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Investigation of the protective mechanism of Neisserial Heparin Binding Antigen (NHBA) induced antibodies

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1. ABSTRACT

Neisserial Heparin Binding Antigen (NHBA) is one of the three main protein antigens of the Bexsero vaccine against *Neisseria meningitidis* serogroup B (MenB). It is a surface-exposed lipoprotein ubiquitously expressed by MenB strains but sparsely distributed on the bacterial surface. NHBA binds heparin and heparan sulfates through an arginine-rich region, it is cleaved by meningococcal and human protease, and its expression is upregulated at 32°C. Recent evidences suggest that NHBA plays a key role in bacterial adherence through its arginine-rich region and is able to affect endothelial permeability. Moreover, NHBA has a direct impact on DNA-dependent biofilm formation.

NHBA induces bactericidal antibodies in humans and confers protective immunity in the infant rat animal model. Anti-NHBA antibodies (either polyclonal or monoclonal) from mice and humans are functional, being able to induce complement-mediated bacterial killing, in the presence of rabbit complement (rSBA). However bactericidal activity is not measurable when human serum is used as a source of complement (hSBA).

The aim of this study was to further elucidate the functional properties that determine the mechanism of protection of anti-NHBA antibodies.

For this purpose, the role of negative regulators of the complement system, such as factor H and vitronectin, have been investigated. The effects of antigen density have also been explored through an NHBA overexpressing strain, used to characterize a panel of anti-NHBA monoclonal antibodies isolated from Bexsero immunized adults. *In vivo* properties of these anti-NHBA antibodies, both polyclonal and monoclonal, have been evaluated through the Infant Rat meningococcal infection model.

Non-specific downregulation of complement-mediated killing due to human factor H was found to result in an underestimation of anti-NHBA antibodies functionality in hSBA. By using the NHBA overexpressing strain, the relevance of antigen density on bactericidal activity was elucidated. Our investigations also highlighted a novel and specific interaction of NHBA with human vitronectin.

Multiple interactions with complement regulators were demonstrated to interfere with the *in vitro* measurement of the bactericidal activity mediated by anti-NHBA antibodies in the presence of human complement. Interestingly NHBA, as the Neisseria Opc and NhhA, interacts with the extracellular matrix component vitronectin. These findings further support the important role played by NHBA in pathogenesis and immunity.

2. ABBREVIATIONS

- BSA = Bovine Serum Albumin
- CDC = Complement Dependent Control
- CEACAM = carcinoembryonic antigen-related cell-adhesion molecule
- CFU = Colony Forming Units
- CIC = Complement Independent Control
- CPS = Capsular Polysaccharide
- CREN = Contact Regulatory Element of Neisseria
- CSF = Cerebrospinal fluid
- ECM = Extracellular Matrix
- eDNA = extracellular DNA
- fHbp = factor H binding protein
- FMS = Fulminant Meningococcal Sepsis
- GNA = Genome-derived Neisserial Antigen
- hK1 = human Kallicrein 1
- hLf = human Lactoferrin
- hSBA = Serum Bactericidal Assay with human complement
- HSPGs = heparan sulfate proteoglycans
- humAbs = human monoclonal Antibodies
- IDP = Intrinsically Disordered Protein
- IMD = Invasive Meningococcal Disease
- IP = intraperitoneally
- KD = Dissociation Constant
- LOS = Lipooligosaccharide

- mAb = monoclonal Antibody
- MATS = Molecular Antigen Typing System
- MenB = *Neisseria meningitidis* serogroup B
- MLST = Multi Locus Sequence Type
- NadA = Neisseria adhesin A
- NHBA = Neisserial Heparin Binding Antigen
- NhhA = N*eisseria H*ia/Hsf *h*omologue
- NMR = Nuclear Magnetic Resonance
- NS = Negative Staining
- OD = Optical Density
- OMVs = Outer Membrane Vesicles
- Opa = Opacity associated protein
- ORFs = Open Reading Frames
- PE = Protein E
- PilA = Pilin A
- PKa = plasma Kallicrein
- Por A = Porin A
- Por B = Porin B
- ROS = Reactive Oxygen Species
- rSBA = rabbit Serum Bactericidal Assay
- RT = Room Temperature
- RU = Resonance Unit
- SPR = Surface Plasmon Resonance
- ST = Sequence Type
- WT = Wild Type

3. INTRODUCTION AND BACKGROUND

3.1 *Neisseria meningitidis* and Meningococcal Disease

Neisseria meningitidis, also known as meningococcus, is a Gram-negative diplococcus, member of the bacterial family of Neisseriaceae. It was identified in late 1800 by Weichselbaum from the cerebrospinal fluid (CSF) of a patient with meningitis (Weichselbaum 1887). This aerobic diplococcus has the peculiar "coffee-bean" shape (Figure 1) and can exist either as encapsulated or unencapsulated. The human **nasopharynx** is considered the natural biological niche colonized by *N. meningitidis* (Kiefer 1896). It has been estimated that up to 10-25% of the population is meningococcus-asymptomatic carrier in the nasopharynx tract (Stephens 2009).

Exposure and acquisition of meningococci are generally mediated by aerosol droplets or close direct contact with carriers (Nelson 1996). Colonization of the nasopharynx is usually asymptomatic, but very rarely in susceptible individuals it may result in penetration of the mucosal epithelium causing local inflammation. The passage into the bloodstream and survival of Neisseria, mostly mediated by the capsule, can lead to different clinical manifestations (McGee et al. 1983).

In some patients with low bacteremia degree, meningococci can be spontaneously cleared, manifesting a transient meningococcemia characterized by a short febrile flu-like episode (Sullivan and LaScolea 1987). If not completely cleared, bacteria can lead in very few hours to fulminant meningococcal sepsis (FMS) or meningitis, as already reported in 1919 by Herrick "no other infection so quickly slays" Herrick (1919).

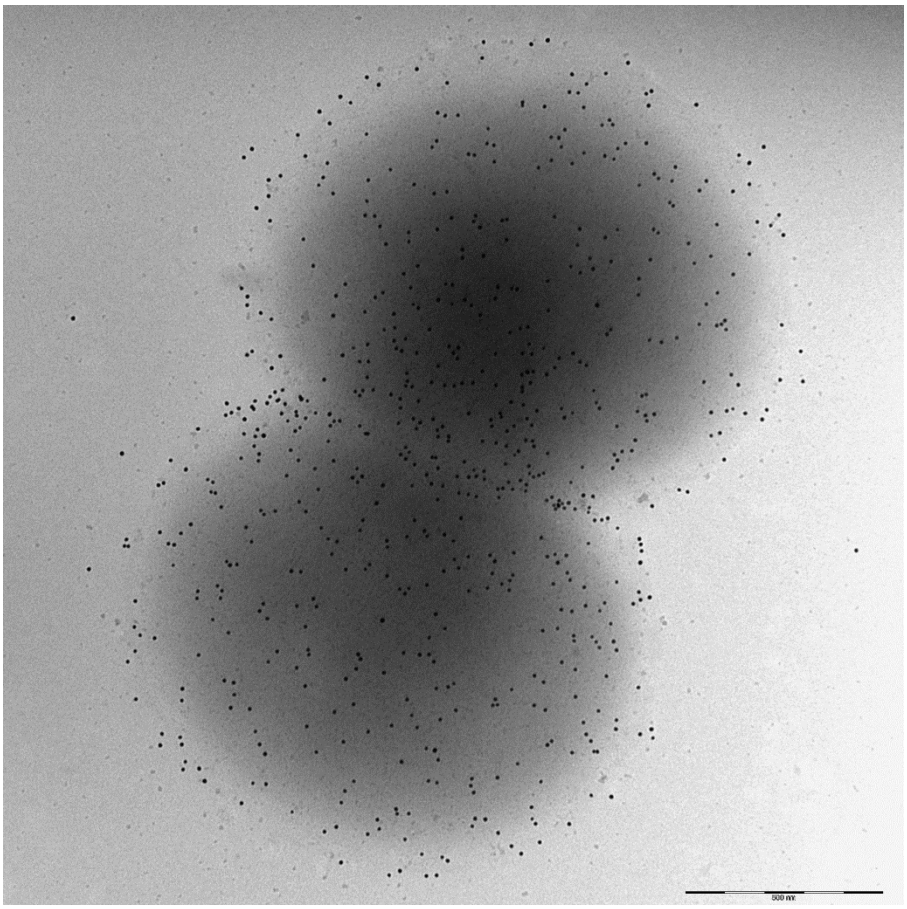


Figure 1. Immuno-gold labelling and transmission electron microscopy images of *Neisseria meningitidis* shows the typical "coffee-bean" shape of the diplococcus. Analysis of the strain was performed with anti-NHBA monoclonal antibody. Scale bar: 500nm.

