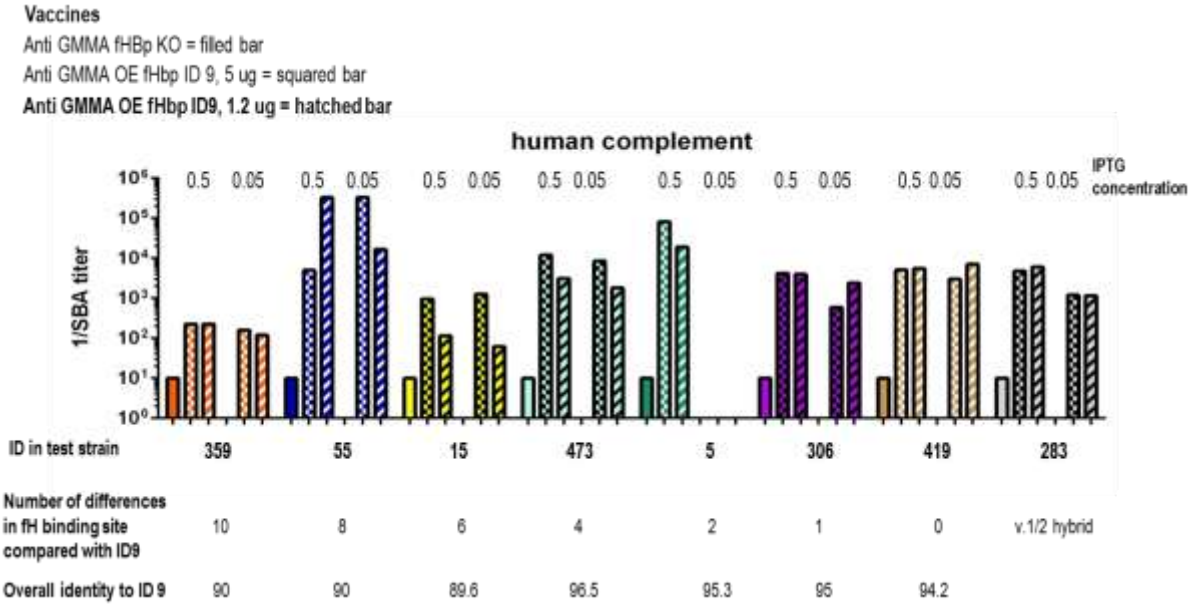


Figure 56: Serum bactericidal antibody responses observed against the isogenic strains generated, in presence of human complement. Mice were previously immunized twice four weeks apart with the vaccines over-expressing variant 1. Serum samples were obtained two weeks after the second dose. A control group of mice received GMMA without over-expressed fHbp. All vaccines were adsorbed on Aluminum hydroxide. Bars show titers obtained with serum pools for eight mice per group. For the assay strains were cultivated at two different concentrations of IPTG (0.5 and 0.05 mM). Sera were from mice immunized with 5 µg or 1.2 µg GMMA OE ID9.

The detection breadth of sequence coverage of antibodies induced by GMMA OE ID5 using rabbit and human complement are reported Figure 57 and Figure 58.
In presence of rabbit complement (Figure 57), no detectable bactericidal responses were obtained in case of control sera against GMMA without fHbp while high SBA titers were obtained against the mutants expressing all the different fHbp sequences indicating activity of anti-fHbp antibodies with a range of titers between 3000 (anti GMMA OE fHbp ID5 0.5 µg against strain expressing hybrid v.1/v.2 ID283 grown in presence of 0.05 mM IPTG) and 400000 (anti GMMA OE fHbp ID5 2.5 µg and 0.5 µg against strain expressing ID5, 15, 55 and 306 grown in presence of both concentrations of IPTG). In general, titers observed were similar against the different strains grown at the two different concentration of IPTG and using the two different doses of vaccines.

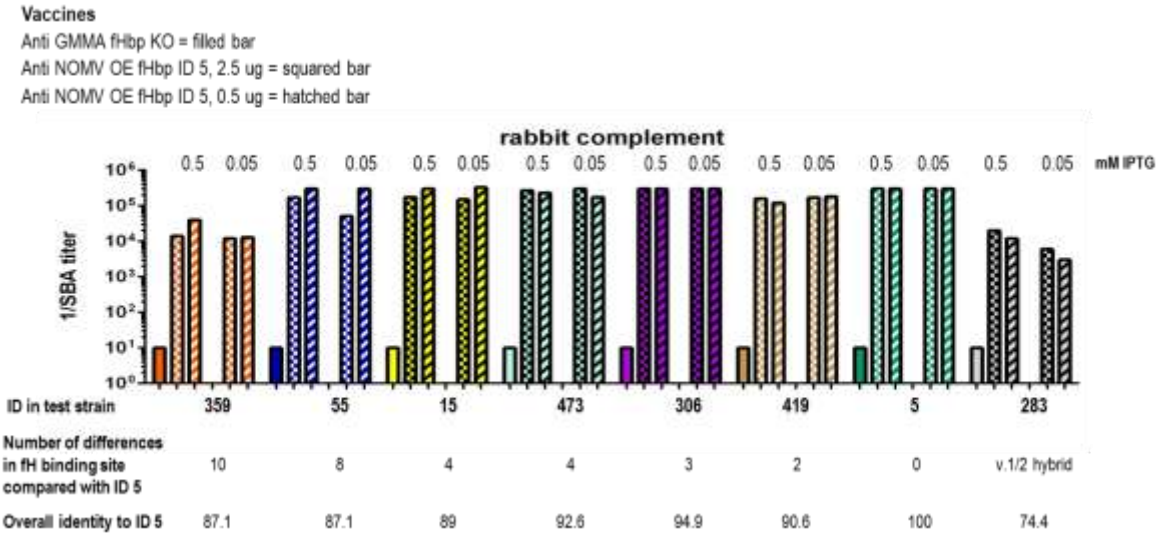


Figure 57: Serum bactericidal antibody responses observed against the isogenic strains generated, in presence of 20% rabbit complement. Mice were previously immunized twice four weeks apart with the vaccines over-expressing variant 1. Serum samples were obtained two weeks after the second dose. A control group of mice received GMMA without over-expressed fHbp. All vaccines were adsorbed on Aluminum hydroxide. Bars show titers obtained with serum pools for eight mice per group. For the assay strains were cultivated at two different concentrations of IPTG (0.5 and 0.05 mM). Sera were from mice immunized with 2.5 µg or 0.5 µg GMMA OE ID5.

When bactericidal activity was measured with human complement (Figure 58), no detectable bactericidal activity was observed in case of sera against GMMA with deleted fHbp. As expected, highest titers were observed against the strain engineered to express ID5 which is identical to the one used for immunization and lowest titers against the strain expressing fHbp v.1 ID 359 with the highest number of differences in the fH binding site and relatively low overall identity to ID5. Also in this experiment, the strain expressing ID15 was more susceptible than the strain expressing ID 359. The two IDs have overall 89 and 87.1 % identity to ID5 but ID359 has 10 amino acid differences in the fH binding site whereas ID 15 has 4. The data indicate that antibodies are directed at the fH binding site and differences contribute to differences in susceptibility to killing.
Overall the SBA data obtained with the isogenic strains suggest that fHbp v.1 expressed in the context of the outer membrane induces antibodies with broad fHbp sequence coverage. Differences in the fH binding site appear to contribute to susceptibility but the overall sequence identity plays a role as well.

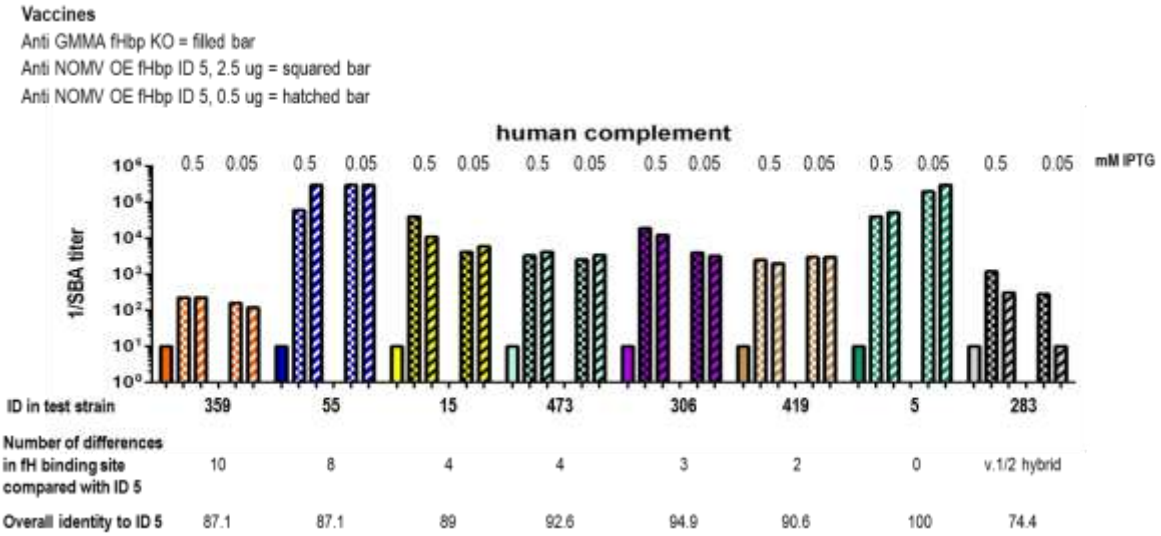


Figure 58: Serum bactericidal antibody responses observed against the isogenic strains generated, in presence of human complement. Mice were previously immunized twice four weeks apart with the vaccines over-expressing variant 1. Serum samples were obtained two weeks after the second dose. A control group of mice received GMMA without over-expressed fHbp. All vaccines were adsorbed on Aluminum hydroxide. Bars show titers obtained with serum pools for eight mice per group. For the assay strains were cultivated at two different concentrations of IPTG (0.5 and 0.05 mM). Sera were from mice immunized with 2.5 µg or 0.5 µg GMMA OE ID5.

