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Dissection of the protective response elicited by the DOMV component of the 4CMenB vaccine

Presentata da: **Viola Viviani**

Coordinatore Dottorato

Chiar.mo Prof

Giovanni Capranico

Supervisore

Chiar.mo Prof.

Vincenzo Scarlato

Co-supervisore

Dott.ssa

Erika Bartolini

Esame finale anno 2021

Abstract

Neisseria meningitidis serogroup B is the major etiological agent of meningitis and life-threatening sepsis, against which two vaccines are licensed. The 4CMenB vaccine is composed of three major protein antigens (fHbp, NHBA and NadA) and detergent-extracted outer membrane vesicles (DOMV) from the NZ98/254 strain. DOMV are safe, immunogenic and able to raise bactericidal antibodies, mainly attributed to the immunodominant PorA protein. Nevertheless, DOMV offer a complex reservoir of potentially immunogenic proteins, whose relative contribution in protection is still poorly characterized.

By testing antisera from vaccinated infants in serum bactericidal assay, we observed that the addition of DOMV in the vaccine formulation enhanced breadth of coverage compared to recombinant proteins alone against a panel of 11 meningococcal strains mismatched for the vaccine antigens. To unravel the DOMV components involved in such protection, 30 DOMV antigens were cloned and expressed in *Escherichia coli* as recombinant proteins and/or in vesicles to maintain their native conformation. Samples obtained were both included in tailor-made protein-microarrays to immunoprofile the antibody repertoire raised by DOMV-containing formulations and were individually used for mouse immunization studies to assess their ability to induce bactericidal antibodies.

The protein-array immunosignature of mouse DOMV/4CMenB antisera unveiled a subset of 8 DOMV-reactive proteins potentially responsible for the additional protective responses. The antisera derived from mouse immunizations showed high levels of antibodies and recognized the corresponding antigen across different meningococcal strains. Among the protein-array reactive antigens, OpcA, NspA and PorB induced antibodies able to kill 10 of the 11 genetically diverse meningococcal strains and the specificity of the protective role of OpcA and PorB was also confirmed in 4CMenB infant vaccinee sera.

In conclusion, we identified additional PorA-independent antigens within DOMV involved in broadening the coverage of 4CMenB, thus supporting the key role played by vesicles in this multivalent formulation.

Disclaimer

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Transparency statement:

Viola Viviani is a PhD student at the University of Bologna Alma Mater Studiorum and participates in a post graduate studentship program at GSK, Siena, Italy. EB is an employee of the GSK group of companies. VS is an employee of University of Bologna, Department of Pharmacy and Biotechnology.

Trademark statement:

Bexsero is a trademark owned by or licensed to the GSK group of companies. *Trumenba* is a trademark owned by Pfizer. *VA-MENGOC-BC* is a trademark of the Finlay Institute, Cuba. *MenBvac* is a trademark of the Norwegian Institute of Public Health. *MeNZB* is a trademark of Novartis.

Human samples:

The human infant samples used in the study were obtained via a Phase II clinical trial (V72P16, NCT00937521) conducted in multiple centres in the Czech Republic, Italy, Hungary, Chile and Argentina between July 2009 and November 2010 in accordance with Good Clinical Practices and according to the Declaration of Helsinki. Parents or guardian of each participant have given their written informed consent to the study. Serum samples used in this analysis are pool of antiserum from 25 subjects. Human complement source used in the study was obtained according to Good Clinical Practice in accordance with the declaration of Helsinki. Patients have given their written consent for the use of samples of study MENB REC 2ND GEN-074 (V72_92).

Animal samples:

All animal sera used in this study derived from mouse immunization experiments performed at the GSK Animal Facility in Siena, Italy, in compliance with the ARRIVE guidelines, the current Italian legislation on the care and use of animals in experimentation (Italian Legislative Decree 116/92) and consecutive ministerial newsletter (Circolare Ministeriale n.8 del 22 Aprile 1994), and with the GSK Animal Welfare Policy and Standards. The animal protocol was approved by the Animal Welfare Body of GSK Vaccines, Siena, Italy, and by the Italian Ministry of Health.

Contents

1. Introduction

1.1 The *Neisseria meningitidis* pathogen

Neisseria meningitidis, also referred to as meningococcus, is a Gram-negative β-proteobacterium member of the Neisseriaceae family. It is a fastidious, aerobic, non-motile and non-sporulating diplococcus usually encapsulated and piliated (Figure 1.1). Meningococcus and the closely related *Neisseria gonorrhoeae* bacterium are the only pathogens of the *Neisseria* genus, which groups several commensal species (Liu *et al.*, 2015).

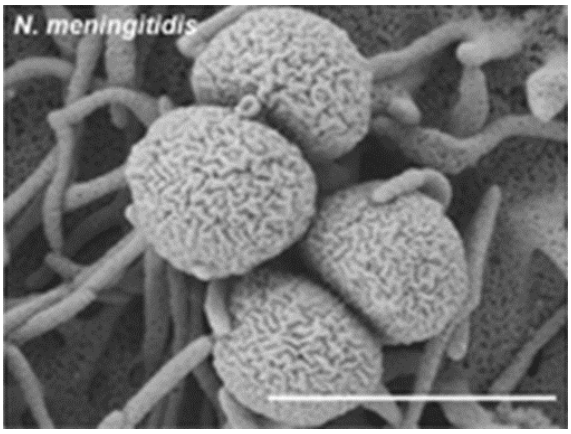

Figure 1.1. Scanning electron microscopy of *Neisseria meningitidis* diplococci. Analysis of epithelial cells infected with *N. meningitidis* 8013. Scale bar: 1 μm (adapted from (Custodio *et al.*, 2020)).

N. meningitidis is an obligate human pathogen and it is usually part of the normal nasopharyngeal mucosa microbiome of healthy young adults (Cartwright *et al.*, 1987, Yazdankhah and Caugant, 2004). However, in susceptible individuals and under rare circumstances still poorly understood, it can switch its attitude of commensal into a life-threatening pathogen responsible for meningitis and septicaemia (Hill *et al.*, 2010).

Depending on the immunological reactivity and the chemical structure of capsular polysaccharides, meningococcal isolates can be subdivided into 13 different serogroups, of which six (A, B, C, W-135, X, Y) account for most disease cases worldwide (Rosenstein *et al.*, 2001, Branham, 1953). Despite serogroup typing is considered the traditional approach, meningococcal strains can be further classified into serosubtype, serotype and immunotype according to class 1 outer membrane proteins (PorA), class 2 or 3 outer membrane proteins (PorB) and lipooligosaccharide (LOS) structure, respectively (Frasch *et al.*, 1985). However, the fact that serological methods rely on surface antigenic determinants which constantly undergo host selective pressure has made them unsuitable for the needs of modern epidemiology. Hence, nucleotide sequence-based approaches for the classification of meningococcal strains have been recently developed. Among them, the Multi Locus Sequence

Typing (MLST) is nowadays considered the gold standard for bacterial classification (Maiden *et al.*, 1998). It indexes single nucleotide variations among a core set of seven housekeeping genes assigning a different allele to each locus. The combination of the given alleles defines the specific allelic profile, which in turn determines the sequence type (ST). Meningococcal isolates that share up to four identical loci are considered closely related, originating from a common ancestor and they are included into a clonal complex (CC) (Maiden, 2006). The establishment of clonal complexes has shed new light on disease-causing strains particularly because mostly of them fall into specific lineages defined thus hyper-invasive (ST-1, ST-4, ST-5, ST-8, ST-11, ST-32, ST-41/44, ST-269) (Caugant, 2008, Brehony *et al.*, 2007).

1.2 Epidemiology of meningococcal disease

N. meningitidis is the etiological agent of meningococcal disease a term used to describe several illnesses which most commonly manifest as meningitis and meningococcemia. Cases of meningococcal infection are estimated to be 1.2 million annually, with a rate of roughly 135,000 victims worldwide (Rouphael and Stephens, 2012). It is worth mentioning that meningococcal geographic distribution is not homogeneous and differs according to serogroups. Indeed, serogroups A and C are predominant across Africa and Asia, serogroup B and C are found in Europe and Americas and serogroup Y spreads throughout United States and Canada. Epidemic outbreaks around the globe are generally due to serogroup W-135 and recently serogroup X was identified as the major cause of meningococcal disease in Africa (Stephens *et al.*, 2007, Peterson *et al.*, 2019, Boisier *et al.*, 2007, Delrieu *et al.*, 2011, Jafri *et al.*, 2013). Despite the huge diffusion, meningococcal infections occur sporadically in high-income countries where the incidence of the disease is falling constantly: from 1996 to 2015 *N. meningitidis* incidence in the United States declined among all age groups with a decrease rate of 93% in infants aged <1 year (from 11.04 cases per 100,000 in 1996 to 0.73 cases per 100,000 in 2015) (MacNeil *et al.*, 2018). On the contrary, meningococcal meningitis remains a high burden in the African meningitis belt, where between 2011 and 2017 17 outbreaks and approximately 32,000 suspected cases have been reported, despite the introduction of a meningococcal serogroup A conjugate vaccine (Fernandez *et al.*, 2019).

As aforementioned, the bacterium inhabits the upper respiratory tract with a prevalence of 10-35% in the global population, phenomenon known as carriage, but the rate of asymptomatic carriers sharply rises in overcrowded-housing like military recruits and university dormitories (Yazdankhah and Caugant, 2004, Christensen *et al.*, 2010, Bruce *et al.*, 2001). Moreover, with a frequency of one case for 100,000 people in the developed world, the bacterium is able to cross the mucosal epithelium, to gain access to the bloodstream and to disseminate systemically throughout the host, giving rise to the

invasive meningococcal disease (IMD) (Nadel and Ninis, 2018). The causes underling IMD are still poorly known, but they are thought to rely on a tight interplay occurring between bacterial factors able to modulate virulence and host susceptibility, like deficiencies in the complement system and asplenia, that favour microbial pathogenesis (Rouphael and Stephens, 2012, Emonts *et al.*, 2003). In general, meningococcal disease has the highest incidence in toddlers under 1 year of age as a result of an immature immune system. Then, disease rate drops down to rise again only during adolescence and early adulthood when peaks of incidence (10-35%) are reached in 20-24 years old individuals, largely due to an increased oropharyngeal carriage in these groups. Generally, the incidence in older individuals decreases to less than 10% (MacNeil *et al.*, 2018, Caugant *et al.*, 2007, Claus *et al.*, 2005). Despite the availability of effective antibiotics, a prompt diagnosis remains crucial. The onset of the disease is rapid and the case-fatality rate of meningococcal meningitis is up to 50% when untreated (Nadel and Ninis, 2018). Even when the disease is diagnosed early and adequate treatments are taken, 5% to 10% of patients die, typically in as short as 24 hours from the initial symptoms (Stephens *et al.*, 2007). Moreover, approximately 10-20% of the survivors develop long-term sequelae including hearing impairments, loss of limbs and neuro-developmental deficits and up to 36% develop physical, cognitive or psychological impairments (Viner *et al.*, 2012, Vyse *et al.*, 2013, Hollingshead and Tang, 2019). However, since disease early symptoms resemble viral infections an early diagnosis is rarely achieved, thus vaccination remains the only efficient approach to reduce disease burden and to limit serious infection outcomes.

1.3 Meningococcal pathogenesis and virulence factors

N. meningitidis pathogenesis is considered a complex multi-stage process. It begins with colonization, when resident, or even inhaled via respiratory droplets, meningococci establish an intimate adherence with the respiratory epithelium (Figure 1.2A). Indeed, a firm host-pathogen interaction is fundamental for the bacteria, which otherwise would be flushed away by the physiological mucus clearance (Hill *et al.*, 2010). Since the capsule may enhance the survival of the bacterium outside the host during transmission, it would appear reasonable that strains primarily encountered by the host are encapsulated (Hill *et al.*, 2010, Virji, 2009). Therefore, the adherence of those encapsulated bacteria to non-ciliated mucosal epithelia is thought to be primarily mediated by capsular-protruding Type IV Pili (Dos Santos Souza *et al.*, 2020), which may interact with CD147 host receptors (Bernard *et al.*, 2014, Le Guennec *et al.*, 2020). Pili are long filamentous appendages whose assembly depend on over 20 different proteins (Kolappan *et al.*, 2016). The pilus shaft is formed by PilE subunits arranged in a helical conformation which protrudes from the bacterial surface beyond the capsule, through a pore formed in the outer membrane by PilQ. Beside their role in adhesion, pili are involved in bacteria

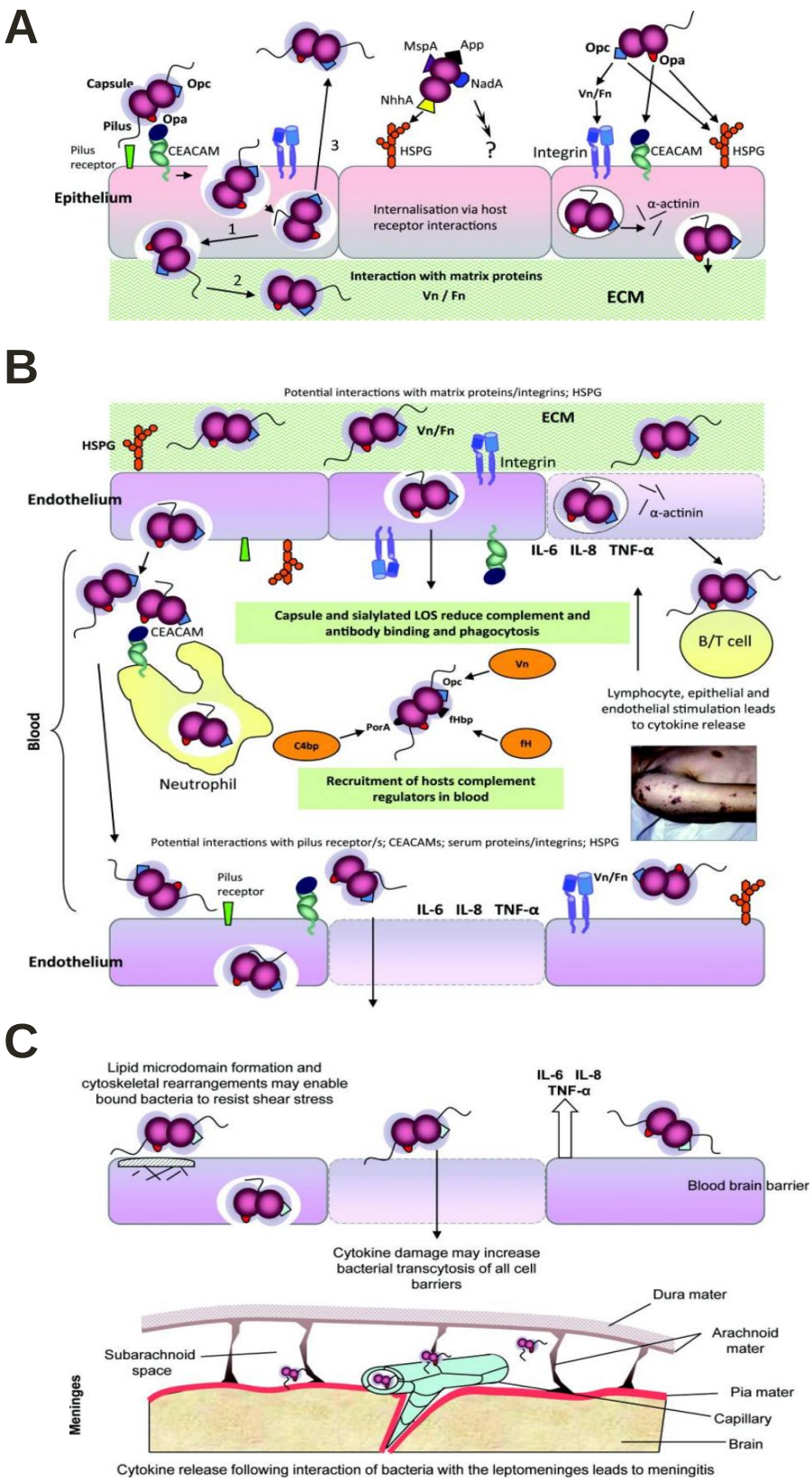

Figure 1.2. Schematic overview of *Neisseria meningitidis* pathogenesis. Representation of the stages that characterize (**A**) meningococcal colonization and interaction with the epithelial barrier, (**B**) entry in the bloodstream and (**C**) penetration of the blood-brain barrier (adapted from (Hill *et al.*, 2010)).

aggregation, twitching motility, protein secretion and natural competence through exogenous DNA uptake (Pelicic, 2008). Upon the attachment, *N. meningitidis* is able to proliferate forming microcolonies. At this stage, the bacterium undergoes the first of several phase-variation processes that involve changes in its surface repertoire. Indeed, although the capsule aids bacterial blood survival by rendering it resistant to complement-mediated lysis and by preventing phagocytosis (Uria *et al.*, 2008), it also adversely influences bacterial virulence. The presence of the capsule around the bacterial surface masks surface-exposed proteins via steric hindrance, hence limiting meningococcal adhesion (Virji, 2009). Consequently, lost or down-regulation of capsule (Deghmane *et al.*, 2002) often occurs at this stage of infection and the resulting unencapsulated phenotype unmasks an array of redundant adhesins that now are able to interact with their cognate host-receptors. Among the major adhesins there are the opacity proteins: Opa and OpcA, which confer the peculiar opaque phenotype to the colonies expressing them (Schubert-Unkmeir, 2017). Opa integral outer membrane proteins are arranged in amphipathic *β*-barrels and present four surface-exposed loops, of which loops 2 and 3 are hypervariable (Carbonnelle *et al.*, 2009). Contrary, OpcA also known as Opc is encoded by the *opcA* gene and its structure, a *β*-barrel with five surface-exposed loops, does not vary greatly among isolates (Prince *et al.*, 2002). Noteworthy, the lack of the *opcA* gene has been described for defined lineages like the CC ET-37 (Seiler *et al.*, 1996), which tends to cause severe sepsis instead of meningitis, leading to the speculation that OpcA may have a role in meningococcal meningitis (Unkmeir *et al.*, 2002). However, during pathogenesis, meningococcal Opa proteins bind several members of the CEACAM (carcino-embryonic antigen-related cell-adhesion molecule) family, of which CEACAM1 is the most widely expressed (Virji *et al.*, 1996, Hammarstrom, 1999). In addition to CEACAMs, human HSPGs (heparan sulfate proteoglycans) can be used as receptors by Opa as well as OpcA proteins. Moreover, OpcA may also form trimolecular complexes interacting with the serum proteins Vitronectin and Fibronectin, which in turn bind epithelial αVβ3-integrin and α5β1-integrin, respectively (Virji *et al.*, 1994). Beside Pili, Opa and OpcA, meningococcal genome mining aimed to identify novel potential vaccine candidates has recently led to the discovery of additional minor adhesins involved in the cell-adhesion. Among them, the Neisserial adhesin A (NadA) is more commonly associated with virulent isolates rather than carrier strains (Comanducci *et al.*, 2002) and NadA deletion lead to a decrease in epithelial adhesion and invasion by encapsulated and unencapsulated strains, respectively (Capecchi *et al.*, 2005). *Neisseria* hia/hsf homologue A (NhhA), formally called Meningococcal surface fibril (Msf), is found in most of the disease-causing meningococci (Peak *et al.*, 2000) and it was described to mediate adhesion to epithelial cells and to components of the extracellular matrix, albeit at low levels (Scarselli *et al.*, 2006). Moreover, it has been reported that the binding of NhhA to the Vitronectin is able to inhibit the complement cascade

(Griffiths *et al.*, 2011). Lastly, Adhesion and penetration protein (App) is described to aid early colonization of the upper-respiratory airways by enhancing adhesion before undergoing an autocleavage event which allows bacteria detachment and thus systemic spread (Serruto *et al.*, 2003). Engagement of CEACAMs, HSPGs and integrins by these adhesins triggers a range of cellular pathways and signalling outcomes, that may aid epithelial/endothelial barrier penetration and meningococcal internalization through the formation of epithelial cell pseudopodia that encircle bacteria into intracellular vacuoles (Eugene *et al.*, 2002) (Figure 1.2B). Notably, under some circumstances, such as during upregulation of host receptors in inflammation, meningococcal invasion has also been reported for fully encapsulated meningococcal isolates (Rowe *et al.*, 2007). Once internalized, bacterial survival and replication depend on their capability to acquire nutrients via specialized transport systems, such as the transferring binding protein (TbpA and TbpB), the lactoferrin binding protein (LbpA and LbpB) and the hemoglobin binding receptor (HmbR) (Perkins-Balding *et al.*, 2004). Moreover, whereas in healthy individuals the bacteria that cross the mucosa can be readily killed by serum bactericidal activity, in susceptible hosts they may survive in the bloodstream and disseminate throughout the body. Meningococcal survival within blood depends on several mechanisms. Aside from the upregulation of the capsule expression that, as said, prevents opsonic and non-opsonic phagocytosis (Uria *et al.*, 2008), other immune evasion strategies evolved by *N. meningitidis* involve the recruitment of complement regulators (Blom *et al.*, 2009, Lewis and Ram, 2020). Indeed, bacterial Factor H binding protein (fHbp) (Madico *et al.*, 2006), Neisserial surface protein A (NspA) (Lewis *et al.*, 2010), Porin B (PorB) (Lewis *et al.*, 2013) and Neisserial Heparin Binding Antigen (NHBA) (Serruto *et al.*, 2010) are all able to bind and sequester directly or indirectly the negative complement regulator Factor H. Likewise, the complement regulators C4-binding protein and Vitronectin are targeted by bacterial Porin A (PorA) (Jarva *et al.*, 2005) and OpcA (Lewis and Ram, 2020), respectively. At this step of pathogenesis, a pivotal role is also played by lipopolysaccharide, more accurately named lipooligosaccharide (LOS) due to the presence of shorts repeating saccharides, instead of long-chain ones. Indeed, LOS has been implicated in triggering the secretion of pro-inflammatory cytokines upon binding with specific receptors present on monocytic and dendritic cells (Brandtzaeg *et al.*, 2001) as well as in avoidance of complement killing, especially when its residues are sialylated, thus mimicking host cell surfaces (Hill *et al.*, 2010). Being a target of host immune response, a constant alteration of LOS antigenic properties is ensured by phase and antigenic variations of the genes involved in LOS biogenesis (Jennings *et al.*, 1999), and the resulting different oligosaccharide chains determine the immunotype (L1–12) used for meningococcal isolate classification (Mandrell and Zollinger, 1977). Moreover, immunotyping screening studies have revealed a trend between LOS immunotype and meningococcal virulence: L3, L7 and L9 are

commonly found in blood isolates during IMD, while L1, L8 and L10 are typically expressed by carrier strains (Jones *et al.*, 1992). Besides the aforementioned virulence factors, *N. meningitidis* in the blood is also characterized by massive vesiculation of its outer membrane, leading to the shed of outer membrane vesicles (OMV) containing lipids, proteins and LOS (Ellis and Kuehn, 2010). These blebs are thought to contribute to the huge amount of circulating LOS found during septicaemia, to act as a decoy for antibodies and complement components and may also be related to the autolysis that occurs during meningococcal stationary phase, resulting in DNA release useful for genetic transformation (Tzeng and Stephens, 2000). Finally, bacteria in the bloodstream can slowly replicate and they may eventually pass across the blood-brain barrier using adhesins and pili, resulting thus in the infection of meninges and cerebrospinal fluid (Schubert-Unkmeir, 2017) (Figure 1.2C). On the contrary, in case of rapid hematogenous multiplication, meningococci cause fulminant sepsis. However, as the fate of the pathogen and that of the host are strictly interconnected, it is noteworthy that the onset of IMD and the subsequent host death represents, from an evolutionary point of view, a failure.

1.4 Surface modulation of virulence factors

A remarkable characteristic of *N. meningitidis* that lays the basis for its success as human-specific pathogen is the ability to continuously and promptly modulate its surface virulence factors. Meningococcus, during its progression from an airway colonizer to a bloodstream invader, encounters different host niches, in terms of nutrient availability, immune responses and environmental stress factors. Thereby, it is not surprising that the bacterium has co-evolved different and redundant virulence factors, which expression undergoes a sophisticated genetic regulation, to create a variability of structures essential for its pathogenesis. Meningococcal phenotypic plasticity, which is intrinsically facilitated by its natural competence, relies on high-frequency (10^{-2} to 10^{-4} variations/cell/generation) (Tzeng and Stephens, 2000) genetic mechanisms including phase and antigenic variation (van der Woude and Baumler, 2004). Through phase variation *N. meningitidis* undergoes stochastic reversible genotypic changes that modulate gene expression between on and off states. It arises primarily from a mechanism, taking place during DNA replication, referred as slipped-strand mispairing, which involves DNA-strand slippage caused by repetitive nucleotide sequences (van der Woude and Baumler, 2004). Variation in sequence repeats occurring within the coding sequence of a gene or in the promoter region results in translational or transcriptional control of expression, respectively (Rotman and Seifert, 2014). In the former, the introduction of frameshifts can lead to the absence or presence of mature proteins, as in the case of the CTCTT pentameric repeats located in the *opa* signal peptide (Stern and Meyer, 1987); in the latter, the variation of the distance

between the -10 and the -35 promoter regulatory elements, changes the expression levels of proteins, as described for the polyC and polyG tracts within the *opcA* and *porA* promoters, respectively (Sarkari *et al.*, 1994, van der Ende *et al.*, 2000, van der Ende *et al.*, 1995). More than 100 genes are described to be phase variable in *Neisseria* genus (Snyder *et al.*, 2001) and, among them, several encode for virulence factors such as PorA, OpcA, Opa, Pili, as well as capsule and LOS (Gasparini *et al.*, 2015). Unlike phase variation, during antigenic variation bacteria express different moieties of molecules that are antigenically distinct (van der Woude and Baumler, 2004). This phenomenon can be caused by horizontal gene transfer, allelic rearrangement or homologous intragenic recombination, which is favoured by the presence of multiple copies of certain genes in the neisserial genome, like *pil* or *opa* (Gasparini *et al.*, 2015). These mechanisms result in a wide variability of surface-exposed determinants exploited by the bacteria during pathogenesis and allow the simultaneous presence of subpopulations with divergent phenotypes that constantly challenge the host immune surveillance.

1.5 Polysaccharide-based ACWY meningococcal vaccines

The earliest attempts to develop meningococcal vaccines were made using heat-killed whole bacteria preparations (Sophian and Black, 1912), but the excessive reactogenicity and the uncertainty of protection ascribed to these formulations limited their spread for long time, during which antibiotics remained the only solution. However, the sulfonamide-resistance arising in meningococci (Millar *et al.*, 1963), prompted renewed interest in developing vaccines. Indeed, pioneered by Gotschlich during 1960s (Gotschlich *et al.*, 1969), purified capsular polysaccharides (CPS), obtained directly from the serogroup of the pathogen, became the main constituent of plain CPS vaccines against meningococcus serogroups A, C, W and Y. Despite their efficacy in adolescent and adults, this class of vaccines failed to protect infants and to induce immunological memory, thus they have been progressively superseded by CPS-conjugate vaccines (Christodoulides and Heckels, 2017). Indeed, the conjugation of the bacterial polysaccharide to a protein carrier induced a stronger and long-lasting antibody response overcoming plain CPS vaccine drawbacks. As a result, conjugate meningococcal vaccines are nowadays the major class of vaccines routinely in use for preventing infections caused by *N. meningitidis* serogroups A, C, W and Y and they are available on the market in different formulations (McCarthy *et al.*, 2018). However, the development of a meningococcal serogroup B (MenB) vaccine has posed several challenges and has lagged behind the others. This scenario has inevitably led to a dramatical increment of IMD cases of serogroup B which accounted for 68% in Europe (2012), 80% in Australia (2014) and 28% in USA (2014) (Toneatto *et al.*, 2017).

1.6 MenB vaccines based on outer membrane vesicles

The reluctance to develop meningococcal B CPS-vaccine was based on the observation that its capsule was structurally similar to the sialic acids found in human neural tissues. Hence, the serogroup B capsule polysaccharide was considered poorly immunogenic and a theoretical autoantigen, rendering it an unsuitable vaccine target (Finne *et al.*, 1983). As a consequence, efforts to develop a vaccine against meningococcus B have shifted focus on non-capsular components like LOS and proteins, and the final solution to circumvent capsular mimicry was developed in response to the tremendous outbreaks that took place worldwide during 1970s. Indeed, as an alternative strategy, vaccines composed of wild-type outer membrane vesicles (OMV) were developed and successfully used to control clonal meningococcal B outbreaks in Cuba (Sierra *et al.*, 1991), Chile (Boslego *et al.*, 1995), Norway (Bjune *et al.*, 1991) and New Zealand (Oster *et al.*, 2005). Noteworthy, all these OMV-based vaccines underwent a detergent extraction process that, reducing LOS toxicity, was fundamental for improving vaccine safety and decreasing formulation reactogenicity. The *VA-MENGOCOC-BC*, at the Finlay Institute in Cuba (Sierra *et al.*, 1991), and the *MenBvac*, at the Norwegian Institute of Public Health (Bjune *et al.*, 1991), were the first meningococcal B vaccines to be manufactured with OMV deriving from their respective outbreak strains. Both tailor-made vaccines were able to successfully tackle the epidemics in their corresponding countries, albeit with different efficacy outcomes mainly due to a longer observational period of the Norwegian trail (Table 1.1) (Holst *et al.*, 2013). More recently, another OMV-based vaccine, the *MeNZB*, was developed to face the New Zealand outbreak dominated by the NZ98/254 isolate (Table 1.1) (Oster *et al.*, 2005). The vaccine was used during a mass-immunization campaign and it demonstrated a well-characterized safety profile and an effectiveness of 73% at 24 months follow-up (Kelly *et al.*, 2007). The huge body of information collected during the New Zealand outbreak, provided an important background and undoubtedly paved the way for the development of following broadly-protective meningococcal vaccines.

Despite these three vaccines resulted to be safe and able to elicit good levels of bactericidal antibodies, their spread has been limited by the relative inability to elicit cross-protective responses against genetically different *N. meningitidis* strains. Indeed, initial vaccine immunogenicity trials revealed higher response rates against strains used for vaccine manufacturing, rather than on heterologous isolates, especially in infants and small children (Tappero *et al.*, 1999). In these age groups, the immune response elicited by the tailor-made vaccines was indeed considered mainly directed against the most abundant and immunodominant PorA protein of OMV (Tappero *et al.*, 1999), which is highly immunogenic, but also characterized by a tremendous antigenic variability. Thus, the protection mounted was considered effective principally against strains expressing the same

Country	Outbreak strain	Vaccine	Doses	Years of trial (number and age of vaccinees)	Efficacy
Cuba	CU385/83 (B:4:P1.19,15:L3,7)	*VA-MENGOCOC-BC* (B:4:P1.19,15:L3,7+MenC CPS)	2	1987–1989 (106,000, 10–14 years; 133,600, 5 months to 24 years)	83%–94% (total estimate)
Norway	H44/76 (B:15:P1.7,16:L3,7,9)	*MenBvac* (B:15:P1.7,16:L3,7,9 OMV)	2	1988–1991 (171,800, 13–16 years)	87% (10 months follow-up) 57% (29 months follow-up)
New Zealand	NZ98/254 (B:4:P1.7-2,4)	*MeNZB* (B:4:P1.7-2,4 OMV)	3	2004–2008 (all under 20 years; 1 million people)	73% (total estimate at 24 months follow-up; 80%–85% for <5 years old children) 69% (total estimate at 42 months follow-up)

Table 1.1. Examples of licensed OMV vaccines used to control meningococcal B outbreaks. Details about countries, strains, vaccine name and composition, doses, years of trials and efficacy are reported (adapted from (Christodoulides and Heckels, 2017)).

serosubtype, despite evidences of cross-protection were observed in older children and adults (Petousis-Harris, 2018). These findings, overlooked for long time, have inevitably limited, but not precluded, the breadth of coverage of OMV-vaccines which were not considered suitable for endemic diseases caused by multiple and different circulating strains. Therefore, second generation OMV-vaccines have been developed in order to broaden the coverage. The strategies explored included the formulation of OMV harvested from multiple strains (Sandbu *et al.*, 2007) or from strains genetically manipulated to express more than one PorA alleles (van der Ley *et al.*, 1995), or to attenuate LOS reactogenicity (Bonvehi *et al.*, 2010). Nevertheless, the advent of the Omics era, which has occurred in recent years, has radically changed the approach to vaccine development, facilitating the identification of surface-antigens with potential broad protection. From these technologies two protein-based vaccines were developed and licensed, the 4CMenB (*Bexsero*) and the rLP2086 (*Trumenba*).