Invasiveness of meningococcus can be influenced by multiple virulence factors (Figure 2):

i) **capsular polysaccharide** expression is essential for the survival of the organism in the blood, providing resistance to complement-mediated killing by antibodies and inhibiting phagocytosis (Uria et al. 2008);

ii) expression of **surface adhesive proteins** mediates meningococcus adhesion to host cells. Piliated meningococci better attach to human nasopharyngeal cells (Stephens and McGee 1981) than meningococci negative for pili expression. In

addition, pili are involved in facilitating DNA uptake by meningococci (Proft and Baker 2009).

iii) **outer membrane proteins** including porins PorA and B, adhesion molecules Opa and Opc interact with multiple members of the CEACAM (carcinoembryonic antigen-related cell-adhesion molecule) family (Virji et al. 1996) and with cell-surface associated HSPGs (heparan sulfate proteoglycans) (Virji et al. 1999);

iv) endotoxin (lipooligosaccharide, **LOS**) (Jennings et al. 1983, Gamian et al. 1992) plays a pivotal role in the adherence of the meningococcus (Kahler and Stephens 1998) and in activation of the innate immune system (Plant et al. 2006)

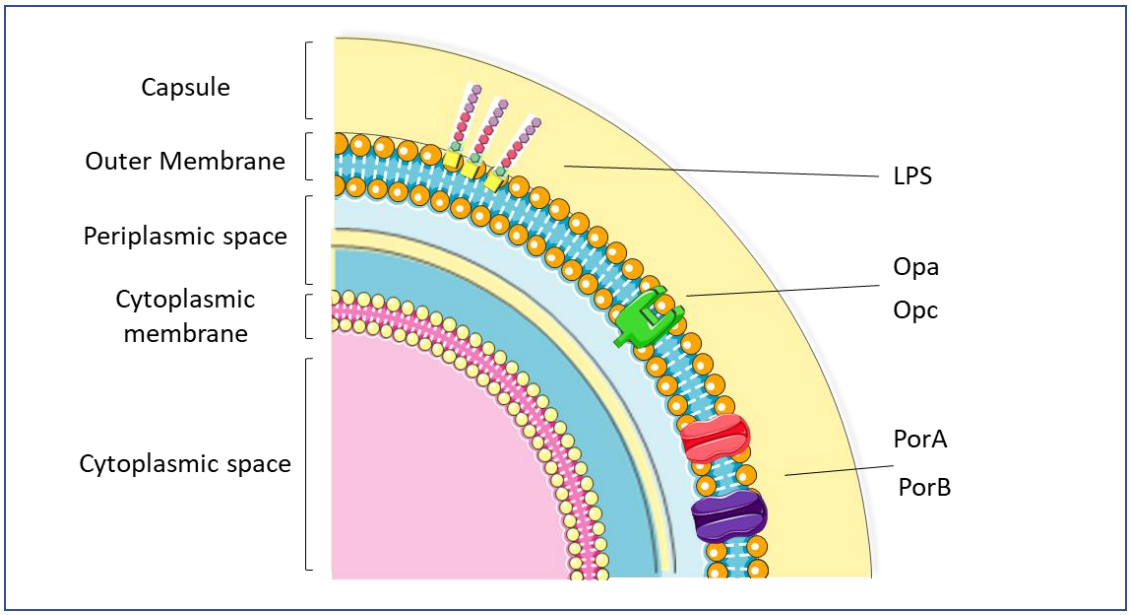


Figure 2. Schematic representation of the main neisserial virulence factors and their localization in the bacterial compartments. Image adapted from Rosenstein NE, 2001

At least 13 distinct meningococcal **serogroups** have been identified based on the capsular polysaccharide (Branham 1953) but only six are considered to cause life-threatening disease

(A, B, C, W-135, X, Y) (Rouphael and Stephens 2012, Stephens et al. 2007, Boisier et al. 2007, Frasch 1989, Jarvis and Vedros 1987).

Molecular genetic typing systems are considered the gold standard for molecular epidemiological typing and identification of clonal groups. Based on detection of polymorphisms in seven house-keeping genes, **MLST** (Multi-locus Sequence Typing) has shown that the majority of disease-associated clinical isolates group into a few sequence types (ST) enabling the definition of hyper-virulent invasive meningococcal lineages (ST-1, ST-4, ST-5, ST-8, ST-11, ST-32, ST41/44 and ST-269) (Yazdankhah et al. 2004, Maiden et al. 1998, Maiden 2008, Caugant 2008).

It is estimated that 1.2 million cases of meningococcal infection occur every year, with a death rate of ~135,000 worldwide. A few clonal complexes that can emerge and spread worldwide (Maiden et al. 1998) cause the great majority of disease cases.

The Epidemiology of meningococcal infection can be sporadic, hyper sporadic or epidemic with variable incidence patterns. Incidence can vary from 1/100,000 in Europe and North America, to 10-1,000 cases/100,000 in an area of sub-Saharian Africa (Jafri et al. 2013), renamed the “meningitis belt” by Lapeyssonnie in 1963 because it is characterized by periodic large epidemics of predominantly meningococcal meningitis (Lapeyssonnie 1963). Serogroup **A** has been associated with the largest and most severe meningococcal outbreaks in sub-Saharan Africa (Hart and Cuevas 1997) and is considered responsible for most of the meningococcal diseases in the early twentieth century but is now more uncommon in the US and Europe.

Serogroup **B** is usually associated with a lower incidence of cases if compared to serogroup A or C, but protracted MenB outbreaks can cause significant morbidity and mortality. Nowadays, MenB is the major cause of endemic meningococcal disease in developed countries, causing 30–40% of the disease in the US and up to 80% in Europe.

Although the geographic distribution is variable, **age distribution** is clear and definite: meningococcus is a common cause of bacterial meningitis in children and teenagers in the USA (Harrison et al. 1999), prevalently affecting children less than 2 years of age (Rosenstein et al. 1999, Kaplan et al. 2006).

Half of the cases in infants are due to serogroup B, while serogroup C is prevalently observed in adolescents and serogroups B and Y in older adults. Even though high incidence occurs among infants and adolescents, sporadic cases are seen in adults older than 18 years (Rouphael and Stephens 2012).

The case-fatality ratio of the meningococcal infection disease is 10% to 15%, increasing up to 40% in septicemia cases (CDC 2015). The most severe clinical forms of invasive meningococcal diseases (IMD) are meningitis (30-60%) and septicemia (20-30%), usually associated with severe manifestations as *Purpura Fulminans* (petechial or purpuric rash) (Pace and Pollard 2012).

Identification and diagnosis of meningococcal disease can be difficult due to similarities with some viral infections such as influenza, but the onset of symptoms may be fulminant. A delay in appropriate therapeutic treatment and rapid progression of the disease from bacteremia and/or meningitis to life-threatening syndrome can occur within the first few hours after the initial stages.

3.2 Meningococcal vaccines and Reverse Vaccinology

Given the rapid progression of the disease and difficulties in diagnosis (Rosenstein et al. 2001, Thompson et al. 2006), meningococcal vaccines are a fundamental need to successfully prevent the spread and control meningococcal disease. So far, no broadly protective vaccine is available to provide protection against all serogroups of *Neisseria meningitidis* although different meningococcal vaccines have been developed against the diverse serogroups. Vaccines against serogroups A, C, Y and W135 were developed in the 1960s by using purified capsular polysaccharide (CPS) as antigen. Subsequently a more effective approach was introduced, in which CPS components were conjugated to carrier proteins such as CRM197, a non-toxic mutant of the diphtheria toxin (Costantino et al. 2011).

When conjugated to a carrier protein, capsule polysaccharides show a significantly improved immunogenicity in young infants (Granoff and Pollard 2007). Formulations of monovalent, bivalent and tetravalent polysaccharide conjugative vaccines are available and effective against meningococcal serogroups A, C, Y and W-135 (Zahlanie et al. 2014) (http://www.who.int/ith/vaccines/meningococcal/en/).

Due to its similarity with human α(2->8)*N*-acetyl neuraminic acid, a polysialic acid present on the surface of human cells, group B polysaccharide capsule is poorly immunogenic and unsafe for potential induction of autoimmunity (Hayrinen et al. 1995); hence, a polysaccharidic approach could not be evaluated for vaccines against serogroups B.

First vaccine approach relied in the use of Outer membrane vesicles (OMVs) preparations.

Surface-exposed proteins contained in the OMVs were demonstrated to be immunogenic in humans in Cuba, Norway and New Zealand, with a valid vaccine efficacy against the respective homologous strain but poor protection against heterologous strains (Rosenstein et al. 2001)

To overcome these limitations, a multicomponent recombinant protein-based vaccine was needed. The *Neisseria meningitidis* serogroup B (virulent strain MC58) genome was sequenced and analysed for the identification of promising vaccine antigens (Tettelin et al. 2000). This novel approach, named "**reverse vaccinology**" led to the identification of 570 Open Reading Frames (ORFs) by bioinformatic analysis (Pizza et al. 2000).

Amplification, cloning and expression of the corresponding genes in *Escherichia coli* allowed the identification of about 600 antigens, 320 of which were used for mice immunization to evaluate their ability to induce functional antibodies (Pizza et al. 2000) (Figure 3).