VI Discussion

Meningococcal disease occurs worldwide in both endemic and epidemic form.
Humans are the only natural reservoir of meningococcus.
In the past, *Neisseria meningitidis* belonging to capsular group A strains were responsible for the majority of cases in the African Meningitis Belt (an area which extends from Ethiopia to Senegal).
In recent years, serogroup W, X and C strains have increasingly contributed to the burden of disease.
The dynamic nature of meningococcal epidemiology is due to environmental, behavioral factors and principally to the antigenic variability that occurs mainly through horizontal gene transfer, which allows the organism to acquire large DNA sequences resulting in capsular switching. Moreover, the meningococcus also uses gene conversion, which is autologous recombination and does not require the acquisition of DNA from another strain and it is also capable of varying its antigenic profile through variable gene expression and phase variation, which can occur through slipped-strand mispairing of variable number tandem repeats, variation in poly-C tracts, and use of insertion sequences.
Even though vaccines against meningococcus are available, an affordable and safe vaccine that protects against strains from all serogroups causing diseases in Africa is still needed.
One meningococcal vaccine approach is based on GMMA (Generalized Modules for Membrane Antigens) from genetically engineered strains.
GMMA are outer membrane blebs release by the bacteria that can be used as “carriers” of protective antigens. Moreover there is the possibility to “design” GMMA by targeted engineering of bacteria such as removing or modifying undesirable antigens, expressing desired antigens or engineering the strain to have increased release of GMMA. In addition, being in a vesicle structure that mimics the surface of the bacterium, antigens are likely to be presented to the host immune system in their native conformation and membrane environment.
One of the main protective meningococcal antigens identified is factor H binding protein (fHbp) which binds human factor H (a negative regulator of the human complement cascade). This binding allows the meningococcus to avoid complement-mediated killing. The protein can be divided into 3 antigenic variants, v.1, v.2 and v.3. Each variant can be further divided into sub-variants (each one identified by a specific ID number) differing in their amino acid sequence. Moreover, fHbp is expressed by almost all strains independent of the serogroup and induces the production of functional bactericidal antibodies in mice and humans. In general antibodies against fHbp show cross protection within each variant group, but limited cross reactivity between variants, e.g. v.1 and v.2/v.3 and viceversa. A second main immunogenic antigen is PorA. However, multiple subtypes of PorA exist and protection is only observed against strain expressing the same subtype as that in the vaccine. African A, W and X strains are relatively conserved in terms of the fHbp and PorA that they express. Most serogroup A and X strains express fHbp belonging to variant 1, while W isolates express fHbp v.1, 2 or 3. The vast majority of W strains, however, expressed a single PorA subtype (1.5,2) while A and X strains showed broader heterogeneity. A single GMMA vaccine has been

developed from a W strain with PorA P1.5,2, engineered to over-express fHbp v.1. To increase safety of the vaccine strain, capsule biosynthesis genes were deleted. In order to increase the tolerability of the vaccine, the genetic modification of Lipooligosaccharide (LOS) is necessary. The lipid A, portion of the LOS is responsible for the endotoxin activity of LOS. Inactivation of *lpxL1* gene that encodes for late-functioning acyltransferases of lipid A biosynthesis results in a penta-acylated lipid A, instead of the wild type hexa-acylated lipid A with lower toxicity, but retaining the adjuvant activity. To increase blebbing *gna33* has been deleted. In mice, GMMA from this mutant elicited bactericidal antibodies against the homologous W strain and also cross protective antibodies against a panel of genetically diverse African A and X strains expressing fHbp v.1.

The primary aim of the project is to systematically investigate and dissect the breadth and nature of fHbp sequence coverage by antibodies against fHbp. This is obtained by using a combination of bioinformatic tools as the basis to analyze functional antibody activity. The analysis can support the selection of fHbp sequences to be included in fHbp based vaccines for sub Saharan Africa.

GMMA have been generated from an African serogroup W (ST11) meningococcus isolate expressing PorA subtype P1.5,2 (highly predominant among African W isolates) and genetically engineered to over-express native fHbp v.2 ID23, represented in 74% of W invasive strains. To attenuate virulence and avoid the possibility of generating serogroup-specific antibodies, mutants lack capsule biosynthesis. Furthermore, the lipooligosaccharide (LOS) has been genetically detoxified by deleting *lpxL1* gene. To increase blebbing *gna33* has been deleted. Once the mutant strain has been generated, GMMA have been collected and the amount of fHbp present has been detected by western blotting, comparing GMMA from mutants and wild type strain (FIG in Results).

We observed more than 10-fold higher presence of the protein in GMMA from mutant strains than in GMMA from wild type meningococcus. We did not detect differences in the level of fHbp in GMMA from different mutants. This suggests that the over-expression system used is a robust method for generating GMMA with high presence of the main vaccine antigen (fHbp).

After separation by SDS PAGE and staining with coomassie blue we looked at the total protein composition of vesicle preparations, comparing GMMA from mutants and wild type strain. We observed that the protein pattern of mutants is similar to that of MenW wild type, however the mutations introduced modify or delete components of the outer membrane and changes in the protein composition would be expected. SDS PAGE may not be sensitive enough to detect differences and quantitative proteomics on the different mutants could reveal differences in the composition after introduction of each of the mutation.

In order to assess the reactogenicity of GMMA with detoxified LOS, we analized the release of cytokines by PBMC. We analyzed by ELISA the release of interleukin 6 (IL-6) from PBMC incubated for 4 hours with serial dilution of GMMA from different mutants. We saw that while non-detoxified GMMA started inducing IL-6 release already at low concentration (10^{-2}ng/mL), detoxified GMMA started stimulating cytokine release at 100-fold higher concentration (10^{1}ng/mL).

We analyzed serogroup W wild type and mutants, having the different combination of mutations described previously, by HPLC-SEC in order to evaluate their soluble protein content, the size and the presence of nucleic acids.
In all the samples analyzed by fluorescence channel, there wasn't the presence of the peaks corresponding to soluble proteins, generally eluating at different times with respect to GMMA. By UV channel, based on the ABS260/ABS280 ratio, we verified also the absence of nucleic acids independently of benzonase treatment. These characterization suggest that the GMMA from the groupW strain with over-expression of the native fHbp v.2 are well purified. A mouse immunogenicity study would be the next step in order to investigate breadth of coverage of African isolates that express fHbp v.2 or 3.
The limited diversity among existing circulating African fHbp IDs and the possibility of changes of the fHbp sequences in the future was the rational for analysis of sequence coverage and test of functionality of anti-fHbp antibodies induced by a vaccine containing fHbp v.1 against a broad panel of highly diverse fHbp sequences IDs, independent of their prevalence.
In order to reach this aim we performed a comparative sequence structure analysis of fHbp v.1, 2 and 3 analyzing the factor H binding site. Being a surface exposed portion of the molecule, the factor H binding site is subject to a balance between immunological selection and functional activity.
By structure analysis and sequence alignment we identified the amino acids that are in contact with factor H in the published fHbp sequences belonging to v.1, 2 and 3.
The analysis of polymorphic sites in factor H binding site using ID9 as reference sequence for variant 1, ID16 for variant 2 and ID28 for variant 3 revealed that there is a similarity in factor H binding site between v.1 and v.2 in terms of number of variable positions and ratio of conservative/not conservative substitutions, not observed between v.1 and v.3, while the matrix showed that there is lower variability overall in factor H binding site v.2 and v.3, compared with v.1.
The differences in the relatively high frequency of conserved fH binding sites in the fHbp v.2 and 3 group versus the lower frequency of conserved amino acids in the variant 1 group was unexpected. The biological reason is unknown, however it could be possible that greater proportion of strains expressing fHbp v.1 are invasive strains which are expected to be under greater immune selection pressure.
However, the number of variable positions for v.1 and v.2 was similar and appears to be in contradiction with that obtained by matrix approach which indicated a relatively high conservation of the factor H binding site among the existing fHbp v.2 sequence IDs. A possible explanation of it is that few sequences in v.2 analysis are characterized by this variability. In fact, by the analysis of polymorphic sites, we looked only at the number of variable positions, without considering the distribution and the frequency of the event for all the sequences existing, differently from informations resulted by matrix. It is possible that v.1 and v.2 have the same number of variable positions, but this variability is most frequent for variant 1, that is for a higher number of sequences IDs (e.g.150 out of 300 sequences compared have the apolar aminoacid isoleucine in place of the polar serine, or the positively charged aminoacid lysine in place of the negatively charged glutamate), with respect to variant 2 (e.g. 25 out of 150 sequences compared have the not conservative substitutions described in the previous example).

Another question has been the conflicting results observed, by analysis of polymorphic sites, between variant 2 and variant 3 (higher number of variable positions in v.2 than v.3), in respect to what we observed by matrix approach, which revealed a similar high conservation within the same variant. It has been observed that frequency with which invasive v.1, v.2 and v.3 occurs is respectively of 65%, 30% and 5%. The discrepancy detected by the analysis of fHbp v.2 and v.3 could be related to the fact that there is a major functional flexibility in factor H binding site of fHbp v.2 in respect to v.3, because most of variant 2 IDs, reported in database and analyzed could be related to invasive (and not carrier) strains and so, affected by a greater immune selection pressure.
Therefore, the above-mentioned approach revealed a novel approach to analyze fHbp functional regions in the v.1, 2 and 3 group and may provide the bases for selection of sequences of fHbp containing vaccines and to provide a rational approach to select fHbp sequences in tests strain used in serum bactericidal assays. In fact, by matrix approach, we selected as model a panel of diverse fHbp sequences based on diversity in the factor H binding site and overall diversity using ID9 as reference in matrix from variant 1. On the basis of the first sequences selection we chose a panel of wild type strains. The isolates belonged to serogroup A, C, W, Y and X that expressed a subset of the selected sequences and a panel of isogenic strains. To generate the last panel we selected one parent strain and generated eight mutants that we engineered to express eight highly diverse fHbp sequences. The advantage of this panel is that it allowed us to specifically investigate functional activity of antibodies against fHbp because the mutants only differed by the fHbp sequence that they expressed, not looking only at sequence prevalence but also specifically at diversity.
Serum bactericidal antibodies responses were measured using baby rabbit serum as complement source against a panel of 11 invasive wild type strains with diverse fHbp sequences and a heterologous PorA compared to that in the vaccine.
High antibodies titers were observed in presence of sera against GMMA over-expressing fHbp v.1 ID9, except for one test strain expressing ID306. By western blotting fHbp expression was detected in *N. meningitidis* wild type strains expressing ID306, so the combination of both results suggest that it is not a feature of the fHbp sequence that this strain is resistant. High antibodies titers that were not significantly different from those induces by GMMA with over-expressed fHbp were observed against 8 strains (expressing fHbp ID9, 328, 15, 258, 37, 321, 5, 1) also in presence of sera against GMMA without fHbp, except for the test strains expressing ID74 and ID14 The observation of bactericidal activity also in presence of sera against GMMA without fHbp indicates that other antigens, in place of fHbp, are responsible for killing. In order to assess directly the contribution of antibodies against fHbp against these strains, the sera will have to be tested by serum bactericidal assay after adsorption of the anti-fHbp antibodies. Moreover immune precipitation experiments and mass spectrometry analysis could support the analysis in order to understand which proteins are involved in inducing the bactericidal activity.
In addition, we applied the selection criteria adopted previously to generate, a panel of eight genetically defined isogenic strains that were engineered to express seven existing diverse fHbp v.1 sequences and one naturally existing fHbp v.1/2 hybrid protein. The advantage of this panel is that it allowed to specifically investigate functional activity of antibodies against fHbp because the mutant only differed by the fHbp sequence that

they expressed. Moreover the sequences chosen for the study were the most diverse in respect to fHbp ID9 (~95% overall identity) except for ID15 (89%) and for the hybrid v.1/v2 ID283. Moreover ID 306 was selected because the single wild type strain available with this ID was resistant to killing by antibodies against GMMA with over-expressed fHbp and we wanted to test if this is because of the feature of this particular sequence ID or the strain.