1.7 4CMenB and rLP2086 MenB vaccines

4CMenB and rLP2086 are two broadly cross-protective vaccines currently available against MenB that were licensed almost simultaneously, despite they have been developed using different approaches for the antigen identification.

4CMenB was developed through an innovative approach known as Reverse Vaccinology (RV) which was propelled by the availability of whole genome sequences and bioinformatics (Rappuoli, 2000). RV has completely overturned the classical laboratory-based analysis applied to vaccine development, preferring the *in silico* mining of genomes aimed to predict suitable vaccine candidates (Masignani *et al.*, 2019). This approach led to the identification of the three genome-derived Neisseria antigens currently included in the 4CMenB vaccine: Factor H binding protein (fHbp; GNA1870; LP2086) (Beernink and Granoff, 2008, Masignani *et al.*, 2003), Neisserial Heparin Binding Antigen (NHBA; GNA2132) (Serruto *et al.*, 2010, Welsch *et al.*, 2003) and Neisserial adhesin A (NadA; GNA1994) (Capecchi *et al.*, 2005, Comanducci *et al.*, 2002). In particular, fHbp and NHBA are present in the vaccine formulation as proteins fused to the conserved meningococcal gene products GNA2091 and GNA1030, respectively. Both these accessory proteins are included in the formulation since they are able to enhance immune responses directed to the main vaccine antigens (Giuliani *et al.*, 2006). On the contrary, NadA tends to lose its immunogenicity when fused to other proteins, probably due to the loss of its trimeric structure, and thus it is formulated as a single polypeptide (Giuliani *et al.*, 2006). Lastly, to provide a broader coverage of serogroup B strains, the 4CMenB vaccine contains a fourth component that are detergent-extracted outer membrane vesicles (DOMV), from the New Zealand epidemic strain, the same used to control the New Zealander outbreak (Serruto *et al.*, 2012). Following clinical trials that demonstrated vaccines safety (Toneatto *et al.*, 2011, Esposito *et al.*, 2014, Prymula *et al.*, 2014) and efficacy in all age groups against the majority of meningococcal B strains (Santolaya *et al.*, 2012, Snape *et al.*, 2010, Vesikari *et al.*, 2013) the 4CMenB was finally licensed in 2013 in Europe and in 2015 in the U.S. To recapitulate, the 4CMenB formulation includes the GNA2091-fHbp fusion protein (fHbp var1.1), the NHBA-GNA1030 fusion protein (NHBA peptide 2), the NadA variant 3.1 and DOMV obtained from the NZ98/254 strain (B:4:P1.7-2,4; ST42 [CC41/44]) (Figure 1.3, green dots).

The vaccine component fHbp is a surface lipoprotein described to specifically bind human factor H and to down-regulate the alternative complement pathway (Madico *et al.*, 2006, Granoff *et al.*, 2009). It shows a wide genetic diversity that, according to public databases, accounts for 1,687 alleles and 1,306 peptides. FHbp allelic peptides are subdivided either into three genetic variants (var1, var2 and var3) further divided into subvariants (var1.x, var2.x and var3.x) or in two subfamilies (A and B, corresponding to variants 2/3 and 1, respectively) (Masignani *et al.*, 2003).

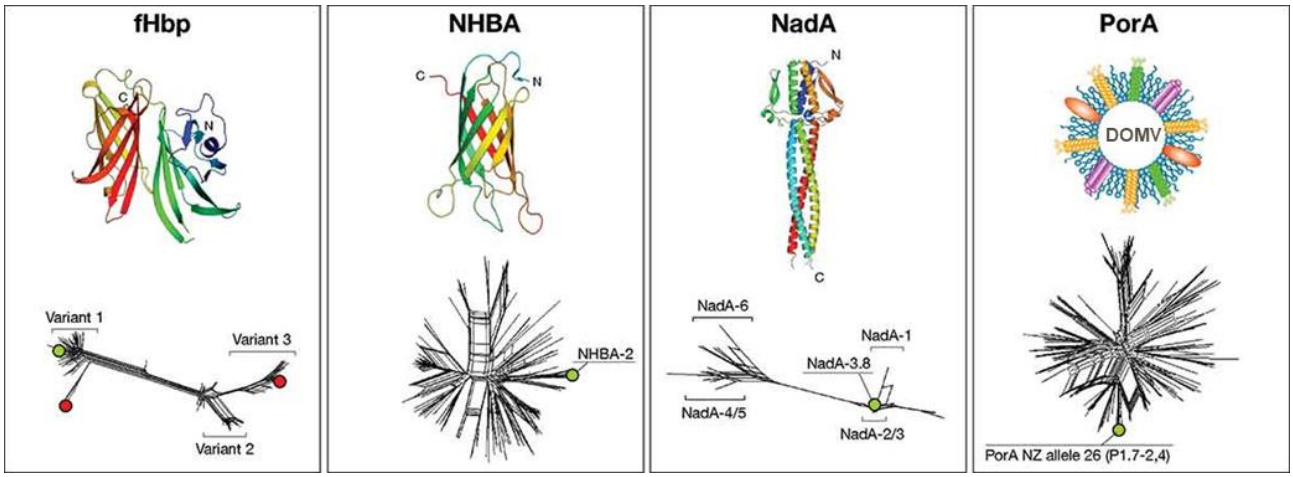

Figure 1.3. Structural characterization and molecular epidemiology of the four components included in the 4CMenB vaccine. The upper panels represent the three-dimensional structure of the full length fHbp var1, the C-terminal portion of NHBA peptide 2, the N-terminal portion of NadA and a schematic representation of DOMV architecture. The lower panels depict the phylogenetic distribution of fHbp, NHBA, NadA and PorA by split tree. Green and red dots indicate the antigen variants present in 4CMenB and rLP2086, respectively (adapted from (Toneatto *et al.*, 2017)).

It has been demonstrated that this diversity has an impact on fHbp immunological properties, indeed fHbp proteins from different variant groups are ineffective in inducing cross-protection against strains that express distantly related variants (Masignani *et al.*, 2003). Like fHbp, also NHBA is a surface-exposed lipoprotein. It is able to bind heparin and heparan-sulfate *in vitro* (Serruto *et al.*, 2010) and it is ubiquitous to all MenB isolates (Bambini *et al.*, 2009). This protein is highly variable with over 1,300 different peptides reported; but despite this variability, preclinical studies have suggested that antibodies elicited against the vaccine peptide are able to cross-protect strains harbouring different peptides (Giuliani *et al.*, 2006). Moreover, antibodies to NHBA and fHbp appear to work cooperatively to induce bactericidal activity (Vu *et al.*, 2011). The third vaccine protein antigen is NadA which is an autotransporter molecule that mainly mediates adhesion to human epithelium (Capecchi *et al.*, 2005). Unlike fHbp and NHBA, only 50% of meningococcal strains harbour the *nadA* gene and six different variants of NadA exist, of which NadA1, NadA2, NadA3 are highly immunogenic and induce cross-reactive bactericidal antibodies (Bambini *et al.*, 2014, Comanducci *et al.*, 2004). The rational for including DOMV in 4CMenB was to provide protection also against strains from CC 41/44 and especially to those expressing the PorA variant P1.4.

A more traditional proteomic approach was instead applied for the development of the rLP2086 vaccine which is composed of equal amounts of two lipidated fHbp variants: variant 3.45/subfamily A05 and variant 1.55/subfamily B01 (Figure 1.3, red dots) (Jiang *et al.*, 2010). rLP2086 has a good breath of coverage and tolerability in adults, adolescents and young children (Jiang *et al.*, 2010), however safety concerns about its use in infants were raised since purified lipoproteins are known to

be TLR-2 antagonist (Richmond *et al.*, 2012). Moreover the recent report that some meningococcal C strains express a truncated form of fHbp, led to the speculation that *fHbp* gene could be dispensable and that a vaccine containing a single antigen could thus lead to the selection of escape mutants, given the high plasticity of meningococcus (Toneatto *et al.*, 2017).

1.8 DOMV composition and protective role within the 4CMenB vaccine

One of the components of the recombinant meningococcal vaccine 4CMenB is DOMV from the NZ98/254 strain. Despite *Neisseria meningitidis* is able to naturally release OMV during its growth, membrane extraction with the deoxycholate detergent is a mandatory step of 4CMenB manufacturing process; it is indeed intended to ensure efficient removal of the otherwise toxic LOS and to increase vesicle yields. However, apart from the extraction of loosely associated membrane lipoproteins, the resulting vesicles resemble the surface of the parental NZ98/254 strain and encompass the same array of outer membrane proteins (OMP). In particular, according to early SDS-PAGE analyses about DOMV composition, five major classes of OMP were identified: class 1 (PorA), class 2/3 (PorB), class 4 (RmpM) and class 5 (Opa) (Tsai *et al.*, 1981). Nevertheless, more recent and sensitive proteomic-based approaches have revealed a far more complex DOMV proteome, which depending on the detection method used, may comprise up to 100 different protein components (Christodoulides, 2014, Lappann *et al.*, 2013, Vipond *et al.*, 2006, Vipond *et al.*, 2005, Wheeler *et al.*, 2007, Tani *et al.*, 2014, Ferrari *et al.*, 2006), mostly represented by OMP, but including also antigens predicted to be periplasmic and cytoplasmic. Despite the DOMV contained in the *Bexsero* formulation may potentially challenge the immune response with a composite mixture of antigens, the protection provided by them has been largely attributed to the immunodominant Porin A (Martin *et al.*, 2006). This protein is arranged in a conserved β-barrel structure spanning the outer membrane, with eight surface-protruding loops. Among them, loop 1 and loop 4, retaining the greatest antigenic diversity, are labelled as variable region 1 and 2 (VR1 and VR2), respectively (Jolley *et al.*, 2007). The peptide sequences of VR1 and VR2 define the PorA-based serosubtyping classification of meningococcal isolates. This nomenclature is indeed described by the prefix P1 for the porin 1 (PorA) followed by the VR1 and VR2 details (variant family name and number spaced by a dash) separated by a comma (Russell *et al.*, 2004), *e.g.*, P1.7-2,4 is the specific designation of the Porin A expressed by NZ98/254. Despite these two PorA regions have been described to provide the major contribution to the protection, their hypervariability inevitably limits the PorA-mediated cross-protection and reflects the serosubtype specificity ascribed to all OMV-based vaccines (Martin *et al.*, 2006). However, a complex reservoir of surface-exposed proteins with potential immunogenic properties is actually offered by DOMV. Among them, highly conserved OMP like PorB, RmpM, OpcA, NspA and

Omp85, whose role in protection has already been hypothesize in other OMV-based vaccines, are likely to contribute to bacteriolysis and to the overall strain coverage mediated by 4CMenB. Therefore, to indicate PorA as the exclusive target of DOMV-raised antibodies is rather reductive and contrasts with several immunoprofiling studies conducted with sera deriving from MenB convalescents or from OMV-vaccinees which assessed the overall presence of a cocktail of functional antibodies targeting different OMV moieties (Awanye *et al.*, 2019, Mendum *et al.*, 2009, Wedege *et al.*, 2007, Wedege *et al.*, 1998, Williams *et al.*, 2009, Williams *et al.*, 2014, Rosenqvist *et al.*, 1995, Rosenqvist *et al.*, 1993). In line with this, *Bexsero* early phase II studies conducted in infants administered with recombinant vaccine antigens alone (rMenB: fHbp, NHBA and NadA) or combined with DOMV (rMenB + DOMV, namely 4CMenB), revealed that the addition of vesicles on top of recombinant antigens was able to confer broader immunogenic benefits that those supposed. In particular, the rMenB + DOMV vaccine, when tested on a panel of 7 MenB indicator strains, was able to mount effective immune responses not only against isolates harbouring the homologous PorA antigen, but also against heterologous ones (Snape *et al.*, 2010, Findlow *et al.*, 2010, Esposito *et al.*, 2014). Multiple explanations have been proposed to support the observed cross-protection, which may be due to: (i) the synergy among antibodies targeting rMenB antigens and/or minor-DOMV antigens, (ii) the intrinsic immunostimulatory effect of DOMV or (iii) minor antigens within the DOMV component that might provide protection, in addition to PorA (Giuliani *et al.*, 2010, Bai *et al.*, 2011). While data about synergies occurring among 4CMenB-elicited antibodies targeting different proteins and epitopes have been previously documented (Giuliani *et al.*, 2010, Giuliani *et al.*, 2018, Vu *et al.*, 2011, Natali *et al.*, 2020), the immunological role played by antigens harboured by the DOMV component of 4CMenB, beside PorA, remains poorly characterized.

Overall, these evidences shed light on the importance of the DOMV within 4CMenB vaccine formulation, but highlighted a marked need to investigate DOMV antigens involved in cross-protection for a better comprehension of vaccine breadth, which otherwise would be underestimated due to the inability to fully take into account the contribution afforded by DOMV proteins.

1.9 Meningococcal correlate of protection

The low incidence of meningococcal disease has posed several challenges for the evaluation of vaccine efficacy in clinical trials. To overcome this limitation, it became necessary to develop a laboratory-based surrogate of protection, able to estimate vaccine efficacy more easily. Indeed, the study conducted by Goldschneider in 1969 (Goldschneider *et al.*, 1969), that showed an inverse correlation between the incidence of meningococcal disease and the presence of circulating bactericidal antibodies, has led to the present use of the serum bactericidal assay (SBA) as the

meningococcal correlate of protection. The mechanism of SBA relies on the killing of *N. meningitidis* target strains in the presence of meningococcal-specific antibodies and a source of complement. It hence depends on the activation of the complement classical pathway. In order to perform SBA, hyperimmune serum is serially diluted and incubated with a bacterial suspension containing the target meningococcal isolate. During incubation, antibodies specifically bind meningococcal surface antigens, and in the presence of exogenous sources of complement coming from rabbit (rSBA) or human (hSBA) the Fc portions of the surface-attached Immunoglobulins recruit the complement Component 1q (C1q). This binding triggers the activation of the classical complement pathway that ultimately leads to bacteriolysis. The SBA titre of each serum is then defined as the highest dilution of sera yielding ≥50% bacterial killing, compared to the number of meningococci found in the experimental controls (Borrow *et al.*, 2005b) (Figure 1.4).

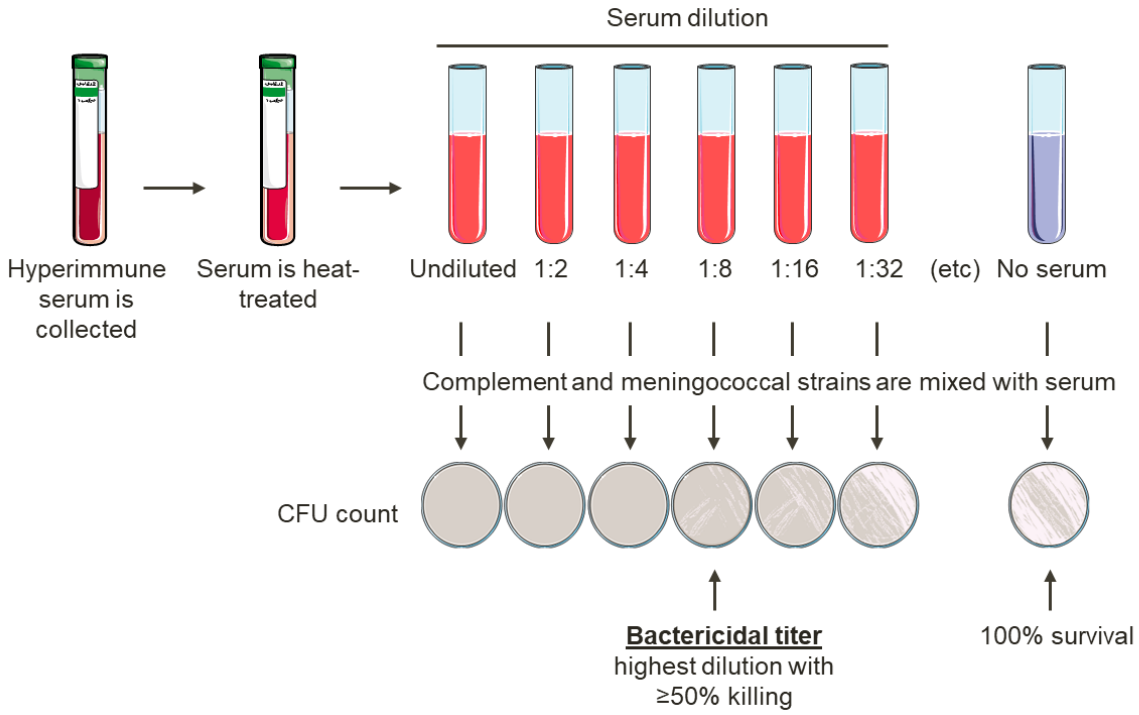

Figure 1.4. Schematic illustration of SBA assay. Hyperimmune serum is collected and it is heath-treated to inactivate endogenous complement. Serum samples are then serially diluted and incubated with bacteria at a given concentration and with exogenous complement. Colony-forming units (CFU) are counted and compared with those found without the addition of serum. The highest dilution of serum that kills ≥50% of the bacteria is then defined as the bactericidal titre (adapted from (Andrews and Pollard, 2014)).

Nowadays, the bactericidal activity of antibodies in presence of human complement, is considered the *in vitro* gold-standard assay for meningococcal protection in human subjects (Borrow *et al.*, 2006) and it is routinely used to support vaccine licensure. Thereby, SBA is used also for testing the seroresponse and the seroconversion upon 4CMenB vaccination. In particular, an hSBA titre ≥4 or,

to be more conservative a ≥4-fold increase in hSBA titres from pre- to post-vaccination, is considered protective (Borrow *et al.*, 2006, McIntosh *et al.*, 2015, Borrow *et al.*, 2005b). Although human complement is the only accepted source for SBA assays aimed to assess vaccine efficacy (Zollinger and Mandrell, 1983), in preclinical studies where hyperimmune sera frequently derive from animal immunizations, SBA in presence of rabbit complement is often used, given also the limited amount of seronegative human complement donors available. In this case, the minimal threshold required for a protective SBA titre is set at ≥16, considering the generally higher titres obtained with this source of complement. However, since protein-based vaccines target the antigenic diversity of disease-causing isolates to provide broad protection, 4CMenB vaccine immunogenicity should be ideally assessed by testing in hSBA multiple strains. Unfortunately this approach was labour-intensive and impracticable, so a minimal panel of *N. meningitidis* isolates mismatched to each of the 4CMenB vaccine antigen, except one, was selected to separately measure the bactericidal antibodies specific to fHbp, NadA, NHBA and PorA (Giuliani *et al.*, 2010). Nonetheless, the requirement of large volumes of human complement and sera, especially limited in the case of infant trials, posed several limitations. Therefore, a novel assay, the Meningococcal Antigen Typing System (MATS), has been successively developed to assess the coverage and to predict the effectiveness of the 4CMenB vaccine (Donnelly *et al.*, 2010). This method allows, for any given meningococcal isolate, to simultaneously quantify the level of expression and the antigenic cross-reactivity for the recombinant 4CMenB protein and correlate it to the probability that the isolate would be killed by vaccinees sera. MATS uses a sandwich ELISA to evaluate the protection induced by NHBA, fHbp and NadA. In particular, by correlating the killing of meningococcal strains in hSBA to their antigen content, it is possible to determine the minimal level of expression of each vaccine antigen required for bactericidal antibody killing induced by 4CMenB. Instead, regarding the contribution of DOMV in vaccine protection, the sequencing of the *porA* locus and the subsequent determination of the serosubtype are retained sufficient for predicting whether a strain would be killed, meaning that strains carrying the P1.4 PorA are considered killed. Using MATS it is hence possible to determine 4CMenB potential coverage in a target geographic area evaluating large panels of isolates (Bettinger *et al.*, 2013, Vogel *et al.*, 2013). However, several evidences demonstrate that this method is a highly conservative predictor of coverage, probably because it cannot take into account any synergistic and/or cumulative effect occurring between vaccine-induced antibodies. Likewise, in MATS-estimated protection the contribution of DOMV-minor antigens is not considered. However, the coverage prediction by MATS is nowadays used to support 4CMenB licensing around the globe, and, according to it, 4CMenB has been predicted to cover 78% of MenB circulating strains in five European countries having at least one antigen rated above the MATS threshold (Vogel *et al.*, 2013).

1.10 Protein-microarray as a tool for the immunological fingerprint of antigens

Despite the serum bactericidal assay is considered a predictive tool of meningococcal protection, this technique is not informative on the bacterial components to which this protection is directed. On the contrary, the identification of immunoreactive antigenic determinants, especially for multicomponent vaccine like 4CMenB, is critical for understanding the mechanisms underlying anti-pathogen immunity. In this context, protein microarray technology represents a high-throughput and versatile approach for the characterization of antigen-antibodies recognition upon vaccination, which may ultimately correlate with functional activities. Requiring minimal volume of biological samples and allowing the simultaneous investigation of thousands of elements, protein microarray-based analyses have been largely used to profile responses against bacterial infections (Lee *et al.*, 2012, Rigat *et al.*, 2019) or as diagnostic tool (Steller *et al.*, 2005). Moreover, being suitable for the screening of polyclonal antibodies (serum and plasma), protein chips have been the assay of choice for the characterization of immunosignatures raised by vaccination (Davies *et al.*, 2008, Price *et al.*, 2013). Recently, tailored protein-microarrays which were hybridized with individual sera from vaccinated infants, adolescents and adults, were used to map the specific epitopes of the 4CMenB antigenic components (fHbp, NHBA and NadA) contributing to protection (Bartolini *et al.*, 2020). Similarly, the same microarray was also used to define the epitopes recognised by a large panel of human monoclonal antibodies elicited by 4CMenB vaccination (Giuliani *et al.*, 2018). Nevertheless, apart from a recent immunoprofiling study conducted with sera from adults vaccinated with an OMV-vaccine (Awanye *et al.*, 2019), the immunological responses induced by the DOMV component of the 4CMenB vaccine have never been investigated with this technique. Indeed, it would represent the first step toward a full characterisation of the contribution that DOMV antigens provide to vaccine protection, beside PorA. In conclusion, protein microarray technology complemented with the serum bactericidal assay have to be considered instrumental for the deconvolution of multicomponent protein-based vaccines, like 4CMenB.

2. Aims of the study

In the present work, we aimed to characterize the immunological properties and the cross-protective ability of antibodies induced by antigens contained into the detergent-extracted outer membrane vesicles (DOMV) component of the 4CMenB vaccine, considering that little is known in literature. In this regard, we firstly evaluated the bactericidal activity of sera derived from infants vaccinated with formulations containing different doses of DOMV by testing them on a panel of strains heterologous for the immunodominant PorA antigen. Then, to specifically identify DOMV antigens involved in such cross-bactericidal activity, the most abundant DOMV-specific proteins, previously identified by proteomic analyses, were cloned and expressed in *Escherichia coli* as recombinant proteins and/or overexpressed as full-length in vesicles, here called GMMA (Generalized Modules for Membrane Antigens). On one hand, the resulting recombinant antigens and engineered GMMA were used for the generation of tailored meningococcal protein-microarrays intended to define the antigen-recognition profile (immunosignature) of antibodies induced in mice by 4CMenB or DOMV. On the other hand, the same samples were singularly used for mice immunization studies and the resulting hyperimmune sera were tested to assess the functionality of each investigated DOMV antigen over the panel of non-vaccine related strains (Figure 2.1).

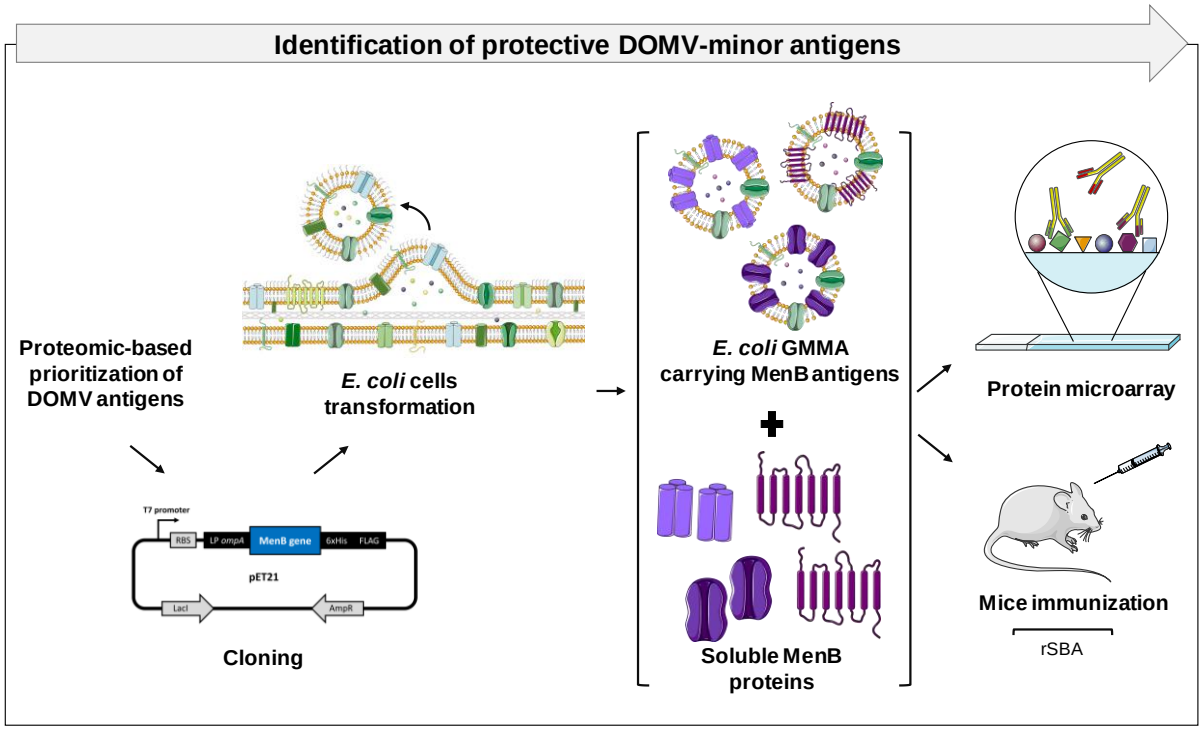

Figure 2.1. Schematic representation of the workflow used for the dissection of the protective response mediated by DOMV antigens. Once prioritized, DOMV-proteins were cloned and expressed in *E. coli* both in GMMA (Generalized Modules for Membrane Antigens) and as recombinant proteins. Samples were then used for the generation of protein microarray as well as for mice *in vivo* studies and the deriving hyperimmune sera were tested in SBA.

3. Results

3.1 The addition of DOMV in the vaccine formulation enhanced the breadth of coverage against a panel of 11 non-vaccine related MenB strains

Detergent-extracted outer membrane vesicles from the epidemic NZ98/254 strain (DOMV) are included, along with the three recombinant meningococcal proteins fHbp, NHBA and NadA (rMenB), in the multicomponent 4CMenB vaccine currently licensed for the active immunization against *Neisseria meningitidis* serogroup B (MenB) (Masignani *et al.*, 2019). While the contribution that rMenB antigens plays in eliciting bactericidal antibodies has been clearly documented in the cohort of vaccinees (Giuliani *et al.*, 2010), the role of DOMV in inducing protective immunity in humans has been poorly investigated. Indeed, early clinical trials of 4CMenB were focused on the possible addition of DOMV on top of rMenB vaccine antigens (Snape *et al.*, 2010, Findlow *et al.*, 2010, Prymula *et al.*, 2014), rather than in the investigation of DOMV immunogenicity alone, principally given the evidences of the safety and efficacy profile previously obtained with the similar OMV-based vaccine *MeNZB* (Arnold *et al.*, 2011). Overall, these clinical experiences confirmed DOMV safety and immunogenicity and demonstrated their beneficial effects in vaccine formulation, which were primarily attributed to the immunodominant PorA protein able to mediate a serosubtype-specific protection. Therefore, the characterization of other DOMV-antigens which may be involved in functionality has always lagged behind.

In order to shed light on vaccine-coverage mediated by the DOMV component of the 4CMenB vaccine, sera from infants immunized with rMenB alone (fHbp var1.1, NadA var3 and NHBA peptide 2) or added with different amounts of DOMV, were tested for their ability to induce complement mediated bactericidal killing of a set of MenB strains. The presence of protective anti-DOMV antibodies was evaluated performing a standardized human serum bactericidal assay (hSBA) using pre- and post-immunization pooled sera derived from 25 infants that received 4 doses of rMenB combined with: i) the full dose of DOMV (equivalent to the final 4CMenB formulation), ii) ¼ dose of DOMV or iii) without DOMV (rMenB alone). Serum samples were assayed against a panel of 11 different strains which were intended to be mismatched for all the 4CMenB antigens, as detailed by the vaccine antigen genotype profile reported in Table 3.1. Given that the study design of the clinical trial did not include any group receiving the DOMV component alone and, on the contrary, all formulations contained rMenB antigens, we selected strains expressing diverse vaccine-antigens variants with the aim to directly tease out the contribution of the solely DOMV-antibodies from the overall functional activity elicited by the vaccination. The genetic diversity of meningococci allowed

the identification of isolates with such characteristics. In particular, taking advantage of the limited cross-reactivity observed for anti-fHbp antibodies against strains carrying different variants (var2 or var3) (Masignani *et al.*, 2003) and considering the absence of the *nadA* gene in half of the circulating MenB population (Comanducci *et al.*, 2004), we selected isolates that express distantly related fHbp variants compared to the one included in the vaccine (except for the MC58 isolate), and that either do not harbour the *nadA* gene or carry a NadA variant not cross-reactive in the SBA. Moreover, to exclude any NHBA-mediated killing, the strains were selected as expressing a NHBA peptide different from peptide 2, with the only exception of NZ98/254 and M10837. Finally, considering the hypervariability of PorA surface-exposed loops and the specificity of the anti-PorA antibodies, we selected isolates with VR1 and VR2 primary sequences different from those of the vaccine isolate NZ98/254. Moreover, being the NZ98/254 the parental strain from which the DOMV are derived, it was included in the panel of isolates bringing the final number of strains to 12.

Isolate description						**Vaccine Antigen Genotype Profile**				
Strain	**Capsular group**	**Year of isolation**	**Country of origin**	**Clonal complex**	**Sequence type**	**fHbp**	***nadA***	**NHBA**	**PorA VR1**	**PorA VR2**
NZ98/254	B	1998	NZL	ST-41/44	42	1.14	absent	2[a]	7-2[b]	4[b]
M10837	B	2003	USA	ST-41/44	409	2.19	absent	2[a]	18-1	34-2
LNP24651	B	2008	FRA	ST-32	32	2.21	yes (1)	47	7	16-26
M08389	B	2001	USA	ST-162	162	2.21	absent	20	22	14
M08117	B	2001	USA	ST-41/44	5879	2.19	absent	29	7-4	1
MC58	B	1985	UK	ST-32	74	1.1[c]	yes (1)	3	7	16-2
M09662	B	2002	USA	ST-60	60	1.13	absent	191	21	16
M14569	B	2005	USA	ST-35	35	2.16	absent	21	22-1	14
M09929	B	2002	USA	ST-35	3592	2.16	absent	19	12-1	16
M07576	B	2000	USA	ST-35	35	2.16	absent	5	22-1	14
M12898	B	2004	USA	ST-35	457	2.16	absent	143	5-1	2-2
M07 241084	B	2007	UK	ST-41/44	1097	2.302	absent	31	19	15
4CMenB						**1.1[c]**	**3**	**2[a]**	**7-2[b]**	**4[b]**

Table 3.1. Characteristics of serogroup B meningococcal strains used as target strains in the SBA analyses. The main characteristics of the isolates are listed. The lowermost row describes the antigen genotype profile of the 4CMenB vaccine. [a, b, c] indicate when the isolate contains variants of NHBA, PorA and fHbp, respectively, homologous to those contained in rMenB or rMenB + DOMV. NZL, New Zealand; USA, United States of America; FRA, France; UK, United Kingdom. fHbp, Factor H binding protein; *nadA*, Neisserial adhesin A, NHBA, Neisserial Heparin Binding Antigen; PorA VR1, Porin A Variable Region 1; PorA VR2, Porin A Variable Region 2.