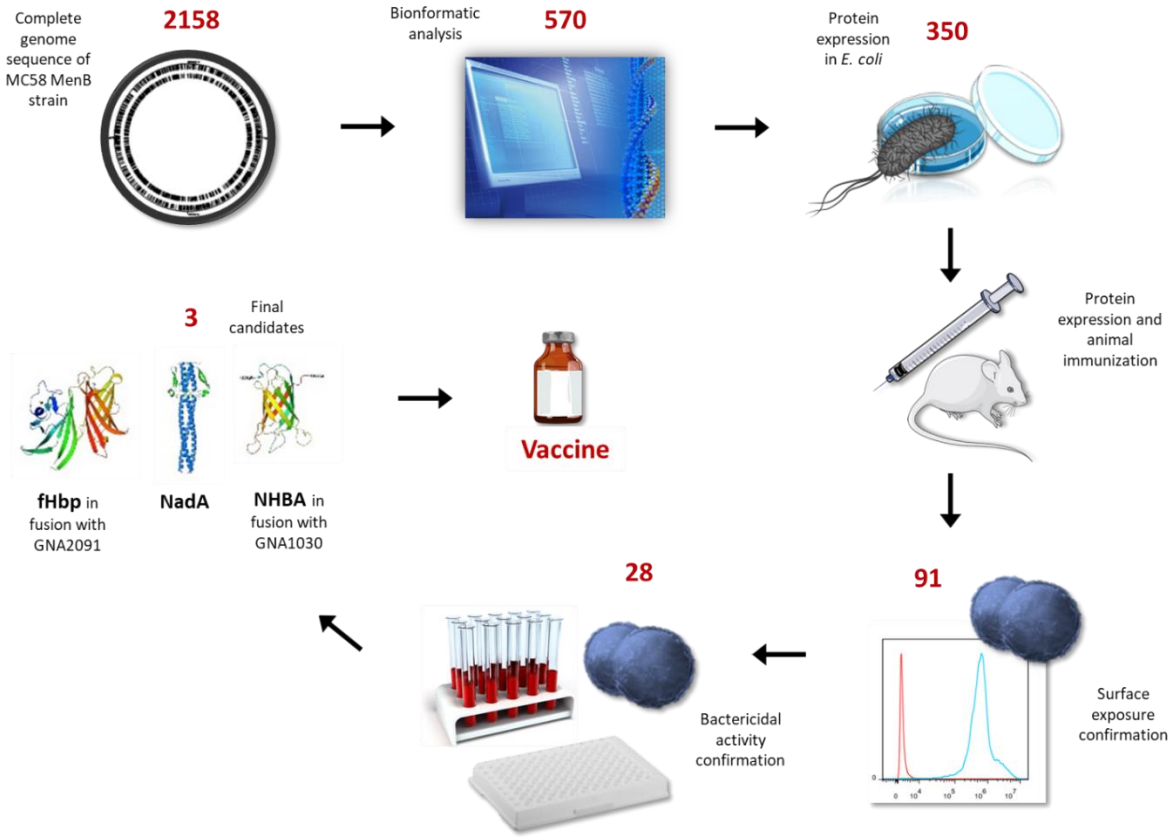


Figure 3. Schematic representation of 4CmenB vaccine development by Reverse Vaccinology

3.3 4CMenB

Among valid candidates, three protein antigens were selected for the final formulation: Genome-derived Neisseria Antigens (GNA) 2132 (Neisseria Heparin Binding Antigen, or **NHBA** as we will discuss later), GNA1870 (factor H binding protein, or **fHbp**) and GNA1994 (Neisseria adhesin A, or **NadA**). FHbp is present in variant 1.1, NHBA is present in peptidic variant p2 and NadA is present in variant 3. Two additional antigens, GNA2091 and GNA1030, were also selected for their ability to induce protective immunity. In order to facilitate large-scale manufacturing of the vaccine, four of the selected antigens were combined as two fusion proteins. The best performing combinations in terms of production and immunogenicity were NHBA fused with GNA1030 and GNA2091 with fHbp. These two fusion proteins were formulated with NadA in combination with Outer Membrane Vescicles (**OMVs**) from the New-Zealand epidemic strain (NZ98/254) (Giuliani et al. 2006) (Figure 4). This novel vaccine named 4CMenB (**4 C**omponents vaccine against **MenB**), was licensed with the trade name of Bexsero and was approved in 2013 in Europe for infants and in 2015 in the US for the age group 10-25 years. Bexsero is now approved in over 40 countries worldwide.

(https://www.ema.europa.eu/en/documents/overview/bexsero-epar-summary-public_en.pdf).

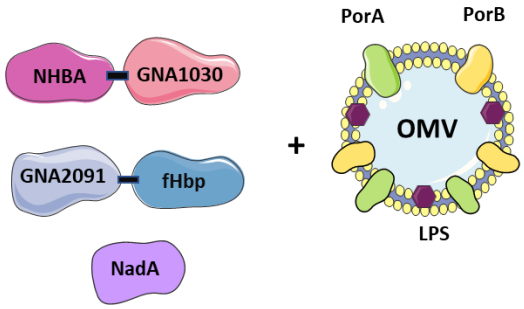


Figure 4. 4CmenB formulation includes two fusion proteins NHBA-GNA1030 and GNA2091-fHbp, NadA and OMVs from NZ98/254 strain, in which the major components are PorA and PorB.

During bioinformatic analysis of the meningococcal genome, GNA2132 was predicted to be a surface lipoprotein with some similarities with transferrin-binding proteins. The gene was ubiquitously expressed in all the MenB strains tested and in the related *N. lactamica*, *N. gonorrhoeae, N. polysaccharea, and N. flavescens* (Pizza et al. 2000, Jacobsson et al. 2006, Bambini et al. 2009) (Muzzi et al. 2013, Lucidarme et al. 2009) .

Gene sequences from genetically diverse group B strains revealed the existence of more than 400 protein sub-variants that have some association with clonal complexes and sequence types (Comanducci et al. 2002, Muzzi et al. 2013).

3.4 Neisserial Heparin Binding Antigen

Only scarce information about the function of this protein were available until 2010, when Serruto et al. reported heparin binding ability of NHBA. The protein exerts its ability to bind heparin and heparin-like molecules through an arginine-rich region (-RSARSRRS-), highly conserved among different Nm strains (Serruto et al. 2010). The ability to bind heparin is considered a virulence factor due to its correlation with increased serum resistance of the bacteria (Rostand and Esko 1997, Schneider et al. 2006, Duensing et al. 1999, Menozzi et al. 2002). On the basis of this evidence, it was suggested to rename GNA2132 as Neisserial Heparin Binding Antigen (NHBA) (Serruto et al. 2010).

NHBA is a 60 kDa protein of about 450 residues, constituted by an extremely variable N-terminal domain (residues 1-230), an arginine-rich region (residues 235–245) and a highly conserved C-terminal domain (residues 305–426) (Pizza et al. 2000).

NHBA is target of cleavage by **NalP**, a bacterial phase variable autotransporter protein with serine protease activity (Turner et al. 2002, van Ulsen et al. 2003). The cleavage site is

upstream of the Arg-rich region and the C-term fragment generated, that still contains the heparin binding site, is named **C2** (Serruto et al. 2010).

Interestingly, NHBA is also target of a human protease known for its ability to cleave the Arg-rich motif of bacterial surface proteins (Hendrixson et al. 2003, Qiu et al. 1998), human **Lactoferrin** (hLf). The cleavage mediated by hLf occurs immediately downstream of the Arg-rich region. The C-term fragment generated does not contain the heparin binding site any longer and is named **C1** (Serruto et al. 2010). Both the cleaved fragments can be released into the culture medium but the ability to bind heparin is limited to the C2 fragment, suggesting a possible protective role of the hLf, avoiding the release of a functional fragment. On the other hand, bacterial proteases such as NalP can release an active fragment and it is reasonable to consider that this fragment might interact with secreted proteoglycans in order to exert its own biological role.

Recently, it has been demonstrated that another human protease present in saliva is able to process NHBA, human **Kallikrein 1** (hK1). The fragments generated by Hk1 cleavage are identical to that generated by hLf. Interestingly, plasma Kallicrein (Pka) was also able to cleave NHBA (Pantano et al. 2019).

A schematic representation of the NHBA multiple cleavage sites is reported in Figure 5.

A study, aimed at verifying whether the two NHBA fragments, C1 and C2, were involved in the alteration of endothelial permeability, demonstrated that only the C2 fragment is able to increases microvasculature endothelial permeability, to rapidly accumulate in the mitochondria of the cells and to enhance production of oxygen radicals. On the contrary, C1 does not reach mitochondria, is unable to trigger ROS production and does not alter endothelial permeability (Casellato et al. 2014). Moreover, alteration of endothelial permeability by the toxic C2 fragment can be associated with vascular leakage, a clinical

feature mostly related to *Purpura fulminans* lesions associated with meningococcemia (Coureuil et al. 2009).