By using the isogenic strains we wanted to test susceptibility to killing under defined conditions having a combination of the different fHbp ID types and different levels of expression, independently of other intrinsic strain specific factors that influence susceptibility to killing. By FACS analysis fHbp is expressed on the surface of the mutants containing the different fHbp IDs at same levels. Therefore, we can control fHbp expression and investigate killing of the different mutants depending purely on the fHbp sequence at a given IPTG concentration.

For SBA both rabbit and human complement were used to investigate potential breadth of coverage against the panel of the 8 isogenic strains. FHbp binds human factor H only and not rabbit factor H and it can be critical because I have selected fhbp sequences that differ in the factor H binding site.

In presence of rabbit complement, no detectable bactericidal responses were obtained in case of control sera against GMMA without fHbp while high SBA titers were obtained against the mutants expressing all the different fHbp sequences. In general, titers observed were similar against the different strains grown at the two different concentration of IPTG and using the two different doses of vaccines.

When bactericidal activity was measured with human complement, again no detectable bactericidal activity was observed in case of sera against GMMA with deleted fHbp while bactericidal activity was observed with sera against GMMA with over-expressed fHbp ID9, indicating activity of anti-fHbp antibodies, dependent on fHbp expression in GMMA.

With human complement the data indicate a trend that with smaller number of differences in the factor H binding site the titers get higher and viceversa. The results observed suggested that amino acids involved in factor H binding played a role giving a contribution in bactericidal activity in respect to whole sequences of fHbp.

In conclusion, antibody titers in presence of human complement (with factor H) are lower than titers in presence of rabbit complement.

Using human complement, a higher number of differences in factor H binding site sequence could result in a lower fHbp antibodies affinity, so that factor H binding has an advantage and therefore the strain results more resistant.

As described previously, with human complement, the data indicate a trend that with smaller number of differences in the factor H binding site the titers get higher and viceversa suggesting that amino acids involved in factor H binding played a role, giving a contribution in bactericidal activity versus the whole sequences. The overall sequence diversity also plays a role. It is not surprising because changes in any part of the molecule can have an effect on the structure and therefore minor changes in antibody susceptibility. However, the data suggest that fHbp sequence diversity plays a role in susceptibility to killing.

It is known that the susceptibility of a strain not only depends on the fHbp sequence but also on fHbp expression level. In the FACS experiment we did not include a reference wild type strain known to express high levels of fHbp. However, the strong shift of the

peak relative to the negative control in the FACS experiment indicates that level of fHbp expression even with 0,05 mM IPTG is relatively high. A high fHbp expression induced in the isogenic strains could have masked to some extend the effect of the sequence diversity and their contribution to killing, Therefore, lower levels of fHbp could demonstrate better the differences between the fHbp IDs in susceptibility. In order to detect if fHbp expression is effectively crucial and if yes, how much it is (more or less than fHbp sequence) and also to detect if effectively the contribution of the factor H binding site differences in the isogenic strains compared with the overall sequence diversity has a most relevant role into strains susceptibility, additional assays could be done (FACS analysis and SBA) using IPTG concentrations lower than 0.05 Mm, as a way to regulate better fHbp expression. In addition individual sera should be used in the SBA, in place of single serum. In fact, for technical reasons, we used pooled sera which didn't allow a statistical analysis of the differences between the strains.

Summarizing and concluding we observed a lower frequency of conserved amino acids in the factor H binding site of fHbp belonging to the variant 1 group, in respect to v.2 and v.3, However, a similar number of variable positions were observed for v.1 and v.2 in respect to variant 3.
The bioinformatic analysis has provided a rational approach to systematically dissect the surface exposed factor H binding site versus the whole sequence of fHbp and their contribution in susceptibility to killing. Serum bactericidal assays revealed that fHbp v.1 in the group W GMMA induce antibodies against a broad range of fHbp sequences. Susceptibility appears to be dependent on differences in the factor H binding site but also the overall fHbp sequence differences contribute.

VII Reference List

1. Peter J. Hotez and Aruna Kamath. (2009) Neglected Tropical Diseases in Sub-Saharan Africa: Review of Their Prevalence, Distribution, and Disease Burden. PLoS Negl Trop Disease.
2. Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, et al. Control of neglected tropical diseases. N Engl J Med. 2007;357(10):1018–1027. 10.1056/NEJMra064142.
3. Chen SH, Ravallion M. The developing world is poorer than we thought, but no less successful in the fight against poverty. World Bank Policy Research Working Paper 4703 (WPS 4703) 2008. Available: http://www-wds.worldbank.org. Accessed November 2, 2008.
4. Palumbo E, Fiaschi L, Brunelli B, Marchi S, Savino S, Pizza M., (2012) Antigen identification starting from the genome: a "Reverse Vaccinology" approach applied to MenB. Methods Mol Biol 799:361-403.
5. Thigpen MC, Whitney CG, Messonnier NE, Zell ER, Lynfield R, Hadler JL, et al. Emerging Infections Programs Network. Bacterial meningitis in the United States, 1998-2007. N Engl J Med. 2011;364:2016-25.
6. Hitchcock P.J., Robinson E.N. (Jr.) and McGee Z.A., "Neisseriae: Gonococcus and Meningococcus", chap. 14 in Schaechter M., Medoff G. and Eisenstein B.I., Mechanisms of Microbial Disease, 2th ed., Baltimore, Williams & Wilkins, 1993., p.231.
7. Maiden, M. C. and Stuart, J. M. (2002) Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination Lancet 359, 1829-1831
8. Baudner, B. and Rappuoli, R. (2008) Neisseria meningitidis. Immunity against mucosal pathogens.
9. Rouphael NG, Stephens DS. (2012) Neisseria meningitidis: biology, microbiology, and epidemiology. Methods Mol Biol. 799:1-20.
10. Fisseha, M., Chen, P., Brandt, B., Kijek, T., Moran, E., and Zollinger, W. (2005) Characterization of native outer membrane vesicles from lpxL mutant strains of Neisseria meningitidis for use in parenteral vaccination Infect. Immun. 73, 4070-4080.
11. Cartwright, K. (1995) Meningococcal Carriage and Disease, John Wiley & Sons, Chichester.
12. Frasch, C. E., Zollinger, W. D. and Poolman, J. T. (1985) Serotype antigens of Neisseria meningitidis and a proposed scheme for designation of serotypes. Rev. Infect. Dis. 7, 504–510.
13. Brehony, C., Jolley, K. A. and Maiden, M. C. (2007) Multilocus sequence typing for global surveillance of meningococcal disease. FEMS Microbiol. Rev. 31, 15–26.
14. Tettelin H, Saunders NJ, Heidelberg J et al (2000) Complete genome sequence of Neisseria meningitidis serogroup B strain MC58. Science 287:1809–15.
15. Parkhill J, Achtman M, James KD et al (2000) Complete DNA sequence of a serogroup A strain of Neisseria meningitidis Z2491. Nature 404:502–6.
16. Schoen C, Blom J, Claus H et al (2008) Whole-genome comparison of disease and carriage strains provides insights into virulence evolution in Neisseria meningitidis. Proc Natl Acad Sci USA 105:3473–8.
17. Uria MJ, Zhang Q, Li Y et al (2008) A generic mechanism in Neisseria meningitidis for enhanced resistance against bactericidal antibodies. J Exp Med 205:1423–34.
18. Frosch M, Weisgerber C, Meyer TF (1989) Molecular characterization and expression in Escherichia coli of the gene complex encoding the polysaccharide capsule of Neisseria meningitidis group B. Proc Natl Acad Sci USA 86:1669–73.
19. Swartley JS, Marfin AA, Edupuganti S, et al (1997) Capsule switching of Neisseria meningitidis. Proc Natl Acad Sci USA 94:271–6.
20. Claus, H., Vogel, U., Muhlenhoff, M., Gerardy-Schahn, R. and Frosch, M. (1997) Molecular divergence of the sia locus in different serogroups of Neisseria meningitidis expressing polysialic acid capsules. Mol. Gen. Genet. 257, 28–34.