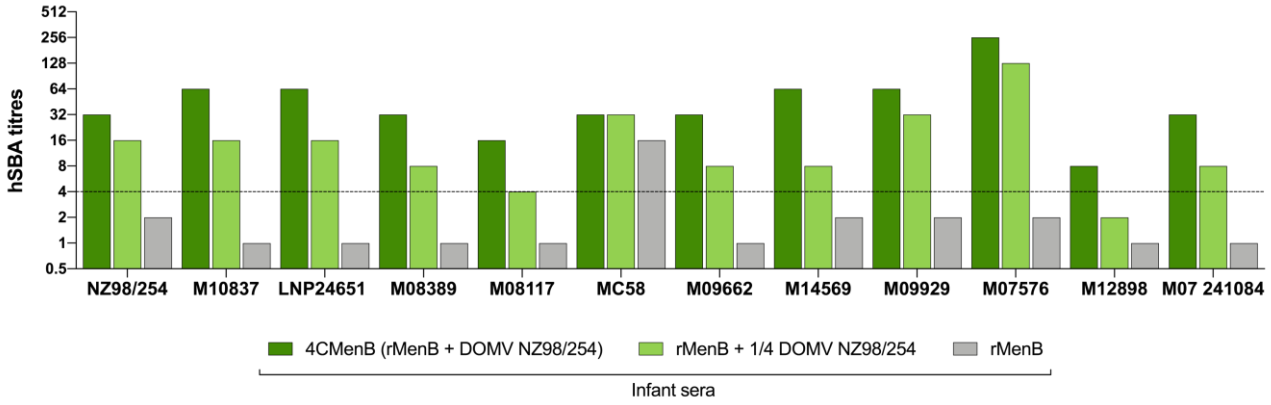

Figure 3.1. The DOMV component of 4CMenB induced protective response in humans. Infant sera (V72P16) were collected before vaccination and after the fourth dose and they were assayed as pool (25 subjects each group). Solid bars describe post-immunization sera. Histograms indicate titres of human SBA (hSBA) obtained with 12 MenB strains using rMenB + DOMV (dark green), rMenB + ¼ DOMV (light green), or rMenB (grey) pooled antisera. Bactericidal titers of preimmune sera was <2 for all groups against all tested strains. Titres ≥4 (dashed line) are considered positive and protective.

Human SBA titres showed that, apart from MC58, none of the strains were susceptible to complement-mediated killing when screened with sera from the rMenB vaccinated cohort (hSBA titres ≤2), confirming the proper selection of the panel of strains for the purpose of the study (Figure 3.1, grey bars). Of note, antibodies to rMenB alone lacked SBA activity against NZ98/254 and M10837 strains, despite expressing NHBA peptide 2. It is known that the mechanisms by which the NHBA antigen elicits SBA are complex and vary depending on: (i) antibody composition of an individual's serum, (ii) NHBA distribution and level of expressions in the strain and (iii) other intrinsic strain factors that scavenge complement regulators, like the binding of NspA or PorB to the factor H (Partridge *et al.*, 2017). Therefore, this result obtained with pooled sera in the cohort of infants, which are usually lower responders to vaccination than older age groups, is not unexpected. On the other hand, the killing of MC58 obtained in presence of rMenB antisera was clearly mediated by anti-fHbp antibodies targeting the vaccine homologous fHbp variant expressed by the isolate. Interestingly, when a full or a quarter-dose of DOMV were added to rMenB formulation and tested on the same set of isolates, the number of strains covered in bactericidal increased to 100% and 92%, respectively (Figure 3.1, green bars). These data suggest that membrane antigens other than PorA and contained in the DOMV component of 4CMenB were able to mount a robust immune response in infants capable to induce complement mediated killing of genetically distinct strains, hence increasing the breadth of coverage with respect to the recombinant protein components alone. Likewise, the differences in SBA titres in the two cohorts of subjects vaccinated with different amounts of DOMV suggest a dose-dependent role of DOMV in meningococcal coverage (Figure 3.1, dark green bars vs

light green bars). In conclusion, the selection of meningococcal strains heterologous for the vaccine antigens and the absence of pre-existing antibodies with functional activity in the infant pre-immune sera enabled to ascribe the cross-bactericidal activity of post-vaccination sera to the presence of the DOMV component, excluding the contribution to the bactericidal killing mediated by fHbp, NHBA or NadA alone. Moreover, vaccines based solely on OMV are also defined as tailor-made, since they are produced from the strains responsible for the outbreaks and used to prevent disease caused by those specific strains (Martin *et al.*, 2006, Tappero *et al.*, 1999). Their protection is usually attributed to bactericidal antibodies targeting PorA, one of the most abundant and immunodominant proteins of the neisserial outer membrane. However, given the limited cross-protection documented for anti-PorA antibodies and considering that the set of strains under investigation express PorA alleles heterologous to the one expressed by NZ98/254 (Table 3.1), our results clearly indicated that bactericidal antibodies raised by DOMV did not uniquely target meningococcal PorA and that other DOMV antigens are instead able to induce functional antibodies accountable for the observed cross-protection.

3.2 Mouse DOMV-antisera confirmed the cross-protective nature of DOMV-raised antibodies and showed a complex pattern of cross-reactivity

Because of the unavailability of sera derived from human subjects vaccinated only with DOMV, an in-depth analysis of the immunological properties of the DOMV component was performed using sera from animals immunized with DOMV-only. CD1 mice were immunized intraperitoneally three times with 8 μg of DOMV and the pooled hyperimmune bleed out sera were used to evaluate the bactericidal killing in presence of baby rabbit complement (rSBA) on the same set of 12 MenB strains described previously. As reported in Figure 3.2, high rSBA titres were obtained for 11 out of the 12 isolates, with titres between 1:2,048 to 1:2,097,152. The only strain resistant to killing was the M07 241084. This result was unexpected considering that the same strain was killed by infant sera immunized with DOMV-containing vaccines (Figure 3.1, green bars). Interestingly, the resistance of M07 241084 to killing by sera containing only antibodies against DOMV suggest that the bactericidal killing is mediated by a cooperative effect of antibodies targeting multiple bacterial antigens, as in the case of those induced by the rMenB plus DOMV formulation. This is in agreement with Giuliani *et al.* who demonstrated that bactericidal killing of some strains was mediated by the synergistic activity of anti-NHBA and anti-DOMV antibodies (Giuliani *et al.*, 2018). Overall, the rSBA results obtained with mouse DOMV antisera validated the conclusions previously drew for human sera about DOMV-mediated protection.

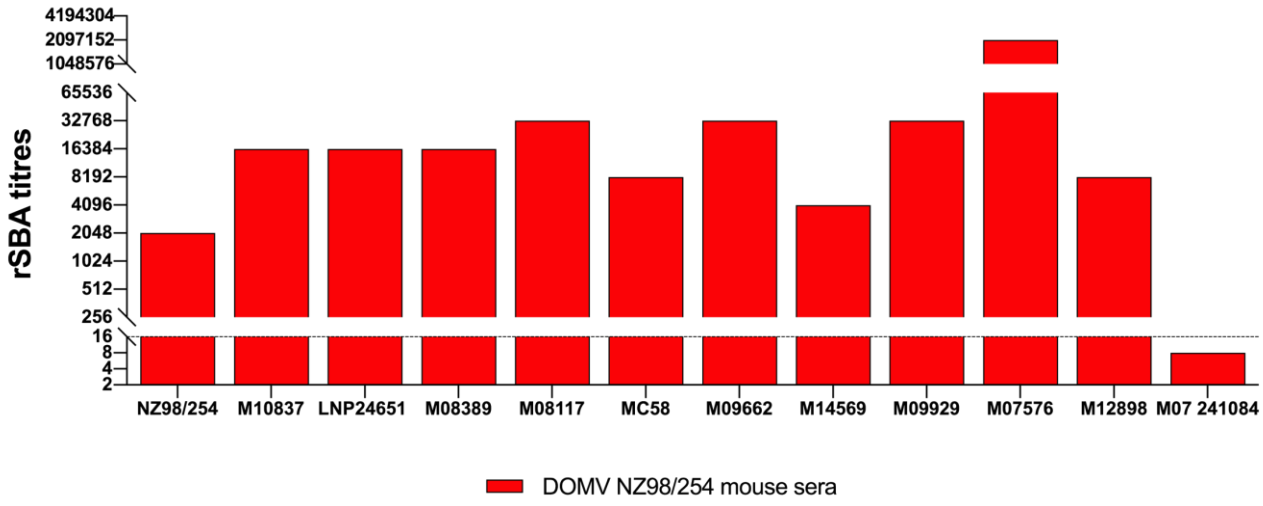

Figure 3.2. The killing ability of DOMV-raised antibodies was confirmed with DOMV-only animal sera. rSBA titres obtained using anti-DOMV mouse sera against the panel of 12 MenB strains under investigation. Eleven groups of 8 mice each deriving from distinct immunization schemes were pooled (n = 88) and used in the analysis. Serum bactericidal titres, indicating the dilution of the pooled mouse sera at which 50% of killing is reached, were determined using baby rabbit complement as source of complement, thereby titres ≥16 (dashed line) are considered positive and protective.

Although the majority of strains was killed in bactericidal, the SBA titres were not identical. To explore the basis of these differences we investigated whether the antibodies induced by the immunization with the DOMV were able to stain the surface of the different strains and to which extent. Differences in recognition could indeed reflect a different membrane composition and in turn, different antibody binding. In particular, the binding of antibodies on the surface of the target bacterium is considered a pre-requisite for the activation of the complement cascade and thus informative of complement-mediated killing. Fluorescence Activated Cell Sorting (FACS) and confocal microscopy were used to measure the binding of anti-DOMV antibodies on the surface of a subset of the MenB strains used in SBA. Seven *N. meningitidis* strains were grown in the same conditions as for the SBA assay and they were incubated with pooled DOMV mouse antisera at different dilutions. FACS profiles (Figure 3.3A) revealed the specific binding of IgG on the surface of all tested isolates and they showed comparable levels of fluorescence intensity for NZ98/254 and the other heterologous strains, confirming again the cross-reactive nature of antibodies elicited by DOMV immunization. Consistently with this, the rSBA titres obtained with α-DOMV sera on these strains were high, ranging from 2,048 to 2,097,152 (Figure 3.2). The ability of DOMV antisera to recognize surface targets on the different bacterial strains was also verified by confocal microscopy. A representative confocal micrograph is shown in Figure 3.3B, where the M07576 strain has been co-stained with DOMV antiserum and FM 4-64, a fluorescent lipophilic dye that selectively

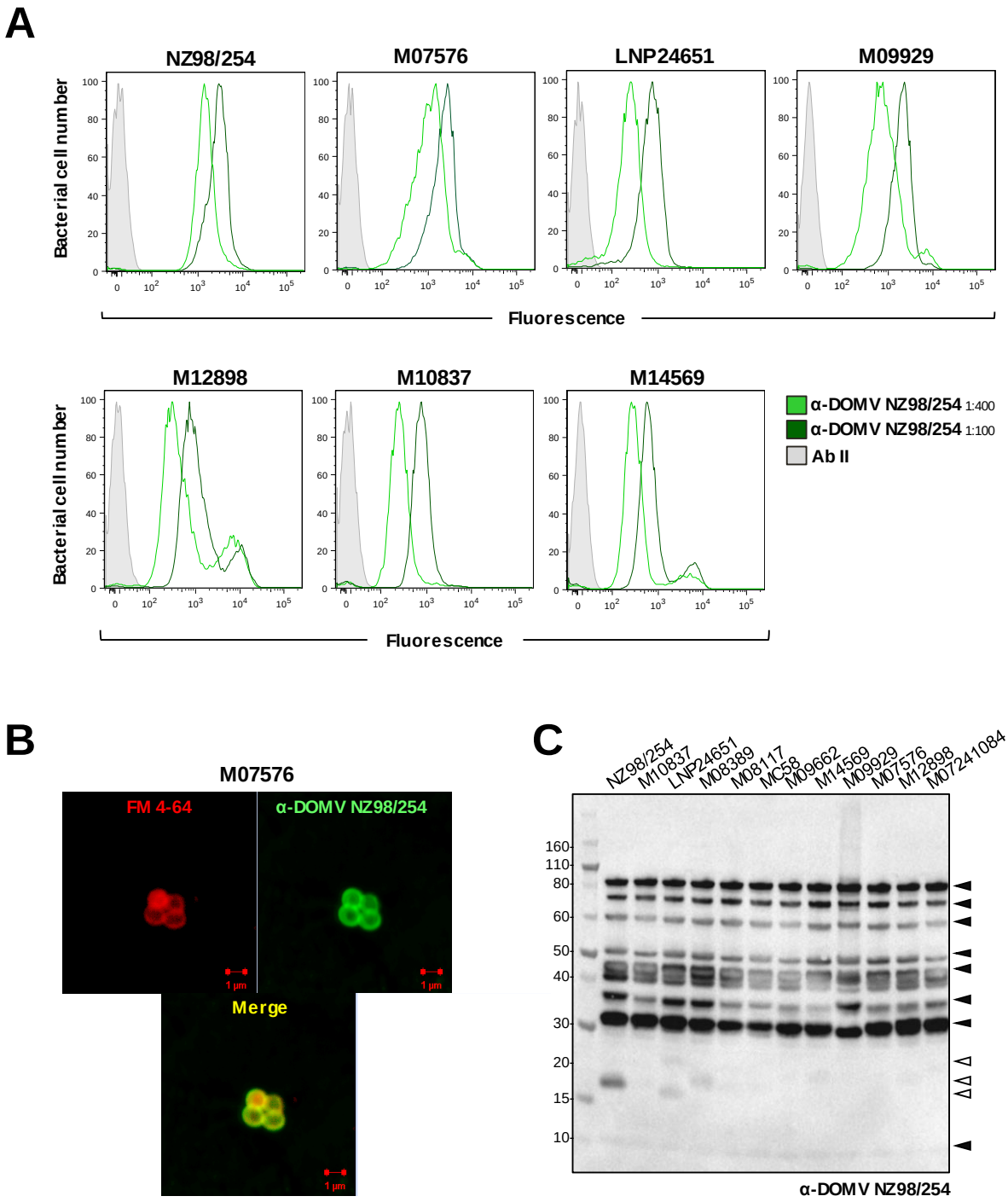

Figure 3.3. Sera raised against DOMV stained the surface of different MenB isolates and revealed a complex recognition profile. (**A**) FACS analyses were performed using mouse anti-DOMV NZ98/254 polyclonal sera against *N. meningitidis* strains grown as for SBA experiments. Dark (1:100) and light (1:400) green profiles represent the binding of antibodies to the surface of the different meningococcal strains at the two indicated dilutions. Grey tinted histograms indicate strains stained only with secondary antibodies which are used as negative controls. (**B**) Immunofluorescence images of the surface of M07576 with FM 4-64 (red) and DOMV antisera (green) were obtained by confocal microscopy. Superimposed image is shown in yellow (scale bar 1µm). (**C**) Normalized neisserial whole-cell lysates were used in Western blot analysis and probed with polyclonal DOMV antibodies. Similar reactive protein pattern among all the MenB strains are pinpointed by closed arrows. Open arrows describe strain-specific reactive proteins. Pooled (n = 8) anti-DOMV NZ98/254 polyclonal sera were used in these analyses.

intercalates within bacterial membranes. As the merge panel shows, the clear colocalization of the signals indicated that DOMV-induced antibodies were able to bind the surface of M07576. Collectivelly, the binding observed in FACS and in confocal microscopy paralleled antibody functional activity in rSBA with DOMV antisera. Having demonstrated that DOMV immunization raised cross-reactive antibodies able to specifically recognize the surface of meningococcal strains, we investigated whether the cross-recognition was directed towards a single antigen or to a set of proteins. Figure 3.3C reported the results of a Western blot analysis in which the whole-cell lysates of the 12 MenB strains were probed with DOMV antisera. Several proteins were recognized by the sera and most of them were consistently recognized in all strains (Figure 3.3C, closed arrows), with only minor differences in the recognition profile of low molecular weight proteins (Figure 3.3C, open arrows). Despite this technique did not take into account antigen surface localization, the blot suggested that the immunization with DOMV induced IgG antibodies directed towards a variety of meningococcal proteins ranging from 10 to 80 kDa.

3.3 The prioritized DOMV-antigens were expressed in *E. coli* vesicles (GMMA) or as recombinant proteins

Considering that DOMV antisera were bactericidal against PorA-heterologous strains and that whole-cell lysates probed with the same antisera were characterized by a complex protein pattern, we investigated which DOMV-minor antigen/s was/were accountable for eliciting protective antibodies against the MenB strains under investigation. To this aim a panel of 30 DOMV-specific antigens was selected and prioritized. The choice of the 30 proteins (Table 3.2) was driven by previous proteomics studies conducted on DOMV. In particular, we specifically selected the 25 most abundant outer membrane proteins (OMP) and lipoproteins which Tani and colleagues consistently found across six different DOMV production lots, excluding periplasmic and cytoplasmic proteins which are known to be less likely targeted by bactericidal antibodies according to their innermost cellular localization (Tani *et al.*, 2014). To these selected antigens, five additional proteins were included in the study as they were identified in the outer membrane vesicles (OMV) of *N. meningitidis* Δ*gna33* derived from different isolates (Ferrari *et al.*, 2006). It is worth noting that the NspA protein was included into the prioritization list despite being poorly detected by the mass-spectrometry (MS) analysis, possibly due to its high number of lysine residues which lead to frequent trypsin miss-cleavage biasing the MS-quantification (Tani *et al.*, 2014). Lastly, the PorA antigen was included as positive control in the final list of 30 DOMV-specific antigens selected as potentially involved in 4CMenB cross-coverage.

Gene accession number		Gene	Protein annotation	Protein abundance across DOMV NZ98/254 lots[a]
PubMLST	MC58			
Outer membrane protein				
NEIS2020	NMB2039	*porB*	Porin B; **PorB**	42,54
NEIS1364	NMB1429	*porA*	Porin A; **PorA**	28,64
NEIS1428	NMB1497	-	TonB-dependent receptor	4,60
NEIS1783	NMB0382	*rmpM*	Class 4 outer membrane protein; **RmpM**	3,08
NEIS0944	NMB0964b	-	TonB-dependent receptor	2,87
NEIS0408	NMB1812	*pilQ*	**PilQ** protein	1,44
NEIS2198	NMB1053	*opcA*	Class 5 outer membrane protein; **OpcA**	0,75
NEIS1963	NMB1988	*frpB*	Iron-regulated outer membrane protein; **FrpB** (FetA)	0,96
NEIS1690	NMB0461	*tbp1*	Transferrin-binding protein 1; **Tbp1** (TbpA)	0,92
NEIS0173	NMB0182	*omp85*	Outer membrane protein **Omp85**	0,87
NEIS0073	NMB0088	-	Outer membrane protein P1; **OmpP1**	0,54
NEIS1468	NMB1540	*lbpA*	Lactoferrin-binding protein A; **LbpA**	0,46
NEIS0275	NMB0280	-	Organic solvent tolerance protein; **OstA**	0,44
NEIS1487	NMB1567	-	Macrophage infectivity potentiator; **MIP**	0,23
NEIS1632	NMB1714	*mtrE*	Multidrug efflux pump channel protein; **MtrE**	0,29
NEIS0101	NMB0109b	-	Hypothetical protein	0,26
NEIS1271	NMB1333	-	Hypothetical protein	0,24
NEIS1687	NMB0464	-	Phospholipase A1	0,09
NEIS0210	NMB0018	*pilE*	Pilin **PilE**	0,08
NEIS2075	NMB2095	-	Adhesin complex protein; **ACP**	n/a[b]
NEIS1246	NMB1309	-	Fimbrial biogenesis and twitching motility protein	n/a[b]
NEIS0612	NMB0663	*nspA*	Outer membrane protein, **NspA**	n/a[b;c]
NEIS1462	NMB1533	-	Outer membrane protein H.8; **OmpH8**	n/a[b]
NEIS0172	NMB0181	-	Outer membrane protein H; **OmpH**	n/a[b]
Lipoprotein				
NEIS1066	NMB1126/NMB1164	-	Hypothetical protein	1,06
NEIS0596	NMB0375/NMB0652	*mafA*	**MafA** protein	0,18
NEIS1634	NMB1716	*mtrC*	Membrane fusion protein; **MtrC**	0,12
NEIS0653	NMB0703	*comL*	Competence lipoprotein; **ComL**	0,14
NEIS0196	NMB0204	*bamE*	Outer membrane protein assembly factor; **BamE**	0,10
NEIS1065	NMB1125/NMB1163	-	Hypothetical protein	0,06

Table 3.2. List of the 30 MenB proteins selected as potentially contributing to 4CMenB coverage. The prioritization was based on protein abundance in 6 different DOMV NZ98/254 production lots (Tani *et al.*, 2014) and on protein presence in OMV from *N. meningitidis* Δ*gna33* strains (Ferrari *et al.*, 2006). Gene accession numbers are provided as NEIS, as described in PubMLST (https://pubmlst.org/neisseria/), and as NMB, as reported in NCBI for MC58 (https://www.ncbi.nlm.nih.gov/nuccore/NC_003112.2).

[a] indicates the average of protein abundance calculated from six production lots of 4CMenB from (Tani *et al.*, 2014).

[b] describes that data from (Tani *et al.*, 2014) are not available (n/a) for those proteins. These antigens were prioritized according to their presence in OMV from *N. meningitidis* Δ*gna33* (Ferrari *et al.*, 2006).

[c] NspA is poorly detected by the proteomic approach, compared with its abundance on SDS-PAGE (Tani *et al.*, 2014).

The genes encoding for the prioritized antigens were amplified from the vaccine-strain NZ98/254 and then cloned into an *ad hoc* vector specifically designed to address meningococcal antigens to the outer membrane of *E. coli* strains (Figure 3.4A). The pET21-LPOmpA plasmid, a pET21 derivative

plasmid, was constructed to have the leader peptide of the *E. coli* OmpA downstream of the T7 IPTG-inducible promoter. This cloning strategy allowed the N-terminal fusion of the *E. coli* periplasmic secretion sequence to the protein of interest. Consequently, apart from NEIS0101 and NEIS0210, all proteins were cloned without their natural secretory signal. Moreover, the hexa-histidine and the FLAG tag sequences were fused both to the N- and to the C-terminal of each antigen and the best cloning strategy was selected based on protein expression (data not shown). The plasmids were then used to transform BL21(DE3)Δ*tolR* or alternatively BL21(DE3)Δ*ompA*, two *E. coli* mutant strains engineered to release large quantities of vesicles, called GMMA (Generalized Modules for Membrane Antigens), into the culture supernatant. The 80% of DOMV-specific antigens prioritized in the study were integral OMP (Table 3.2) whose expression and refolding into their native three-dimensional structure was considered rather challenging because of their hydrophobic nature. In this context, GMMA represented an alternative valuable approach not only by offering an ideal scaffold for the expression of heterologous proteins, but also allowing the presentation of the antigens in their native orientation and conformation to the immune system, which is critical to stimulate an effective humoral immune response (Gerritzen *et al.*, 2017, van der Pol *et al.*, 2015). Once transformed, the strains were grown to late-logarithmic phase and meningococcal antigen expression was induced for 6 hours by supplementing the culture with 1 mM IPTG. GMMA were purified from bacterial culture supernatants as described in the Materials and Methods section. BL21(DE3)Δ*tolR* and BL21(DE3)Δ*ompA* strains transformed with vector devoid of any insert were instead used to produce GMMA Empty (negative controls).

The expression of MenB antigens in *E. coli* GMMA was verified by Coomassie blue stained gels and Western blots probed with anti-FLAG antibodies (Figure 3.4B and C, respectively). As shown by the SDS-PAGE, 21 of the 30 prioritized proteins were expressed at different levels in *E. coli* GMMA once fused to amino- or carboxy-terminal tags. Meningococcal antigen expression of other five proteins was instead confirmed by immunoblots, for a total of 26 antigens of the 30 selected. Of note, the expression of the antigen NEIS0596 was clearly visible in the stained SDS-PAGE, while only a faint band was present at the corresponding molecular-weight in the immunoblot. The explanation may lay in the inaccessibility of the FLAG tag of the protein to antibodies.

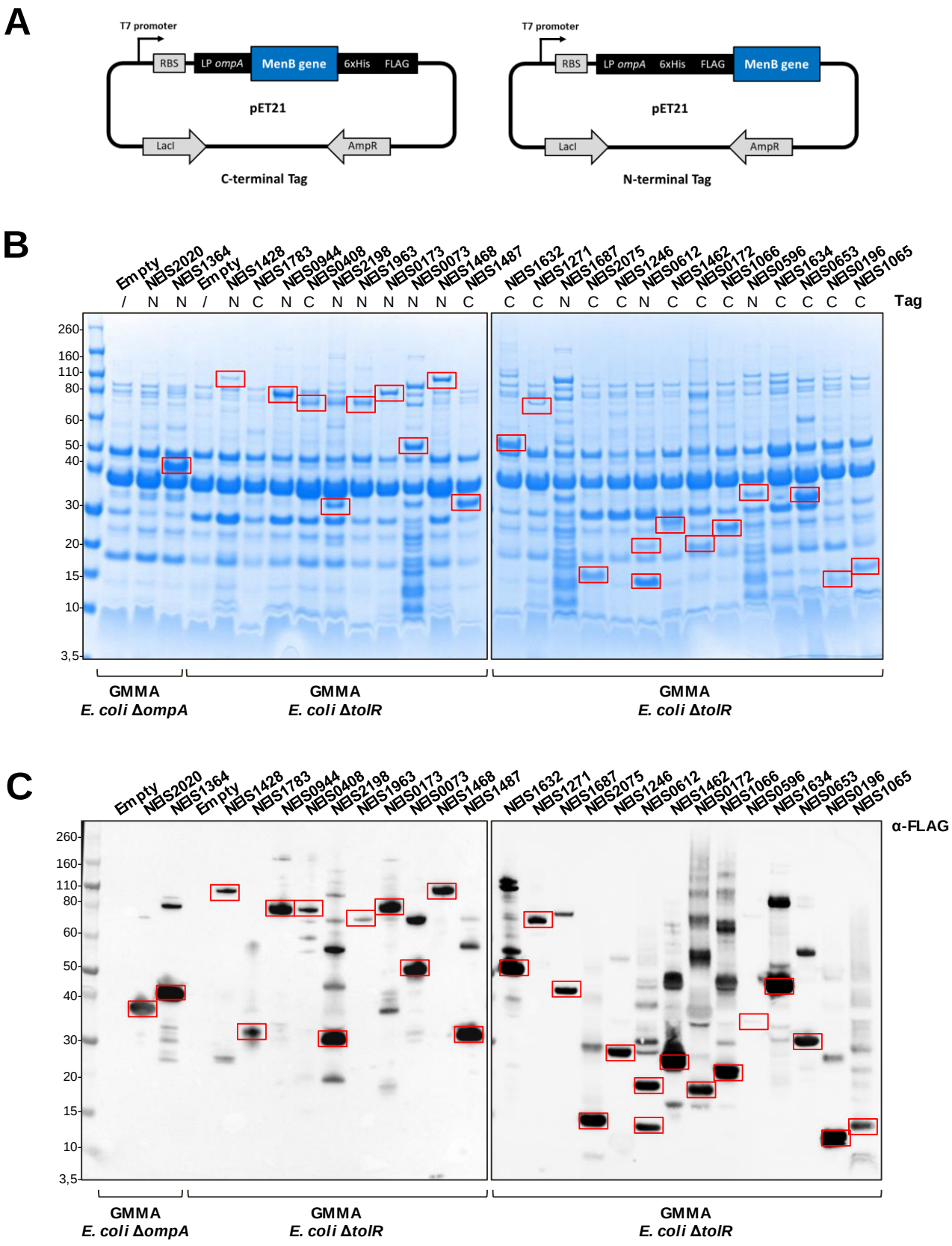

Figure 3.4. DOMV antigens were expressed in GMMA obtained from different *E. coli* mutants engineered to hyperbleb. (**A**) Two different strategies were adopted for the cloning of meningococcal proteins into the expression vectors. The two vector maps differ only in the location of the tags, either carboxy- or amino-terminal (left and right

maps, respectively). The vectors carry the IPTG-inducible T7 promoter, the leader peptide (LP) of the *E. coli ompA* and tag sequences composed of six-histidine (6xHis) and the FLAG. The cloning site of heterologous antigens is represented by the blue box labelled "MenB gene". RBS, ribosome binding site; LacI, *lac* repressor; AmpR, ampicillin resistance. Recombinant *E. coli* GMMA decorated with meningococcal antigens were analysed by (**B**) Coomassie blue stained SDS-PAGE and (**C**) Western blot probed with α-FLAG antibodies. Bands corresponding to recombinant antigens are boxed in red. Final location of the tag sequence (N, N-terminal and C, C-terminal) as well as *E. coli* strains used for GMMA production are indicated.

Moreover, despite both cloning strategies have been adopted, we were unable to express NEIS0210, NEIS1690, NEIS0275 and NEIS0101 proteins in GMMA. According to anti-tag immunoblots performed on post-induction *E. coli* whole cell-lysates, different levels of NEIS0210, NEIS1690 and NEIS0275 expression were detected both in the total and soluble fractions, but the antigens were not localized into the recombinant vesicles (Figure 3.5). The reason for this was not investigated further. On the contrary, the antigen NEIS0101 was expressed at very low levels in the cell lysates (Figure 3.5) which may explain the lack of expression in the derivative *E. coli* GMMA.

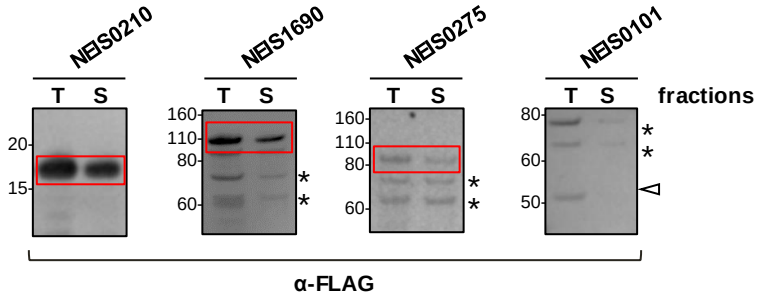

Figure 3.5. Four MenB antigens were expressed in *E. coli* cell lysates but they were not taken up by the corresponding GMMA. Western blots probed with anti-FLAG antibodies were carried out on *E. coli* BL21Δ*tolR* total (T) and soluble (S) cell-extract fractions expressing the specific MenB antigens with C-terminal tags. Bands corresponding to recombinant antigens are boxed. The open arrow indicates the expression of NEIS0101 exclusively in the total cell extract fraction. * indicates not specific band.

Parallelly to the expression of antigens in *E. coli* vesicles we explored the possibility of obtaining meningococcal proteins in their recombinant form as His-tag fusions. During the production of recombinant *E. coli* GMMA, total and soluble fractions of whole-cell lysates from the parental cultures were screened in Western blot for protein expression (data not shown). From them, only proteins whose presence was visible in the soluble fraction of lysates were selected for purification by metal chelate affinity chromatography, followed by size exclusion chromatography. Conversely, PorA and PorB which did not match the aforementioned criteria, were expressed in the inclusion bodies, re-solubilized and refolded by dilution into buffers containing detergents before purification.

From the panel of the 30 MenB antigens 13 different recombinant proteins were successfully purified as shown in the SDS-PAGE of Figure 3.6.

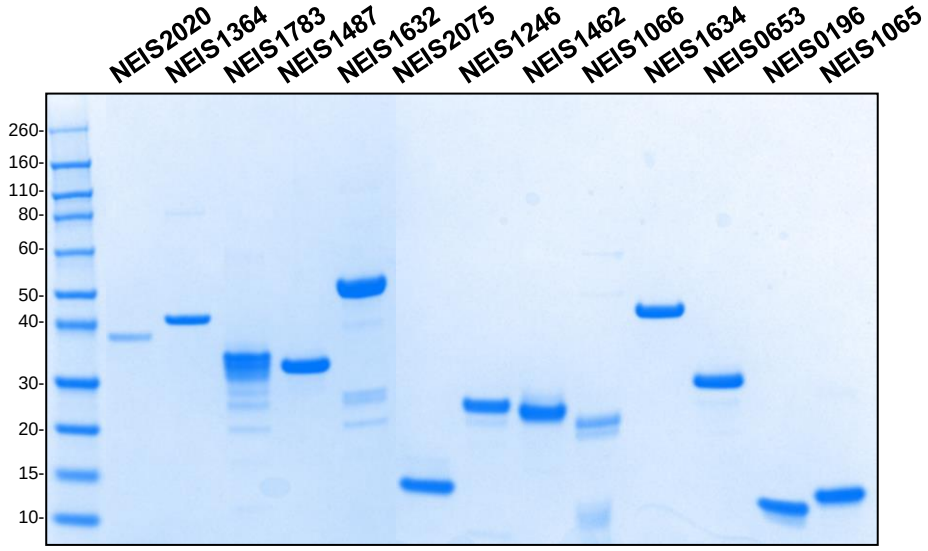

Figure 3.6. Thirteen different meningococcal proteins were obtained as recombinant antigens. Equal amounts of meningococcal antigens (2 µg/lane, except for NEIS2020 and NEIS1066 1 µg/lane) were resolved in a Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis and were stained with Coomassie blue.