Increased expression of NHBA, as well as increased aggregation, biofilm formation and cellular adherence, have been observed at 32°C versus 37°C. In fact, 32°C is a condition more similar to the nasopharynx environment encountered during initial colonization by *N. meningitidis* (Lappann et al. 2016) compared to 37°C, as commonly used in *in vitro* experiments to investigate pathogen-host interactions. These observations suggested that the effect of the C2 fragment on epithelial cells of the naso-pharynx should be investigated. Surprisingly, a study aimed to evaluate the effect of C2 on a cell line resembling the morphological features of human respiratory tract epithelium revealed no influence of the C2 fragment on epithelium permeability, but cleavage ability of epithelial cells was detected. Incubating both the C2 fragment and full-length NHBA with Normal Human Bronchial Epithelial, a smaller fragment was found lacking the Arg-rich region motif, consequently abrogating the toxic effect of the C2 fragment. C3-convertase of the alternative complement pathway was demonstrated to be responsible for this inactivating cleavage (Di Fede et al. 2018).

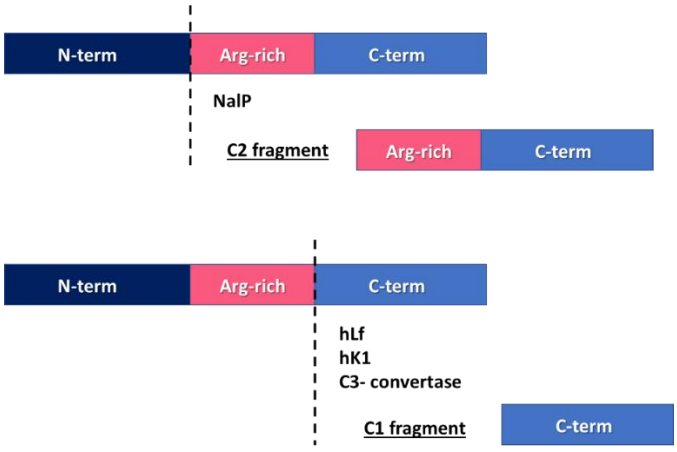


Figure 5. **NHBA processing**. NHBA can be cleaved by bacterial NalP releasing a toxic C2 fragment that still includes the Arg-rich domain. Downstream from the Arg-rich motif, NHBA can be cleaved by human Lactoferrin (hFl), human Kallikrein 1 (Hk1) and C3-convertase releasing an inactivated C1 fragment.

The C-term, initially solved by Nuclear Magnetic Resonance (NMR) spectroscopy, is constituted by a β-barrel with 8 anti-parallel strands stabilized by a hydrogen bond network that links the β-strands (Esposito et al. 2011). Interestingly, this region of NHBA shares significant homology with, factor H binding protein (fHbp), another protein included in the 4CMenB vaccine, suggesting an ancestral precursor. The common architecture comprises a C-terminal domain consisting of 8 anti-parallel β-strands. The N-term of NHBA comprises regions possibly intrinsically unfolded, rich in small hydrophilic and hydrophobic amino acids and various low complexity sequences (e.g. polyglycine and polyserine motifs) (Esposito et al. 2011). Since intrinsically disordered proteins (IDPs) are predicted to lack a stable secondary structure, crystallization of the N-term of NHBA is challenging.

Two human monoclonal antibodies (humAbs **12E1** and **10C3**) isolated from Bexsero vaccinees (Giuliani et al. 2018), targeting N-term of NHBA were used in the attempt to characterize the three-dimensional structure of NHBA. Fab fragments from these human mAbs were cloned and then used for binding, crystallization and co-crystallization studies (Maritan et al. 2017). More recently, an unnoticed NHBA fragment with 2-stranded ß-hairpin packing against the C-terminal domain was observed, with the putative role of stabilizing the barrel. Due to the Fab fragment of the humAb **5H2,** the presence of a large cross-reactive conformational epitope on NHBA was observed (Maritan et al. 2018). Currently available NHBA 3D structures are reported in Figure 6.

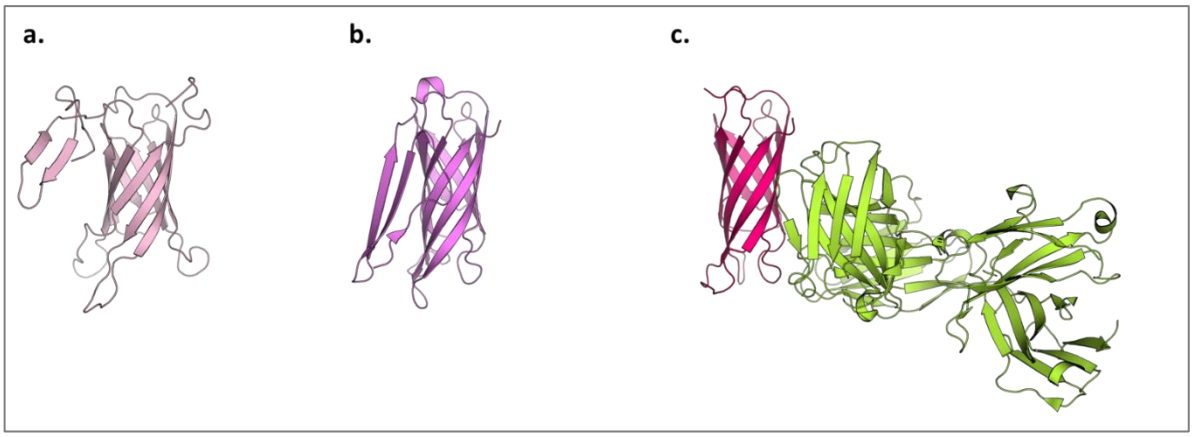


Figure 6. Currently available 3D structures of NHBA from the RCSB Protein Databank: **a.** three-dimensional structure of the C-terminal region solved by nuclear magnetic resonance (Esposito et al. 2011) (PDB code 2LFU); **b.** Crystal structure of the C-terminal domain of NHBA (Maritan et al. 2018) (PDB code 6CUJ); **c**. human Fab 5H2 bound to NHBA (Maritan et al. 2018) (PDB code 5O1R); The antibody is shown in green. The structures were prepared with PyMOL (http://www.pymol.org).

3.5 Biological functions of NHBA

Many functions have been associated with NHBA, suggesting its key role as a virulence factor in multiple steps of meningococcal colonization, infection and survival.

As mentioned above, the name Neisserial Heparin Binding Antigen was assigned to the GNA2132 after the discovery of its ability to **bind heparin** and heparan sulphates (Serruto et al. 2010). This ability is commonly associated with increased bacterial survival and serum resistance (Chen et al. 1995, Menozzi et al. 2002, Duensing et al. 1999, Rostand and Esko 1997, Dubreuil et al. 2004). It has been proposed that binding of heparin could be an efficient strategy used by pathogens to recruit several human heparin binding proteins to their surfaces, bypassing the need to synthesize individual receptors for each of these proteins (Duensing et al. 1999).

The ability of NHBA to bind heparin or heparin-like molecules present on the cell surface and in the extracellular matrix suggested that it may also be involved in bacterial **adhesion to the epithelium**. In fact, NHBA was confirmed to bind epithelial cells through its Arg-rich region via direct interaction with heparan sulphate proteoglycans (HSPGs) present on the cell surface and in the extracellular matrix (ECM) (Vacca et al. 2016).

It is noteworthy that, in some *N. meningitidis* strains belonging to a hypervirulent clonal complex, a 150-bp region known as a **Contact Regulatory Element of Neisseria** (CREN) is present upstream of the NHBA coding sequence. This regulatory element in the promoter region, specific for pathogenic Neisseria species, is involved in the induction of the downstream-associated genes when bacteria are in contact with target eukaryotic cells. It is considered essential for effective meningococcal adhesion to epithelial cells (Deghmane et al. 2003, Deghmane et al. 2002).

It has been demonstrated that NHBA plays a key role in **biofilm formation**, highly structured microbial communities (Costerton et al. 1995). Characterized by the irreversible binding to surfaces, interfaces or other bacterial cells, provides the pathogen a protective niche enhancing resistance to antimicrobial agents, antibodies and host defence molecules (Leid et al. 2005, Jensen et al. 2010). *In vitro* biofilm formation is a typical feature of unencapsulated meningococci and is mainly dependent on the presence of eDNA released from bacterial autolysis (Lappann et al. 2010). Moreover, the lack of capsule could be considered as an advantage since unencapsulated cells bind to the epithelium more efficiently than capsulated cells (Stephens et al. 1983, Hammerschmidt et al. 1996, Virji et al. 1992). Since NHBA can bind heparin and heparan sulphates, that are negatively charged molecules, its ability to bind extracellular DNA (eDNA) and take part in biofilm formation was also investigated. Indeed, it was confirmed that NHBA can bind DNA and participates in eDNA-dependent biofilm

formation. In fact, inactivation of the *nhba* gene drastically affects the initiation of this type of biofilm formation. On the contrary, it does not influence eDNA-independent initiation of biofilm formation that occurs in some meningococcal strains (Arenas et al. 2013).