21. Warren, L. and Blacklow, R. S. (1962) Biosynthesis of N-acetyl-neuraminic acid and cytidine-5 -monophospho-N-acetyl-neuraminic acid in Neisseria meningitidis. Biochem. Biophys. Res. Commun. 7, 433–438.
22. Swartley, J. S., Liu, L. J., Miller, Y. K., Martin, L. E., Edupuganti, S. and Stephens, D. S. (1998) Characterization of the gene cassette required for biosynthesis of the (α1→6)-linked N-acetyl-dmannosamine-1-phosphate capsule of serogroup A Neisseria meningitidis. J. Bacteriol. 180, 1533–1539.
23. Estabrook MM, Griffiss JM, Jarvis GA (1997) Sialylation of Neisseria meningitidis lipooligosaccharide inhibits serum bactericidal activity by masking lacto-N-neotetraose. Infect Immun 65:4436–44.
24. Kahler CM, Martin LE, Shih GC et al (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 66:5939–47.
25. Hobb RI, Tzeng YL, Choudhury BP et al (2010) Requirement of NMB0065 for connecting assembly and export of sialic acid capsular polysaccharides in Neisseria meningitidis. Microbes Infect 12:476–87.
26. Zimmer SM, Stephens DS (2006) Serogroup B meningococcal vaccines. Curr Opin Investig Drugs 7:733–9.
27. Nikaido H (1999) Microdermatology: cell surface in the interaction of microbes with the external world. J Bacteriol 181:4–8.
28. Girardin SE, Boneca IG, Viala J et al (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J Biol Chem 278:8869–72.
29. Antignac A, Rousselle JC, Namane A et al (2003) Detailed structural analysis of the peptidoglycan of the human pathogen Neisseria meningitidis. J Biol Chem 278:31521–8.
30. Jennings HJ, Johnson KG, Kenne L (1983) The structure of an R-type oligosaccharide core obtained from some lipopolysaccharides of Neisseria meningitidis. Carbohydr Res 121:233–41.
31. Gamian A, Beurret M, Michon F et al (1992) Structure of the L2 lipopolysaccharide core oligosaccharides of Neisseria meningitidis. J Biol Chem 267:922–5.
32. Kahler CM, Stephens DS (1998) Genetic basis for biosynthesis, structure, and function of meningococcal lipooligosaccharide (endotoxin). Crit Rev Microbiol 24:281–334.
33. Plant L, Sundqvist J, Zughaier S et al (2006) Lipooligosaccharide structure contributes to multiple steps in the virulence of Neisseria meningitidis. Infect Immun 74:1360–7.
34. Jennings, M. P., Srikhanta, Y. N., Moxon, E. R., Kramer,M., Poolman, J. T., Kuipers, B. and van der Ley, P. (1999) The genetic basis of the phase variation repertoire of lipopolysaccharide immunotypes in Neisseria meningitidis. Microbiology 145, 3013–3021.
35. Wright, J. C., Hood, D.W., Randle, G. A., Makepeace, K., Cox, A. D., Li, J., Chalmers, R., Richards, J. C. and Moxon, E. R. (2004) lpt6, a gene required for addition of phosphoethanolamine to inner-core lipopolysaccharide of Neisseria meningitidis and Haemophilus influenzae. J. Bacteriol. 186, 6970–6982.
36. Mandrell, R. E. and Zollinger, W.D. (1977) Lipopolysaccharide serotyping of Neisseria minigitidis by hemaglutination inhibition, Infect. Immun., 16, 471-475.
37. Zollinger, W.D. and Mandrell, R.E. (1977) Outer membrane protein and lipopolysaccharide serotyping of Neisseria meningitidis by inhibition of a solid-phase radioimmunoassay, Infect. Immun., 18, 424-433.
38. Zollinger, W.D. and Mandrell, R.E. (1980) Type-specific antigens of group A Neisseria meningitidis: lipopolysaccharide and heat modifiable outer membrane proteins, Infect. Immun., 28, 451-458.
39. Tsai, C.M., Mocca, L.F., and Frasch, C.E. (1987) Immunotype epitopes of Neisseria meningitidis lipooligosaccharide types 1 through 8, Infect. Immun., 55, 1652-1656.

40. Griffiss, J. M., Brandt, B., Engstrom, J., Schneider, H., Zollinger, W., and Gibson, B. (1994) Structural relationships and aialylation among meningococcal lipopolysaccharide (LOS) serotypes, in Proceedings of the Ninth International Pathogenic Neisseria Conference, Evans, J.S., Yost, S., Maiden, M. C. J., and Feavers, I. M., Eds. Winchester, England.
41. Mandrell, R. E., Griffiss, J. M., and Macher, B. A. (1988) Lipooligosaccharides (LOS) of Neisseria gonorrhoeae and Neisseria meningitidis have components that are immunochemically similar to precursors of human blood group antigens, J Exp. Med., 168, 107-126.
42. Tsai, C.M. and ivin, C.I. (1991) Eight lipooligosaccharides of Neisseria meningitidis react with a monoclonal antibody which binds lacto-N-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), Infect. Immun., 59, 3604-3609.
43. Verheul, A.F.M., Snippe, H., and Poolman, J.T. (1993) Meningococcal lipopolysaccharides: virulence factor and potential vaccine component, Micobiol. Rev., 57, 34-49.
44. Zughaier SM, Tzeng YL, Zimmer SM et al (2004) Neisseria meningitidis lipooligosaccharide structure-dependent activation of the macrophage CD14/Toll-like receptor 4 pathway.Infect Immun 72:371–80.
45. Zughaier S, Steeghs L, van der Ley P et al (2007) TLR4-dependent adjuvant activity of Neisseria meningitidis lipid A. Vaccine 25:4401–9.
46. Braun JM, Blackwell CC, Poxton IR et al (2002) Proinflammatory responses to lipooligosaccharide of Neisseria meningitidis immunotype strains in relation to virulence and disease. J Infect Dis 185:1431–8.
47. Brandtzaeg P, Kierulf P, Gaustad P et al (1989) Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J Infect Dis 159:195–204.
48. Brandtzaeg P, Bryn K, Kierulf P et al (1992) Meningococcal endotoxin in lethal septic shock plasma studied by gas chromatography, mass-spectrometry, ultracentrifugation, and electron microscopy. J Clin Invest 89: 816–23.
49. N.G. Rouphael and D.S. Stephens (2012) Neisseria meningitidis: biology, microbiology, and epidemiology. Methods Mol Biol. 799:1-20.
50. Pinner RW, Spellman PA, Stephens DS (1991) Evidence for functionally distinct pili expressed by Neisseria meningitidis. Infect Immun 59:3169–75.
51. Virji M, Alexandrescu C, Ferguson DJ et al (1992)Variations in the expression of pili: the effect on adherence of Neisseria meningitidis to human epithelial and endothelial cells. Mol Microbiol 6:1271–9.
52. Virji M, Watt SM, Barker S et al (1996) The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of Neisseria meningitidis and Neisseria gonorrhoeae. Mol Microbiol 22:929–39.
53. Hill DJ, Griffiths NJ, Borodina E et al (2010) Cellular and molecular biology of Neisseria meningitidis colonization and invasive disease. Clin Sci (Lond) 118:547–64.
54. Perkins-Balding D, Ratliff-Griffin M, Stojiljkovic I (2004) Iron transport systems in Neisseria meningitidis. Microbiol Mol Biol Rev 68:154–71.
55. Schryvers AB, Stojiljkovic I (1999) Iron acquisition systems in the pathogenic Neisseria. Mol Microbiol 32:1117–23.
56. Massari P, Ram S, Macleod H et al (2003) The role of porins in neisserial pathogenesis and immunity. Trends Microbiol 11:87–93.
57. Vermont CL, van Dijken HH, Kuipers AJ et al (2003) Cross-reactivity of antibodies against PorA after vaccination with a meningococcal B outer membrane vesicle vaccine. Infect Immun 71:1650–5.
58. Jafri RZ, Ali A, Messonnier NE, et al. Global epidemiology of invasive meningococcal disease. Popul Health Metr 2013;11:17. doi: 10.1186/1478-7954-11-17.
59. Chang Q, Tzeng YL, Stephens DS. Meningococcal disease: changes in epidemiology and prevention. Clin Epidemiol 2012;4:237-45. doi: 10.2147/CLEP.S28410.

60. Harrison LH. Epidemiological profile of meningococcal disease in the United States. Clin Infect Dis 2010;50:S37-S44.
61. Dwilow R, Fanella S. Invasive meningococcal disease in the 21st century-an update for the clinicians. Curr Neurol Neurosci Rep 2015;15:2-9.
62. Halperin SA, Bettinger JA, Greenwood B, et al. The changing and dynamic epidemiology of meningococcal disease. Vaccine 2012;30:B26-36.
63. Lapeyssonnie L. La m´eningite c´er´ebrospinale en Afrique. Bull World Health Organ 1963;28:1–114.
64. Funk A, Uadiale K, Kamau C, et al. Sequential outbreaks due to new strain of Neisseria Meningitidis Serogroup C in Northern Nigeria, 2013-2014. PLOS Currents Outbreaks 2014;6.
65. Xie O, Pollard AJ, Mueller J, et al. Emergence of serogroup X meningococcal disease in Africa: need for a vaccine. Vaccine 2013;31:2852-61.
66. Törös B, Thulin Hedberg S, Jacobsson S, et al. Surveillance of invasive Neisseria meningitidis with a serogroup Y update, Sweden 2010 to 2012. Euro Surveill 2014;19: pii 20940.
67. Rosenstein NE, Perkins BA, Stephens DS, Lefkowitz L, Cartter ML, Danila R, et al. The changing epidemiology of meningococcal disease in the United States, 1992–1996. J Infect Dis 1999;180(6):1894–901.
68. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. I. The role of humoral antibodies. J Exp Med 1969;129:1307–26.
69. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. II. Development of natural immunity. J Exp Med 1969;129(6):1327–48.
70. Jackson LA,Wenger JD. Laboratory-based surveillance formeningococcal disease in selected areas, United States, 1989–1991. MMWR CDC Surveill Summ 1993;42(2):21–30.
71. Harrison LH, Broome CV. The epidemiology of meningococcal meningitis in the U.S. civilian population. In: Vedros NA, editor. Evolution of Meningococcal Disease. Boca Raton, FL: CRC Press; 1987. p. 27–45.
72. Jones IR, Urwin G, Feldman RA, Banatvala N. Social deprivation and bacterial meningitis in north east Thames region: three year study using small area statistics. BMJ 1997;314(7083):794–5.
73. Platonov AE, Vershinina IV, Kuijper EJ, Borrow R, Kayhty H. Long term effects of vaccination of patients deficient in a late complement component with a tetravalent meningococcal polysaccharide vaccine. Vaccine 2003;21(27–30):4437–47.
74. Stephens DS, Hajjeh RA, Baughman WS, Harvey RC, Wenger JD, Farley MM. Sporadic meningococcal disease in adults: results of a 5-year populationbased study. Ann Intern Med 1995;123(12):937–40.
75. Holmes FF,Weyandt T, Glazier J, Cuppage FE, Moral LA, Lindsey NJ. Fulminant meningococcemia after splenectomy. JAMA 1981;246(10):1119–20.
76. Figueroa JE, Densen P. Infectious diseases associated with complement deficiencies. Clin Microbiol Rev 1991;4(3):359–95.
77. Francke EL, Neu HC. Postsplenectomy infection. Surg Clin North Am 1981;61(1):135–55.
78. Salimans MM, Bax WA, Stegeman F, van Deuren M, Bartelink AK, van Dijk H. Association between familial deficiency of mannose-binding lectin and mutations in the corresponding gene and promoter region. Clin Diagn Lab Immunol 2004;11(4):806–7.
79. Kuipers S, Aerts PC, Cluysenaer OJ, Bartelink AK, Ezekowitz RA, BaxWA, et al. A case of familial meningococcal disease due to deficiency in mannose-binding lectin (MBL). Adv Exp Med Biol 2003;531:351–5.
80. Emonts M, Hazelzet JA, de Groot R, Hermans PW. Host genetic determinants of Neisseria meningitidis infections. Lancet Infect Dis 2003;3(9):565–77.