3.4 DOMV-proteins shared a high level of amino acid identity within the collection of 11 MenB strains

To evaluate the cross-protective ability of the DOMV-antigens under investigation against the collection of genetically different strains, we investigated the conservation of the 30 proteins compared to the NZ98/254 strain, from which the DOMV of the 4CMenB vaccine are derived. Possibly due to incomplete sequence assembly, the PubMLST database did not contain the allele of the NEIS1690 gene (*tbp1*) of the NZ98/254 reference strain; therefore, for this locus we used the sequence of the NZ05/33 isolate, which shares 99.4% sequence identity and 1,595/1,605 core genome loci with NZ98/254 (Rodrigues *et al.*, 2018). Moreover, although PilE (NEIS0210) and MafA (NEIS0596) proteins were amongst the group of antigens under investigation, they were excluded from the analysis for different reasons. Indeed, the former was not present in the public database probably due to sequencing/assembly difficulties since its sequence consists of highly repetitive DNA stretches with multiple copies within the meningococcal genome (Rotman and Seifert, 2014); the latter was found fragmented across multiple contigs in several of the MenB genomes under investigation. The percentage of amino acid sequence identity obtained from the bioinformatic analysis is described in Figure 3.7. The degree of diversity of the proteins with respect to the DOMV-

vaccine strain varied, ranging from the most conserved peptides (NEIS0101, 99.26-100% amino acid identity) to the most variable (PorB, NEIS2020 60.17%-100%). However, the majority of NZ98/254 OMP (20 of 28) displayed from 95.74 to 100% sequence conservation within *N. meningitidis* isolates, with the lowest percentage of sequence identity observed for the PorB antigen (NEIS2020) expressed by M09662 (60.17%). Among the most dissimilar proteins compared to NZ98/254 there were: PorB (NEIS2020), FrpB (NEIS1963), Tbp1 (NEIS1690), LbpA (NEIS1468), PorA (NEIS1364), OmpP1 (NEIS0073) and TonB-dependent receptor (NEIS0944). Surprisingly, from our analysis BamE (NEIS0196) resulted as one of the most diverse proteins; however, almost all the strains, with the exception of M09662, harboured an identical BamE peptide, which was different from that expressed by NZ98/254, biasing thus the analysis.

	M10837	LNP24651	M08389	M08117	MC58	M09662	M14569	M09929	M07576	M12898	M07241084
(PorB) NEIS2020	94.56	97.58	96.07	97.89	92.15	60.17	97.58	100.00	97.58	97.58	99.39
(PorA) NEIS1364	87.98	93.89	90.84	95.42	93.89	92.09	89.54	90.86	89.80	90.28	91.58
(TonB-dep rec) NEIS1428	98.37	98.15	98.81	98.37	97.94	98.81	98.70	97.83	98.70	98.70	100.00
(RmpM) NEIS1783	100.00	98.76	98.76	100.00	98.76	100.00	99.17	99.17	99.17	99.17	100.00
(TonB-dep rec) NEIS0944	95.55	95.94	90.59	95.68	95.94	94.99	95.94	94.99	95.16	95.16	95.55
(PilQ) NEIS0408	98.57	98.70	98.83	99.74	99.48	98.83	98.83	98.70	98.83	96.10	98.96
(OpcA) NEIS2198	98.53	98.16	98.53	98.90	98.16	98.53	98.16	98.16	98.16	98.16	98.90
(FrpB) NEIS1963	98.04	93.21	90.91	91.30	98.04	94.83	95.01	94.97	95.71	91.85	97.48
(Tbp1) NEIS1690*	96.73	95.43	95.75	95.33	94.78	96.96	na	96.09	96.84	96.08	96.41
(Omp85) NEIS0173	98.87	99.62	98.87	99.62	99.62	99.50	99.37	99.12	99.37	99.37	100.00
(OmpP1) NEIS0073	100.00	93.15	93.58	100.00	93.15	94.86	93.15	93.15	93.15	93.15	100.00
(LbpA) NEIS1468	97.67	96.19	94.50	98.09	96.08	97.03	97.04	98.94	97.46	97.46	96.08
(OstA) NEIS0275	99.75	98.00	99.63	98.88	98.75	99.38	99.38	99.00	99.38	99.38	99.38
(MIP) NEIS1487	100.00	100.00	99.63	100.00	100.00	99.63	100.00	97.79	100.00	100.00	100.00
(MtrE) NEIS1632	100.00	99.38	98.76	100.00	99.38	99.38	99.38	98.96	99.38	99.38	100.00
(Hp) NEIS0101	100.00	99.26	99.51	100.00	99.26	99.26	99.26	99.26	99.26	99.26	100.00
(Hp) NEIS1271	100.00	99.67	99.67	100.00	98.67	98.67	99.67	99.67	99.67	99.67	100.00
(Phosph A1) NEIS1687	99.73	99.73	100.00	99.73	99.73	99.73	99.73	99.47	99.47	99.73	99.20
(ACP) NEIS2075	100.00	99.19	100.00	100.00	99.19	100.00	100.00	100.00	100.00	100.00	100.00
(Fimbrial) NEIS1246	100.00	100.00	99.62	100.00	100.00	99.25	99.62	99.62	100.00	100.00	100.00
(NspA) NEIS0612	99.43	98.28	97.70	100.00	98.28	100.00	100.00	100.00	100.00	100.00	97.70
(OmpH8) NEIS1462	95.74	100.00	100.00	99.45	100.00	99.45	98.91	99.45	98.91	98.91	99.45
(OmpH) NEIS0172	100.00	100.00	100.00	100.00	100.00	99.40	100.00	100.00	100.00	100.00	100.00
(Hp) NEIS1066	99.10	100.00	100.00	99.10	100.00	99.55	99.55	99.55	99.55	99.55	99.10
(MtrC) NEIS1634	99.76	98.54	99.51	99.76	98.54	99.27	99.03	99.51	99.03	99.03	99.76
(ComL) NEIS0653	100.00	98.13	100.00	100.00	96.25	100.00	100.00	99.63	100.00	100.00	100.00
(BamE) NEIS0196	95.20	95.20	95.20	95.20	95.20	89.60	95.20	95.20	95.20	95.20	95.20
(Hp) NEIS1065	100.00	100.00	100.00	100.00	100.00	99.19	100.00	100.00	100.00	100.00	100.00

60 65 70 75 80 85 90 95 100

Figure 3.7. High percentage of amino acid identity of the major DOMV-proteins among the MenB collection. Multiple sequence alignments of 28 DOMV-specific proteins in 12 MenB strains were obtained and percentages of identity for each antigen with respect to NZ98/254 are reported. Blue scale represents the degree of identity, from dark blue (60%) to white (100%). The asterisk indicates that the reference sequence of the locus was retrieved from NZ05/33. na describes the unavailability of nucleotide sequence due to the multiple fragmentation of the gene among different contigs.

Moreover, during the multiple alignment analyses we observed that for some of the proteins, such as PorA, PorB, FrpB, Tbp1, LbpA and OmpP1, the peptide sequence variability was not distributed evenly across the full amino acid sequence and the differences were concentrated in peculiar regions. These data were not surprising, considering that it is well-documented that in bacteria the mutation hotspots are usually localized in regions encoding for surface-exposed epitopes which are constantly under selective pressure by the immune system (Pajon *et al.*, 1997, van der Ley *et al.*, 1991). An example is provided by the PorA alignment shown in Figure 3.8, where the hypervariable regions VR1 and VR2, correspond to loop I and loop II, respectively.

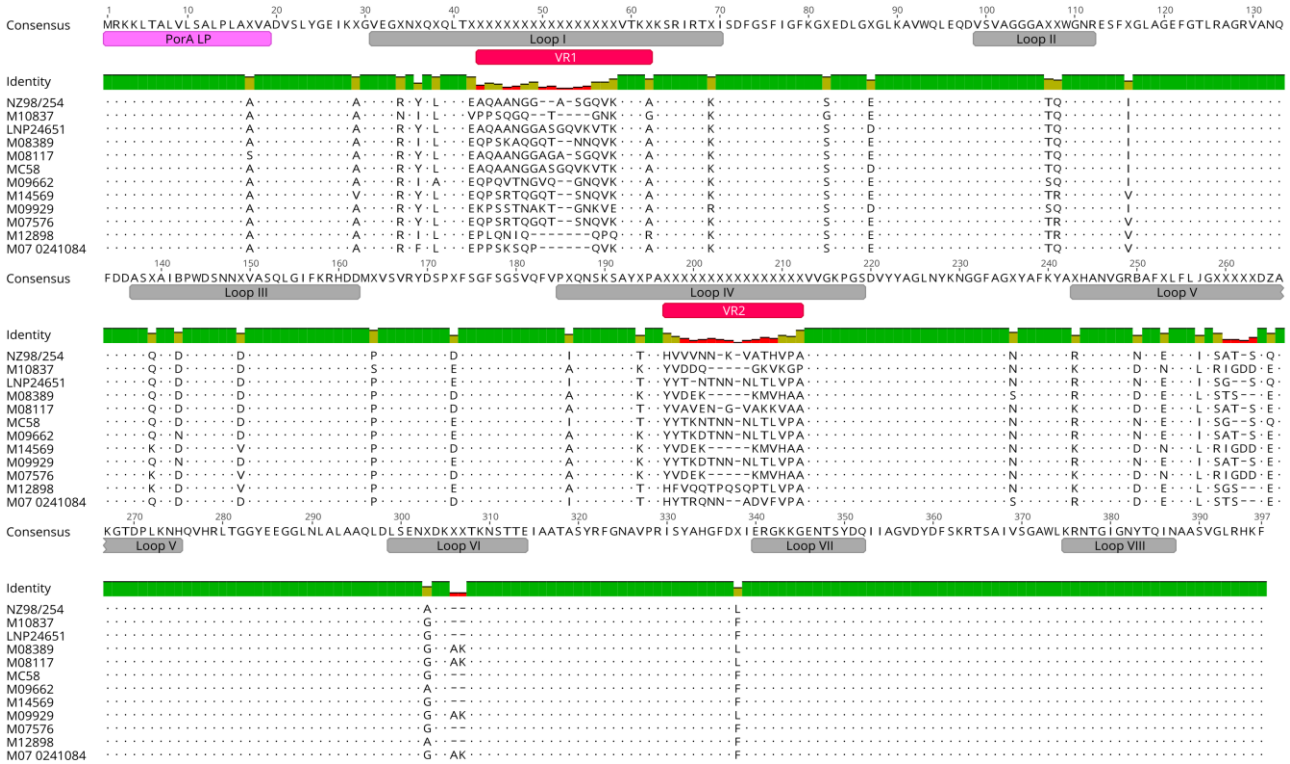

Figure 3.8. Multiple sequence alignment of the different PorA peptides carried by the 12 *N. meningitidis* strains confirmed that hypervariable regions correspond to surface exposed loops. The consensus sequence is shown above the aligned sequences and the coloured bar represents the identity across all sequences: in green 100% identity, in yellow lower identity and in red very low identity. Within the multiple sequence alignment, conserved amino acids are represented by dots, mismatches are indicated with the amino acid and gaps are illustrated by dashes. The locations of the Variable Region 1 (VR1) and 2 (VR2) (in red), the leader peptide (in pink) as well as the putative surface-exposed loop sequences (I–VIII) (in grey) are indicated.

Lastly, in the alignment of PorB sequences we observed that, while M09662 carried the *porB2* allele (encoding PorB2 or class 2 protein), all other strains, including NZ98/254, harboured the *porB3* allele (encoding for PorB3 or class 3 protein). These two PorB protein classes exhibit high levels of genetic diversity, especially in the surface-exposed regions, with longer extracellular loops present in PorB2 respect to PorB3. Thereby, as suggested by the phylogenetic tree constructed from the multiple

sequence alignment of the 12 PorB peptides, the allele of the strain M09662 was the most distantly related (Figure 3.9), while the PorB3 alleles carried by the other isolates were all clustered in a separated branch of the tree. This result was in line with the low percentage of PorB amino acid identity observed between NZ98/254 and M09662 (Figure 3.7).

Figure 3.9. The PorB2 expressed by M09662 was distantly related to that carried by the other strains. Phylogenetic tree reconstructed from the multiple sequence alignment of PorB peptides identified in the 12 MenB strains. The reference isolate NZ98/254 is highlighted in red, whereas the outlier M09662 is in blue. Tree scale is represented as a bar.

3.5 Protein microarray analysis enabled the identification of highly reactive MenB antigens in sera from mice immunized with DOMV

Once the cloning, expression and purification of the MenB antigens were achieved, we included those samples in *ad hoc* MenB protein microarrays to characterize the antibody response directed towards the different components of the serogroup B meningococcal vaccine. In particular, the dissection of vaccine-induced humoral response was aimed to the identification of the immunogenic antigens present into the DOMV which could play a role in the DOMV-mediated protection.

In order to optimize the spotting conditions of both substrates, GMMA and recombinant proteins, two separate MenB protein microarrays were manufactured. The former was realized spotting onto the slide the 26 *E. coli* GMMA containing each DOMV antigen, two GMMA Empty and the DOMV from NZ98/254 as control, whereas the latter was generated by printing the purified recombinant proteins. The rMenB components of 4CMenB (NadA, GNA2091-fHbp and NHBA-GNA1030) were spotted onto both microarrays as positive controls. All samples were printed randomly in replicates onto nitrocellulose-coated glass slides. A preliminary screening with specific cocktails of antibodies was performed to assure that the replicate spots were efficiently immobilized on nitrocellulose slides

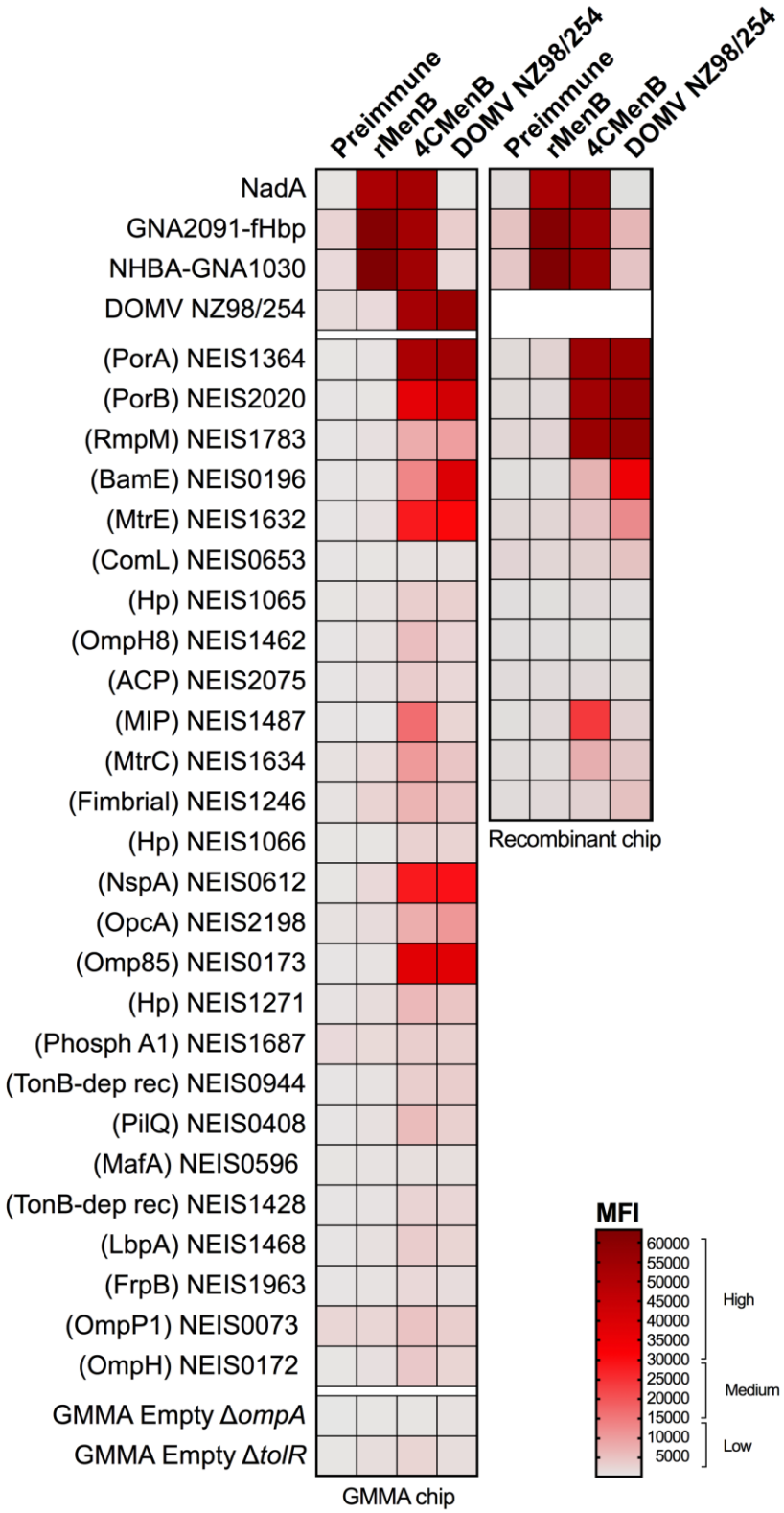

Figure 3.10. The immunosignature of DOMV and 4CMenB antisera revealed eight immunogenic DOMV-proteins. Results of the immunoscreening obtained on GMMA and recombinant protein microarrays are represented by distinct heatmaps. Pre- and post-immunization sera were pooled (preimmune pool *n* = 80, rMenB pool *n* = 10, 4CMenB pool *n* = 54, DOMV NZ98/254 pool *n* = 88) and used in the analysis. Sera are arranged in columns and samples spotted onto the chip in rows. Each tinted block represents the averaged reactivity of the replicated spots for each sera screened and results are expressed as MFI values. Signals were considered positive when their MFI were greater than 5,000,

corresponding to the MFI of control protein-spots after detection with fluorescent-labelled anti-mouse antibodies, plus 10 times the standard deviation. Three arbitrary MFI thresholds were also assigned for low (5,000≤ MFI >15,000), medium (15,000≤ MFI >30,000 MFI) and high (MFI ≥30,000) reactivities. Colour scale of signal intensity is reported on bottom-right of the heatmap. Sample "rNEIS1066 (Hp)" was excluded from the analysis since the protein was found degraded at the time of this particular experiment.

with excellent reproducibility. Sera reactivity was assessed by detecting total IgG bound to each protein spot using fluorescently labelled secondary antibodies and measuring the Fluorescence Intensity (FI) values of each protein. For the analysis, the FI data of the replicates of each antigen were subtracted by local background and averaged, hence resulting in a single data point per sample named Mean Fluorescence Intensity (MFI).

Once designed, manufactured and validated the meningococcal protein chips were used to immunoprofile the antibody repertoire raised by different vaccine formulations. Pooled sera from final bleed of mice immunized with 4CMenB, DOMV or rMenB antigens were used to hybridize the slides, using preimmune (PI) sera as control. The serum IgG reactivity of both protein chips are summarized in the heatmaps of Figure 3.10. From the screening, PI sera resulted consistently not reactive, while, as expected, the other three tested sera reacted differentially with antigens upon immunizations. Indeed, IgG antibodies raised by rMenB were exclusively directed against NadA, GNA2091-fHbp and NHBA-GNA1030. On the other hand, following immunization with DOMV-containing vaccine formulations, a more complex pattern of recognition was observed. Despite slight differences in MFI values between the two arrays mainly due to the difficulties to normalize the spots on the basis of the amount of antigen contained in the GMMA, according to both protein chips the most reactive antigens which were recognized by α-DOMV and α-4CMenB antibodies corresponded to PorA, PorB, RmpM, BamE and MtrE. On the contrary, by having only the GMMA-based form of NspA, OpcA and Omp85 such antigens resulted highly immunogenic exclusively on GMMA-chip. Moreover, the reactive profile observed with the 4CMenB total formulation resembled that of DOMV-single component, with even greater MFI values observed for MIP and MtrC using 4CMenB antisera. The protein microarray technology was thus instrumental to measure the immune responses against DOMV proteins and allowed a detailed dissection of the subset of DOMV-antigens able to stimulate a robust response both when administered alone and when contained in the *Bexsero* formulation, moreover, it pinpointed a potential role of such proteins in the protection. Noteworthy, we did not observe a clear correlation between immunogenicity and antigen amount in DOMV as was the case of BamE and MtrE, present in DOMV in very low percentage, but showing an high IgG reactivity (Tani *et al.*, 2014). Thus, these results suggest that the most abundant antigens are not exclusively the most immunogenic ones.

3.6 Western blot and ELISA confirmed the induction of specific antibodies in sera collected from *in vivo* studies

Parallelly to the immunosignature of antisera by protein array, we investigated whether the DOMV-specific proteins expressed in *E. coli* GMMA or as recombinants were able to induce specific antibody responses. Thereby, mice were immunized intraperitoneally three times at 2 weeks apart with 20 µg of recombinant antigens or 8 µg of *E. coli* GMMA (Figure 3.11). Along with the twenty-six recombinant *E. coli* GMMA expressing MenB proteins and the whole set of recombinant proteins (except PorA), we used for immunizations two GMMA Empty derived from BL21(DE3) Δ*tolR* and Δ*ompA* which did not express any antigen (control groups). As our final goal was to evaluate the functionality of the generated antibodies, we decided to administer to mice uniquely PorA expressed in *E. coli* GMMA rather than the refolded one, considering that it has been previously shown that SDS-denatured recombinant PorA failed to induce bactericidal immune responses (Nurminen *et al.*, 1992). Indeed, PorA reconstituted in vesicles were more likely to elicit functional antibodies compared to refolded antigens (Arigita *et al.*, 2003).

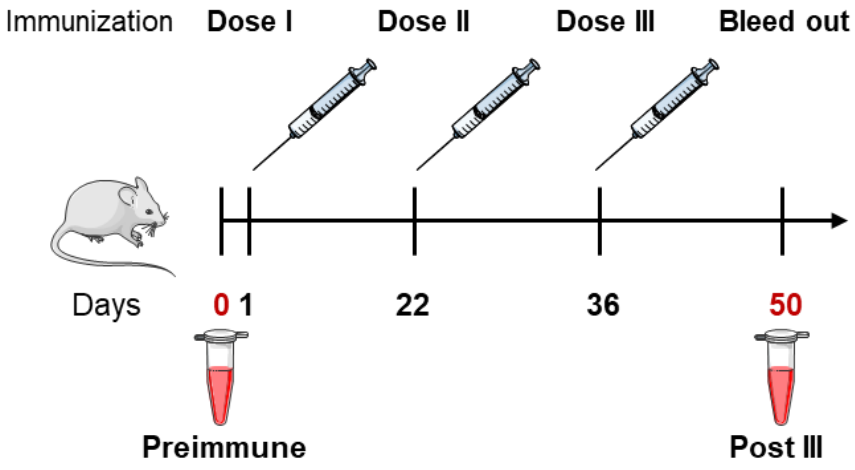

Figure 3.11. Mice immunization schedule. Eight CD-1 mice per group were immunized intraperitoneally three times at days 1, 22 and 36. Sera were collected the day before the first immunization (Day 0) and 2 weeks after the third immunization (Day 50). The 12 recombinant antigens used for *in vivo* studies (20 µg) were: rNEIS2020, rNEIS1783, rNEIS1487, rNEIS1632, rNEIS2075, rNEIS1246, rNEIS1462, rNEIS1066, NEIS1634, rNEIS0653, rNEIS0196 and rNEIS1065. The 26 recombinant GMMA (8 µg) were: *E. coli* GMMA NEIS2020 Δ*ompA*, *E. coli* GMMA NEIS1783 Δ*tolR*, *E. coli* GMMA NEIS1487 Δ*tolR*, *E. coli* GMMA NEIS1632 Δ*tolR*, *E. coli* GMMA NEIS2075 Δ*tolR*, *E. coli* GMMA NEIS1246 Δ*tolR*, *E. coli* GMMA NEIS1462 Δ*tolR*, *E. coli* GMMA NEIS1066 Δ*tolR*, *E. coli* GMMA NEIS1634 Δ*tolR*, *E. coli* GMMA NEIS0653 Δ*tolR*, *E. coli* GMMA NEIS0196 Δ*tolR*, *E. coli* GMMA NEIS1065 Δ*tolR*, *E. coli* GMMA NEIS1364 Δ*ompA*, *E. coli* GMMA NEIS1428 Δ*tolR*, *E. coli* GMMA NEIS0944 Δ*tolR*, *E. coli* GMMA NEIS0408 Δ*tolR*, *E. coli* GMMA NEIS2198 Δ*tolR*, *E. coli* GMMA NEIS1963 Δ*tolR*, *E. coli* GMMA NEIS0173 Δ*tolR*, *E. coli* GMMA NEIS0073 Δ*tolR*, *E. coli* GMMA NEIS1468 Δ*tolR*, *E. coli* GMMA NEIS1271 Δ*tolR*, *E. coli* GMMA

NEIS1687 Δ*tolR*, *E. coli* GMMA NEIS0612 Δ*tolR*, *E. coli* GMMA NEIS0172 Δ*tolR*, *E. coli* GMMA NEIS0596 Δ*tolR*. The two negative controls used for immunizations were: *E. coli* GMMA Empty Δ*ompA* and *E. coli* GMMA Empty Δ*tolR*. All samples were formulated with 3 mg/ml alum.

The polyclonal antibodies derived from mice immunizations were then characterized performing different serological assays. Western blot analyses were performed to parallelly assess the presence, the specificity and the possible cross-reactivity of raised-antibodies in each of the 40 sera. Whole-cell extract and DOMV from the NZ98/254 strain were used as reference, whereas the other two genetically distinct strains (M07576 and M09929) were selected amongst those more susceptible to DOMV-mediated killing. As shown in Figure 3.12A, sera raised against GMMA Empty, regardless the parental strains, lacked reactive antibodies, thus indicating that endogenous *E. coli* proteins bared by GMMA did not induce cross-reactive inter-specie antibodies neither against *Neisseria meningitidis* lysates nor against vesicles. Therefore, all signals observed might be ascribed to the MenB antigens expressed in engineered GMMA. The screening of the coupled GMMA-recombinant sera, revealed that the IgG elicited by each immunization were able to recognize the proteins expressed by the different meningococcal isolates, highlighting the cross-reactive nature of antibodies (Figure 3.12B). On the contrary, the remaining engineered GMMA raised specific antibodies in 10 out of 14 specimens (Figure 3.12C), while with GMMA-NEIS1963, -NEIS1687,-NEIS1428 and -NEIS1271 antisera no signals were observed (Figure 3.12D), possibly due to the low expression levels of those heterologous antigens in GMMA. Among the antibodies elicited by the immunizations, some of them have peculiar patterns of cross reactivity. In particular, GMMA-NEIS1364 (anti-PorA) sera mainly recognized the homologous P1.7-2,4 expressed by NZ98/254 and present in DOMV, while little reactivity is detected for M07576 and M09929 expressing P1.22-1,14 and P1.12-1,16, respectively. A similar recognition profile was also observed for GMMA-NEIS2198 (anti-OpcA) sera which reacted uniquely with NZ98/254 and DOMV. Considering the high conservation of OpcA primary sequence within our strain collection (Figure 3.7) the absence of signal in those isolates might be due to differences in expression levels. Surprisingly, we observed a signal with the GMMA-NEIS0612 (anti-NspA) sera only in M07576 and DOMV, but not in the parental NZ98/254. Given the 100% protein identity of NZ98/254 with M07576 and M09929 (Figure 3.7), the absence of cross-recognition was unexpected. Sera collected after GMMA-NEIS0073 and GMMA-NEIS1468 immunization recognized only the corresponding antigens carried by DOMV, possibly as a result of the enrichment of these OMP during the bulging process with respect to crude total extracts. Finally, GMMA-NEIS0408, -NEIS0172, -NEIS0173, -NEIS0596 and -NEIS0944 sera were able to cross-recognize the specific proteins in all tested strains (Figure 3.12C).

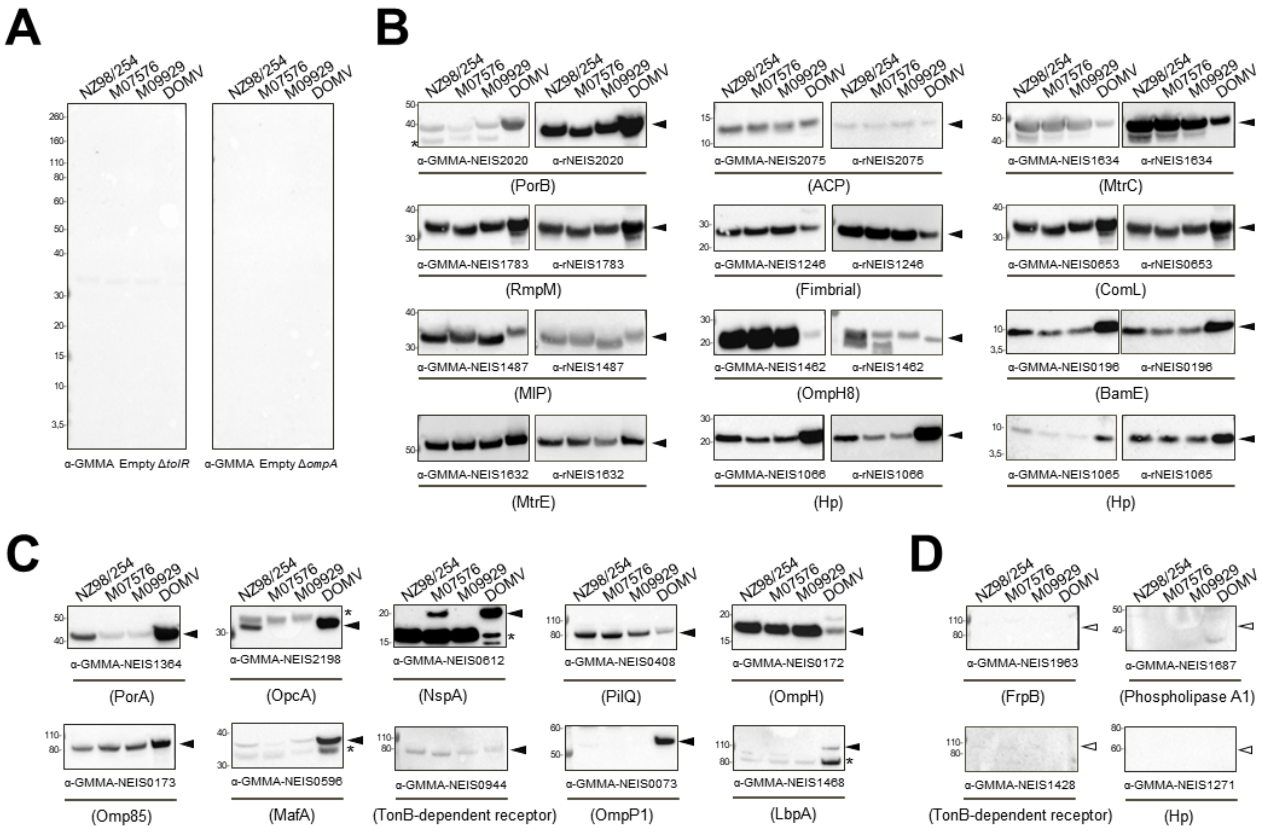

Figure 3.12. Western blot analyses revealed that sera raised by mouse immunizations recognized the specific antigen expressed by different meningococci. Equal amounts of whole cell lysates as well as DOMV were separated by SDS-PAGE prior to Western blotting. The specific antisera raised against (**A**) GMMA Empty (negative control); (**B**) coupled GMMA-recombinant and (**C**-**D**) engineered GMMA used to probe each membrane are listed beneath each blot. All specific sera used for the analysis are pooled (n = 8). Protein bands corresponding to the expected molecular size of the protein are indicated by closed arrows. Open arrows indicate the molecular weight at which the specific-reactive band was expected. The asterisk pinpoints non-specific bands.

Moreover, when recombinant proteins were available, ELISA experiments were performed to measure the antigen specific IgG titres in the corresponding bleed out serum. Thereby, each of the 13 MenB recombinant proteins produced was used to coat a different ELISA plate. As suggested by the geometric mean ELISA titres shown in Figure 3.13, the induction of specific antibodies was confirmed for all the antisera raised both by recombinant GMMA and proteins, in agreement with the reactivity previously observed in Western blot analyses. On the contrary, little cross-reactivity was detected when mice were immunized with Δ*tolR* and Δ*ompA* GMMA Empty indicating that the endogenous *E. coli* proteins did not induce high levels of antibodies cross-reacting with MenB DOMV-specific antigens used for the coating, with the exception of PorB (Figure 3.13). Indeed, 10 of the 13 recombinant GMMA (Figure 3.13, dark blue bars) induced specific IgG titres which were significantly higher respect to the negative control represented by GMMA Empty antisera (Figure

3.13, light blue bars). Finally, it is noteworthy that recombinant proteins (Figure 3.13, green bars) raised antigen-specific antibody responses which, in almost all the cases, were in the same range as the titres elicited by the corresponding recombinant GMMA (Figure 3.13, dark blue bars), despite an almost 2.5-fold difference in the final administered doses, 20 µg vs 8 µg of total protein content, respectively. In particular, the amount of recombinant antigens expressed in *E. coli* vesicles represented a small percentage (data not shown) of total *E. coli* proteins carried by GMMA. Hence, these data suggest an adjuvant effect of vesicles on MenB antigen immunogenicity, as already reported elsewhere (Fantappie *et al.*, 2014, Micoli *et al.*, 2020).