As mentioned above, NHBA has been recently associated with **vascular leakage**. Endothelium permeability can be affected by the C2 fragment (the C-term fragment that still includes the Arg-rich region. Figure 5) generated by the NalP- mediated cleave of NHBA. By localizing in the mitochondria, C2 induces phosphorylation and degradation of the adherents-junction protein VE-cadherin in a ROS-dependent manner.

I**mmunological** studies of sera from mice immunized with GNA2132 have demonstrated that they bind to the surface of live *N. meningitidis* cells and elicit deposition of human C3b and iC3b. When the sera were incubated in the presence of baby rabbit complement, they showed high bactericidal activity, specific for the antigen. Moreover, anti-GNA2132 sera showed complete passive protection in the Infant Rat infection model of meningococcal bacteraemia (Welsch et al. 2003) and opsonophagocytic activity (Plested and Granoff 2008). Furthermore, anti-NHBA antibodies were able to inhibit meningococcal adhesion to epithelial cells (Vacca et al. 2016).

4. AIM OF THE STUDY

NHBA induces bactericidal antibodies in humans and confers protective immunity in the *in vivo* animal model. Anti-NHBA antibodies (polyclonal and monoclonal) from mice and humans are functional, being able to induce complement-mediated bacterial killing, in the presence of rabbit complement. However bactericidal activity is not measurable when human serum is used as a source of complement (hSBA).

The aim of this study was to elucidate further the functional properties of anti-NHBA antibodies. To this end, a number of aspects have been examined.

1. The possible effects of standard Serum Bactericidal Assay **read-out** on low-expression antigen testing, given the scarce distribution of NHBA on the bacterial surface in *in vitro* conditions.

2. To analyze the mechanism of action of these antibodies, the influence of **complement negative regulators**, such as Factor H and Vitronectin, was evaluated.

3. The role of **antigen density** in the activation of the complement cascade was considered, enabling an elucidation of the antibody-mediated killing mechanism in a high-NHBA-expression environment through the generation of an NHBA overexpressing strain.

4. The ***in vivo* model** for meningococcal infection has been used to analyze the immunological properties of polyclonal and monoclonal anti-NHBA sera.

5. MATERIALS AND METHODS

5.1 Bacterial Strains

NGH38 is a WT strain isolated in 1988 from a carrier in Norway and belongs to ST- 36. It expresses NHBA variant p2, fHbp variant 2.24. It does not express NadA and mismatches for PorA from OMVnz. It is considered the MATS-NHBA reference strain (Medini et al. 2015).

2996 is a WT invasive strain isolated in 1975 in the United Kingdom and belongs to ST-540. It expresses NHBA variant p20 and fHbp variant 2. It expresses NadA variant 3.8 and it mismatches for PorA from OMVnz.

Variants are named according to the classification reported on the Neisseria Multi Locus Sequence Type (PubMLST) database (https://pubmlst.org/neisseria/).

5.2 Generation of NGH38 NHBA-Over Expressing strain (NGH38OE)

With the aim of generating a meningococcal strain that overexpresses NHBA, a stabilized version of the PorA promoter was used to drive NHBA expression (Delany I). The plasmid for the generation of the mutant consisted of a 459 bp region (NMB2133) upstream of *nhba* amplified with KAPA Hi-Fi polymerase (KAPA Biosystems, Sigma), using primers AR_nmb2133_mc58_iPCR_Fwd/AR_nmb2133_mc58_ iPCR_Rev from a template vector containing the NEIS2110 gene (Serruto et al. 2010)), the P_{PorA}^{st2} promoter fused to the intergenic region upstream of *nhba*, which included also the Contact Regulatory Element of *Neisseria* (CREN) (Deghmane et al. 2003), and part of the NHBA coding sequence. These regions were subcloned in a different plasmid (Redsted et al., paper under submission) and amplified with KAPA Hi-Fi polymerase (KAPA Biosystems, Sigma) using primers

AR_constructs_vPCR_Fwd/AR_constructs_vPCR_Rev (see Table 7). The two PCR products were mixed and cloned with the PIPE method (Klock and Lesley 2009). The XmaI-linearized vector was then used to transform the NGH38 strain, according to a previously reported procedure (Masignani et al. 2003), taking advantage of the NEIS2110 and N-terminus *nhba* regions as homologous recombination sites and selecting the mutated clone using kanamycin.

_nmb2133_mc58_IPCR_Fwd	gaaccgctggcgcgttaaggcgtttatgccttctttacccgttc
_nmb2133_mc58_ IPCR_Rev	tttagatgtctaaaaagcttattccgcccggttcctctgtcg
_constructs_vPCR_Fwd	ggaaccgggcggaataagcttttagacatctaaatctaggtactaaaacaattcatc
_constructs_vPCR_Rev	aaagaaggcataaacgccttaacgcgccagcggttcggtg

Table 7. Primers used for NGH38 OE strain generation

5.3 Generation of luciferase reporter strains

With the aim of generating *N. meningitidis* mutant lux reporter strains, a series of plasmids containing the bacterial luciferase expression under the control of the promoters under investigation was generated. Bacterial luciferase transcriptional fusions of the promoter under study at a chromosomal location between the two converging ORFs NMB1074 and NMB1075, flanked on both sides with transcriptional terminators, plasmid pSL-LuxFla was constructed for allelic exchange in *N. meningitidis* 2996 strains. Following a previously reported procedure (Fagnocchi et al. 2013), the promoterless *luxCDABE* operon and *cat* cassette were subcloned from pSB1075 into pBluescript II as an EcoRI-BamHI fragment and then cloned as a 6.5-kb XhoI-BamHI fragment into pSL-*furlacZ*, replacing a 4.7 kb fragment containing an erythromycin cassette and the *fur-lacZ* fusion, generating pSL-LuxFla. Either the *nhba* or the *nadA* promoters were cloned as a 250-bp Xho-KpnI fragment upstream of the *luxCDABE* operon, generating the pSLPnhba-lux and the pSLPnadA-lux, respectively. The pSL-LuxFla, pSLPnhba-lux and pSLPnadA-lux plasmids were used for transformation of the 2996 strain, generating the

isogenic recombinant mutants 2996-*lux*, 2996-P*nhba-lux* and 2996-P*nadA-lux*, respectively, for the *in vivo* reporter analysis.

5.4 Human monoclonal Antibodies (HumAbs)

Anti-NHBA human monoclonal Antibodies (humAbs) were selected from a panel of recently isolated humAbs (Giuliani et al. 2018). Peripheral Blood Mononuclear Cells (PBMCs) from three adult subjects immunized with the 4CMenB vaccine were collected 8 days after the second dose of vaccine. Plasma blasts were isolated as single cells and used to obtain monoclonal antibodies (mAbs) as previously described by Beernink and co-workers (Beernink et al. 2015).

5.5 Hexabody generation

The VH regions of 3 selected anti-NHBA mAbs were cloned in a vector containing an IgG1 constant region carrying 2 point-mutations, namely E345R and S440Y. Transfection were performed in Expi 293 expression system following manufacturer instructions (de Jong et al. 2016).

5.6 Animal Polyclonal sera

To prepare antisera, 20 µg of NadA, NHBA-GNA1030, or GNA2091-fHbp antigen or a combination of 20 µg each of NHBA-GNA1030, GNA2091-fHbp, and NadA with or without 10 µg of deoxycholate-extracted OMVs derived from the NZ98/254 strain were used to immunize 6-week-old CD1 female mice (Charles River Laboratories International, Inc, Wilmington, MA,

USA). Five to ten mice per group were used. The antigens were administered intraperitoneally (i.p.), together with aluminum hydroxide (3 mg/ml), on days 0, 21 and 35.

5.7 Sources of complement

Pooled baby rabbit sera (Cedarlane, Burlington, Canada) or human serum obtained from volunteer donors under informed consent, were used as a complement source for rSBA or hSBA respectively.

5.8 Serum Bactericidal Assay

The Serum Bactericidal assay (SBA) was performed in 96-well plates (Thermo Scientific, Waltham, MA, USA). From frozen glycerol stocks, bacteria were seeded and grown overnight on chocolate agar plates at 37°C in 5% CO_2. The day after 10-15 colonies were inoculated in Müller-Hinton broth containing 0.25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C with shaking until the OD_{600} reached 0.25. Bacteria were diluted 10,000-fold in Dulbecco's phosphate buffered saline (DPBS), 1% (w/v) Bovine Serum Albumin (BSA), 0.1% glucose (w/v) and added to a reaction mix with a serial two-fold dilution of serum and complement. The plate was incubated for 1 hour at 37°C on a shaker; 7 µl of mix were spotted on Müller-Hinton agar plates that were incubated overnight at 37°C, and the Colony Forming Units (CFU) were counted the day after. Bactericidal titers were defined as the serum dilution resulting in 50% decrease in CFU compared to the negative controls, the Complement Dependents Control (CDC) and the Complement Independent Control (CIC). Figure 8 shows a schematic representation of the Serum Bactericidal Assay plate layout.