81. Smirnova I, Mann N, Dols A, Derkx HH, Hibberd ML, Levin M, et al. Assay of locus-specific genetic load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility. Proc Natl Acad Sci USA 2003;100(10): 6075–80.
82. Tappero JW, Reporter R,Wenger JD,Ward BA, Reeves MW, Missbach TS, et al. Meningococcal disease in Los Angeles County, California, and among men in the county jails. N Engl J Med 1996;335(12):833–40.
83. Imrey PB, Jackson LA, Ludwinski PH, England 3rd AC, Fella GA, Fox BC, et al. Outbreak of serogroup C meningococcal disease associated with campus bar patronage. Am J Epidemiol 1996;143(6):624–30.
84. Imrey PB, Jackson LA, Ludwinski PH, England 3rd AC, Fella GA, Fox BC, et al. Meningococcal carriage, alcohol consumption, and campus bar patronage in a serogroup C meningococcal disease outbreak. J Clin Microbiol 1995;33(12):3133–7.
85. Fischer M, Hedberg K, Cardosi P, Plikaytis BD, Hoesly FC, Steingart KR, et al. Tobacco smoke as a risk factor for meningococcal disease. Pediatr Infect Dis J 1997;16(10):979–83.
86. Cookson ST, Corrales JL, Lotero JO, Regueira M, Binsztein N, Reeves MW, et al. Disco fever: epidemic meningococcal disease in northeastern Argentina associated with disco patronage. J Infect Dis 1998;178(1):266–9.
87. Froeschle JE. Meningococcal disease in college students. Clin Infect Dis 1999;29(1):215–6.
88. Harrison LH, Dwyer DM, Maples CT, Billmann L. Risk of meningococcal infection in college students. JAMA 1999;281(20):1906–10.
89. Bruce MG, Rosenstein NE, Capparella JM, Shutt KA, Perkins BA, Collins M. Risk factors for meningococcal disease in college students. JAMA 2001;286(6):688–93.
90. MacLennan J, Kafatos G, Neal K, Andrews N, Cameron JC, Roberts R, et al. Social behavior and meningococcal carriage in British teenagers. Emerg Infect Dis 2006;12(6):950–7.
91. Neal KR, Nguyen-Van-Tam JS, Jeffrey N, Slack RC, Madeley RJ, Ait-Tahar K, et al. Changing carriage rate of Neisseria meningitidis among university students during the first week of term: cross sectional study. Br Med J 2000;320(7238):846–9.
92. Olcen P, Kjellander J, Danielsson D, Lindquist BL. Epidemiology of Neisseria meningitidis; prevalence and symptoms from the upper respiratory tract in family members to patients with meningococcal disease. Scand J Infect Dis 1981;13(2):105–9.
93. Young LS, LaForce FM, Head JJ, Feeley JC, Bennett JV. A simultaneous outbreak of meningococcal and influenza infections. N Engl J Med 1972;287(1): 5–9.
94. Harrison LH, Armstrong CW, Jenkins SR, HarmonMW, Ajello GW,Miller Jr GB, et al. A cluster of meningococcal disease on a school bus following epidemic influenza. Arch Intern Med 1991;151(5):1005–9.
95. Moore PS, Hierholzer J, DeWittW, Gouan K, Djore D, Lippeveld T, et al. Respiratory viruses andmycoplasma as cofactors for epidemic groupAmeningococcal meningitis. JAMA 1990;264(10):1271–5.
96. Krasinski K, Nelson JD, Butler S, Luby JP, Kusmiesz H. Possible association of mycoplasma and viral respiratory infections with bacterial meningitis. Am J Epidemiol 1987;125(3):499–508.
97. Cartwright KA, Jones DM, Smith AJ, Stuart JM, Kaczmarski EB, Palmer SR. Influenza A and meningococcal disease. Lancet 1991;338(8766):554–7.
98. Greenwood BM, Bradley AK, Cleland PG, Haggie MH, Hassan-King M, Lewis LS, et al. An epidemic of meningococcal infection at Zaria. Northern Nigeria. 1. General epidemiological features. Trans R Soc Trop Med Hyg 1979;73(5):557–62.
99. Brundage JF, Zollinger WD. Evolution of meningococcal disease epidemiology in the US army. In: Vedros NA, editor. Evolution of Meningococcal Disease. Boca Raton, FL: CRC Press; 1987. p. 5–25.

100. Andrews TD, Gojobori T. Strong positive selection and recombination drive the antigenic variation of the PilE protein of the human pathogen Neisseria meningitidis. Genetics 2004;166(1):25–32.
101. Howell-Adams B, Seifert HS. Molecular models accounting for the gene conversion reactions mediating gonococcal pilin antigenic variation. Mol Microbiol 2000;37(5):1146–58.
102. Dolan-Livengood JM,Miller YK, Martin LE,Urwin R, Stephens DS. Genetic basis for nongroupable Neisseria meningitidis. J Infect Dis 2003;187(10):1616–28.
103. Saunders NJ, Jeffries AC, Peden JF, Hood DW, Tettelin H, Rappuoli R, et al. Repeat-associated phase variable genes in the complete genome sequence of Neisseria meningitidis strain MC58. Mol Microbiol 2000;37(1): 207–15.
104. Claus H, Borrow R, Achtman M, Morelli G, Kantelberg C, Longworth E, et al. Genetics of capsule O-acetylation in serogroup C,W-135 and Y meningococci. Mol Microbiol 2004;51(1):227–39.
105. Stephens DS,Swartley JS, Kathariou S, Morse SA. Insertion of Tn916 in Neisseria meningitidis resulting in loss of group B capsular polysaccharide. InfectImmun 1991;59(11):4097–102.
106. Uria MJ, Zhang Q, Li Y, ChanA, Exley RM, Gollan B, et al.Ageneric mechanism in Neisseria meningitidis for enhanced resistance against bactericidal antibodies. J Exp Med 2008;205(6):1423–34.
107. Swartley JS, Marfin AA, Edupuganti S, Liu LJ, Cieslak P, Perkins B, et al. Capsule switching of Neisseria meningitidis. Proc Natl Acad Sci USA 1997;94(1):271–6.
108. Aguilera JF, Perrocheau A, Meffre C, Hahne S. Outbreak of serogroup W135 meningococcal disease after the Hajj pilgrimage, Europe, 2000. Emerg Infect Dis 2002;8(8):761–7.
109. VogelU, Claus H, FroschM.Rapid serogroup switching inNeisseria meningitidis. N Engl J Med 2000;342(3):219–20.
110. Kertesz DA, Coulthart MB, Ryan JA, Johnson WM, Ashton FE. Serogroup B, electrophoretic type 15 Neisseria meningitidis in Canada. J Infect Dis 1998;177(6):1754–7.
111. Mayer LW, Reeves MW, Al-Hamdan N, Sacchi CT, Taha MK, Ajello GW, et al. Outbreak of W135 meningococcal disease in 2000: not emergence of a new W135 strain but clonal expansion within the electophoretic type-37 complex. J Infect Dis 2002;185(11):1596–605.
112. Tzeng YL, Stephens DS, 2000 Epidemiology and pathogenesis of Neisseria meningitidis. Microbes Infect. 2(6):687-700.
113. SP Yazdankhah, D Caugant Neisseria meningitidis: An overview of the carriage state J Med Microbiol, 53 (2004), pp. 821–832.
114. DS Stephens, LH Hoffman, ZA McGee Interaction of Neisseria meningitidis with human nasopharyngeal mucosal: attachment and entry into columnar epithelial cells J Infect Dis, 148 (1983), pp. 369–376.
115. DS Stephens Uncloaking the meningococcus: dynamics of carriage and disease Lancet, 353 (1999), pp. 941–942.
116. KA Cartwright, JM Stuart, DM Jones, ND Noah The Stonehouse survey: nasopharyngeal carriage of meningococci and Neisseria lactamica Epidemiol Infect, 99 (1987), pp. 591–601.
117. K Cartwright Meningococcal carriage and disease K Cartwright (Ed.), Meningococcal Disease, Wiley & Sons, Chichester (1994), pp. 115–146.
118. JE Mueller, S Yaro, Y Traore, et al. Neisseria meningitidis serogroups A and W-135: carriage and immunity in Burkina Faso, 2003 J Infect Dis, 193 (2006), pp. 812–820.
119. Goldschneider, EC Gotschlich, MS Artenstein Human immunity to the meningococcus. I. The role of humoral antibodies J Exp Med, 129 (1969), pp. 1307–1326.