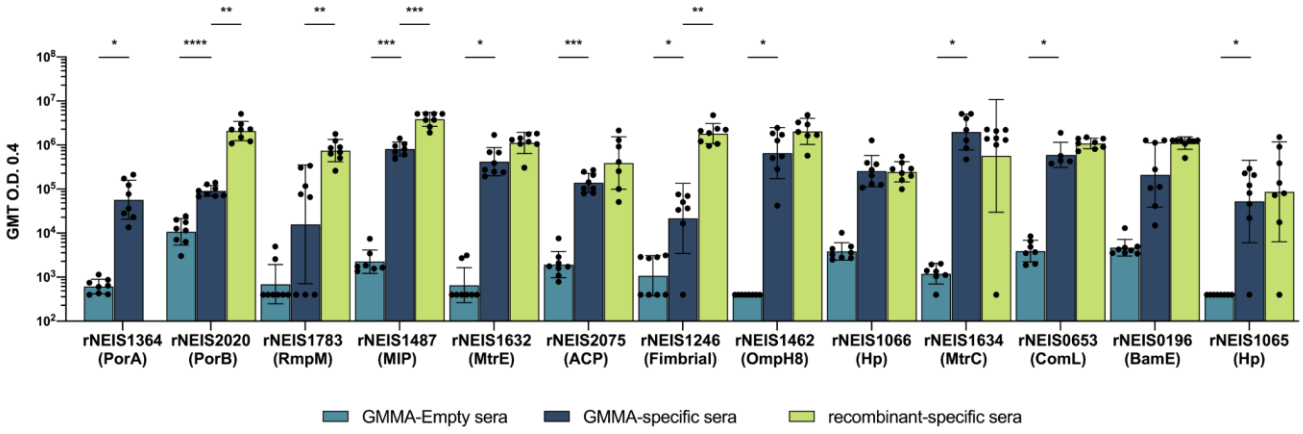

Figure 3.13. ELISA measured the specific IgG titres in mice immunized with different *E. coli* GMMA and recombinant proteins. IgG titres were determined by ELISA using plates coated with the 13 recombinant antigens, listed on the X axis. Light blue bars indicate GMMA Empty antisera used as negative control, in particular *E. coli* GMMA Empty Δ*ompA* antisera was used for the plates coated with PorA and PorB antigens, whereas all the other coatings were probed with *E. coli* GMMA Empty Δ*tolR* antisera. Dark blue and green bars indicate the GMMA-specific and recombinant antigen-specific antisera which correspond to antigen used to coat the plates, respectively. Each dot represents the titre obtained from an individual mouse serum, while histograms indicate the geometric mean (GMT) value within each immunization, expressed as the reciprocal of the serum dilution yielding an OD value of 0.4. Error bars of each mouse group describe the geometric standard deviation. Statistical analysis was performed applying the t-student test and *p*-values ≤ 0.05 were considered statistically significant (* $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$, **** $p \leq 0.0001$).

3.7 The characterization of sera revealed three novel DOMV-functional antigens in addition to PorA

Once demonstrated that antigen-specific antibodies were raised following immunization with engineered vesicles as well as with recombinant proteins in 36 out of 40 antisera, we then assessed the surface localization of each meningococcal protein, since it is considered a major determinant of bactericidal killing. The binding of IgG antibodies to the surface of the NZ98/254 encapsulated strain was measured by FACS surface staining. From the analysis conducted with the specific antisera

generated, of the 26 antigens analysed only antibodies against three of them - PorA (NEIS1364), OpcA (NEIS2198) and NspA (NEIS0612) – were able to stain the NZ98/254 surface. On the contrary, PorB (NEIS2020) seemed only moderately accessible to the binding of polyclonal sera as demonstrated by a modest positive shift compared to meningococci exclusively stained with secondary antibodies and measurable only with sera raised against the recombinant PorB antigen (Figure 3.14A). Surprisingly, no significant fluorescent signals were observed for all the other 22 antigens (two representative FACS profiles are shown in Figure 3.14B), where the shifts in FACS were almost identical to the staining of the NZ98/254 strain by the anti-GMMA empty sera, used as negative control (Figure 3.14C).

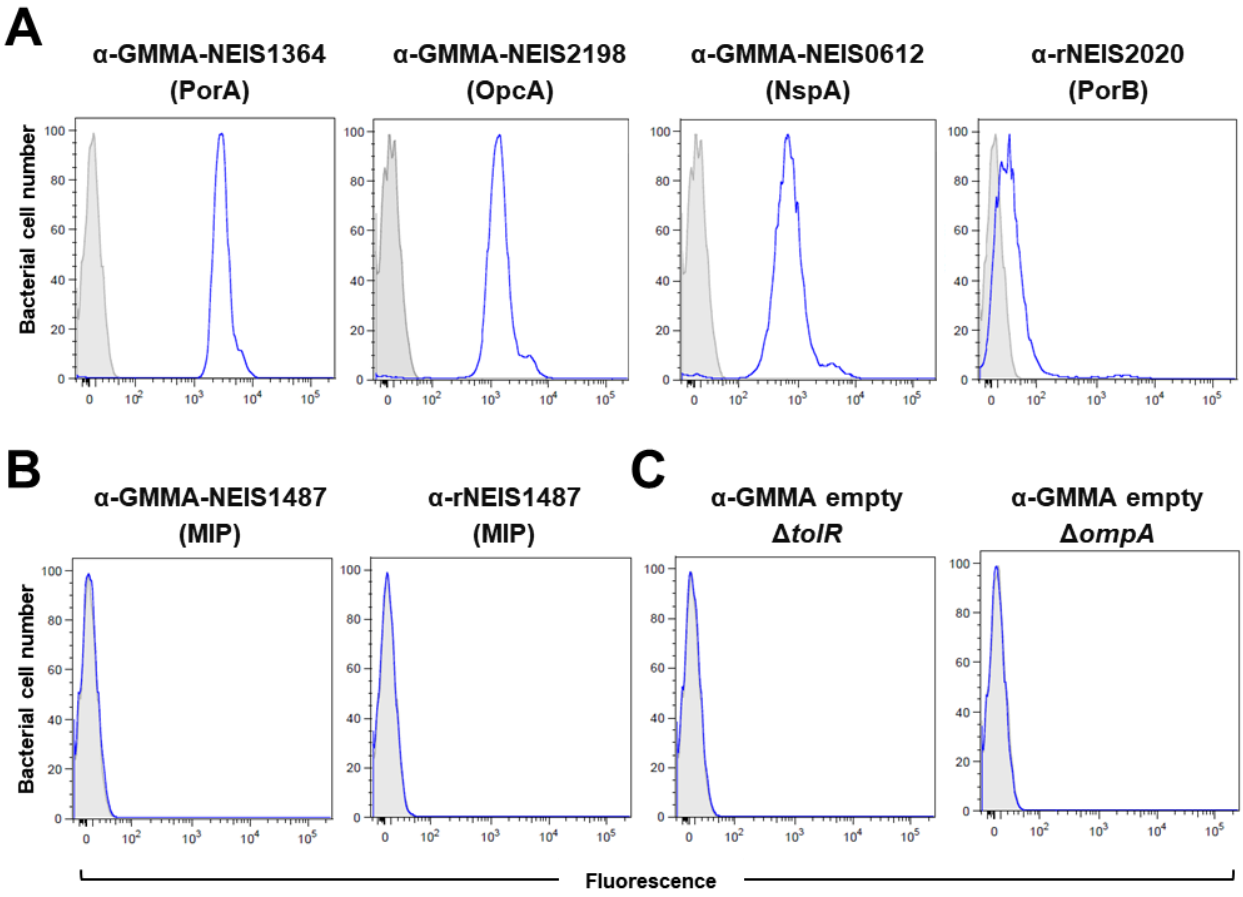

Figure 3.14. PorA, OpcA, NspA and PorB were surface exposed in NZ98/254 and accessible to specific antibodies. FACS surface staining analyses were carried out on live NZ98/254 meningococcal cells using pool (n = 8) polyclonal mouse sera raised by antigen immunization. (**A**) FACS positive results obtained for 4 out of the 26 antigens tested. Sera used were: α-GMMA-PorA, α-GMMA-OpcA, α-GMMA-NspA, α-recombinant PorB. (**B**) Representative FACS of 1 out of the 22 not-surface exposed antigens obtained probing bacteria with GMMA and recombinant MIP immune sera. (**C**) FACS profiles of the negative controls obtained with α-GMMA Empty sera from both genetic backgrounds. Blue profiles represent bacteria incubated with hyperimmune sera, while shaded grey those stained with fluorescent secondary antibodies alone (negative controls).

These results were quite unexpected and suggest that, at least in this experimental settings and for this isolate, either those proteins were not surface exposed on live meningococcal cells, which in part contrasts with their predicted outer membrane localization or, more likely, that in the growth conditions used for the analysis they were partially shielded by the polysaccharide capsule and thus relatively less accessible to antibodies on the cell surface. Having confirmed the surface exposure of some antigens, we then verified the ability of the antibodies to induce complement-mediated *in vitro* killing of meningococci, a test which correlates with vaccine efficacy in humans. In order to perform a rapid screening of the panel of sera, SBA was initially carried out on three of the 12 MenB natural strains under investigation in this study. The bactericidal activity of sera was tested against NZ98/254, M07576 and M09929 isolates in presence of 25% of baby rabbit complement. Among the list of potentially protective candidates, in line with flow cytometry results, PorA, OpcA, NspA and PorB were able to induce bactericidal antibodies as reported in Table 3.3. The differences in PorA-, OpcA- and NspA-mediated killing against the three strains perfectly correlated with the reactivity detected in Western blot in the corresponding isolates (Figure 3.12). On the contrary, sera raised by the recombinant form of PorB showed functional activity exclusively against M07576 and M09929, although PorB antisera were cross-reactive with all crude cell preparations (Figure 3.12). Presumably, the lack of protection against NZ98/254 is attributable to the lower PorB expression on bacterial surface and/or to a limited accessibility of antibodies as revealed by FACS. Overall, these results revealed that the inclusion of PorA, NspA and OpcA within *E. coli* vesicles favoured the production of functional antibodies; the reasons why this did not occur with PorB remains unclear. Notably, all these four proteins were amongst those with the highest reactivity according to protein array analysis carried out with DOMV-antisera (Figure 3.10), supporting our initial speculation for the DOMV-protection. Moreover, these results further confirm that surface accessibility to antibody binding is fundamental for an antigen to be a good protective candidate. Indeed, despite most of the antibodies were able to recognize the antigen in the total extracts (Figure 3.12), none of the other antisera negative in FACS showed significant bactericidal activity, in agreement with the lack of surface detection. Although we cannot exclude that the same antigens could be surface-exposed in different growth conditions, the results of this analysis allowed us to exclude them from the panel of potential candidates responsible for the DOMV-mediated protection and thus to focus the attention on the four promising antigens.

	rSBA on three MenB strains		
Sera	**NZ98/254**	**M07576**	**M09929**
DOMV NZ98/254	+	+	+
GMMA empty Δ*tolR*	-	-	-
GMMA empty Δ*ompA*	-	-	-
GMMA-NEIS2020 (PorB)	-	-	-
rNEIS2020 (PorB)	-	+	+
GMMA-NEIS1364 (PorA)	+	-	-
GMMA-NEIS1783 (RmpM)	-	-	-
rNEIS1783 (RmpM)	-	-	-
GMMA-NEIS0944 (TonB)	-	-	-
GMMA-NEIS0408 (PilQ)	-	-	-
GMMA-NEIS2198 (OpcA)	+	-	-
GMMA-NEIS0173 (Omp85)	-	-	-
GMMA-NEIS0073 (OmpP1)	-	-	-
GMMA-NEIS1468 (LbpA)	-	-	-
GMMA-NEIS1487 (MIP)	-	-	-
rNEIS1487 (MIP)	-	-	-
GMMA-NEIS1632 (MtrE)	-	-	-
rNEIS1632 (MtrE)	-	-	-
GMMA-NEIS2075 (ACP)	-	-	-
rNEIS2075 (ACP)	-	-	-
GMMA-NEIS1246 (Fimbrial)	-	-	-
rNEIS1246 (Fimbrial)	-	-	-
GMMA-NEIS0612 (NspA)	-	+	-
GMMA-NEIS1462 (OmpH8)	-	-	-
rNEIS1462 (OmpH8)	-	-	-
GMMA-NEIS0172 (OmpH)	-	-	-
GMMA-NEIS1066 (Hp)	-	-	-
rNEIS1066 (Hp)	-	-	-
GMMA-NEIS0596 (MafA)	-	-	-
GMMA-NEIS1634 (MtrC)	-	-	-
rNEIS1634 (MtrC)	-	-	-
GMMA-NEIS0653 (ComL)	-	-	-
rNEIS0653 (ComL)	-	-	-
GMMA-NEIS0196 (BamE)	-	-	-
rNEIS0196 (BamE)	-	-	-
GMMA-NEIS1065 (Hp)	-	-	-
rNEIS1065 (Hp)	-	-	-

Table 3.3. SBA pre-screening revealed that PorA, PorB, NspA and OpcA antisera were bactericidal in presence of baby rabbit complement. NZ98/354, M07576 and M09929 were analysed for their susceptibility to killing by different polyclonal sera through rSBA. Each sample tested is a pool made from 8 individual mouse serum. Resistance (titres <16, grey boxes with "-") and susceptibility to killing (titres ≥16, green boxes with "+") are indicated.

3.8 In-depth characterization of DOMV-functional antigens

The preliminary DOMV dissection conducted against three MenB strains for the identification of DOMV-antigen(s) conferring protection disclosed four candidates. Therefore, the characterization of PorA, OpcA, NspA and PorB was extended to the whole set of initially investigated strains that were specifically killed by DOMV rather than by the recombinant 4CMenB antigens.

3.8.1 PorA protein displayed in heterologous GMMA raised functional antibodies against the homologous serosubtype strain

Considering the well-documented protection attributed to PorA antibodies against *Neisseria meningitidis* serosubtype-matched strains (Martin *et al.*, 2006), antisera from *E. coli* GMMA-expressing PorA P1.7-2,4 were included in the analysis as control. Prior to the evaluation of anti-PorA mediated protection against different serosubtypes, both the expression of the antigen, which is known to be phase variable (van der Ende *et al.*, 1995), and the cross-reactivity of the polyclonal sera were investigated. As shown by the different band intensities in the immunoblot of crude cell extracts of Figure 3.15A, the antisera mainly recognized the homologous PorA expressed by NZ98/254 or carried by DOMV, whereas little recognition was observed for the other protein alleles, which were characterized by different VR1 and VR2 epitopes (Table 3.1). Signals were indeed completely absent in M09662 and in the PorA deletion mutant generated in the NZ98/254 genetic background. Moreover, while the antisera raised against GMMA-PorA preparations showed a very strong binding ability against the homologous PorA expressed on the surface of NZ98/254 (Figure 3.14), it failed to bind the other mismatched serosubtype isolates tested (as shown in the M09929 representative FACS) and the NZ98/254Δ*porA* (Figure 3.15B). Finally, the same antisera was tested on the complete panel of MenB strains to investigate its bactericidal ability and it showed high titres against NZ98/254, but not against all the other natural and engineered strains (Figure 3.15C), in correlation to what previously observed with Western blot and surface cell-labelling experiments. Collectively, these results suggested that for this antigen mice immune responses were mainly directed toward the more variable surface exposed loops of PorA, rather than to the conserved transmembrane domains and confirmed the protective role of PorA antibodies against strains carrying the homologous antigen. Moreover, these evidences validated the use of *E. coli* GMMA as a tool for the expression of heterologous antigens in the native orientation and conformation critical to stimulate protective immune responses.

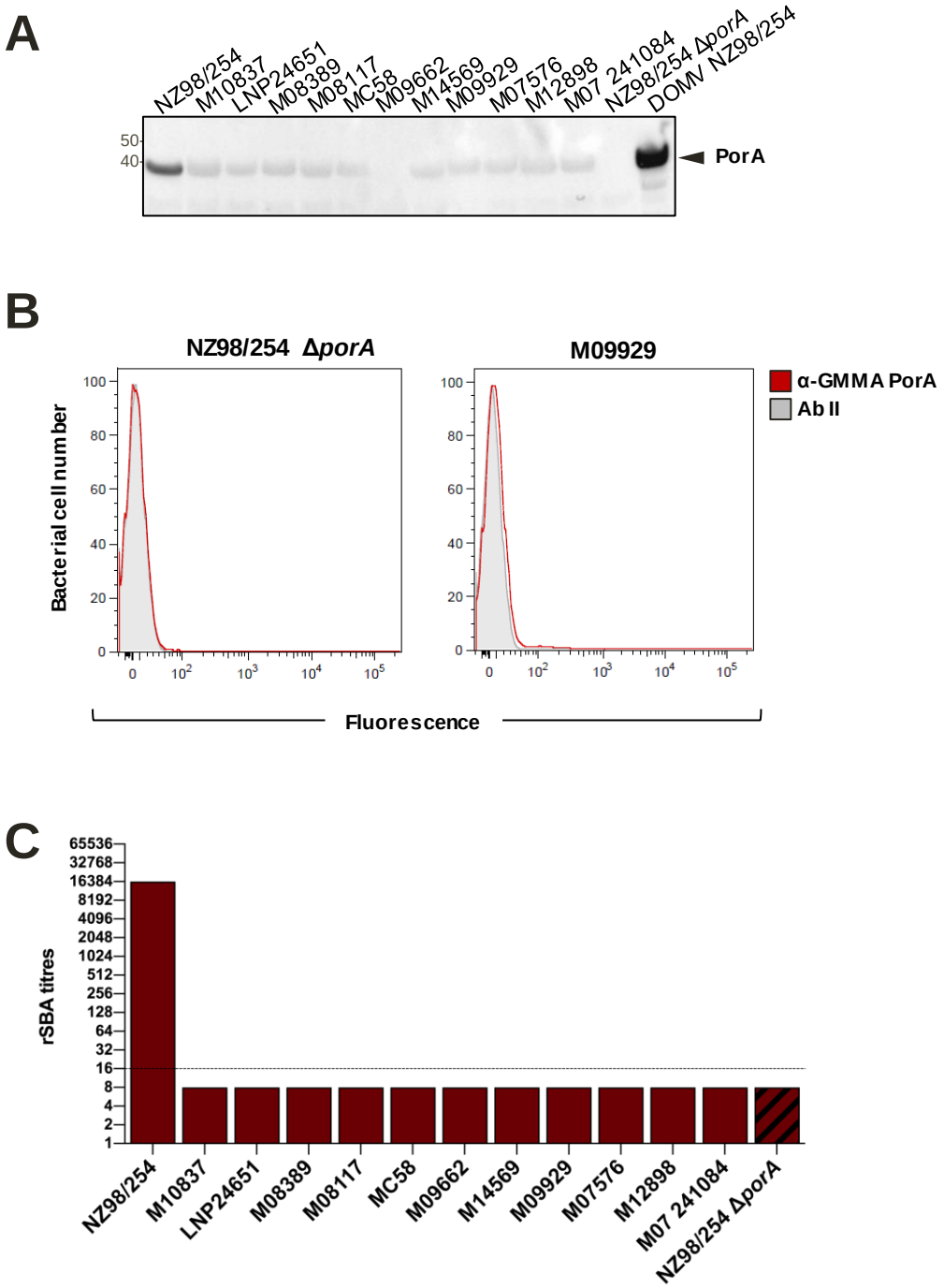

Figure 3.15. Genetically engineered PorA GMMA elicited antibodies able to recognize and to specifically kill the homologous NZ98/254 isolate. (**A**) Western blot analysis was performed using PorA polyclonal antibodies on crude cell extracts of MenB natural strains, NZ98/254 PorA deletion mutant and DOMV. The closed arrow indicates the PorA protein. (**B**) FACS analyses of NZ98/254Δ*porA* and M09929 were carried out using α-GMMA PorA antibodies. Shaded profiles represent bacterial cells exclusively stained with secondary antibodies, while non-shaded red profiles show the reaction with anti-PorA immune sera. (**C**) rSBA titres obtained using GMMA-PorA antisera are represented by histograms. Filled bars represent natural strains, while the stripped bar indicates *porA* knock-out mutant in the NZ98/254 background, used as negative control. The dashed line indicates positive and protective threshold in presence of rabbit complement (rSBA ≥16). Sera used in all the analyses are pool of 8 mice.

3.8.2 The highly conserved OpcA protein induced functional immunity against eight of the 11 DOMV-specific meningococcal strains

The preliminary screening of DOMV-specific sera provided insight onto the pivotal role played by the minor antigen OpcA. Before extending the functional characterization to the whole set of isolates, we assessed the expression levels of the protein in the total cell extracts of natural strains as well as in the LNP24651Δ*opcA* mutant generated by the insertion of a resistance cassette into the open reading frame of the *opcA* gene (Figure 3.16A). The analysis revealed that the amount of protein across the strains was variable and ranged from high ($OpcA^{++}$) to intermediate ($OpcA^{+}$) levels and, although all these natural strains carried the *opcA* gene, three of them, along with the *opcA* deletion mutant, resulted negative in Western blot ($OpcA^{-}$). Given the high percentage of amino acid sequence identity shared by OpcA in our bacterial collection (98.16-98.90% of amino acid identity, Figure 3.7) we excluded any implication of amino acid variability in the differential recognition pattern observed among the strains. Moreover, we were able to confirm through flow cytometry that OpcA expression levels in meningococcal total fractions were overall correlated with its exposure on the bacterial cell surface (Figure 3.16B). After the identification of several OpcA phenotypes, we investigated the possible reasons beneath this differential expression. Taking advantage of the studies of Sarkari and colleagues which demonstrated that OpcA phase variation is dependent on the length of a poly-cytidine (poly-C) stretch in the *opcA* promoter (Sarkari *et al.*, 1994), we decided to analyse the promoter region of this gene in the panel of MenB isolates. The bioinformatic analysis of the 12 *opcA* promoters revealed that the number of cytidines within the promoter was correlated with the OpcA expression detected by Western blot (Figure 3.16C and A, respectively) in accordance to what already published: 12 or 13 nucleosides were predictive of an efficient expression, 11 or 14 of an intermediate one and ≤10 or ≥15 characterized the lack of expression (Sarkari *et al.*, 1994). The only discrepancy with the predicted expression was observed in the strain M12898, which carried a 13 nt-long poly-C and was expected to express high levels of OpcA, but it expressed intermediate levels both in Western blot and in FACS. However, as also suggested by Sarkari and co-workers, additional factors, still unknown, could potentially be involved in *opcA* down-regulation *in vitro* and *in vivo*. Finally, in the case of M07 241084 the location of *opcA* promoter at the edge of contigs prevented us to draw any conclusion on the length of the cytidine stretch, which was at least 7 residues-long.

Once characterized the OpcA expression and surface exposure of the different meningococcal isolates, we investigated whether anti-OpcA antibodies were capable of inducing complement-mediated bactericidal killing. The rSBA titres shown in Figure 3.16D revealed that antibodies against OpcA were specifically able to kill eight of the 12 strains with titres ranging from 25 to 8,192; data

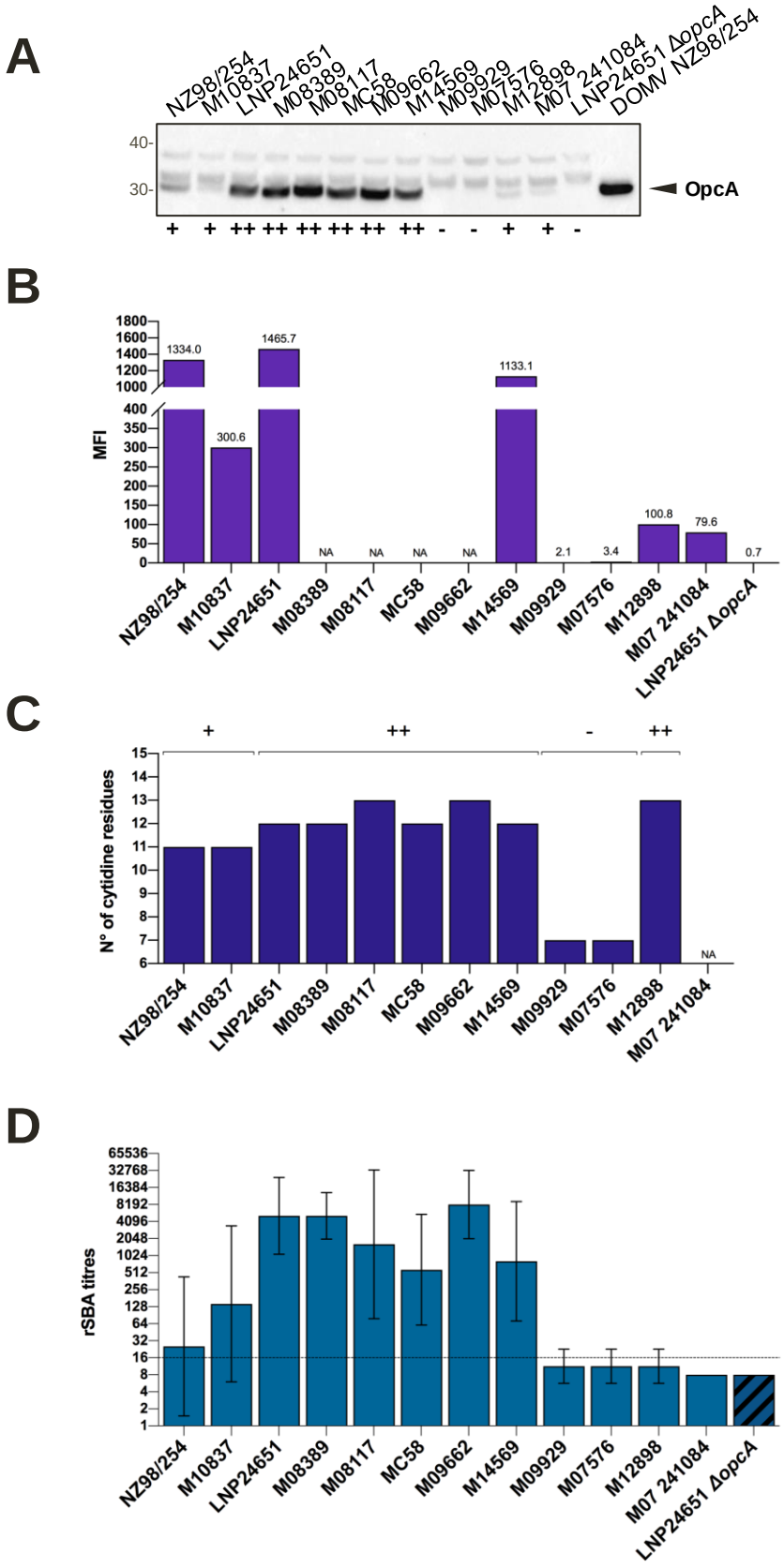

Figure 3.16. OpcA expression and surface exposure varied among strains and isolates that expressed higher amount of the protein were more sensitive to OpcA-mediated killing. (**A**) DOMV and whole cell lysates, prepared from bacteria culture grown in SBA-like conditions, were resolved in SDS-PAGE prior to Western blotting. OpcA protein, detected by pooled GMMA-OpcA antisera, is indicated by an arrow. Specificity of the signal is confirmed by the *opcA*

deletion mutant. Levels of expression are indicated below the blot as: ++ for OpcA^{++}, + for OpcA^{+} and – for OpcA^{-}. (**B**) The expression of OpcA protein was determined by flow cytometric analysis on live bacteria. Bars describe the Mean Fluorescent Intensity (MFI) values of OpcA subtracted by the MFI of each corresponding negative control (bacteria + Ab II). MFI values are reported above each bar. NA describes isolates for which OpcA surface exposure was not investigated. Sera used in all the analyses are pool of 8 mice. (**C**) Histograms represent the number of cytidines residues located in the *opcA* promoter region of each isolate. High (++), intermediate (+) and lack (–) of OpcA expression levels are indicated above each bar as predicted form the analysis of the promoter in agreement to (Sarkari *et al.*, 1994). NA refers to the impossibility to determine poly-C length due to the location of the *opcA* promoter of M07 241084 at the edge of contigs. (**D**) rSBA titres using pooled murine GMMA-OpcA antisera are represented by filled bars on natural strains and by a stripped bar on the *opcA* null mutant. Data are represented as GMT of the titres obtained testing sera from 6 independent immunization schemes and error bars describe the geometric standard deviation. rSBA values ≥16 (dashed lines) are considered protective.

further corroborated by the absence of functionality in the OpcA null mutant. Furthermore, a correlation between OpcA expression levels and killing was detected. Meningococcal isolates that expressed higher amount of total protein were indeed more susceptible to the killing than strains expressing lower amounts and, as expected, OpcA^{-} phenotype prevented any OpcA-mediated protection (M09929 and M07576). Lastly, two outliers, M12898 and M07 241084, were resistant to OpcA-dependent killing, despite being OpcA^{+}, like NZ98/254 and M10837. However, this phenotype may be correlated with a less surface accessibility of OpcA to antibodies or to a lower expression, as suggested by MFI results (Figure 3.16B). To test this hypothesis, we measured the deposition of the complement factor C3 onto the surface of live *N. meningitidis* cells characterized by different survival outcomes in SBA (M10837, OpcA^{+}, rSBA positive and M12898, OpcA^{+}, rSBA negative) in presence of anti-OpcA antibodies (Figure 3.17).

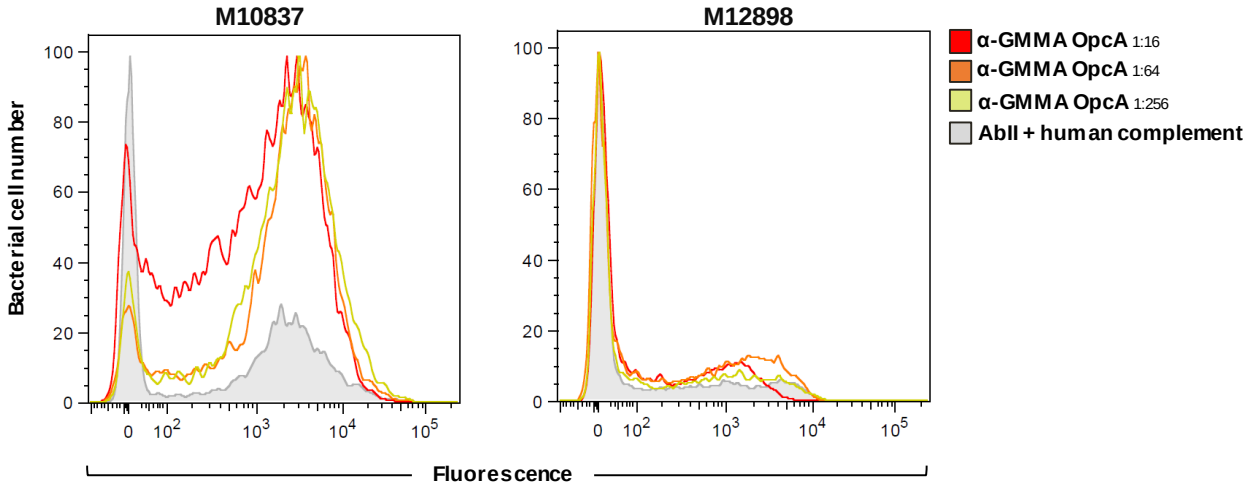

Figure 3.17. OpcA-antibodies triggered differential C3 deposition on meningococcal cells characterized by diverse SBA survival outcomes. The amount of C3 bound onto the surface of M10837 and M12898 was measured by indirect

fluorescence through flow cytometry. Different dilutions of pooled GMMA-OpcA sera are showed by red, orange and yellow lines (1:16; 1:64 and 1:256, respectively). Grey filled profiles represent negative controls obtained incubating bacteria exclusively with human plasma and anti-human C3 fluorescent-labelled antibodies.

In the strain M10837 the C3 was recruited even at high dilution of anti-OpcA antibodies (1:256), whereas no C3 was attached to the surface of M12898, neither at low sera dilutions (1:16). Therefore, the C3 deposition was confirmed as a marker of the activation of the classical complement pathway and this result revealed that the different susceptibility to killing in $OpcA^+$ isolates might be due to intrinsic features of strains.