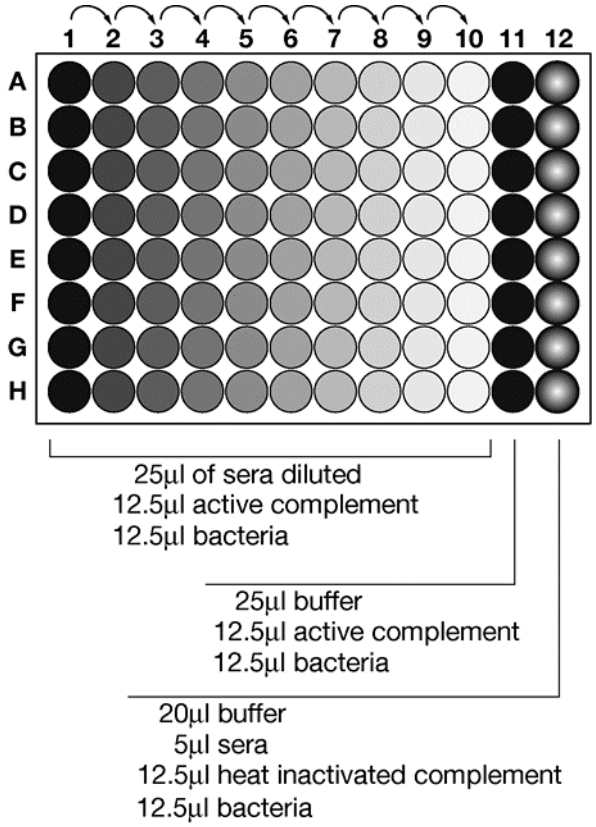


Figure 8. Schematic representation of the Serum Bactericidal Assay plate layout.

5.9 Flow cytometry for protein surface exposure detection

From frozen glycerol stocks, bacteria were seeded and grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Müller-Hinton broth containing 0.25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C 5% CO_2 with shaking until an OD_{600} of 0.25 or 0.5 was obtained. Bacterial cells were centrifuged at 3500 rpm for 10 min. The supernatant was discarded, and the pellet suspended in 5ml PBS 1% BSA (w/v).

The sample were sequentially incubated with mouse primary antibody (1:100, 1:200 and 1:400) for 1 hour at room temperature (RT). Murine monoclonal Antibody against *Neisseria meningitidis* serogroup B capsule (Remel Microbiology Product, Thermo Scientific, Waltham, MA, USA) was used as positive control. Bacterial cells were centrifuged at 3500 rpm for 10 min.

The binding was detected by using either anti-mouse or anti-human (whole-molecule) FITC-conjugated antibodies (Sigma-Aldrich, Saint Louis, Missouri, USA) at a 1:100 dilution in PBS 1% BSA (w/v) with 30-minutes incubation at RT. After the secondary antibody incubation step bacteria were fixed with formaldehyde and plated to check bacterial inactivation. The samples were analyzed with a Canto II cell counter (FACSCanto II, BD Biosciences, San Jose, CA, USA) and histograms generated using Flowjo 8.60 software (BD Biosciences, San Jose, CA, USA).

5.10 Human C3 deposition assay

From frozen glycerol stocks, bacteria were seeded and grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Müller-Hinton broth containing 0.25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C 5% CO_2 with shaking until an OD_{600} of 0.25 or 0.5 was obtained. Bacterial cells were centrifuged at 3500rpm for 10 min. The supernatant was discarded, and the pellet suspended in PBS 1% BSA (w/v).

50 µl of bacterial suspension was incubated with 25 µl of human complement were added resulting in a concentration of 10 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 5 U/ml heparin: i.e 10 µl of salt and 10 µl of heparin in 1 ml of plasma) for 60 minutes at 37˚C with 5% CO_2 with gentle shaking. Bacterial cells were centrifuged 3500 rpm for 10 min, the supernatant was discarded, the pellet was washed in PBS and sequentially incubated with anti-Human C3_FITC antibody (1:100) (Cederlane, Burlington, Canada) 30 minutes at room temperature (RT). After incubation the bacteria were fixed with formaldehyde and plated to check bacterial inactivation. The samples were analyzed with a Canto II cell counter (FACS Canto II, BD Biosciences, San Jose, CA, USA) and histograms were generated using Flowjo 8.60 software (BD Biosciences, San Jose, CA, USA).

5.11 Human C9 deposition assay

From frozen glycerol stocks, bacteria were seeded and grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Müller-Hinton broth containing 0.25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C 5% CO_2 with shaking until OD_{600} of 0.25 or 0.5 was obtained. Bacterial cells were centrifuged at 3500 rpm for 10 min, the supernatant was discarded, and the pellet was suspended in PBS 1% BSA (w/v).

50 µl of bacterial suspension was incubated with 25 µl of human complement were added resulting in a concentration of 10 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 5U/ml Heparin: i.e 10ul of salt and 10 µl of heparin in 1 ml of plasma) for 60 minutes at 37˚C with 5% CO_2 with gentle shaking. Bacterial cells were centrifuged at 3500 rpm for 10 min, the supernatant was discarded, the pellet was washed in PBS and sequentially incubated with mouse anti-Human C9 (1:200) (Thermo Scientific, Waltham, MA, USA) 30 minutes at room temperature (RT).

The binding was detected using anti-mouse (whole-molecule) FITC-conjugated antibodies (Sigma-Aldrich, Saint Louis, Missouri, USA) at a 1:100 dilution in PBS 1% BSA (w/v) with 30-minutes incubation at RT. After the secondary antibody incubation bacteria were washed in PBS, fixed with formaldehyde and plated to check bacterial inactivation. Samples were analyzed with a Canto II cell counter (FACS Canto II, BD Biosciences, San Jose, CA, USA) and histograms generated using Flowjo 8.60 software (BD Biosciences, San Jose, CA, USA).

5.12 Factor H deposition assay

From frozen glycerol stocks, bacteria were seeded and grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Müller-Hinton broth containing 0,25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C 5% CO_2 with shaking until OD_{600} of 0.25 or 0.5. Bacterial cells were centrifuged 3500 rpm for 10 min, the supernatant was discarded, and the pellet was suspended in PBS 1% BSA (w/v).

50 µl of bacterial suspension was incubated with 25 µl of human complement or with human factor H (50 µg/ml) (Calbiochem, San Diego, CA, USA).

Bacterial cells were centrifuged at 3500rpm for 10 min, the supernatant was discarded, the pellet was washed in PBS and sequentially incubated with goat anti-human fH (1:100) (Calbiochem, San Diego, CA, USA) for 30 minutes at RT. The binding was detected using a donkey anti-goat FITC-conjugated antibody (Jackson Immuno Research Europe Ltd, Cambridge House, St. Thomas' Place, UK) at a 1:100 dilution in PBS 1% BSA (w/v) with 30-minutes incubation at RT. After the secondary antibody incubation bacteria were washed in PBS, fixed with formaldehyde and plated to check bacterial inactivation. The samples were analyzed with a Canto II cell counter (FACS Canto II, BD Biosciences, San Jose, CA, USA) and histograms generated using Flowjo 8.60 software (BD Biosciences, San Jose, CA, USA).

5.13 Confocal Microscopy

From frozen glycerol stocks, bacteria were seeded and grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Müller-Hinton broth containing 0.25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C 5% CO_2 with shaking until OD_{600} of 0.25 or 0.5. 1 ml of bacterial growth at OD_{600} of 0.25 or 0.5 was incubated with FM4-64FX (Thermo Fisher Scientific Inc., Waltham, MA USA) and then fixed with 4% Formaldehyde (Carlo Erba Reagents S.r.l., Milano, Italy). 150 µl were used for each staining condition and spotted on a glass slide (Polysine Corning® microscope slides, Sigma-Aldrich, Saint Louis, Missouri, USA). Staining was performed on the slide with 100 µl of mouse polyclonal antibodies diluted 1:250 and incubated for 1 h at RT. After several wash steps, the slides were incubated in 100 µl of rabbit anti-mouse antibody Alexa fluor 488 conjugated (Life technologies, Carlsbad, California, USA), diluted 1:1000 for 30 minutes at RT. The samples were dried under vacuum, mounted using ProLong gold antifade reagent with diamidino-2-

phenylindole (DAPI; Invitrogen-Thermo Fisher Scientific Inc., Waltham, MA USA) and analyzed by confocal microscopy using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany).

5.14 Transmission Electron Microscopy

From frozen glycerol stocks, bacteria were seeded and grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Müller-Hinton broth containing 0.25% glucose to reach OD_{600} of 0.05 to 0.06 and incubated at 37°C 5% CO_2 with shaking until OD_{600} of 0.25 or 0.5.