120. Carbonnelle E et al.: A systematic genetic analysis in Neisseria meningitidis defines the Pil proteins required for assembly: functionality, stabilization and export of Type IV pili. Mol Microbiol 2006, 61:1510-1522.
121. Coureuil M et al.: Meningococcus Hijacks a beta2-adrenoceptor/beta-Arrestin pathway to cross brain microvasculature endothelium. Cell 2010, 143:1149-1160.
122. Lecuyer H, Nassif X, Coureuil M: Two strikingly different signaling pathways are induced by meningococcal Type IV pili on endothelial and epithelial cells. Infect Immun 2012, 80:175-186.
123. Bernard SC et al.: Pathogenic Neisseria meningitidis utilizes CD147 for vascular colonization. Nat Med 2014, 20:725-731.
124. DS Stephens, (2009) Biology and pathogenesis of the evolutionarily successful, obligate human bacterium Neisseria meningitidis Vaccine, 27 Suppl 2:B71-77.
125. C.M. Kahler, L.E. Martin, G. Shih, R.W. Carlson, D.S. Stephens The (α2 → 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 (12) (1998), pp. 5939–5947.
126. D.S. Stephens, B. Greenwood, P. Brandtzaeg Epidemic meningitis, meningococcemia and Neisseria meningitidis Lancet, 369 (9580) (2007), pp. 2196–2210.
127. Brandtzaeg, P., Bjerre, A., Ovstebo, R., Brusletto, B., Joo,G. B. and Kierulf, P. (2001) Neisseria meningitidis lipopolysaccharides in human pathology. J. Endotoxin Res. 7, 401–420.
128. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J. and Gusovsky, F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J. Biol. Chem. 274, 10689–10692.
129. Fransen, F., Heckenberg, S. G., Hamstra, H. J., Feller, M., Boog, C. J., van Putten, J. P., van de Beek, D., van der Ende, A. and van der Ley, P. (2009) Naturally occurring lipid A mutants in Neisseria meningitidis from patients with invasive meningococcal disease are associated with reduced coagulopathy. PLoS Pathog. 5, e1000396.
130. Geoffroy, M. C., Floquet, S., Metais, A., Nassif, X. And Pelicic, V. (2003) Large-scale analysis of the meningococcus genome by gene disruption: resistance to complement-mediated lysis. Genome Res. 13, 391–398.
131. Madico, G., Welsch, J. A., Lewis, L. A., McNaughton, A., Perlman, D. H., Costello, C. E., Ngampasutadol, J., Vogel, U., Granoff, D. M. and Ram, S. (2006) The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. J. Immunol. 177, 501–510.
132. Jarva, H., Ram, S., Vogel, U., Blom, A. M. and Meri, S. (2005) Binding of the complement inhibitor C4bp to serogroup B Neisseria meningitidis. J. Immunol. 174, 6299–6307.
133. Nassif, X., Pujol, C., Morand, P. and Eugene, E. (1999) Interactions of pathogenic Neisseria with host cells. Is it possible to assemble the puzzle? Mol. Microbiol. 32, 1124–1132.
134. Massari, P., Henneke, P., Ho, Y., Latz, E., Golenbock, D. T. and Wetzler, L. M. (2002) Cutting edge: Immune stimulation by neisserial porins is toll-like receptor 2 and MyD88 dependent. J. Immunol. 168, 1533–1537.
135. Massari, P., Ho, Y. and Wetzler, L. M. (2000) Neisseria meningitidis porin PorB interacts with mitochondria and protects cells from apoptosis. Proc. Natl. Acad. Sci. U.S.A. 97, 9070–9075.
136. Virji, M. and Griffiths, N. J. (2008) Binding of Opc to vitronectin contributes to increased serum resistance of Neisseria meningitidis isolates, Sixteenth International Pathogenic Neisseria Conference 2008, Rotterdam, The Netherlands, P096.
137. Wiertz, E. J., Delvig, A., Donders, E. M., Brugghe, H. F., van Unen, L. M., Timmermans, H. A., Achtman, M., Hoogerhout, P. and Poolman, J. T. (1996) T-cell responses to outer membrane proteins of Neisseria meningitidis: comparative study of the Opa, Opc, and PorA proteins. Infect. Immun. 64, 298–304.

138. Lee, H. S., Boulton, I. C., Reddin, K., Wong, H., Halliwell, D., Mandelboim, O., Gorringe, A. R. and Gray-Owen, S. D. (2007) Neisserial outer membrane vesicles bind the coinhibitory receptor carcinoembryonic antigen-related cellular adhesion molecule 1 and suppress CD4+ T lymphocyte function. Infect. Immun. 75, 4449–4455.
139. Wedege, E., Bolstad, K., Aase, A., Herstad, T. K., McCallum, L., Rosenqvist, E., Oster, P. and Martin, D. (2007) Functional and specific antibody responses in adult volunteers in new zealand who were given one of two different meningococcal serogroup B outer membrane vesicle vaccines. Clin. Vaccine Immunol. 14, 830–838.
140. Youssef, A. R., van der Flier, M., Estevao, S., Hartwig, N. G., van der Ley, P. and Virji, M. (2009) Both Opa+ and Opa- pathogenic Neisseria induce sustained proliferative response in human CD4+ T cells. Infect. Immun. 77, 5170–5180.
141. Chen, T., Bolland, S., Chen, I., Parker, J., Pantelic, M., Grunert, F. and Zimmermann, W. (2001) The CGM1a (CEACAM3/CD66d)-mediated phagocytic pathway of Neisseria gonorrhoeae expressing opacity proteins is also the pathway to cell death. J. Biol. Chem. 276, 17413–1741954.
142. Franzoso, S., Mazzon, C., Sztukowska, M., Cecchini, P., Kasic, T., Capecchi, B., Tavano, R. and Papini, E. (2008) Human monocytes/macrophages are a target of Neisseria meningitidis adhesin A (NadA). J. Leukocyte Biol. 83, 1100–1110.
143. Tavano, R., Franzoso, S., Cecchini, P., Cartocci, E., Oriente, F., Arico, B. and Papini, E. (2009) The membrane expression of Neisseria meningitidis adhesin A (NadA) increases the proimmune effects of MenB OMVs on human macrophages, compared with NadA-OMVs, without further stimulating their proinflammatory activity on circulating monocytes. J. Leukocyte Biol. 86, 143–153.
144. Stephens DS, Greenwood B, Brandtzaeg P: Epidemic meningitis: meningococcaemia, and Neisseria meningitidis. Lancet 2007, 369:2196-2210.
145. Zimmer, S. M. and Stephens, D. S. (2006) Serogroup B meningococcal vaccines Curr. Opin. Investig. Drugs 7, 733-739.
146. Stein DM et al.: Are antibodies to the capsular polysaccharide of Neisseria meningitidis group B and Escherichia coli K1 associated with immunopathology? Vaccine 2006, 24:221-228.
147. Holst J et al.: Properties and clinical performance of vaccines containing outer membrane vesicles from Neisseria meningitidis. Vaccine 2009, 27:B3-B12.
148. Giuliani MM et al.: A universal vaccine for serogroup B meningococcus. Proc Natl Acad Sci U S A 2006, 103: 10834-10839.
149. Martin NG, Snape MD: A multicomponent serogroup B meningococcal vaccine is licensed for use in Europe: what do we know, and what are we yet to learn? Expert Rev Vaccines 2013, 12:837-858.
150.Plotkin SA (2008) Correlates of vaccine-induced immunity. Clin Infect Dis 47: 401–409.
151. Richmond PC et al.: Safety, immunogenicity, and tolerability of meningococcal serogroup B bivalent recombinant lipoprotein 2086 vaccine in healthy adolescents: a randomised, singleblind, placebo-controlled, phase 2 trial. Lancet Infect Dis 2012, 12:597-607.
152. Koeberling O., Ispasanie E., Hauser J., Rossi O., Pluschke G., Caugant DA., Saul A., MacLennan CA., (2014) A broadly-protective vaccine against meningococcal disease in sub-Saharan Africa based on generalized modules for membrane antigens (GMMA). Vaccine 32(23):2688-95.
153. Berlanda Scorza F, Colucci AM, Maggiore L, Sanzone S, Rossi O, Ferlenghi I, Pesce I, Caboni M, Norais N, Di Cioccio V, Saul A, Gerke C. High yield production process for Shigella outer membrane particles. PLoS One. 2012;7(6):e35616. doi: 10.1371/journal.pone.0035616. Epub 2012 Jun 6.
154. Ferrari, G., Garaguso, I., Adu-Bobie, J., Doro, F., Taddei, A. R., Biolchi, A., Brunelli, B., Giuliani, M. M., Pizza, M., Norais, N., and Grandi, G. (2006) Outer membrane vesicles from

group B Neisseria meningitidis delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles Proteomics. 6, 1856-1866.
155. Maiden CJ, Stuart JM, for the UK Meningococcal Carriage Group. (2002) Carriage of serogroup C meningococci 1 year after meningococcal C conjugate polysaccharide vaccination. Lancet 359: 1829–30.
156. Kuehn, M. J. and Kesty, N. C. (2005) Bacterial outer membrane vesicles and the host-pathogen interaction Genes Dev. 19, 2645-2655.
157. Beveridge, T. J. (1999) Structures of gram-negative cell walls and their derived membrane vesicles J. Bacteriol. 181, 4725-4733.
158. Kulp, A. and Kuehn, M. J. (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles Annu. Rev. Microbiol. 64, 163-184.
159. McBroom, A. J., Johnson, A. P., Vemulapalli, S., and Kuehn, M. J. (2006) Outer membrane vesicle production by Escherichia coli is independent of membrane instability J. Bacteriol. 188, 5385-5392.
160. Henry, T., Pommier, S., Journet, L., Bernadac, A., Gorvel, J. P., and Lloubes, R. (2004) Improved methods for producing outer membrane vesicles in Gram-negative bacteria Res. Microbiol. 155, 437-446.
161. Collins, B. S. (2011) Gram-negative outer membrane vesicles in vaccine development Discov. Med. 12, 7-15.
162. Kesty, N. C. and Kuehn, M. J. (2004) Incorporation of heterologous outer membrane and periplasmic proteins into Escherichia coli outer membrane vesicles J. Biol. Chem. 279, 2069-2076.
163. Alaniz, R. C., Deatherage, B. L., Lara, J. C., and Cookson, B. T. (2007) Membrane vesicles are immunogenic facsimiles of Salmonella typhimurium that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity in vivo J. Immunol. 179, 7692-7701.
164. Parker, H., Chitcholtan, K., Hampton, M. B., and Keenan, J. I. (2010) Uptake of Helicobacter pylori outer membrane vesicles by gastric epithelial cells Infect. Immun. 78, 5054-5061.
165. Schild, S., Nelson, E. J., Bishop, A. L., and Camilli, A. (2009) Characterization of Vibrio cholerae outer membrane vesicles as a candidate vaccine for cholera Infect. Immun. 77, 472-484.
166. Leitner, D. R., Feichter, S., Schild-Prufert, K., Rechberger, G. N., Reidl, J., and Schild, S. (2013) Lipopolysaccharide modifications of a cholera vaccine candidate based on outer membrane vesicles reduce endotoxicity and reveal the major protective antigen Infect. Immun. 81, 2379-2393.
167. Mitra, S., Barman, S., Nag, D., Sinha, R., Saha, D. R., and Koley, H. (2012) Outer membrane vesicles of Shigella boydii type 4 induce passive immunity in neonatal mice FEMS Immunol. Med. Microbiol. 66, 240-250.
168. Koeberling, O., Seubert, A., and Granoff, D. M. (2008) Bactericidal antibody responses elicited by a meningococcal outer membrane vesicle vaccine with overexpressed factor H-binding protein and genetically attenuated endotoxin J. Infect. Dis. 198, 262-270.
169. Zollinger, W. D., Donets, M. A., Schmiel, D. H., Pinto, V. B., Labrie, J. E., III, Moran, E. E., Brandt, B. L., Ionin, B., Marques, R., Wu, M., Chen, P., Stoddard, M. B., and Keiser, P. B. (2010) Design and evaluation in mice of a broadly protective meningococcal group B native outer membrane vesicle vaccine Vaccine 28, 5057-5067.
170. Park, S. B., Jang, H. B., Nho, S. W., Cha, I. S., Hikima, J., Ohtani, M., Aoki, T., and Jung, T. S. (2011) Outer membrane vesicles as a candidate vaccine against edwardsiellosis PLoS. One. 6, e17629.