3.8.3 Antibodies to Neisserial surface protein A displayed bactericidal activity against two of the DOMV-specific MenB isolates

The deconvolution of the DOMV-mediated protection revealed that NspA is another pivotal antigen. In line with what has been done for OpcA, the expression of NspA protein was investigated in meningococcal lysates obtained from cultures grown in SBA-mimicking conditions. According to Western blots probed with GMMA-NspA antisera and contrary to what expected, the major reactive band corresponded to a ~15 kDa protein that had the same mobility across all lysates, but which was not NspA since present also in the *nspA* deletion mutant (Figure 3.18A). In the lanes of M07576 and DOMV, the presence of NspA was evident by the appearance of a second reactive band at ~18,5 kDa corresponding to the molecular mass of NspA predicted from the amino acid sequence. Moreover, the occurrence of a second immunoreactive band in the DOMV sample, with a different molecular weight, was conceivably related to the nature of NspA, a heat modifiable protein, which has been described as present in SDS-PAGE analysis as two forms: a denatured protein running at lower molecular weight and a folded form at higher molecular weight (Martin *et al.*, 2000, Moe *et al.*, 1999, Martin *et al.*, 1997). These electrophoretic mobilities are also consistent with the presence of two distinct forms identified by SDS-PAGE and Western blot when the expression of NspA was assessed in *E. coli* GMMA (Figure 3.4B and C) and with data obtained by Lujana and colleagues (Lujan *et al.*, 2016). However, given the very high amino acid sequence identity of NspA among our strains (97.70-100%, Figure 3.7), we excluded differences in cross-recognition caused by polymorphisms of NspA protein. Moreover, the anti-GMMA NspA sera was not able to recognize the protein in the homologous NZ98/254 strain in immunoblot, but NspA surface-exposure was detected in the same strain by cytofluorimetry analysis (Figure 3.14). Therefore, we speculated that the absence of cross-reactive bands corresponding to NspA in Western blot could be due to a bias of the analysis resulting from the unfolding and the loss of conformational epitopes during sample preparations. To this purpose, we carried out NspA-sera surface staining by flow cytometry of other isolates to enable the

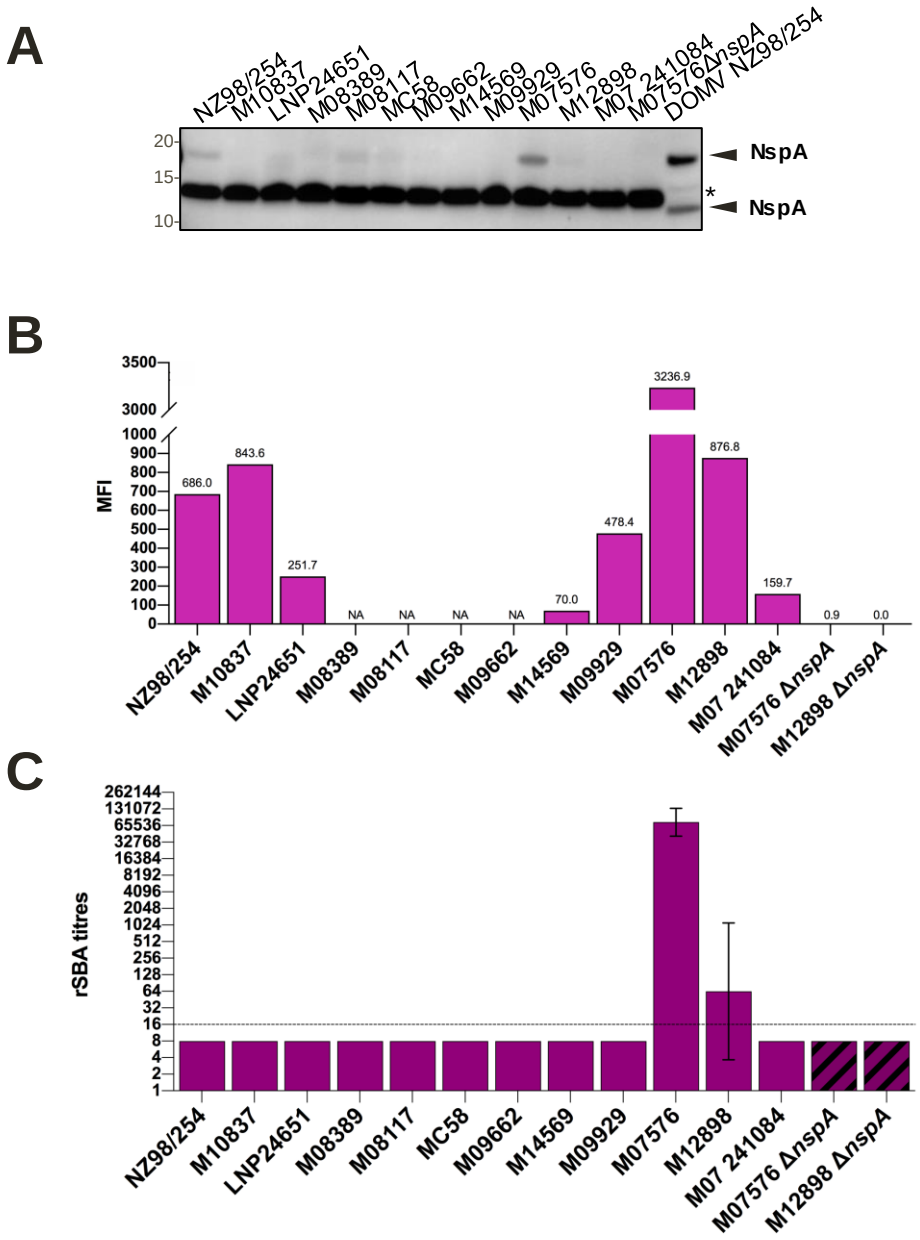

Figure 3.18. The NspA protein elicited bactericidal antibodies able to kill M12898 and M07576. (**A**) Meningococcal crude lysates prepared from cultures grown in SBA-like conditions as well as DOMV were separated in SDS-PAGE prior to Western blotting. Pooled GMMA-NspA sera was used to probe the membrane. The two forms of NspA are indicated by arrows. Asterisk refers to the unspecific band used as loading control among strain lysates. (**B**) Bacterial cells stained with NspA-antibodies were analysed by FACS and MFI values used to quantify antibody-cell surface binding are represented as histograms. MFI values are listed above each bar. MFI of NspA was subtracted with the value of each corresponding negative control (bacteria + Ab II) before plotting. NA labels isolates for which the surface exposure of NspA was not investigated. Sera used in all the analyses are pool of 8 mice. (**C**) Serum bactericidal antibody titres were measured in presence of rabbit complement using anti-NspA pooled mouse sera. Data are represented as GMT of the titres obtained testing sera from 5 independent immunization schemes and error bars describe the geometric standard deviation. Histograms represent GMT of SBA titres against wild-type strains (filled bars) and *nspA* knock-out mutants (stripped bars). SBA titres ≥16 are considered protective (dashed line).

detection of NspA antigen in a native conformation. The FACS MFI results (Figure 3.18B) revealed that NspA was exposed on the surface of all the isolates tested although at different levels, and sera specificity was also corroborated by negative results obtained on the M07576 Δ*nspA* as well as on another *nspA* deletion mutant generated (M12898Δ*nspA*). The positive FACS results obtained for the strains compared to the negative Western blots, support the hypothesis that NspA antisera may recognize conformational epitopes rather than linear ones. Moreover, the total absence of the "non-specific" fluorescence signal during surface staining of the two knock-out strains suggest that the ~15 kDa reactive-protein in Western blot, would presumably be a cytoplasmic contaminant. This assumption is also corroborated by the absence of the uncharacterized band in immunoblots resolving DOMV (Figure 3.18A), where the protein content is skewed towards OMP rather than soluble cytoplasmic proteins.

Lastly, to clearly establish the protective potential of NspA, rSBA was performed against the panel of 12 strains as shown in Figure 3.18C. NspA-mediated bacteriolysis was observed for M07576 with very high titres (65,536) and for M12898, whereas the other strains resulted not susceptible. However, at least in the strains analysed, NspA was surface-exposed and the polyclonal sera were able to bind the protein, suggesting that in this case other unknown factors are responsible for the resistance to bacteriolysis.

3.8.4 Antibodies raised against PorB mediated the complement dependent killing of M07576 and M09929

The functional screening of sera identified as fourth protective antigen PorB, the most abundant outer membrane protein in *Neisseria meningitidis* serogroup B. Unlike PorA, for which most of the antigenic variability reside in two loops resulting in limited cross reactivity of PorA-raised antibodies, little is known about the inter-species reactivity of the PorB counterpart. To clarify this aspect, MenB total protein extracts of the 12 strains were separated in SDS-PAGE prior to the blotting and probing with recombinant PorB-raised antibodies. As shown in Figure 3.19A, sera were able to recognize the different variants of PorB3 expressed by almost all meningococcal isolates, but not the PorB2 harboured by the M09662. This result was consistent with the low percentage of PorB amino acid identity observed between NZ98/254 and M09662 (60.17%, Figure 3.7). However, the surface exposure of PorB measured by FACS analysis on a subpanel of strains (Figure 3.19B) showed different results compared to Western blot. Aside from the little PorB exposure previously documented on the homologous NZ98/254 (Figure 3.14), high binding levels of PorB antibodies were observed for M09929 and M07576 isolates, whereas both M14569 and M09662 were PorB-negative. While the result of the former strain was unforeseen, the latter, carrying the natural and dissimilar

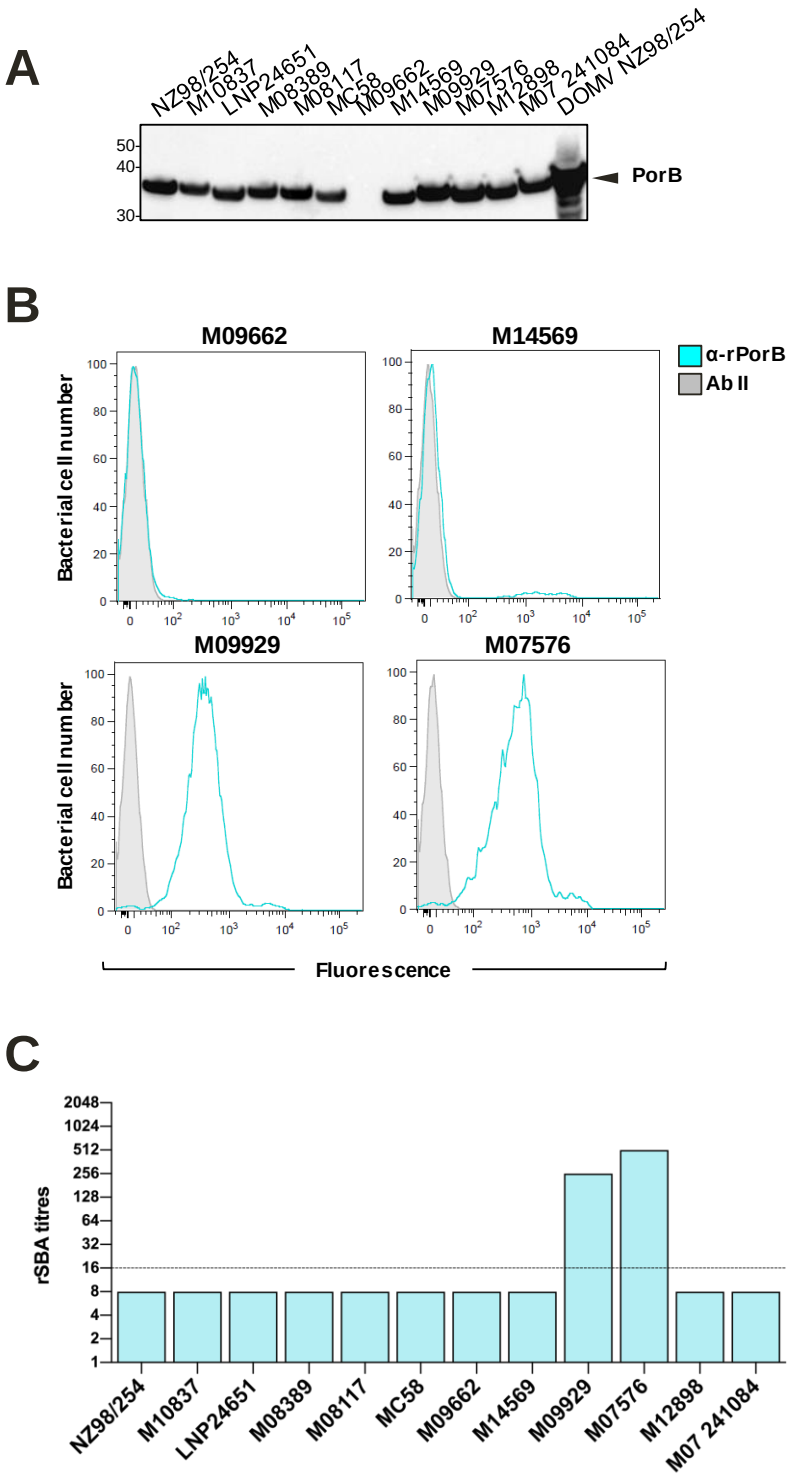

Figure 3.19. PorB expression levels as measured in total cell extracts did not correlate with protein surface exposure and PorB-dependent killing. (**A**) Western blot of solubilized cells deriving from 12 *Neisseria meningitidis* strains and DOMV were probed with recombinant PorB antisera. Arrow indicates PorB molecular weight. (**B**) The binding of anti-PorB antibodies to live bacteria was assessed by flow cytometry. Shaded profiles represent bacterial cells exclusively stained with secondary antibodies, while non-shaded light blue profile shows the reaction with anti-PorB immune sera. (**C**) rSBA titres obtained with murine pooled anti-recombinant PorB sera upon designed strains are represented by bars. The dashed line represents the minimal threshold considered protective in presence of rabbit complement. Sera used in all the analyses are pool of 8 mice.

porb2 allele, was included in the analysis as negative control due to the inability to generate PorB knock-out viable colonies on different genetic backgrounds (M07576 and M09929). The discrepancies of sera reactivity between Western blot and surface live meningococci staining conceivably reflect the different accessibility for antibody binding, which are likely to result from hindrance of capsules/LOS molecules as well as from the short surface-exposed loops which characterize PorB3 variants, as previously discussed in (Michaelsen *et al.*, 2001). Of the whole collection of MenB strains tested in rSBA using the polyclonal PorB sera, only M07576 and M09929 isolates were killed (Figure 3.19C, rSBA titres of 512 and 256, respectively), suggesting that the indirect-fluorescence cell binding assay may give information on the surface accessibility of PorB epitopes which are crucial for the interaction with protective antibodies.

3.9 PorA, PorB and OpcA antigens contained in the DOMV-component of the 4CMenB vaccine elicited protective immunity in humans

The dissection of the DOMV protection confirmed the serosubtype specificity of PorA antibodies and identified OpcA, NspA and PorB as three vesicular antigens able to mediate complement dependent killing in 10 out of the 11 genetically diverse MenB strains. However, respect to our experimental approach based on overexpression of antigens for mice immunization, only low amounts of the identified four protective antigens are contained within the DOMV component of 4CMenB, along with a plethora of other proteins. Hence, it was fundamental to evaluate the ability of these antigens to still elicit functional antibodies when included in the complex vaccine-like environment of the DOMV. With the purpose to tease out the single contribution in the protection of each of the four antigens within DOMV-raised antisera, we individually evaluated their specificity and functionality performing flow cytometry and SBA analysis on wild-type (WT) and knock-out (KO) strains. In particular, NZ98/254, LNP24651, M07576 and M09929 were chosen to measure the specific contribution of PorA, OpcA, NspA and PorB, respectively. FACS profiles shown in Figure 3.20A, in which DOMV antibodies bound the surface of NZ98/254Δ*porA* and LNP24651Δ*opcA* in a lower extent than the corresponding WT parental strains, reinforced protein-chip data (Figure 3.10) about the presence of anti-PorA and anti-OpcA antibodies in the complex cocktail of immunoglobulins elicited by DOMV. Likewise, the significant reduction of fluorescent intensity observed in the deletion mutants, unveiled PorA and OpcA antigens among the major targets of DOMV antibodies expressed by the corresponding isolates. On the contrary, the nearly identical reactive profile of M07576 wild-type and mutant strains, emphasised that, although present within the DOMV-sera (Figure 3.10), NspA antibodies were not contributing to the fluorescent signals, probably due to their

low amount. Furthermore, the lack of PorB-deficient mutants precluded us to investigate the reactivity of PorB-specific antibodies, encompassed within the DOMV antisera, upon live meningococci.

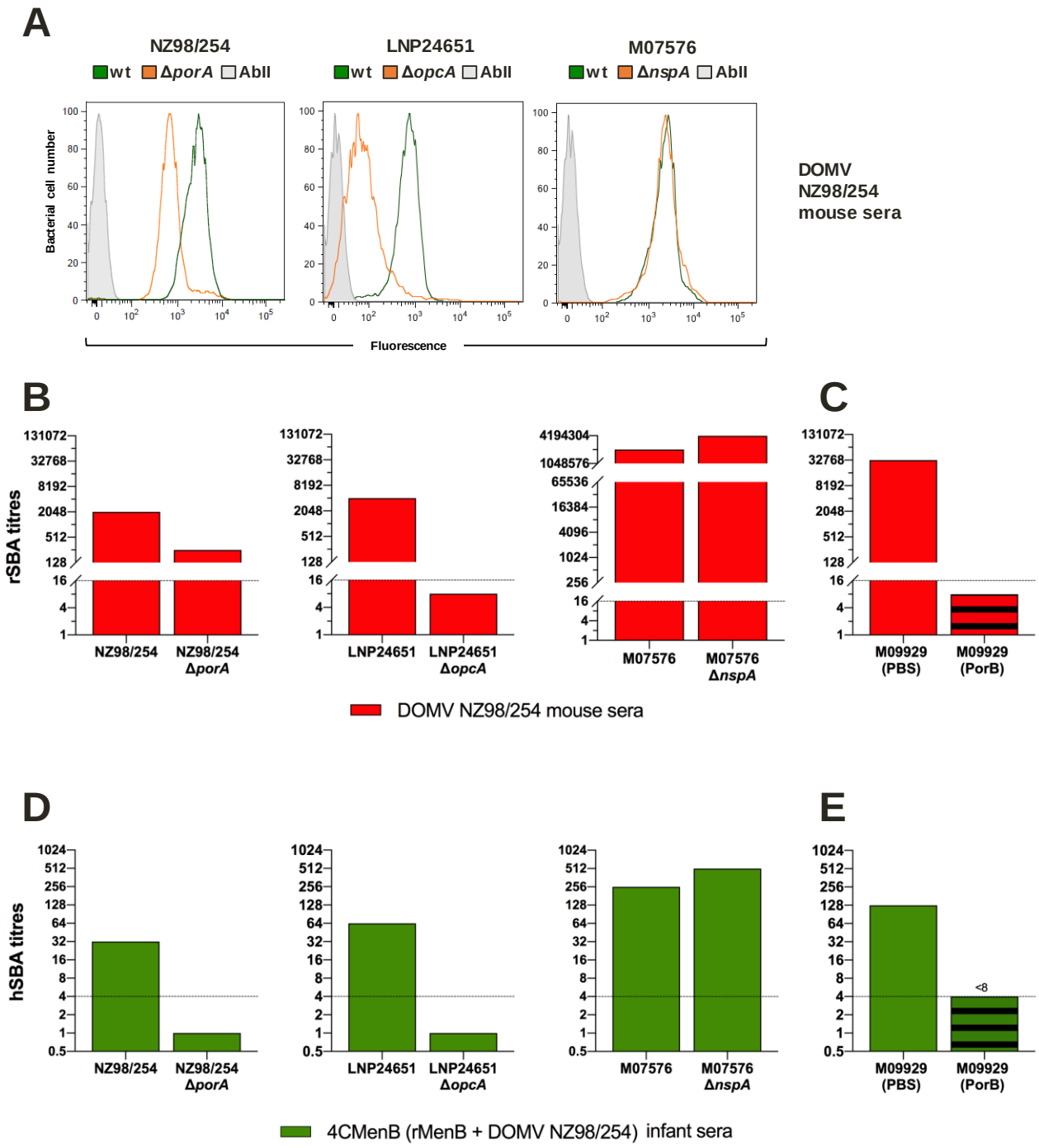

Figure 3.20. Anti-PorA, -OpcA and -PorB functional antibodies were raised by the DOMV component of the 4CMenB vaccine in humans. (**A**) Flow cytometric analyses were carried out on NZ98/254, LNP24651 and M07576 natural strains (green profiles) and on *porA*, *opcA* and *nspA* respective derivatives knock-out strains (orange profiles) using pooled sera from mice immunized with DOMV (n = 8). Grey filled profiles represent bacteria stained only with fluorescent secondary antibodies used as negative control, while the non-shaded profiles show the reaction with DOMV immune sera. (**B**) rSBA titres obtained with pooled DOMV mice sera (n = 8) against wild-type and knock-out strains are

represented by columns. Dashed lines represent the minimal threshold of protection in presence of baby rabbit complement. (**C**) The ability of PBS buffer alone or purified recombinant PorB protein to inhibit the killing of M09929 in presence of DOMV pooled sera (n = 8) was tested by competitive SBA and the resulting rSBA titres are illustrated as filled or striped histograms, respectively. Competitors are indicated in brackets. rSBA ≥16 are considered protective (dashed lines). (**D**) The ability of pooled (n = 25) infant immune sera collected from 2-months old infants who received 4 doses of 4CMenB (V72P16) to kill wild-type and knock-out *N. meningitidis* strains was tested in hSBA. Bactericidal titres are described as columns. hSBA ≥4 are considered protective (dashed line). (**E**) Competitive hSBA was performed on M09929 using PBS or PorB protein as competitor (filled and striped bars, respectively). The V72P16 sera used in this analysis was a pool comprising serum samples from 25 infants who received 4 doses (2, 3, 4 and 12 months) of 4CMenB. The not titrated SBA value for the M09929 preincubated with PorB protein is represented as 4 in the graph, but the first tested dilution (8) is indicated upon the corresponding column. hSBA ≥4 are considered protective (dashed line).

However, in order to investigate whether the diminished surface reactivity observed for MenB mutant strains echoed a reduced functionality, serum bactericidal assays in presence of rabbit complement were performed upon the same set of strains and using DOMV antisera as source of antibodies (Figure 3.20B). Although rSBA titres between NZ98/254 and the corresponding PorA-mutant differed only of 3-fold (2,048 and 256, respectively), the specificity and functionality of anti-PorA antibodies was further confirmed by the linearity and the parallelism of the two survival curves obtained from the colony counts at each serum dilution (Figure 3.21). Indeed, the percentages of survival of both strains after 60 minutes of incubation with DOMV sera provided clear hints that the differences in rSBA titres were due to the removal of the Porin A, even though the fact that DOMV are derived from the New Zealand strain prevented the reduction of rSBA titres to baseline in the NZ98/254Δ*porA*. The residual killing ability of the sera can hence be interpreted as the cumulative effect of bactericidal antibodies raised against DOMV-minor antigens able to target the homologous vaccine strain, confirming again the protective role played by non-PorA DOMV-antigens. As for OpcA, the bactericidal activity was completely abolished on the *opcA* KO mutant generated in the LNP24651 background, whereas the corresponding natural strain was killed with high efficiency with sera diluted up to 1/4,096. Based on this evidence, we confirmed the specificity of the protective role of the opacity protein within the complex DOMV-antisera, and parallelly we demonstrated that OpcA-targeting antibodies are the unique responsible of the DOMV-mediated killing on LNP24651. In contrast, NspA deletion in M07576 background did not seem to influence DOMV functionality as described by very high bactericidal titres on both strains (M07576 WT and M07576Δ*nspA*) and as also predicted by the overlapping profiles observed in FACS analysis. Finally, a subtractive approach, was used to measure PorB-specific bactericidal responses within DOMV-raised sera. Competitive inhibition SBA was performed on the M09929 isolate incubating bacteria and DOMV antisera in presence or absence of the PorB protein as competitor (Figure 3.20C). A 12-fold decrease in rSBA

titres was observed between PBS (32,768) and PorB (8) pre-treated sera: the recombinant PorB protein was able to specifically reduce the titres to baseline levels. This result provided an indirect evidence of the immunogenic activity of PorB within DOMV and unveiled this antigen as the unique DOMV-component responsible for M09929 killing.

Finally, having demonstrated the direct role of anti-PorA, -OpcA and -PorB antibodies in DOMV-mediated killing, we tried to confirm their ability to induce protective responses also in human subjects upon 4CMenB vaccination. NZ98/254, LNP24651, M07576, M09929 and their corresponding null mutants were tested against post-immunization pooled sera obtained from 25 infants vaccinated with 4CMenB (Figure 3.20D and E). In particular, this assay performed in presence of human complement is considered the surrogate of MenB protection. Despite hSBA titres demonstrated that the single contribution of NspA in vaccine was not evident, the absence of SBA activity against NZ98/254Δ*porA* and LNP24651Δ*opcA* confirmed the specificity of PorA and OpcA protective role in 4CMenB vaccines. Likewise, the protective role of PorB was demonstrated by the pre-treatment of human sera with exogenous PorB protein which completely abolished the protection on M09929 (Figure 3.20E). These results allowed us to prove that three DOMV components, despite being present in low amounts, were able to induce bactericidal antibodies in infants. Moreover, they paralleled denoted that the protection of NZ98/254, M07576 and M09929 was totally dependent upon one antigen contained exclusively within DOMV, supporting the pivotal role played by vesicles in the 4CMenB multivalent formulation.

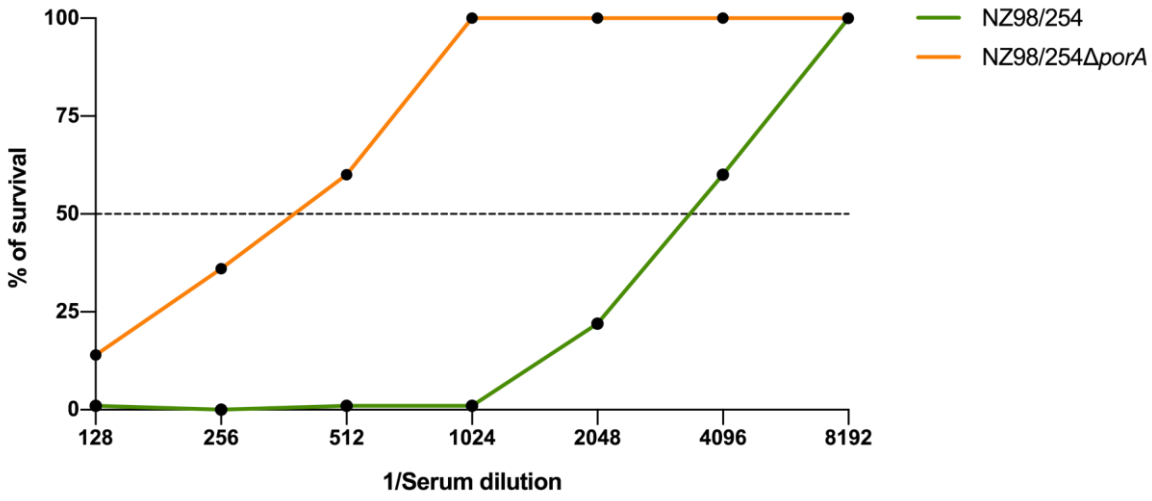

Figure 3.21. The lack of PorA antigen compromised the survival of the mutant strain in DOMV sera. Results of the rSBA analysis obtained incubating NZ98/254 (green line) and NZ98/254Δ*porA* (orange line) in presence of 25% baby rabbit complement as source of exogenous complement and serial dilutions of pooled DOMV antisera (n = 8). For each strain percentages of survival are plotted according to the inverse of the serum dilution. The dashed line indicates the half of the survival from which SBA titres are obtained.