For **Negative Staining** (NS) a 5 µl aliquot of a formaldehyde (Carlo Erba Reagents S.r.l., Milano, Italy) fixed bacterial culture with a final concentration of 1 OD/ml was loaded for 30 seconds onto a glow discharged copper 300-square mesh grid. After blotting the excess, the grid was negatively stained using NanoW (Nanoprobes, Yaphank, NY, USA) for 30 seconds. The samples were analyzed using a Tecnai G2 spirit and TEM FEI Tecnai G2 spirit microscope operating at 100kV and equipped with an 2k × 2k CCD Emsis Veleta camera (Emsis, Germany). The images were acquired using a Veleta CCD camera.

For **Immunogold** (Ig) a 5 µl aliquot of a formaldehyde (Carlo Erba Reagents S.r.l., Milano, Italy) fixed bacterial culture with a final concentration of 1 OD/ml was adsorbed on a 300-mesh formvar/carbon coated nickel grid (Agar Scientific Ltd, United Kingdom), blocked in PBS with 0.5% bovine serum albumin for 1 h and incubated with primary antibody (diluted 1:50 in in PBS with 0.5% bovine serum albumin) for 1 h. Grids were washed and incubated with 5- or 10-nm gold-labeled secondary antibody (Sigma-Aldrich, Inc. Merck, Germany) (diluted 1:20 in PBS with 0.5% bovine serum albumin) for 1 h. After washing with distilled water, the grids were negatively stained and observed using a TEM FEI Tecnai G2 spirit microscope operating at

100kV and equipped with an 2k × 2k CCD Emsis Veleta camera (Emsis, Germany). The images were acquired and processed using iTem (OSIS, Olympus, Shinjuku, Tokyo, Japan) software.

5.15 Enzyme-Linked Immunosorbent Assay

Vitronectin-coated 96 well plates (R&D Systems, Inc. Minneapolis, USA) were used for a direct binding assay. NHBA, NHBA-GNA1030 and GNA1030 serial two-fold dilutions were incubated for 2 h at 37°C. After 3 washes (PBS + Tween 0.05%), anti-NHBA-GNA1030, anti-NHBA and anti-GNA1030 (diluted 1:200 in PBS + Tween 0.05% + BSA 1%) mouse sera (100 μl/well) were incubated for 1 h at 37°C. After 3 washes a goat anti-mouse IgG-ALP conjugated secondary antibody (Sigma Aldrich. Inc. Merck, Germany) was added (100 μl/well) and incubated 1 h at 37°C. The signal was detected using a PNPP substrate (50 μl/well) after incubating for 30 min at room temperature and reading the plate at 405nm on a Spectramax reader (Molecular Devices, California, USA).

5.16 Surface Plasmon Resonance

For Surface plasmon resonance (SPR) analysis human Vitronectin (Life Technologies, Carlsbad, CA, USA) was immobilized on the surface of a CM5 sensor chip (see Figure 9 for the chip characteristics) using an amine coupling procedure. The proteins were immobilized in the flow cells 2 while flow cells 1 were used as reference.

Chip: CM5
Flow cells per cycle: 1

Flow cell	Procedure	Method	Ligand	Response Bound (RU)	Response Final (RU)	Target Reached
1	Blank	Amine			175.6	N/A
2	Target level	Amine	Vitronectin 20 ug/mL pH4.5	1621.4	1622.7	No - Response level decrease

Figure 9. Properties of the CM5 sensor chip

The recombinant NHBA-GNA1030, NHBA p2 and GNA1030 proteins were diluted in HBS-EP+ buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005 % P20 surfactant, pH 7.4) to reach a final concentration of 300 nM and injected onto the chip. Finally, the chip was regenerated by injection of 3 M Magnesium chloride.

The final read out of the analysis is the sensorgram, a plot of response (measured in *resonance units* [RU]) against time, that shows the progress of the interaction. The response is directly proportional to the concentration of biomolecules on the surface.

To **assess the affinity** of the binding, maintaining the same chip set up, five increasing concentrations (6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM) of each protein were injected for 60 seconds each on the surface of the CM5 sensor chip. After the last injection, dissociation of the protein was followed for 120 seconds. After each analysis cycle, the chip was regenerated injecting 3 M Magnesium chloride. This analysis enabled the kinetic binding parameters to be measured: the ligand-analyte association (Kon) (described by the ascendant tract of the curve, 60 seconds, 30µl/min), the ligand-analyte dissociation (Koff) (described by the descendant tract of the curve during buffer injection into the system, 240-300 seconds of injection time) and the equilibrium dissociation constant (Kd) (the lower the Kd value, the higher is the binding affinity of the ligand for the analyte).

5.17 *In vivo* infection model

The ability of anti-NHBA antibodies to confer passive protection against *N. meningitidis* bacteremia was tested using the Infant Rat infection model, intraperitoneal (i.p.) challenging 5-7 days Wistar Han rat pups (Charles River Laboratories International, Inc, Wilmington, MA, USA) as previously described (Moe et al. 1999).

Bacteria from frozen glycerol stocks were seeded and grown overnight on chocolate agar plates at 37°C in 5% CO_2. 10-15 colonies were inoculated in Müller-Hinton broth containing 0.25% glucose to reach OD_{600} 0.05-0.06 and incubated at 37°C 5% CO_2 with shaking until OD_{600} 0.25 was reached.

For passive protection experiments, animals were treated i.p. at time zero with 100 µl of different dilutions of test or control antisera. Three hours later, the same animals were i.p. challenged with 10^4 or 10^6 CFU dose of *N. meningitidis* strains, in a final volume of 100 µl. Eighteen hours after the bacterial challenge, blood samples were obtained by cheek puncture and collected in Eppendorf tubes containing 25 U of heparin (Eparina Vister, Teva Italia S.r.l., Milano). 100 µl of collected blood were plated on Agar Columbia (5% blood) plates and incubated O/N at 37°C in 5% CO_2 and the day after the CFUs were counted. Rats were considered infected when >10 CFUs were counted. Counts above the threshold were verified for positivity by examining plates carrying 10- and 100-fold dilutions of blood. Workflow of the experimental protocol is schematically represented in Figure 10.

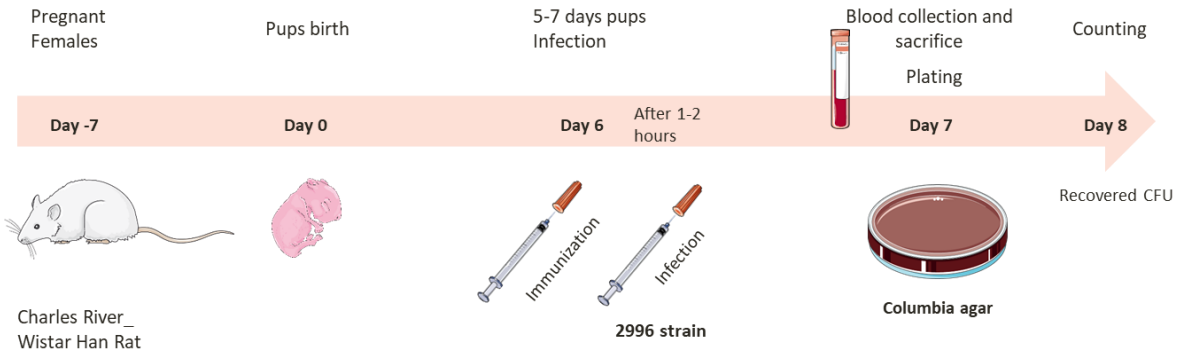


Figure 10. Workflow of the Infant Rat challenge protocol

In vivo imaging of the bioluminescence of the strains was monitored in infant rats i.p. infected. Bioluminescence measurements of ventral views of each group of rats were taken at time

zero, 3 h and 20 h post-challenge, using an IVIS 100 system (Xenogen Corp., Alameda, CA, USA) according to the manufacturer's instructions. Analysis was performed using Living Image 3.1 software (Xenogen Corp., Alameda, CA, USA). Quantification was performed using the photons emitted per second by each rat. Rats infected with the 2996 wild-type strain under the same conditions of acquisition were used for background signal subtraction. Blood samples were collected at 20 h post infection and plated to allow CFU counting, as for standard Infant Rat challenge.

6. RESULTS

6.1. Serum Bactericidal assay read out

The Serum Bactericidal Assay (SBA) is considered the gold standard for correlate of protection of meningococcal vaccines (Frasch et al. 2009). This assay, commonly used to investigate the ability of anti-meningococcal antibodies to elicit complement deposition and bacterial killing, has a classical read out of 7 µl reaction drop on Mueller-Hinton Agar. The colony count is performed the next day, after an O/N incubation at 37 °C 5% CO_2. In this experiment the same reaction (Rabbit Anti-NHBA-GNA1030 serum against the Nm strain NGH38 WT, at OD_{600} = 0.25, in the presence of the same Human complement) was used to evaluate the effect of changing the bacterial dilution (1:10000 vs 1:15000), the medium (MHA vs Agar Chocolate) or the plating mode (7 µl reaction drop spot vs whole reaction volume using spread-plate method of isolation) (Figure 11).