171. Berlanda Scorza, F., D'Oro, F., Rodriguez-Ortega, M. J., Stella, M., Liberatori, S., Taddei, A. R., Serino, L., Gomes, M. D., Nesta, B., Fontana, M. R., Spagnuolo, A., Pizza, M., Norais, N., and Grandi, G. (2008) Proteomics characterization of outer membrane vesicles from the extraintestinal pathogenic Escherichia coli DeltatolR IHE3034 mutant Mol. Cell Proteomics. 7, 473-485.
172. Kuehn, M. J. and Kesty, N. C. (2005) Bacterial outer membrane vesicles and the host-pathogen interaction Genes Dev. 19, 2645-2655.
173. Feavers IM, Pizza M, (2009) Meningococcal protein antigens and vaccines. Vaccine 27 Suppl 2:B42-50.
174. Pizza M, Scarlato V, Masignani V, Giuliani MM, Aricò B, Comanducci M, Jennings GT, Baldi L, Bartolini E, Capecchi B, Galeotti CL, Luzzi E, Manetti R, Marchetti E, Mora M, Nuti S, Ratti G, Santini L, Savino S, Scarselli M, Storni E, Zuo P, Broeker M, Hundt E, Knapp B, Blair E, Mason T, Tettelin H, Hood DW, Jeffries AC, Saunders NJ, Granoff DM, Venter JC, Moxon ER, Grandi G, Rappuoli R. (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science. 287(5459):1816-20.
175. Masignani V, Comanducci M, Giuliani MM, Bambini S, Adu-Bobie J, Arico B, Brunelli B, Pieri A, Santini L, Savino S, Serruto D, Litt D, Kroll S, Welsch JA, Granoff DM, Rappuoli R, Pizza M. 2003. Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870. J. Exp. Med. 197:789-799.
176. Gossger N, Snape MD, Yu LM, Finn A, Bona G, Esposito S, Principi N, Diez-Domingo J, Sokal E, Becker B, Kieninger D, Prymula R, Dull P, Ypma E, Toneatto D, Kimura A, Pollard AJ. 2012. Immunogenicity and tolerability of recombinant serogroup B meningococcal vaccine administered with or without routine infant vaccinations according to different immunization schedules: a randomized controlled trial. JAMA 307:573-582.
177. Richmond PC, Marshall HS, Nissen MD, Jiang Q, Jansen KU, Garces-Sanchez M, Martinon-Torres F, Beeslaar J, Szenborn L, Wysocki J, Eiden J,Harris SL, Jones TR, Perez JL. 2012. Safety, immunogenicity, and tolerability of meningococcal serogroup B bivalent recombinant lipoprotein 2086 vaccine in healthy adolescents: a randomised, single-blind, placebo-controlled, phase 2 trial. Lancet Infect. Dis. 12:597-607.
178. Santolaya ME, O'Ryan ML, Valenzuela MT, Prado V, Vergara R, Munoz A, Toneatto D, Grana G, Wang H, Clemens R, Dull PM. 2012. Immunogenicity and tolerability of a multicomponent meningococcal serogroup B (4CMenB) vaccine in healthy adolescents in Chile: a phase 2b/3 randomised, observer blind, placebo-controlled study. Lancet 379:617-624.
179. Marshall HS, Richmond PC, Nissen MD, Wouters A, Baber J, Jiang Q, Anderson AS, Jones TR, Harris SL, Jansen KU, Perez JL. 2013. A phase 2 open-label safety and immunogenicity study of a meningococcal B bivalent rLP2086 vaccine in healthy adults. Vaccine.
180. Bai X, Findlow J, Borrow R. 2011. Recombinant protein meningococcal serogroup B vaccine combined with outer membrane vesicles. Expert Opin. Biol. Ther. 11:969-985.
181. Schneider MC, Prosser BE, Caesar JJ, Kugelberg E, Li S, Zhang Q, Quoraishi S, Lovett JE, Deane JE, Sim RB, Roversi P, Johnson S, Tang CM, Lea SM. 2009. Neisseria meningitidis recruits factor H using protein mimicry of host carbohydrates. Nature 458:890-893.
182. Cantini F, Veggi D, Dragonetti S, Savino S, Scarselli M, Romagnoli G, Pizza M, Banci L, Rappuoli R. (2009) Solution structure of factor H binding protein, a survival factor and protective antigen of Neisseria meningitidis J Biol Chem. 284(14):9022-6.
183. Mascioni A, Jacob J, Moy F, Dilts D, Fink P, Malakian K, Sigethy S, Wen Y, Novikova E, Zlotnick GW, Tsao DH, (2009) Biomol NMR Assign. Backbone and side-chain assignment of the lipidated and non-lipidated forms of the meningococcal outer membrane protein LP2086. 3(1):111-3.
184. Mascioni A, Bentley BE, Camarda R, Dilts DA, Fink P, Gusarova V, Hoiseth SK, Jacob J, Lin SL, Malakian K, McNeil LK, Mininni T, Moy F, Murphy E, Novikova E, Sigethy S, Wen Y,

Zlotnick GW, Tsao DH. (2009) Structural Basis for the Immunogenic Properties of the Meningococcal Vaccine Candidate LP2086. J Biol Chem. 284(13):8738-46.
185. Fletcher LD, Bernfield L, Barniak V, Farley JE, Howell A, Knauf M, Ooi P, Smith RP, Weise P, Wetherell M, Xie X, Zagursky R, Zhang Y, Zlotnick GW. 2004. Vaccine potential of the Neisseria meningitidis 2086 lipoprotein. Infect. Immun. 72:2088-2100.
186. Murphy E, Andrew L, Lee KL, Dilts DA, Nunez L, Fink PS, Ambrose K, Borrow R, Findlow J, Taha MK, Deghmane AE, Kriz P, Musilek M, Kalmusova J, Caugant DA, Alvestad T, Mayer LW, Sacchi CT, Wang X, Martin D, von Gottberg A, du Plessis M, Klugman KP, Anderson AS, Jansen KU, Zlotnick GW, Hoiseth SK. 2009. Sequence diversity of the factor H binding protein vaccine candidate in epidemiologically relevant strains of serogroup B Neisseria meningitidis. J. Infect. Dis. 200:379-389.
187. Pajon R, Beernink PT, Harrison LH, Granoff DM. 2010. Frequency of factor H binding protein modular groups and susceptibility to cross-reactive bactericidal activity in invasive meningococcal isolates. Vaccine 28:2122-2129.
188. Wang X, Cohn A, Comanducci M, Andrew L, Zhao X, MacNeil JR, Schmink S, Muzzi A, Bambini S, Rappuoli R, Pizza M, Murphy E, Hoiseth SK, Jansen KU, Anderson AS, Harrison LH, Clark TA, Messonnier NE, Mayer LW. 2011. Prevalence and genetic diversity of candidate vaccine antigens among invasive Neisseria meningitidis isolates in the United States. Vaccine 29:4739-4744.
189. Seib KL, Brunelli B, Brogioni B, Palumbo E, Bambini S, Muzzi A, DiMarcello F, Marchi S, van der Ende A, Arico B, Savino S, Scarselli M, Comanducci M, Rappuoli R, Giuliani MM, Pizza M. 2011. Characterization of diverse subvariants of the meningococcal factor H (fH) binding protein for their ability to bind fH, to mediate serum resistance, and to induce bactericidal antibodies. Infect. Immun. 79:970-981.
190. Pajon R, Fergus AM, Koeberling O, Caugant DA, Granoff DM. (2011) Meningococcal factor H binding proteins in epidemic strains from Africa: implications for vaccine development. PLoS Negl Trop Dis. 5(9):e1302.
191. De Cordoba SR, de Jorge EG. 2008. Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. Clin. Exp. Immunol. 151:1-13.
192. Perkins SJ, Nan R, Li K, Khan S, Miller A. 2012. Complement factor H-ligand interactions: self-association, multivalency and dissociation constants. Immunobiology 217:281-297.
193. Madico G, Welsch JA, Lewis LA, McNaughton A, Perlman DH, Costello CE, Ngampasutadol J, Vogel U, Granoff DM, Ram S. (2006) The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. J Immunol. 177(1):501-10.
194. Schneider MC, Exley RM, Chan H, Feavers I, Kang YH, Sim RB, Tang CM. 2006. Functional significance of factor H binding to Neisseria meningitidis. J. Immunol. 176:7566-7575.
195. Beernink PT, LoPasso C, Angiolillo A, Felici F, Granoff D. 2009. A region of the N-terminal domain of meningococcal factor H-binding protein that elicits bactericidal antibody across antigenic variant groups. Mol. Immunol. 46:1647-1653.
196. Malito E, Faleri A, Lo Surdo P, Veggi D, Maruggi G, Grassi E, Cartocci E, Bertoldi I, Genovese A, Santini L, Romagnoli G, Borgogni E, Brier S, Lo Passo C, Domina M, Castellino F, Felici F, van der Veen S, Johnson S, Lea SM, Tang CM, Pizza M, Savino S, Norais N, Rappuoli R, Bottomley MJ,Masignani V. 2013. Defining a protective epitope on factor H binding protein, a key meningococcal virulence factor and vaccine antigen. Proc. Natl. Acad. Sci. U. S. A. 110:3304-3309.
197. Vu DM, Pajon R, Reason DC, Granoff DM. 2012. A Broadly Cross-Reactive Monoclonal Antibody Against an Epitope on the N-terminus of Meningococcal fHbp. Sci. Rep. 2:341.