4. Discussion

Detergent-extracted outer membrane vesicles (DOMV) from the NZ98/254 strain are one of the four components contained in the broad-spectrum 4CMenB vaccine, currently licensed for the prevention of invasive meningococcal disease caused by *Neisseria meningitidis* serogroup B. Preclinical and clinical studies post-vaccine licensure have deepened the knowledge on the three recombinant antigens of 4CMenB (rMenB: fHbp, NHBA and NadA), regarding their ability to induce functional antibodies in vaccinees (Findlow *et al.*, 2010, Martin and Snape, 2013), their role in pathogenesis (Pizza and Rappuoli, 2015), *in vivo* regulation (Biagini *et al.*, 2016), their interplay with host complement components (Serruto *et al.*, 2010) and the extent of cross-protection (Serruto *et al.*, 2012), until the recent identification of specific epitopes involved in the protection among different age groups of vaccinated (Bartolini *et al.*, 2020). On the contrary, the role of vesicular component of 4CMenB has not been fully elucidated, especially for what concern its contribution to vaccine protection. This was mostly due to the presence of PorA, pinpointed as the immunodominant DOMV antigen, and to the complex nature of DOMV, which posed several challenges for the deconvolution of antigen-specific contributions to the overall vaccine efficacy. Nevertheless, 4CMenB early phase II trials in infants have tended to explain the cross-protection observed on vaccine-heterologous strains to the presence of DOMV minor antigens, rather than PorA (Findlow *et al.*, 2010, Esposito *et al.*, 2014). Thus, although DOMV-antibodies are retained responsible of protective responses in humans, there is still little knowledge about the specific DOMV antigens contributing to bacterial clearance. Thereby, in this work we determined the impact of the DOMV component in the vaccine performance over a panel of non-vaccine related MenB strains and we exactly defined the implications of those DOMV antigens in vaccine coverage. The multicomponent nature of the 4CMenB vaccine intrinsically triggers a wide array of antibodies that may target multiple bacterial antigens and that overall contribute to the protection demonstrated through the Serum Bactericidal Assay (SBA), that for MenB is the accepted surrogate of protection. Therefore, the first step for the characterization of DOMV protective immunity was a judiciously chosen panel of strains. By exploiting the genetic diversity of *Neisseria meningitidis* population, we narrowed the scope of potential cross-reactivity due to major antigenic vaccine components and we selected a panel of strains in which measure the DOMV-specific bactericidal responses. Despite these strains have not to be intended as representative of the whole set of circulating vaccine-heterologous isolates, they were killed by antibodies targeting the vesicular component of 4CMenB in the cohort of vaccinated infants, suggesting that DOMV-containing formulations confer a more robust and a broader range of protection, than the solely rMenB. Overall, these findings were in line with previous studies carried

out on seven MenB indicator strains (Findlow *et al.*, 2010, Snape *et al.*, 2010, Esposito *et al.*, 2014), but our *ad hoc* panel of strains reinforces on a broader scale the immunological benefit of including DOMV in vaccine formulation. Moreover, the increase in hSBA titres observed with the full-dose of DOMV respect to a quarter dose, tends to suggest a DOMV dose-dependence for the coverage. The relevance of these results is also highlighted by the age group of vaccinees on which these conclusions are derived. Indeed, pre-existing immunity to meningococci is considered a process acquired during childhood and youth by repeating colonization of neisserial commensal species, like *Neisseria lactamica* (Troncoso *et al.*, 2000). In these naturally primed older age groups, even small amounts of minor-DOMV antigens contained in the vaccine formulation may be enough to boost protective response. On the contrary, this is rather unlikely in naïve two-month-old infants. Hence, these findings represent a breakthrough in the comprehension of the DOMV-mediated protection, especially in light of the disproportion of infants affected by meningococcal disease. Moreover, the susceptibility to the killing of all these PorA-mismatched strains points out the functional role of DOMV minor antigens in mediating protection. Nevertheless, our analysis suffers from the lack of DOMV-only vaccinated human subjects in which straightforward confirming the functionality of antibodies raised by DOMV. Therefore, the mouse model was used to obtain DOMV antisera that were able to recapitulate the same evidences indirectly gained with infant sera, with the only exception of the M07 241084 isolate. Noteworthy, while this isolate was resistant to complement mediated killing of anti-rMenB and anti-DOMV antibodies alone, 4CMenB vaccinees sera mediated its bacteriolysis. This different outcome may be explained by the cooperativity of antibodies elicited by 4CMenB which target different bacterial antigens (Vu *et al.*, 2011, Natali *et al.*, 2020, Giuliani *et al.*, 2018). Indeed, in competitive-SBA experiments, Giuliani and colleagues demonstrated that, although each rMenB antigen was able to significantly inhibit the killing of the corresponding reference strain, higher levels of inhibitions were obtained when the relevant antigens were combined with DOMV (Giuliani *et al.*, 2010), hence suggesting a synergistic effect of antibodies raised against rMenB and DOMV antigens. Furthermore, considering that the inhibition observed in these experiments was stronger on the NHBA reference strain with the simultaneous addition of DOMV and NHBA, and that the M07 241084 strain of our analysis harbours a distantly related fHbp variant from that of the vaccine and does not contain the *nadA* gene, we speculate that this specific cooperativity, involving NHBA, might also occur for the aforementioned isolate. Overall, these results underline the functional role of DOMV-minor antigens, but they do not provide information about which specific protein/s is responsible for the protective immunity onto the defined panel of strains. To this aim, we decided to elucidate the immunological repertoire of DOMV by dissecting its associated antigens and investigating their role in protection. As DOMV are complex mixtures of

lipids, OMP, periplasmic and cytoplasmic proteins, we narrowed the investigation onto those antigens that are more likely related to protection according to their presence, abundance and cellular localization, namely the major OMP. Our prioritized list of antigens included 30 distinct proteins, 21 of which were identified as the DOMV-specific core set since they comprised approximately 90% of DOMV content, according to qualitative and quantitative proteomic analysis performed on six different DOMV production lots (Tani *et al.*, 2014). This led us to hypothesize that such proteins consistently found across lots were presumably responsible for the DOMV-dependent cross-protection. In support of this theory, several of them were able to trigger an antibody response in adults upon *MeNZB* and *MenBvac* vaccination (Wedege *et al.*, 2007). Similarly, when human vaccinee sera were used to screen a complex microarray composed of 90 OMV-proteins, only about 10% of them were seroreactive, suggesting that among the plethora of antigens expressed by DOMV a defined subpanel is more likely to provide protection (Awanye *et al.*, 2019). However, despite cost-effective, our approach may suffer from *a priori* protein selection constraints. By prioritizing DOMV antigens according to proteomic-based studies, we did not take into account the involvement of the membrane-bound LOS into vaccine-induced protection. Indeed, although residual amounts of LOS contained into DOMV after bacterial treatment with detergent represent roughly the 5-8% of original LOS (Durand *et al.*, 2009), we cannot exclude their role in providing protection in vaccine individuals. Parallelly, the hydrophobic nature of selected proteins posed a challenge for their expression and folding in a native conformation. Bacterial surface-exposed proteins, like those contained in DOMV, are often characterised by extracellular domains in which protective epitopes are localized, usually made of few amino acids arranged in a defined conformation. Consequentially, in order to induce protective immunity these antigens need to be properly folded (Micoli *et al.*, 2020, Rossi *et al.*, 2020). Here, we used *E. coli* outer membrane vesicles, named GMMA (Generalized Modules for Membrane Antigens), for reconstitute proteins that require membrane interactions and we exploited the resulting bioengineered GMMA to raise functional immune responses that otherwise would be difficult to evoke. Previous studies have proven vesicle success in the expression and immunogenicity of bacterial (Bartolini *et al.*, 2013), viral (Watkins *et al.*, 2017) and even cancer antigens (Grandi *et al.*, 2017). Moreover, *E. coli* represents a very easy to handle model and an efficient tool for obtaining recombinant vesicles: in our study 26 of the 30 heterologous meningococcal antigens were obtained in GMMA, compared to only 13 recombinant counterparts. Moreover, their ability to carry membrane associated proteins in native conformation and orientation providing them natural immunogenicity, is supported by the specific-serosubtype functionality of antibodies raised against GMMA-PorA that we observed. Indeed, although expected, it provides a further validation of our approach. Vesicles built-in adjuvanticity was also useful for boosting the

immune response in mice even when sub-optimal expression of OMP was gained in GMMA (Micoli *et al.*, 2020). This was particularly evident for NEIS1634 antigen, whose expression in vesicles was not visible from SDS-PAGE analysis, but ELISA and Western-blot confirmed the presence of specific antibodies in the raised sera. In agreement with previous studies (Bartolini *et al.*, 2013), our data demonstrate that recombinant antigens used to decor GMMA induce high antibody responses. Indeed, despite a 2.5-fold difference in the formulation doses used for mouse immunization, 20 µg for recombinant protein and 8 µg for engineered GMMA, antibody titres were often similar between the corresponding sera. Moreover, the singular dissection of major DOMV antigens in heterologous vesicles was instrumental to realize a microarray which contained the repertoire of DOMV antigens and that shed light onto antigen ability to elicit IgG responses, at least for those protein components included into the protein chip. By comparing the immunoreactive profile of different DOMV-containing formulations, this tailored protein chip allowed a detailed assessment of the antibody responses in mice directed against the DOMV component of the meningococcal 4CMenB vaccine. As a result, we defined PorA, PorB, RmpM, BamE, MtrE, NspA, OpcA and Omp85 as the highest reactive DOMV antigens. Overall, these findings were consistent with a recent work in which OMV from the 44/76 MenB strain were used to immunize mice and to vaccinate humans (Awanye *et al.*, 2019). By performing protein array serumprofiling studies, authors identified PorA, PorB, RmpM, OpcA, PilQ, BamC and GNA1162 as the top responding antigens in humans. However, among them, only PorA, PorB and PilQ were targeted by mouse immunoglobulins, which in part contrast with our results obtained with animal sera. Nonetheless, this difference is well justified by the dose of OMV used for mice immunization studies, which was more than threefold-lower than the one used in our work (2.5 µg vs 8 µg). On the contrary, we did not detect PilQ among our immunoreactive spots, presumably due to its low level of expression in *E. coli* vesicles. Lastly, while Awanye and co-workers confidently demonstrated that two lipoproteins, BamC and GNA1162, were able to elicit immunity in humans, no information on them can be derived with our protein chip since they were not among the antigens spotted onto the slides. Nevertheless, according to proteomic studies of DOMV (Tani *et al.*, 2014), GNA1162 was not among the top 50 DOMV-antigens identified, whereas BamC was not identified in the whole DOMV proteome. Consequently, we can conclude that BamC is unlikely to elicit antibodies upon vaccination with 4CMenB. On the contrary, although its low abundance in DOMV, we cannot rule out GNA1162 involvement in vaccine immunogenicity, particularly in light of the BamE seroreactivity obtained in our analysis: it represents on average 0,1% of the total DOMV protein content, but it is amongst the main DOMV-immunoreactive proteins (Tani *et al.*, 2014). This suggests that antigen abundance is not always a prerequisite for immunogenicity. Contrary to RmpM, Omp85 and NspA, whose ability to mount immunity is well documented in

literature (Ying *et al.*, 2014, Rosenqvist *et al.*, 1999, Wedege *et al.*, 2013), an unanticipated outcome of this protein chip study was the identification of MtrE, for which very little is known. Lastly, it is noteworthy that the results obtained hybridizing the chip with DOMV antisera matched those obtained with 4CMenB. Despite a marked discrepancy between the two formulations in the amount of DOMV antigens present, they were still able to evoke immunity, at least in mice. Protein array resulted thus a valuable technique to shed light on DOMV immunogenic antigens within 4CMenB vaccine and to define an immunosignature that overall allowed us to fish antigens to be further characterized. Indeed, among all the specific DOMV-antisera tested in SBA those targeting the immunoreactive PorA, PorB, OpcA and NspA were able to differently kill the panel of meningococcal strains on which we had the first evidence about DOMV functionality. Conversely, although immunoreactive on our tailored protein-chip, Omp85 and RmpM were not able to induce functional antibodies. Interestingly, antisera from individuals vaccinated with the Norwegian OMV-based vaccine have been described to recognize a 80-kDa protein no better characterized (Rosenqvist *et al.*, 1995), that in a following study has been hypothesized to be Omp85 (Weynants *et al.*, 2007). Despite considered an immunogen, when Omp85 was overexpressed in meningococcal DOMV, it failed to induce higher bactericidal antibodies compared to wild-type vesicles, in mice (Wedege *et al.*, 2013). These results suggested that this protein induces antibodies poorly bactericidal on their own. Nevertheless, in the work of Weynants and co-workers the combination of Omp85 antisera with antibodies raised against other OMP prior to SBA, resulted in high levels of functional activity, suggesting a putative mechanism of synergy between antibodies, that can also occur following 4CMenB vaccination (Weynants *et al.*, 2007). Less understood is instead the immunogenicity of RmpM. It is considered to bind PorA and PorB through its N-terminal domain and the peptidoglycan layer with its N-region (Maharjan *et al.*, 2016), though it is not believed to be exposed to the extracellular environment, unless bacteria integrity is compromised during detergent treatment. Regardless of its cellular localization, OMV-vaccinees sera have been shown to contain antibodies reactive to RmpM (Awanye *et al.*, 2019, Wedege *et al.*, 2007, Williams *et al.*, 2014), but once purified they had not bactericidal activity (Rosenqvist *et al.*, 1999). Taken together, these results confirmed the lack of bactericidal activity that we got also in our experiments.

Unlike PorA, PorB has deserved little attention over the past years, probably due to its hypervariability localized in six of the eight protein loops (Stefanelli *et al.*, 2016), which render it a putative unsuitable vaccine candidate. Our studies, demonstrated that, despite all the isolates expressed comparable levels of the protein PorB3, only M07576 and M09929 were susceptible to PorB-dependent killing. Overall, the lack of bactericidal activity seems to be influenced by a decreased surface accessibility of the respective epitopes, as measured by FACS surface staining on

a subpanel of strains, rather than on amino acid sequence polymorphisms. Indeed, despite M09929 and NZ98/254 share 100% amino acid identity, the different ability of antibodies to stain their surface correlates with different SBA outcomes. The same observation can be deduced from the comparison of M07576 and M14569. In line with these findings, Michaelsen and colleagues elegantly showed that the PorB3 porin is poorly accessible for the antibody binding on live meningococci, compared to the PorB2 counterpart. Moreover, the authors clarified that the reason behind the PorB2 not binding-phenotype may be short extracellular loops and long carbohydrate chains of LPS molecules presented by strains (Michaelsen *et al.*, 2001). Therefore, we can speculate that the underlying reasons for the different SBA outcomes observed in our analysis may reside in an LPS shielding effect. In light of the foregoing results, the failure to elicit bactericidal antibodies of PorB expressed on GMMA, was rather unexpected and contrast with previous works. Indeed, preclinical studies have demonstrated that purified recombinant PorB was immunogenic in mice, but only PorB antigens included in micelles and/or liposomes were protective, despite in a serotype-specific manner (Wright *et al.*, 2002). However, taken together, these results validate the protocol used in our studies for PorB refolding which resulted essential to elicit functional antibodies and which is markedly different from the direct re-solubilization in urea used by Wright (Wright *et al.*, 2002).

Through our DOMV dissection analysis, Neisserial surface protein A was identified as another protective antigen contained within DOMV, confirming previous findings about NspA ability to elicit bactericidal antibodies (Moe *et al.*, 1999, Ying *et al.*, 2014) and to protect animals during *in vivo* meningococcal challenges (Martin *et al.*, 2000, Martin *et al.*, 1997). In our study, only two strains, M07576 and M12898, out of the whole panel were killed by NspA antisera, but the high percentage of identity of NspA primary sequence among isolates ruled out the involvement of protein variability in the different susceptibility to the killing observed. As already pointed out for PorB, several evidences in literature reported an inverse correlation between polysaccharide capsule amounts and surface accessibility also for NspA, which might be related with the limited length of NspA surface exposed loops and with a reduced bactericidal activity of anti-NspA antibodies in highly encapsulated isolates (Moe *et al.*, 1999, Moe *et al.*, 2001, Vandeputte-Rutten *et al.*, 2003). Despite the levels of NspA surface expression varied in the panel of strains under investigation, poor correlation has been observed among protein expression levels, surface exposure and bactericidal activity. Therefore, although the reasons behind this resistant phenotype remain still unknown, this result suggests that an interplay between more factors may contribute to the serum resistance observed in such meningococcal strains.

Lastly, OpcA was identified as an additional DOMV antigen responsible for the killing of 8 out of 12 DOMV-specific isolates. According to our analysis, the levels of OpcA expression have a profound

effect on OpcA-dependent bacteriolysis. Likewise, the OMV contained in the Norwegian vaccine has been demonstrated to raise anti-OpcA bactericidal antibodies in humans (Rosenqvist *et al.*, 1995, Rosenqvist *et al.*, 1993, Wedege *et al.*, 2007) and, in agreement with our results, vaccine elicited antibodies were able to kill uniquely those strains expressing high levels of OpcA, while they failed to protect against intermediate or low OpcA expressor isolates (Rosenqvist *et al.*, 1993). Therefore, considering that OpcA expression is phase-variable and that its transcription is regulated by the length of a poly-C stretch within *opcA* promoter (Sarkari *et al.*, 1994), the knowledge on its promoter nucleotide sequence could be instrumental for the prediction of potentially OpcA-covered strains following 4CMenB vaccination.

Notably, among the whole repertoire of antigens displayed by the DOMV, the proteins found immunogenic and protective in this study, PorA, PorB, OpcA and NspA, are all involved in the interaction with different host complement factors (Lewis and Ram, 2020, Lewis *et al.*, 2010, Lewis *et al.*, 2013, Jarva *et al.*, 2005). Likewise, also the recombinant vaccine components fHbp and NHBA are able to recruit complement regulators during pathogenesis (Madico *et al.*, 2006, Serruto *et al.*, 2010). Indeed, *N. meningitidis* scavenges host complement inhibitors as an important mechanism for subverting complement attack (Lewis and Ram, 2020). Therefore, the possibility to elicit antibodies towards these virulence factors is pivotal both for the complement-mediated killing of bacteria and to potentially compete with the binding to complement inhibitors, thereby enhancing bacterial clearance *in vivo*, as it was already suggested for fHbp (Beernink *et al.*, 2011).

Lastly, the generation of specific meningococcal knock-out mutants as well as the use of competitive SBA were instrumental to understand the protective role of PorA, PorB, OpcA and NspA in humans upon 4CMenB vaccination. Indeed, we were able to demonstrate that, apart from NspA, the other three DOMV antigens were present in DOMV in sufficient amounts to elicit protective antibodies in infants. Consistent with this evidence, Halperin *et al*., showed that recombinant NspA protein was immunogenic following a phase I-clinical trial, but it failed to provide protection against *N. meningitidis* (Halperin *et al.*, 2007). The unfolded nature of the vaccine protein, which may lack important conformational epitopes essential for the production of functional antibodies might have contributed to such failure (Halperin *et al.*, 2007); however, more recent data suggested that binding of complement factor H to NspA may impair elicitation of functional NspA antibodies (Lujan *et al.*, 2016). While for PorA and OpcA a protective role has been shown upon *MenBvac* vaccination (Rosenqvist *et al.*, 1993), for PorB the ability to elicit protective immunity in humans has been poorly characterized, despite it was recognized as an immunogen (Rosenqvist *et al.*, 1995, Wedege *et al.*, 2007, Wedege *et al.*, 1998).

In conclusion, we showed that the inclusion of DOMV within 4CMenB is pivotal to enhance the breadth of coverage against a panel of non-vaccine related strains, hence confirming the key role played by vesicles within this multicomponent vaccine. Moreover, through the dissection of the DOMV immunological repertoire, we were able to identify specific non-PorA antigens involved in such broad cross-protection and to further confirm their protective role in 4CMenB vaccinees sera.

5. Material and Methods

5.1 Bacterial strains and culture conditions

Escherichia coli and *Neisseria meningitidis* strains used in this study are listed un Table 5.1.

E. coli strains used for cloning and expression experiments were routinely grown in Luria-Bertani (LB) agar plates or medium at 37°C. When required ampicillin, chloramphenicol and kanamycin, were added to achieve a final concentration of 100,20 and 50 µg/ml, respectively.

N. meningitidis strains were routinely cultured overnight on Gonococcus (GC) agar medium (Difco) with Kellogg's supplement I (Kellogg *et al.*, 1963) or on chocolate agar plates (Biomérieux) at 37°C in an atmosphere of 5% CO_2. Liquid cultures were grown under the same conditions in Mueller Hinton (MH) broth plus 0.25% glucose. When required, erythromycin (5 µg/ml), kanamycin (150 µg/ml) or spectinomycin (50 µg/ml) were added to culture media at the indicated final concentrations.

5.2 Construction of plasmids for heterologous antigen expression in *E. coli*

DNA manipulations were carried out using standard laboratory methods (Sambrook, 2001). All cloning steps were performed using the PIPE (Polymerase Incomplete Primer Extension) method (Klock and Lesley, 2009). The amplification of all meningococcal antigens listed in Table 5.2 was performed by PCR using the genomic DNA of *N. meningitidis* NZ98/254 isolate as template and primers (Table 5.3) that amplified the genes without their natural leader sequence for secretion (iPCR). NZ98/254 gene sequences were obtained from the public database PubMLST (https://pubmlst.org/neisseria/), except for NEIS1690 and NEIS0210 which were not available, and sequences were extracted from the New Zealand isolate NZ05/33. The presence of the leader peptide and location of its cleavage site were determined using Signal P 4.1 (http://www.cbs.dtu.dk/services/SignalP-4.1/) (Nielsen, 2017); details about the removal of the leader peptide are listed in Table 5.2. The pET21-LPOmpA vector, a pET21 derivative plasmid carrying the sequence encoding the *E. coli* K-12 OmpA leader peptide (MKKTAIAIAVALAGFATVAQA), 6-histidine tag sequence (HHHHHH) and the FLAG tag (DYKDDDDK), was used for the heterologous antigen expression. It was amplified (vPCR) using different primers, depending on the desired location of the Tag: HISFLAG Fw/OmpRev for C-terminal tags, while For-pet21/HisFlagRev-pet21 for N-terminal ones. Unpurified vPCR and iPCR DNA fragments were mixed in a 1:2 ratio and directly transformed into chemically competent Stellar cells (Takara). All recombinant proteins were thus expressed fused to the OmpA leader sequence, in order to address the meningococcal antigens

on the outer membrane of *E. coli* mutant strains. Cells were plated on LB containing 10 µg/ml of ampicillin and grown at 37°C overnight (ON). Positive clones were selected by PCR analysis. Plasmid DNA were isolated (Table 5.4), the correctness of the cloning was verified by sequence analysis and they were used to transform BL21(DE3)Δ*tolR* (Berlanda Scorza *et al.*, 2008) or BL21(DE3)Δ*ompA* (Fantappie *et al.*, 2014) strains made chemically-competent. Transformants were grown on LB plates containing 100 µg/ml of ampicillin. pET15 vector with OmpA leader sequence and without insert was instead used to transform BL21(DE3)Δ*tolR* and BL21(DE3)Δ*ompA* strains as negative control.

5.3 Expression of the heterologous meningococcal proteins in *E. coli* mutant strains and GMMA preparation

The recombinant strains were pre-cultured over-day in 5 ml of LB supplemented with 100 µg/ml of ampicillin and growth at 37°C, in a rotary shaker (180 rpm). Pre-cultures were then diluted 1:100 in 50 ml High Throughput Medium Complex (HTMC) (3% yeast extract, 1.5% glycerol, 40 mM KH_2PO_4, 90 mM K_2HPO_4, 2 mM $MgSO_4 \cdot 7H_2O$, pH 7.4) with 100 µg/ml of ampicillin and grown ON at 20°C, 160 rpm. The cultures were then centrifuged 10 min at 1800 x*g* at 20°C, the supernatant was discarded to removed GMMA Empty and the pellet was resuspended in 60 ml of fresh HTMC supplied with 100 µg/ml of ampicillin. The expression of the recombinant proteins was induced with iso-propyl β-d-1-thiogalactopyranoside (IPTG) (Sigma Aldrich) at a final concentration of 1 mM. Culture were then incubated for 6 h at 37°, 180 rpm. Induced bacterial samples were collected to verify meningococcal protein expression in total as well as in soluble protein fractions. The cultures were clarified by centrifugation for 20 min at 3000 x*g* and the culture media were filtered through a 0.22-µm pore size filter (Millipore). The supernatants were subjected to high-speed centrifugation at 119000 x*g* for 2 h at 4°C (Beckman Coulter Optima Ultracentrifuge) and the pellets containing the GMMA were washed with phosphate buffer saline (PBS), ultracentrifuged again as above and, finally resuspended in PBS after being filtered with 0,22-µm filter. GMMA total protein content was quantified through the Lowry assay (DC Protein Assay, BioRad) following manufacturer's instructions.

5.4 Recombinant protein production and purification

Single colonies of recombinant *E. coli* BL21(DE3)Δ*tolR* or *E. coli* BL21(DE3)Δ*ompA* strains were inoculated in 5 ml of LB added with 100 µg/ml ampicillin and grown for 7 h at 37°C, 160 rpm. The cultures were then diluted 1:100 in 75 ml of HTMC medium with 100 µg/ml ampicillin, and they

were grown ON at 20°C, 160 rpm. The following day, protein expression was induced by the addition of 1 mM IPTG and cultures were grown at 20°C, 160 rpm for 24 h.

For protein purification, bacterial pellets were resuspended in equilibration buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0) supplemented with a tablet of protease inhibitor cocktail (cOmplete, EDTA-free, Roche) each 50 ml of equilibration buffer, before mechanical lysis by sonication (amplitude 65%, 30sec ON/OFF, total 30 minutes). Alternatively, bacterial pellets were resolubilized in B-PER Bacterial Protein Extraction Reagent (B-PER, Thermo Fisher, 1g/10ml) or Cell-Lytic Express (Sigma Aldrich) and incubated for 1 h at RT and periodically mixed. The unbroken cells and bacterial debris were removed by centrifugation and the recombinant His_6-tagged meningococcal proteins were purified from the supernatant by IMAC (immobilized metal-ion-affinity chromatography) purification using cobalt-based chelating columns (Hitrap TALON Crude; Merck) according to the manufacturer's instructions. Columns were washed with equilibration buffer and proteins bound to the resin were then eluted with imidazole gradient (from 0 mM to 250 mM imidazole) in equilibration buffer. After this first purification step, sample purity was assessed on an analytical size-exclusion by Ultra Performance Liquid chromatography (SE-UPLC) using BEH200 4.6 x 300 mm columns (Waters) and using as running buffer 10 mM NaH_2PO_4, 400 mM $(NH_4)_2SO_4$, pH 6.0. Samples whose purity was higher than 80% were dialyzed in PBS or loaded in a desalting column (GE Healthcare) and eluted in PBS. When sample purity was lower than 80%, samples were further purified by preparative size-exclusion chromatography (SEC) (Superdex 75/200) columns (HiLoad, GE Healthcare) in PBS. Endotoxin quantity was detected using LAL-test (Charles River) and when endotoxin levels were higher than >0.5U/µg, another purification step was performed with cHT (Bio-Rad) following the manufacturing protocols. Finally, proteins were analysed by SDS-PAGE followed by Coomassie blue staining (Giotto), and SE-UPLC to evaluate sample purity. Protein content was quantified by the BCA assay (Thermo Scientific).

The recombinant PorA and PorB proteins were instead purified from inclusion bodies using a modification of the method described in (Zhu *et al.*, 2018). Briefly, PorA bacterial pellets were dissolved in Guanidine 6 M, 50 mM Tris-HCl, pH 8.5, whereas PorB bacterial pellets were lysed in 8 M Urea, 50 mM Tris-HCl, pH 8,0. Undissolved particles were removed by centrifugation at 12000 x*g* for 30 min at 4°C. The solubilized inclusion bodies were concentrated in an Amicon concentrator,10 kDa cutoff (Millipore). Then samples were diluted into a refolding buffer (200 mM CAPS, 400 mM NaCl, 50 mM Tris-HCl, 0.3% Lauryl-N,N-Dimethylamine-N-Oxide [LDAO], pH 11). The mixtures were stirred ON at 4°C and the pH was adjusted to 8.0 with 1 N HCl. The refolded porins were loaded onto an IMAC column (HisTrap HP, GE Healthcare), washed with PBS + 0,1% LDAO (PBSL) and eluted with PBSL containing 300 mM imidazole. The samples were concentrated

with Amicon (10 kDa cutoff) and then loaded onto an S-300 Sephacryl column (GE Healthcare) and the protein was eluted in 0,1% PBSL. Sample purity was determined on an SE-HPLC (Superdex 200 5/150 GL) using as running buffer PBSL and the endotoxin content quantified as above stated. Finally, protein quantity was determined using Nanodrop (Thermo Fisher).

5.5 Protein microarray design, generation, validation and immunogenicity probing

Two different protein microarrays were manufactured *in house* to ensure the best spotting conditions of vesicles and recombinant proteins. The former protein chip encompassed 26 recombinant *E. coli* GMMA, two GMMA empty and DOMV from NZ98/254 (1mg/ml in 20% final glycerol). The latter included 13 MenB recombinant soluble proteins (0.5mg/ml in a final glycerol concentration of 40%). NHBA-GNA1030, GNA2091-fHbp and NadA were spotted onto both microarrays (0.5mg/ml in a final glycerol concentration of 40%). Controls consisted of 8 serial 2-fold dilutions of mouse IgG (from 0.5 mg/ml to 0.004mg/ml in 40% glycerol), MenB unrelated proteins (0.5 mg/ml in 40% glycerol) and PBS + 40% glycerol spots. All samples were printed randomly in at least 4 replicates onto ultra-thin nitrocellulose-coated glass slides (FAST slides; Maine Manufacturing) which enabled the immobilization of proteins within its fibrous matrix, offering optimal binding capacity, assuring the preservation of protein conformation and providing long shelf-life stability. Printing was performed with the ink-jet spotter Marathon Argus (Arrayjet) (two drops of 100 pl each spot) in a cabinet with controlled temperature and humidity (18°C and 50-55% respectively). Preliminary validation screenings were carried out to assure that replicate spots were efficiently immobilized on nitrocellulose slides with excellent reproducibility. Some test slides were probed with: i) anti-FLAG antibodies (Thermo Fisher) 1:5000, ii) rabbit anti-His6 tag polyclonal antibodies (Genetex) 1:1000, iii) pool made of antisera from the immunization of mice with recombinant antigens (1:2000), iv) mouse sera raised against GMMA Empty (1:2000), and followed by detection with an AlexaFluor 647-conjugated anti-rabbit or anti-mouse IgG secondary antibody (Jackson Immunoresearch) - 1:800. Preliminary experiments with animal sera showed that 1:1000 dilutions of sera corresponded to the best signal to noise ratio.

For sera profiling studies, nonspecific binding was minimized by preincubating arrays with a blocking solution (BlockIt, ArrayIt) for 1 hour at RT. Sera samples were diluted 1:1000 in Block-It buffer and incubated for 1 h prior to undergo two washes with Tween 0.1% in PBS (TPBS). Slides were incubated in the dark with 1:800 diluted AlexaFluor 647-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch) another hour. Then, after two final washes with TPBS and one in PBS, slides were rinsed in de-ionized water and dried. Fluorescence images were obtained using

Power scanner (Tecan Trading) and the 16-bit images were generated with PowerScanner software v1.2 at 10 µm/pixel resolution. Spot fluorescence intensities were determined using ImaGene 9.0 software (Biodiscovery Inc.) and microarray data analysis was performed using *in-house* developed R scripts. For each protein the fluorescence intensity data of the replicates was subtracted by local background values surrounding each spot and averaged, hence resulting in a single data point per sample named Mean Fluorescence Intensity (MFI). Signals were considered positive when their MFI were greater than 5,000, corresponding to the MFI of control protein-spots after detection with fluorescent-labelled anti-mouse antibodies, plus 10 times the standard deviation. All obtained MFI score were then classified in four categories: i) high reactivity; MFI ≥30,000; ii) medium reactivity; 15,000≤ MFI >30,000; iii) low reactivity; 5,000≤ MFI >15,000; (d) no reactivity; MFI<5,000.

5.6 Mice immunization

Four-six weeks-old CD1 female mice (8 per group, Charles River) were immunized intraperitoneally on days 1, 22 and 36 with 8 µg of recombinant *E. coli* GMMA or DOMV NZ98/254 and with 20 µg of recombinant proteins. Prior to immunization, samples were formulated with 3 mg/ml Aluminium hydroxide as adjuvant in a volume of 200 µl each dose. Mouse sera were collected the day before the first immunization (day 0) and 2 weeks after the third immunization (day 50). To prepare 4CMenB and rMenB antisera CD1 female mice (8-10 per group), were immunized as above using 20 µg of each individual antigen (GNA2091-fHbp, NHBA-GNA1030, NadA) + 10 µg DOMV or 20 µg of each individual antigens, respectively.

5.7 Ethical statement

All animal sera used in this study derived from mouse immunization experiments performed were performed at the GSK Animal Research Centre in Siena, Italy, in compliance with the ARRIVE guidelines, the current Italian legislation on the care and use of animals in experimentation (Italian Legislative Decree 116/92) and consecutive ministerial newsletter (Circolare Ministeriale 8/94), and with the GSK Animal Welfare Policy and Standards. The animal protocol was approved by the Animal Welfare Body of GSK Vaccines, Siena, Italy, and by the Italian Ministry of Health (Approval number AWB 2018_02, AWB2013_11 and AWB2017_04).

5.8 Enzyme-linked immunosorbent assay

Serum IgG titres directed to DOMV-specific antigens were evaluated by enzyme-linked immunosorbent assay (ELISA). Ninety six-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with 1 µg/ml of purified recombinant antigens in a buffer of 0.115% Na_2HPO_4, 0.02% KCl, 0.02% KH_2PO_4, 0.8% NaCl, pH 7.4 and incubated ON at 4°C. Plates were then washed three times with 0.05% Tween-20 in PBS 0.074M and blocked with 250 µl/well of 2.7% polyvinylpyrrolidone (Sigma-Aldrich) for 2 h at 37°C. Each incubation step was followed by triple washes in 0.05% Tween-20 in PBS 0,074M. Serum samples were diluted in 1%BSA (Bovine Serum Albumin) + 0.05% Tween-20 in PBS 0.074M buffer, transferred to coated-blocked plates and 2-fold serially diluted, then incubated 2 h at 37°C. One hundred microliter of 1:2000 alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) diluted in 1% BSA in 0.05% Tween-20 in PBS 0.074M were added to each well and left for 90 min at 37°C. Bound alkaline phosphatase was visualized by the addition of 100 µl/well of p-nitrophenyl phosphate (Sigma-Aldrich) and incubation for 30 min at room temperature (RT). Then 100 µl of 4N NaOH were added to each well and plates were analysed at 405/620-650 nm in a microplate spectrophotometer. Antibody titres were quantified as the dilution of serum that gives an absorbance of 0.4 OD.

Results were analysed with GraphPad Prism Software using t-student test to calculate statistical significance (*$p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.9 Human serum samples

The human serum samples used in this study were obtained via a Phase II clinical trial (V72P16, NCT00937521) conducted in multiple centres in the Czech Republic, Italy, Hungary, Chile and Argentina between July 2009 and November 2010 in accordance with Good Clinical Practices and according to the Declaration of Helsinki. Parents or guardian of each participant have given their written informed consent to the study. The subjects, healthy infants aged approximately 2 months at the time of enrolment, received at 2, 3, 4 and 12-months of age four doses of different formulation: i) rMenB + DOMV (namely 4CMenB, 50 µg each recombinant protein + 25 µg DOMV from NZ98/254 epidemic strains), ii) rMenB + ¼ DOMV (50 µg each recombinant protein + 6.25 µg DOMV), or iii) rMenB alone (50 µg each recombinant protein). Sera samples used in this analysis are anonymous pool of 25 subjects.

5.10 Serum bactericidal assay

Serum bactericidal activity against *N. meningitidis* strains was assessed as previously described (Borrow *et al.*, 2005a) with minor modifications. Briefly, bacteria were grown on chocolate agar plates at 37°C in 5% CO_2, ON. Colonies were inoculated in 7 ml of MH broth, plus 0.25% glucose (OD of ~0.05) and allowed to grow with shaking until OD of ~0.25 at 37°C. Bacteria were diluted in Dulbecco's saline phosphate buffer (Sigma) with 0.1% glucose and 1% BSA to a working dilution of 10^4–10^5 CFU/ml. All sera screened were heat inactivated for 30 min at 56°C. Bacteria were incubated with serial 2-fold dilutions of sera and complement (25% of the final reaction volume). The two different complement sources used in the analyses were: pooled baby rabbit serum (Cedarlane) or exogenous human complement deriving from donors with no detectable intrinsic bactericidal activity. Human complement source used in the study was obtained according to Good Clinical Practice in accordance with the declaration of Helsinki. Patients have given their written consent for the use of samples of study MENB REC 2ND GEN-074 (V72_92). The activity of plasma complement was reconstituted by the addition of heparin and divalent cations immediately before use. Experimental controls included bacteria incubated with complement serum alone (Complement Dependent Control) and immune sera incubated with bacteria and heat-inactivated complement (Complement Independent Control). Immediately after the addition of complement, controls were plated on MH agar plates using the tilt method (T_0). The reaction mixture was incubated at 37°C for 60 min and aliquots were plated in duplicate after incubation (T_{60}). The agar plates were incubated for 18 h at 37°C, and the colonies corresponding T_0 and T_{60} were counted. Serum bactericidal titres were defined as the serum dilution resulting in a 50% decrease in the number of viable colonies after 60 min of incubation relative to baseline colony counts at T_0.