198. Van der Ley P, Steeghs L, Hamstra HJ, ten Hove J, Zomer B, van Alphen L. (2001) Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. Infect Immun. 69(10):5981-90.
199. Ferrari G, Garaguso I, Adu-Bobie J, Doro F, Taddei AR, Biolchi A, Brunelli B, Giuliani MM, Pizza M, Norais N, Grandi G. (2006) Outer membrane vesicles from group B Neisseria meningitidis delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. Proteomics. 6(6):1856-66.
200. Tikhomirov E., Santamaria M., Esteves K., (1997)Meningococcal disease: public health burden and control, Wld Hlth Statist. Quart. 50 (1997).
201. Pinner R.W., Onyango F., Perkins B.A., Mirza N.B., Ngacha D.M., Reeves M., Dewitt W., Njeru E., Agata N.N., Broome C.V., (1992) Epidemic meningococcal disease in Nairobi, Kenya, 1988. The Kenya/Centers for Disease Control (CDC) Meningitis Study Group, J. Infect. Dis. 359–364.
202. Jackson L.A., Schuchat A., Reeves M.W., Wenger J.D., (1995) Serogroup C meningococcal outbreaks in the United States. An emerging threat, JAMA 383–389.
203. Maiden MC, Malorny B, Achtman M. (1996) A global gene pool in the Neisseriae. Mol Microbiol 21(6):1297–8.
204. Linz B, Schenker M, Zhu P,Achtman M. (2000) Frequent interspecific genetic exchange between commensal Neisseriae and Neisseria meningitidis. Mol Microbiol 36(5):1049–58.
205. Artenstein MS, Gold R, Zimmerly JG, Wyle FA, Schneider H, Harkins C. (1970) Prevention of meningococcal disease by group C polysaccharide vaccine. N Engl J Med 282: 417–20.
206. Gold R, Lepow ML, Goldschneider I, Draper TL, Gotschlich EC. (1975) Clinical evaluation of group A and group C meningococcal polysaccharide vaccines in infants. J Clin Invest 56: 1536–47.
207. Committee on Infectious Diseases. (2000) Meningococcal disease prevention and control strategies for practice-based physicians (Addendum: recommendations for college students). Pediatrics 106: 1500–04.
208. Trotter CL, Andrews NJ, Kaczmarski EB, Miller E, Ramsay ME. (2004) Effectiveness of meningococcal serogroup C conjugate vaccine 4 years after introduction. Lancet 364: 365–67.
209. Ramsay ME, Andrews NJ, Trotter CL, Kaczmarski EB, Miller E. (2003) Herd immunity from meningococcal serogroup C conjugate vaccination in England: database analysis. BMJ 326: 36 566.
210. Larrauri A, Cano R, Garcia M, Mateo S. (2005) Impact and eff ectiveness of meningococcal C conjugate vaccine following its introduction in Spain. Vaccine 23: 4097–100.
211. Bilukha OO, Rosenstein N. (2005) Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep 54: 1–21.
212. Trotter CL, Edmunds WJ, Ramsay ME, Miller E. (2006) Joint Committee on Vaccines and Immunisation. Proposed changes to the routine childhood immunization schedule, 2006. Hum Vaccin 2: 68–73.
213. Snape MD, Pollard AJ. Meningococcal polysaccharide-protein conjugate vaccines. Lancet Infect Dis 5: 21–30.
214. Adu Bobie et al. Infect. Immun. 2004, 72(4); 1914-1919.
215. Koeberling et al. 2008. J Infect Dis 19:262-70.
216. Kahler et al. 1998; Mackinnon et al. 1993.
217. Vogel et al. 1996, 1997.
218. Koeberling et al., CVI 2011.
219. Iewa et al., J Bacteriology 2005.

220. Stoddard, M. B., Pinto, V., Keiser, P. B., and Zollinger, W. (2010) Evaluation of a whole-blood cytokine release assay for use in measuring endotoxin activity of group B Neisseria meningitidis vaccines made from lipid A acylation mutants Clin. Vaccine Immunol. 17, 98-107.
221. Köller M., Wick M., Muhr G. (2001). Decreased leukotriene release from neutrophils after severe trauma: role of immature cells. Inflammation. 25(1):53-9.
222. Ramadas Bath U., Kontrohr T., and Mayer H. (1987) Structure of Shigella sonnei lipid A FEMS microbiology 40, 189-192.
223. Caugant, D. A., Hoiby, E. A., Magnus, P., Scheel, O., Hoel, T., Bjune, G., Wedege, E., Eng, J., and Froholm, L. O. (1994) Asymptomatic carriage of Neisseria meningitidis in a randomly sampled population J. Clin. Microbiol. 32, 323-330.
224. Koeberling, O., Seubert, A., and Granoff, D. M. (2008) Bactericidal antibody responses elicited by a meningococcal outer membrane vesicle vaccine with overexpressed factor H-binding protein and genetically attenuated endotoxin J. Infect. Dis. 198, 262-270.
225. Ferrari, G., Garaguso, I., Adu-Bobie, J., Doro, F., Taddei, A. R., Biolchi, A., Brunelli, B., Giuliani, M. M., Pizza, M., Norais, N., and Grandi, G. (2006) Outer membrane vesicles from group B Neisseria meningitidis delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles Proteomics. 6, 1856-1866.
226. Van der Ley P, Steeghs L, Hamstra HJ, ten Hove J, Zomer B, van Alphen L. Modification of lipid A biosynthesis in Neisseria meningitidis lpxL mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. Infect Immun. 2001 Oct;69(10):5981-90.
227. Vorachek-Warren, M. K., Ramirez, S., Cotter, R. J., and Raetz, C. R. (2002) A triple mutant of Escherichia coli lacking secondary acyl chains on lipid A J. Biol. Chem. 277, 14194-14205.

Ringraziamenti

Alla fine del mio percorso formativo, vorrei ringraziare tutte le persone che hanno contribuito ad arricchire il mio bagaglio di studi e di vita.

Grazie innanzitutto a colui che mi ha seguito in questi anni in modo umanamente e scientificamente impeccabile. Ringrazio il mio mentore: il Dr. Oliver Koeberling.

Ringrazio il Prof. Giovanni Sannia per la grande disponibilità dimostratami e per l'opportunità offertami di vivere questa ricca esperienza formativa.

Ringrazio il Prof. Calman MacLennan, il Dr. Allan Saul e Glaxo Smith Kline Vaccines Institute for Global Health (GVGH), con il suo efficiente e disponibile apparato amministrativo, per aver supportato e supervisionato i miei studi.

Grazie a coloro che, nonostante le disavventure, le sfide e i segni del tempo e della vita stessa, sono stati sempre presenti, sin dal primo concorso, dal famoso 26 novembre 2012 e dall'avventurosa ricerca della casa, al supporto di ogni tipo, soprattutto morale. Ringrazio i pilastri e le costanti della mia vita: i miei genitori, Beatrice e Peppino, i miei tre fratelli, Alessandro, Ettore e Roberto, la mia sorella adottiva, Valeria. Ringrazio le persone che purtroppo ho perso, in particolare i miei nonni i quali non smetteranno mai di essere il mio faro.

Grazie a colui che ha sempre creduto in me, sostenendo ogni mia scelta ad occhi chiusi e che con pazienza, dopo 9 anni, ancora è qui, pronto ad incoraggiarmi. Ringrazio l'uomo che non ha mai camminato davanti a me, né dietro di me, ma sempre al mio fianco: Ciro. Un grazie speciale va alla sua meravigliosa famiglia che sento ormai un po' come la mia seconda famiglia.

Grazie agli amici di sempre, immutati e immutabili: Lucia, Rosaria, Ilaria, Maria Teresa e Ciro.

Grazie alle due prime persone incontrate a Siena con le quali mi sono sempre sentita a casa. Ringrazio coloro su cui ho sempre potuto contare, a prescindere da tempo e distanze: Deborah e Lucia.

Grazie a chi c'è stato dal mio primissimo giorno in azienda: persone sincere che mi hanno sempre incoraggiato a dare il meglio facendo crescere la mia autostima, e che hanno reso straordinarie, indimenticabili, divertenti e irripetibili le mie giornate in azienda, così come al di fuori di essa. Ringrazio i miei amici, Francesco e Angela, con la speranza che il rapporto quasi simbiotico che si è creato in questi anni possa non perdersi mai.

Grazie a chi è arrivato, rivelandosi una piacevole scoperta. Ringrazio Arianna che, forse inconsapevolmente, mi ha fatto crescere tanto, da tutti i punti di vista. Ringrazio

Gianluigi per la sua costante disponibilità e innata genuinità. Ringrazio Federico per il suo grande supporto morale e scientifico.

Grazie a chi ho sempre trovato ad accogliermi al ritorno da lavoro, regalandomi un sorriso, per nulla scontato. Fortunata ad averle incontrate sul mio cammino, ringrazio Agnese per le sue spassose “performance teatrali”, Giovanna per le sue lungimiranti “sedute psicologiche”, Martina per la sua “rigorosa ecosostenibilità” ed Emanuela per le interminabili chiacchierate serali. Ringrazio tutte loro per essere state non soltanto semplici coinquiline, bensì amabili sorelle.

Grazie ancora a tutte le persone di cui porterò con me sempre un piacevole ricordo. Ognuno ha contribuito con il proprio tassello a comporre un memorabile puzzle della mia esperienza: Oscar, Omar, Valerio, Francesca, Martina, Luisa, Giuseppe, Mariaelena, Roberto, Gabriella, Alessia, Lynda, Emilia, Melissa, Antonio, Giusy, Francesco, Melina, Michele, Agnese, Pasquale e Nunzio.

Chi c’era, continuando ad esserci, mi ha reso più sicura.

Chi è arrivato ex novo, ogni singola persona incontrata, comprese quelle non citate, mi ha regalato, non sapendo, una parte del suo essere, permettendomi di allargare gli orizzonti e tirare fuori, maieuticamente, uno spirito critico ad ampio raggio.

Con questi nuovi occhi, farò tesoro delle mille sfaccettature di ogni singola personalità conosciuta, verso ciò che sarà.

Quindi, semplicemente **GRAZIE**!