Competition SBAs were performed by adding recombinant protein to the serum samples to a final concentration of 1500 μg protein/ml serum, incubating overnight at 4°C, and then diluting and testing the sample in SBA as above. Sera pre-treated with equal protein-volume of PBS were used as controls for mimicking the "dilution-effect".

5.11 Construction of meningococcal deletion mutants

Plasmids for the allelic replacement of *opcA* and *nspA* coding sequences were obtained from (Echenique-Rivera *et al.*, 2011). PorA knock-out was generated by replacing the promoter region and nucleotides 1-512 of *porA* CDS with a negative selection cassette (*tetR-sacB*) from pJJ260 vector (Johnston, 2012) and a spectinomycin resistance cassette through homologous recombination. About 500 basepair upstream and downstream to the target *locus* were PCR amplified using porAkoUPF/

porAkoDOR from NZ98/254 genomic DNA and inserted into pET15 vector by PIPE cloning. The resulting plasmid was used as template for the vPCR using porAkoUPR and porAkoDOF. In a second step, the iPCR positive-negative selection cassette (*tetR-sacB-specR*) was inserted between the recombination fragments (vPCR). Finally, the obtained construct was PCR amplified using porAkoUPF and porAkoDOR, purified and used for the transformation. Strains, primers and plasmids used are listed in Table 5.1, Table 5.3 and Table 5.4, respectively. Linearized plasmids were used to transform *Neisseria meningitidis* strains as in (Masignani *et al.*, 2003). Briefly, four to seven colonies of a freshly grown ON plate were resuspended in 100 µl of PBS, mixed with 5 µg of DNA and spotted onto GC agar plates, allowed to dry, and incubated for 6 h at 37°C, 5% CO_2. Spots were then plated onto selective GC agar plates. Single colonies were further selected and amplified onto another plate with antibiotics. Correct clones were confirmed by PCR and western blotting analysis.

5.12 Flow cytometry assay

Five millilitres of *N. meningitidis* cells in early exponential phase were harvested by centrifugation at 1500 x*g* for 15 min and pellets were resuspended in PBS plus 1% BSA. Aliquots of 50 µl were dispensed in a round bottom 96-well plate (Corning Costar) and incubated for 1 h at RT with antibodies of interest (1:100). Plates were centrifuged at 1400 x*g* for 5 min, washed twice with PBS + 1% BSA, and then added with anti-mouse (whole-molecule) FITC-conjugated antibody (Sigma Aldrich) at a 1:100 dilution and incubated for 30 min. Each well was then washed with PBS, and the plates centrifuged at 1400 x*g* for 5 min. Before flow cytometry analysis, bacteria were fixed with a 0.5% formaldehyde solution in PBS buffer for 1 h. After a final washing step with PBS, cells were re-suspended in filtered PBS. Bacterial fluorescence was recorded with BD FACS CANTO II (BD Bioscience) acquiring 10,000 events, and data were analysed using Flow-Jo v.3 (FlowJo, LLC). This software was also used to calculate the mean fluorescence intensity of each bacterial population, which correlated to the amount of protein bound to the surface of bacteria. Bacteria incubated with buffer alone were used as negative controls.

5.13 C3 deposition on *N. meningitidis* surface

To measure C3 and IgG binding to the surface of *Neisseria meningitidis*, samples were prepared as for the FACS analysis. Aliquots of 25 µl of resuspended bacteria were dispensed in a round bottom 96-well plate, supplemented with GMMA-OpcA antisera at different dilutions and human plasma from MENB REC 2ND GEN-074 (V72_92), which was reconstituted by the addition of heparin and divalent cations immediately before use. Samples were incubated 15 min at 37°C. C3 deposition was

detected using FITC-conjugated anti-human C3 antibody (Cedarlane) (1:100). Fluorescence of 5,000 bacteria was measured with BD FACS CANTO II and analysed using Flow-Jo.

5.14 *N. meningitidis* confocal immunofluorescence microscopy

Bacteria used for confocal microscopy were grown as for SBA experiments until early exponential phase. One hundred and fifty microliters of each bacteria cultures were incubated 10 min at 37°C with FM 4-64FX (Thermo Fisher) diluted 1:100. Then, bacteria were diluted 1:2 with 4% formaldehyde and 150 µl of solution spread on polylysine-coated slides (Thermo Scientific) and incubated for 30 min. Bacteria were washed extensively with PBS and incubated with DOMV NZ98/254 antisera (1:250) for 1h at RT. Unbound antibodies were removed by washing and cells were stained with Alexa Fluor 488 anti-mouse IgG (Invitrogen) for 30 min (diluted 1:1000). After a final wash, labelled preparations were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen), analysed with a Zeiss LSM-710 confocal microscope and images were captured using ZEN software (Carl Zeiss).

5.15 Protein sample preparation

Recombinant meningococcal antigens, *E. coli* GMMA and DOMV NZ98/254 were prepared in 4X NuPAGE LDS Sample Buffer (Thermo Fisher) and 10X NuPAGE Sample Reducing Agent (Thermo Fisher).

E. coli cell lysates used to verify meningococcal protein expression were prepared from bacteria grown in LB. Five hundred microliters were removed from induced cultures and pelleted in a bench-top centrifuge 10 min at 9000 x*g*. Pellets were resuspended in 300 µl of B-PER (Thermo Fisher) and mix for 1 h at RT. The lysed solutions were added with 600 µl of binding buffer (20mM $NaPO_4$, 300mM NaCl, 10mM imidazol, pH=8). Sixty microliters of the mixture, corresponding to the total fractions, were added with 20 µl of 4X NuPAGE LDS Sample Buffer + 10X Sample Reducing Agent. The remaining lysed solutions were centrifuged 20 min at 9000 x*g*. Sixty microliters of the supernatant, corresponding to the soluble fractions, were mixed with 20 µl of 4X NuPAGE LDS Sample Buffer + 10X Sample Reducing Agent.

N. meningitidis total crude lysates were prepared from strains grown in the same conditions as for the SBA assay to an OD of 0.25. Bacteria suspensions were then centrifuged 15 min at 1800 x *g* and the pellet resuspended in lysis buffer (5 % SDS, 100 mM Tris-HCl, pH 8, 50 mM DTT, and protease inhibitor mixture (Roche) to a final concentration of 0.007 OD/µl.

5.16 Polyacrylamide gel electrophoresis and Western blotting

Recombinant meningococcal antigens (2 µg/lane or 1 µg/lane), *E. coli* GMMA (10 or 15 µg/lane, Western-blot or SDS-PAGE, respectively), DOMV NZ98/254 (10 µg/lane), *E. coli* total and soluble fractions (12 µl/lane) and *N. meningitidis* lysates (0.07 OD/lane) were boiled 10 min, separated by SDS-PAGE on NuPAGE Novex 4-12% Bis-Tris Protein Gels in MES 1X (Life Technologies) and stained with ProBlue Safe Stain (Giotto Biotech). For Western blot, gels were transferred to nitrocellulose membranes using iBlot Dry Blotting System (Invitrogen). After 1 h saturation with 5% powdered milk (Sigma Aldrich) in TPBS, the filters were incubated with specific antisera (1:2000) or with anti-FLAG antibodies (1:5000, Thermo Fisher) in 3% powdered milk in TPBS and incubated for 1 h. A horseradish peroxidase-conjugated anti-mouse IgG antibody (Invitrogen) and the Pierce ECL Western Blotting Substrate (Thermo Fisher) were used according to the manufacturer's instructions.

5.17 Bioinformatic analysis

Nucleotide sequences of the thirty DOMV-loci and of the promoter of *opcA* were extracted from the genomes of the 12 MenB strains under investigation. Amino acid sequences were then aligned using the CLUSTAL Omega algorithm incorporated in the Geneious software (Biomatters) (Kearse *et al.*, 2012), and the percentages of amino acid identity with the respect of NZ98/254 were calculated. The phylogenetic tree of PorB was reconstructed using the Neighbor-Joining method. The multiple sequence alignment of the *opcA* promoter was performed using MUSCLE and manually curated.

Table 5.1. Strains used in this study.

Name	Description	Antibiotic resistance	Reference
	***Escherichia coli* strains**		
BL21(DE3)Δ*tolR*	BL21(DE3) derivative, lacking *tolR* gene	Kanamycin	Berlanda *et al.* , 2008
BL21(DE3)Δ*ompA*	BL21(DE3) derivative, lacking *ompA* gene	Chloramphenicol	Fantappiè *et al* ., 2014
BL21(DE3)Δ*tolR* Empty	BL21(DE3)Δ*tolR* derivative, carryng the pET15b plasmid	Kanamycin	This study
BL21(DE3)Δ*ompA* Empty	BL21(DE3)Δ*ompA* derivative, carryng the pET15b plasmid	Chloramphenicol	This study
BL21(DE3)Δ*ompA* NEIS2020	BL21(DE3)Δ*ompA* derivative, carryng the pET21-LPompA NEIS2020_N plasmid for the expression of NEIS2020	Chloramphenicol, Ampicillin	This study
BL21(DE3)Δ*ompA* NEIS1364	BL21(DE3)Δ*ompA* derivative, carryng the pET21-LPompA NEIS1364_N plasmid for the expression of NEIS1364	Chloramphenicol, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1428	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1428_N plasmid for the expression of NEIS1428	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1783	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1783_C plasmid for the expression of NEIS1783	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0944	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0944_N plasmid for the expression of NEIS0944	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0408	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0408_C plasmid for the expression of NEIS0408	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS2198	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS2198_N plasmid for the expression of NEIS2198	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1963	BL21(DE3)Δ*tolR* derivative, carryng thepET21-LPompA NEIS1963_N plasmid for the expression of NEIS1963	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1690_C	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1690_C plasmid for the expression of NEIS1690	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1690_N	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1690_N plasmid for the expression of NEIS1690	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0173	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0173_N plasmid for the expression of NEIS0173	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0073	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0073_N plasmid for the expression of NEIS0073	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1468	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1468_N plasmid for the expression of NEIS1468	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0275_C	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0275_C plasmid for the expression of NEIS0275	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0275_N	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0275_N plasmid for the expression of NEIS0275	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1487	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1487_C plasmid for the expression of NEIS1487	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1632	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1632_C plasmid for the expression of NEIS1632	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0101_C	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0101_C plasmid for the expression of NEIS0101	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0101_N	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0101_N plasmid for the expression of NEIS0101	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1271	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1271_C plasmid for the expression of NEIS1271	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1687	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1687_N plasmid for the expression of NEIS1687	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0210_C	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0210_C plasmid for the expression of NEIS0210	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0210_N	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0210_N plasmid for the expression of NEIS0210	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS2075	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS2075_C plasmid for the expression of NEIS2075	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1246	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1246_C plasmid for the expression of NEIS1246	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0612	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0612_N plasmid for the expression of NEIS0612	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1462	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1462_C plasmid for the expression of NEIS1462	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0172	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0172_C plasmid for the expression of NEIS0172	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1066	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1066_C plasmid for the expression of NEIS1066	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0596	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0596_N plasmid for the expression of NEIS0596	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1634	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1634_C plasmid for the expression of NEIS1634	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0653	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0653_C plasmid for the expression of NEIS0653	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS0196	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS0196_C plasmid for the expression of NEIS0196	Kanamycin, Ampicillin	This study
BL21(DE3)Δ*tolR* NEIS1065	BL21(DE3)Δ*tolR* derivative, carryng the pET21-LPompA NEIS1065_C plasmid for the expression of NEIS1065	Kanamycin, Ampicillin	This study

Table 5.1 (continued). Strains used in this study.

Name	Description	Antibiotic resistance	Reference
	***Neisseria meningitidis* strains**		
NZ98/254	Clinical isolate	/	
M08389	Clinical isolate	/	
LNP24651	Clinical isolate	/	
M08117	Clinical isolate	/	
M10837	Clinical isolate	/	
MC58	*Neisseria meningitidis* laboratory-adapted reference strain	/	
M09662	Clinical isolate	/	
M14569	Clinical isolate	/	
M07576	Clinical isolate	/	
M09929	Clinical isolate	/	
M12898	Clinical isolate	/	
M07 241084	Clinical isolate	/	
NZ98/254Δ*porA*	NZ98/254 derivative, lacking *porA* gene	Spectinomycin	This study
LNP24651Δ*opcA*	LNP24651 derivative, lacking *opcA* gene	Kanamycin	This study
M07576Δ*nspA*	M07576 derivative, lacking *nspA* gene	Erythromycin	This study
M12898Δ*nspA*	M12898 derivative, lacking *nspA* gene	Erythromycin	This study

Table 5.2. Meningococcal antigens cloned in this study.

PubMLST	Leader sequence[a]		Mw (kDa)	Tag (6xHis + FLAG)	*E. coli* cells used for transformation
	Removed	Amino acid residues removed			
NEIS2020	yes	1-19	35,7	N-term	BL21(DE3)Δ*ompA*
NEIS1364	yes	1-19	41,6	N-term	BL21(DE3)Δ*ompA*
NEIS1428	yes	1-25	103	N-term	BL21(DE3)Δ*tolR*
NEIS1783	yes	1-22	25,7	C-term	BL21(DE3)Δ*tolR*
NEIS0944	yes	1-24	84,3	N-term	BL21(DE3)Δ*tolR*
NEIS0408	yes	1-24	82	C-term	BL21(DE3)Δ*tolR*
NEIS2198	yes	1-19	30	N-term	BL21(DE3)Δ*tolR*
NEIS1963	yes	1-22	78,7	N-term	BL21(DE3)Δ*tolR*
NEIS1690[b]	yes	1-24	101,3	C-term/N-term	BL21(DE3)Δ*tolR*
NEIS0173	yes	1-21	88,2	N-term	BL21(DE3)Δ*tolR*
NEIS0073	yes	1-24	50,2	N-term	BL21(DE3)Δ*tolR*
NEIS1468	yes	1-24	105,2	N-term	BL21(DE3)Δ*tolR*
NEIS0275	yes	1-23	88	C-term/N-term	BL21(DE3)Δ*tolR*
NEIS1487	yes	1-22	28,7	C-term	BL21(DE3)Δ*tolR*
NEIS1632	yes	1-39	50,2	C-term	BL21(DE3)Δ*tolR*
NEIS0101	no	-	52	C-term/N-term	BL21(DE3)Δ*tolR*
NEIS1271	yes	1-20	66,2	C-term	BL21(DE3)Δ*tolR*
NEIS1687	yes	1-22	41,5	N-term	BL21(DE3)Δ*tolR*
NEIS0210[b]	no	-	19,4	C-term/N-term	BL21(DE3)Δ*tolR*
NEIS2075	yes	1-21	13,2	C-term	BL21(DE3)Δ*tolR*
NEIS1246	yes	1-37	27,6	C-term	BL21(DE3)Δ*tolR*
NEIS0612	yes	1-19	18,5	N-term	BL21(DE3)Δ*tolR*
NEIS1462	yes	1-23	18,2	C-term	BL21(DE3)Δ*tolR*
NEIS0172	yes	1-23	18,8	C-term	BL21(DE3)Δ*tolR*
NEIS1066	yes	1-22	23,8	C-term	BL21(DE3)Δ*tolR*
NEIS0596	yes	1-25	33,2	N-term	BL21(DE3)Δ*tolR*
NEIS1634	yes	1-26	42,3	C-term	BL21(DE3)Δ*tolR*
NEIS0653	yes	1-18	31	C-term	BL21(DE3)Δ*tolR*
NEIS0196	yes	1-18	14,01	C-term	BL21(DE3)Δ*tolR*
NEIS1065	yes	1-24	13,1	C-term	BL21(DE3)Δ*tolR*

[a] According to Signal P; [b] nucletode sequece from NZ05/033

Table 5.3. Primers used in this study

Name	Sequence[a]	Application	Reference
HISFLAG Fw	catcaccatcaccatCACGATTACAAAGAC	vPCR for cloning the antigens with a C-terminal Tag (Fw)	This study
OmpRev	ggcctgcgctacggtAGCGAAA	vPCR for cloning the antigens with a C-terminal Tag (Rev)	This study
For-pet21	tgagatccggctgcTAACAAAGCCCGAAAGG	vPCR for cloning the antigens with a N-terminal Tag (Fw)	This study
HisFlagRev-pet21	cttgtcatcatcgtcTTTGTAATCGTGATGGTGATGG	vPCR for cloning the antigens with a N-terminal Tag (Rev)	This study
NEIS2020-Fw_n	gacgatgatgacaagGACGTTACCCTGTACGGCACCA	iPCR for cloning of NEIS2020 with the N-terminal Tag (Fw)	This study
NEIS2020-Rv_n	gcagccggatctcaGAATTTGTGGCGCAGACCGACA	iPCR for cloning of NEIS2020 with the N-terminal Tag (Rev)	This study
NEIS1364-Fw_n	gacgatgatgacaagGATGTCAGCCTATACGGCGAAAT	iPCR for cloning of NEIS1364 with the N-terminal Tag (Fw)	This study
NEIS1364-Rv_n	gcagccggatctcaGAATTTGTGGCGCAAACCGACG	iPCR for cloning of NEIS1364 with the N-terminal Tag (Rev)	This study
NEIS1428-Fw_n	gacgatgatgacaagGAAGATGCAGGGCGCGCGGG	iPCR for cloning of NEIS1428 with the N-terminal Tag (Fw)	This study
NEIS1428-Rv_n	gcagccggatctcaAAACTTGTAGCTCATCGTTATCAAAA	iPCR for cloning of NEIS1428 with the N-terminal Tag (Rev)	This study
NEIS1783-Fw_c	accgtagcgcaggccGGCGAGGCGTCCGTTCAG	iPCR for cloning of NEIS1783 with the C-terminal Tag (Fw)	This study
NEIS1783-Rv_c	atggtgatggtgatgGTGTTGGTGATGATTGTG	iPCR for cloning of NEIS1783 with the C-terminal Tag (Rev)	This study
NEIS0944-Fw_n	gacgatgatgacaagCATGAAACCGAGCAGTCGGTGG	iPCR for cloning of NEIS0944 with the N-terminal Tag (Fw)	This study
NEIS0944-Rv_n	gcagccggatctcaAAACTTCACGTTCACGCCGCCG	iPCR for cloning of NEIS0944 with the N-terminal Tag (Rev)	This study
NEIS0408-Fw_c	accgtagcgcaggccGGAAACATTACAGACATCAAAGTTTC	iPCR for cloning of NEIS0408 with the C-terminal Tag (Fw)	This study
NEIS0408-Rv_c	atggtgatggtgatgATAGCGCAGGCTGTTGCCGGC	iPCR for cloning of NEIS0408 with the C-terminal Tag (Rev)	This study
NEIS2198-Fw_n	gacgatgatgacaagGCA CAAGAGCTTC AAACCGC	iPCR for cloning of NEIS2198 with the N-terminal Tag (Fw)	This study
NEIS2198-Rv_n	gcagccggatctca GAATTTTATGCCGACGCGCAAG	iPCR for cloning of NEIS2198 with the N-terminal Tag (Rev)	This study
NEIS1963-Fw_n	gacgatgatgacaagGCAGAAAATAATGCCAAGGTCGTA	iPCR for cloning of NEIS1963 with the N-terminal Tag (Fw)	This study
NEIS1963-Rv_n	gcagccggatctcaGAACTTGTAGTTCACGCCCAAGC	iPCR for cloning of NEIS1963 with the N-terminal Tag (Rev)	This study
NEIS1690-Fw_c	accgtagcgcaggccGAAAATGTGCAAGCCGGACAAG	iPCR for cloning of NEIS1690 with the C-terminal Tag (Fw)	This study
NEIS1690-Rv_c	atggtgatggtgatgGAACTTCATTTCCAAGCTAAATG	iPCR for cloning of NEIS1690 with the C-terminal Tag (Rev)	This study
NEIS1690-Fw_n	gacgatgatgacaagGAAAATGTGCAAGCCGGACAAG	iPCR for cloning of NEIS1690 with the N-terminal Tag (Fw)	This study
NEIS1690-Rv_n	gcagccggatctcaGAACTTCATTTCCAAGCTAAATG	iPCR for cloning of NEIS1690 with the N-terminal Tag (Rev)	This study
NEIS0173-Fw_n	gacgatgatgacaagGACTTCACCATCCAAGACATCCG	iPCR for cloning of NEIS0173 with the N-terminal Tag (Fw)	This study
NEIS0173-Rv_n	gcagccggatctcaGAACGTCGTGCCGAGTTGGAATT	iPCR for cloning of NEIS0173 with the N-terminal Tag (Rev)	This study
NEIS0073-Fw_n	gacgatgatgacaagTCCGGCTACCACTTCGGCACA	iPCR for cloning of NEIS0073 with the N-terminal Tag (Fw)	This study
NEIS0073-Rv_n	gcagccggatctcaTTTGAATTTGTAGGTGTATTGCAGG	iPCR for cloning of NEIS0073 with the N-terminal Tag (Rev)	This study
NEIS1468-Fw_n	gacgatgatgacaagGCAAACCCTGAAACGGCGGCA	iPCR for cloning of NEIS1468 with the N-terminal Tag (Fw)	This study
NEIS1468-Rv_n	gcagccggatctcaAAACTTCATTTCGAGCGCGAGGC	iPCR for cloning of NEIS1468 with the N-terminal Tag (Rev)	This study
NEIS0275-Fw_c	accgtagcgcaggccGCCGCCGATGCCGTTGCG	iPCR for cloning of NEIS0275 with the C-terminal Tag (Fw)	This study
NEIS0275-Rv_c	atggtgatggtgatgGGGTCGTTTGTTGCGTCCGG	iPCR for cloning of NEIS0275 with the C-terminal Tag (Rev)	This study
NEIS0275-Fw_n	gacgatgatgacaagGCCGCCGATGCCGTTGCG	iPCR for cloning of NEIS0275 with the N-terminal Tag (Fw)	This study
NEIS0275-Rv_n	gcagccggatctcaGGGTCGTTTGTTGCGTCCGG	iPCR for cloning of NEIS0275 with the N-terminal Tag (Rev)	This study
NEIS1487-Fw_c	accgtagcgcaggccAAAAAAGAAGCCGCCCCCGCAT	iPCR for cloning of NEIS1487 with the C-terminal Tag (Fw)	This study
NEIS1487-Rv_c	atggtgatggtgatgATTTACTTTTTTGATGTCGACTTGAG	iPCR for cloning of NEIS1487 with the C-terminal Tag (Rev)	This study
NEIS1632-Fw_c	accgtagcgcaggccATTCCCCAATACGAGCAGC	iPCR for cloning of NEIS1632 with the C-terminal Tag (Fw)	This study
NEIS1632-Rv_c	atggtgatggtgatgTTTGTCGGTTTGGGTATCC	iPCR for cloning of NEIS1632 with the C-terminal Tag (Rev)	This study
NEIS0101-Fw_c	accgtagcgcaggccTTAAAATGCGGAACATTTTTTATC	iPCR for cloning of NEIS0101 with the C-terminal Tag (Fw)	This study
NEIS0101-Rv_c	atggtgatggtgatgTTCGGAGCGGTTGAAGCCAAAC	iPCR for cloning of NEIS0101 with the C-terminal Tag (Rev)	This study
NEIS0101-Fw_n	gacgatgatgacaagTTAAAATGCGGAACATTTTTTATC	iPCR for cloning of NEIS0101 with the N-terminal Tag (Fw)	This study
NEIS0101-Rv_n	gcagccggatctcaTTCGGAGCGGTTGAAGCCAAAC	iPCR for cloning of NEIS0101 with the N-terminal Tag (Rev)	This study
NEIS1271-Fw_c	accgtagcgcaggccGCCCACGACGCGGCACACAA	iPCR for cloning of NEIS1271 with the C-terminal Tag (Fw)	This study
NEIS1271-Rv_c	atggtgatggtgatg ACGTATCCAGCTCGAAGGGTTCA	iPCR for cloning of NEIS1271 with the C-terminal Tag (Rev)	This study
NEIS1687-Fw_n	gacgatgatgacaagGAGACCGCGCTGCAATGCGC	iPCR for cloning of NEIS1687 with the N-terminal Tag (Fw)	This study
NEIS1687-Rv_n	gcagccggatctcaGATGCCGTCCAAGTCGTTGAACA	iPCR for cloning of NEIS1687 with the N-terminal Tag (Rev)	This study
NEIS0210-Fw_c	accgtagcgcaggccAACACCCTTCAAAAAGGTTTTAC	iPCR for cloning of NEIS0210 with the C-terminal Tag (Fw)	This study
NEIS0210-Rv_c	atggtgatggtgatgGCTGGCAGATGCTGTGTCG	iPCR for cloning of NEIS0210 with the C-terminal Tag (Rev)	This study
NEIS0210-Fw_n	gacgatgatgacaagAACACCCTTCAAAAAGGTTTTAC	iPCR for cloning of NEIS0210 with the N-terminal Tag (Fw)	This study
NEIS0210-Rv_n	gcagccggatctcaGCTGGCAGATGCTGTGTCG	iPCR for cloning of NEIS0210 with the N-terminal Tag (Rev)	This study
NEIS2075-Fw_c	accgtagcgcaggccGCCGGCACGAACAACCCCAC	iPCR for cloning of NEIS2075 with the C-terminal Tag (Fw)	This study
NEIS2075-Rv_c	atggtgatggtgatg ACGTGGGGAACAGTCTTTGAAGA	iPCR for cloning of NEIS2075 with the C-terminal Tag (Rev)	This study
NEIS1246-Fw_c	accgtagcgcaggccCCCTCGCGGGCAGAAAAAGCC	iPCR for cloning of NEIS1246 with the C-terminal Tag (Fw)	This study
NEIS1246-Rv_c	atggtgatggtgatgTTGACCGGTGAGGACGGTTTGC	iPCR for cloning of NEIS1246 with the C-terminal Tag (Rev)	This study
NEIS0612-Fw_n	gacgatgatgacaagGAAGGCGCATCCGGCTTTTACG	iPCR for cloning of NEIS0612 with the N-terminal Tag (Fw)	This study
NEIS0612-Rv_n	gcagccggatctcaGAATTTGACGCGCACACCGGC	iPCR for cloning of NEIS0612 with the N-terminal Tag (Rev)	This study
NEIS1462-Fw_c	accgtagcgcaggccGCGCCTGCTGCCGAGGCAAC	iPCR for cloning of NEIS1462 with the C-terminal Tag (Fw)	This study
NEIS1462-Rv_c	atggtgatggtgatgATCGACCAAAGTCACTTTGCCGTT	iPCR for cloning of NEIS1462 with the C-terminal Tag (Rev)	This study
NEIS0172-Fw_c	accgtagcgcaggccGACACCTTCCAAAAAATCGGCTTT	iPCR for cloning of NEIS0172 with the C-terminal Tag (Fw)	This study
NEIS0172-Rv_c	atggtgatggtgatgGCGGGCGTTCATTTCTTTAATGAC	iPCR for cloning of NEIS0172 with the C-terminal Tag (Rev)	This study
NEIS1066-Fw_c	accgtagcgcaggccGAATCCTCACGCAGTCTCG	iPCR for cloning of NEIS1066 with the C-terminal Tag (Fw)	This study
NEIS1066-Rv_c	atggtgatggtgatgACGGTTGGGTTGCCATG	iPCR for cloning of NEIS1066 with the C-terminal Tag (Rev)	This study
NEIS0596-Fw_n	gacgatgatgacaagGGCGGCAAACGCTTTGCCG	iPCR for cloning of NEIS0596 with the N-terminal Tag (Fw)	This study
NEIS0596-Rv_n	gcagccggatctcaTCCTCCTTTGCGGCGGCGG	iPCR for cloning of NEIS0596 with the N-terminal Tag (Rev)	This study
NEIS1634-Fw_c	accgtagcgcaggccAAAGGCGGAGACGCGGCGC	iPCR for cloning of NEIS1634 with the C-terminal Tag (Fw)	This study
NEIS1634-Rv_c	atggtgatggtgatgTTCCGCTTCAGAAGCAGTTTTGG	iPCR for cloning of NEIS1634 with the C-terminal Tag (Rev)	This study
NEIS0653-Fw_c	accgtagcgcaggccACTCAAGGTACGGTCGATAAAGATG	iPCR for cloning of NEIS0653 with the C-terminal Tag (Fw)	This study
NEIS0653-Rv_c	atggtgatggtgatgATGCCAGTAACGCCACCAGGGC	iPCR for cloning of NEIS0653 with the C-terminal Tag (Rev)	This study
NEIS0196-Fw_c	accgtagcgcaggccAGCGTCGAACGCGTTTCAC	iPCR for cloning of NEIS0196 with the C-terminal Tag (Fw)	This study
NEIS0196-Rv_c	atggtgatggtgatgGAATTTTATGCCGACGCG	iPCR for cloning of NEIS0196 with the C-terminal Tag (Rev)	This study
NEIS1065-Fw_c	accgtagcgcaggccAACGGGCA AAAATCCCTG TA	iPCR for cloning of NEIS1065 with the C-terminal Tag (Fw)	This study
NEIS1065-Rv_c	atggtgatggtgatgTCGCTTGCCTCCTTTAC	iPCR for cloning of NEIS1065 with the C-terminal Tag (Rev)	This study
porAkoUPF	ctgtacttccagggcGAATCTAGGACGCAGGGTTAAG	Amplification of *porA* promoter and CDS (Fw)	This study
porAkoDOR	aattaagtcgcgttaAAGTCCGGAGAGTCGTAGCGTAC	Amplification of *porA* promoter and CDS (Rev)	This study
porAkoUPR	gctgctgcccggatgCAGTCTC	vPCR for *porA* knock-out mutant (Rev)	This study
porAkoDOF	tattactagtcgacaACTTACCGCCCTCGTATTGTC	vPCR for *porA* knock-out mutant (Fw)	This study
porAkoDOR	aattaagtcgcgttaAAGTCCGGAGAGTCGTAGCGTAC	Amplification of porA promoter and CDS (Rev)	This study
CSporAF	catccgggcagcagcGACATCTTGGTTACCGTGAAG	iPCR of *tetR-sacB-specR* selection cassette (Fw)	This study
CSporAR	cggtattgcgtttcaTTCGCGGTGGCTGAGATCAG	iPCR of *tetR-sacB-specR* selection cassette (Rev)	This study

Capital letters correspond to nucleotides of the meningococcal sequence or of the vectors, whereas small letters of each primer sequence correspond to the 15 base complementary overlaps used for PIPE-cloning

Table 5.4. Plasmids used in this study

Name	Description	Antibiotic resistance	Reference
pET15-LPompA 6xHis+FLAG	pET15 derivative containing the LP of *E. coli* ompA followed by 6 Histidine residues and the FLAG tag	Ampicillin	This study
pET21-LPompA 6xHis+FLAG	pET21 derivative containing the LP of *E. coli* ompA followed by 6 Histidine residues and the FLAG tag	Ampicillin	This study
pET21-LPompA NEIS2020_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS2020 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1364_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1364 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1428_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1428 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1783_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1783 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0944_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0944 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0408_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0408 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS2198_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS2198 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1963_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1963 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1690_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1690 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1690_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1690 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0173_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0173 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0073_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0073 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1468_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1468 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0275_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0275 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0275_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0275 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1487_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1487 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1632_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1632 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0101_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0101 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0101_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0101 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1271_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1271 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1687_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1687 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0210_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0210 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0210_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0210 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS2075_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS2075 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1246_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1246 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0612_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0612 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1462_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1462 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0172_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0172 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1066_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1066 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0596_N	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0596 expression with N-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1634_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1634 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0653_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0653 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS0196_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS0196 expression with C-terminal tag	Ampicillin	This study
pET21-LPompA NEIS1065_C	pET21-LPompA 6xHis+FLAG. Costruct for NEIS1065 expression with C-terminal tag	Ampicillin	This study
pET15-Δ*porA* SpecR	pET15 with the UP and DOWN por*A* -regions for the recombination which contains a Spec resistance cassette	Ampicillin, Spectinomycin	This study
pBS-Δ*opcA* KanR	pBS with the UP and DOWN *opcA* -regions for the recombination which contains a Kan resistance cassette	Ampicillin, Kanamycin	Echenique-Rivera *et al* ., 2011
pBS-Δ*nspA* EryR	pBS with the UP and DOWN *nspA* -regions for the recombination which contains a Ery resistance cassette	Ampicillin, Erythromycin	Echenique-Rivera *et al* ., 2011

KanR, kanamycin; EryR, erytromycin; SpecR, spectinomycin; LP, leader peptide

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