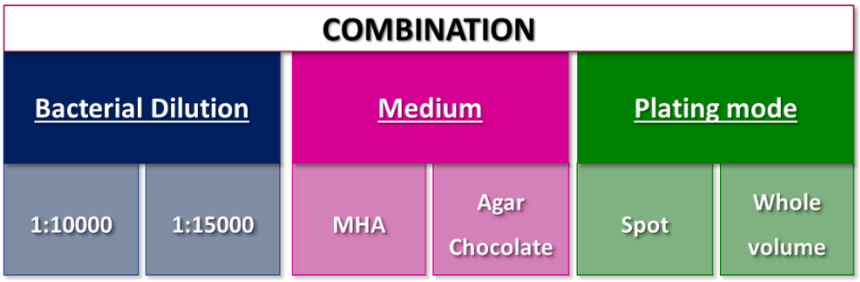


Figure 11. In the image are reported the variables combined to test SBA read out variability: bacterial dilution (1:10000 or 1:15000); medium (Mueller Hinton Agar or Agar Chocolate); plate condition (7 µl spot or whole plating);

High variability of hSBA results was observed when the bacterial dilution, the plating mode or the medium ere changed. The best combination to achieve maximum killing was showed to be 1:10000 bacterial dilution, on Agar Chocolate and plating the whole volume using spread-

plate method of isolation. By combining these conditions, a rabbit anti-NHBA-GNA1030 serum tested against the Nm strain NGH38 WT showed the bactericidal titer of 32 in the presence of human complement, compared to the negative titer obtained with the standard read out (Figure 12).

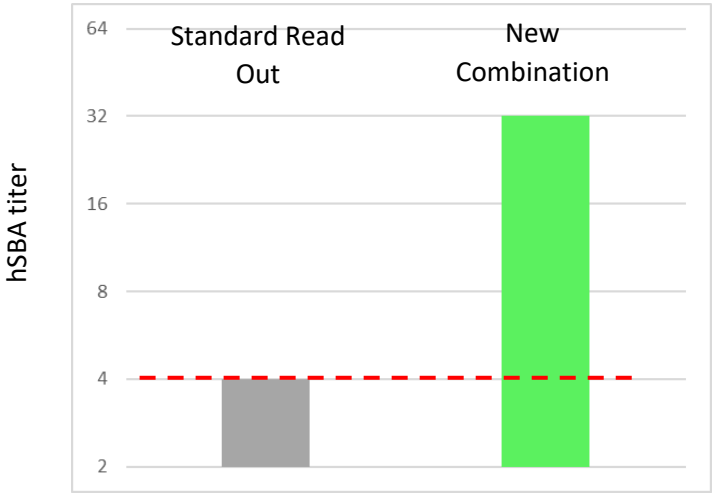


Figure 12. The graph reports the hSBA titers obtained with standard read out (1:10000 bacterial dilution, 7 µl spot on MHA) and with the new combination (1:10000 bacterial dilution, whole volume with spread-plate method, on Agar Chocolate).

6.2. Factor H interaction

The factor H negative regulation of the Alternative Pathway was investigated to evaluate the impact of this down-regulation on the bactericidal activity of anti-NHBA-GNA1030 antibodies in the serum bactericidal assay. Factor H was sequestered from the reaction by the addition of fHbp at scalar concentrations. At a high sequestrator concentration, the amount of factor H available in the serum was dramatically decreased and no down regulation of the Alternative Pathway took place. As a direct effect, the anti-NHBA-GNA1030 antibodies showed strong

bactericidal activity in the presence of human complement, directly proportional to the concentration of fHbp added to the reaction (Figure 13).

To demonstrate that removal of factor H did not make the bacteria susceptible to killing in absence of Ab, anti-NHBA-GNA1030 antibodies were tested against NHBA Knock-Out strain, and no killing was still observed in the presence of different fHbp amounts.

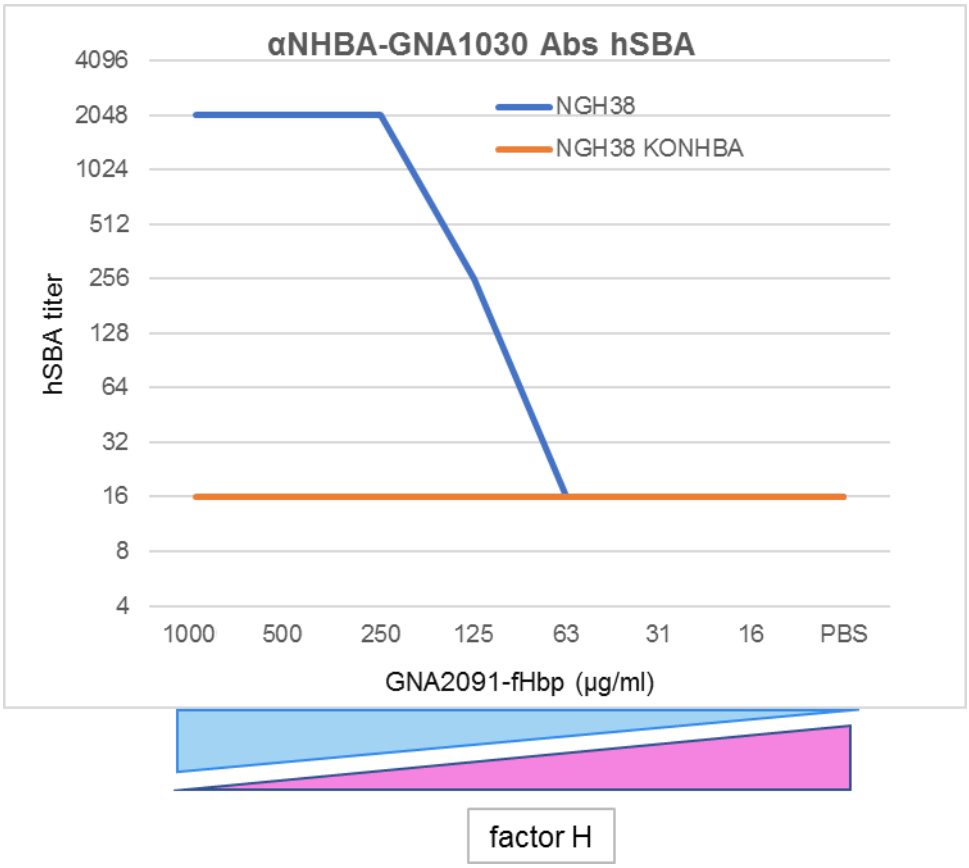


Figure 13. hSBA titers obtained by testing the anti-NHBA-GNA1030 serum against NGH38 WT (blue) and NGH39KONHBA (orange) in presence of scalar concentrations of GNA2091-fHbp added to the reaction.

On the contrary, when human Factor H was added to rabbit SBA, a decrease of the titers was observed, mimicking the results obtained for hSBA. This effect was observed non-specifically testing anti-NadA serum, anti-NHBA serum or anti-fHbp serum, but the impact was more

evident in the case of a poorly expressed antigen on the bacterial surface, as for NHBA. In fact, for fHbp and NadA, which are highly expressed on the surface, the titers moved from very high to high (65536 to 16384) and from high to medium (4096 to 256), respectively. For anti-NHBA Abs, the titer decreased from medium to negative (1024 to <16) (Table 14). This underlines the significant impact of unspecific down regulation of factor H in the *in vitro* testing of anti-meningococcal antibodies, especially if they are raised against poorly expressed antigens.

Strain	Mouse anti serum	rSBA + fH added (µg/ml)		hSBA
		--	40	
BZ83	NadA	4096	256	256
BZ83	NHBA-GNA1030	1024	<16	<16
BZ83	GNA2091-fHbp	65536	16384	8192

Table 14. The table reports the rSBA and hSBA titers obtained testing anti-NadA, anti-NHBA-GNA1030 and anti-GNA2091-fHbp sera against the BZ83 strain. 40 µl of Human factor H was added to rSBA.

6.3. Vitronectin interaction

To investigate whether Vitronectin and NHBA interact, an ELISA-based assay was performed. Serial two-fold dilutions of NHBA-GNA1030, NHBA or GNA1030 were alternatively incubated on Vitronectin-coated 96-well commercial ELISA plates. Anti-NHBA-GNA1030, anti-NHBA and anti GNA1030 sera were used for primary incubation and the signal was detected through ALP-conjugated secondary antibodies (Figure 15).

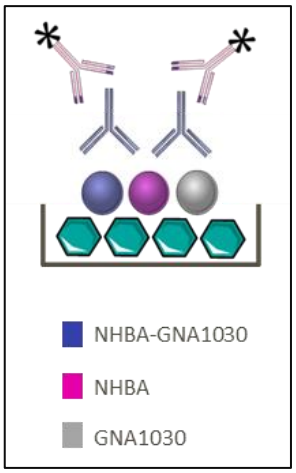
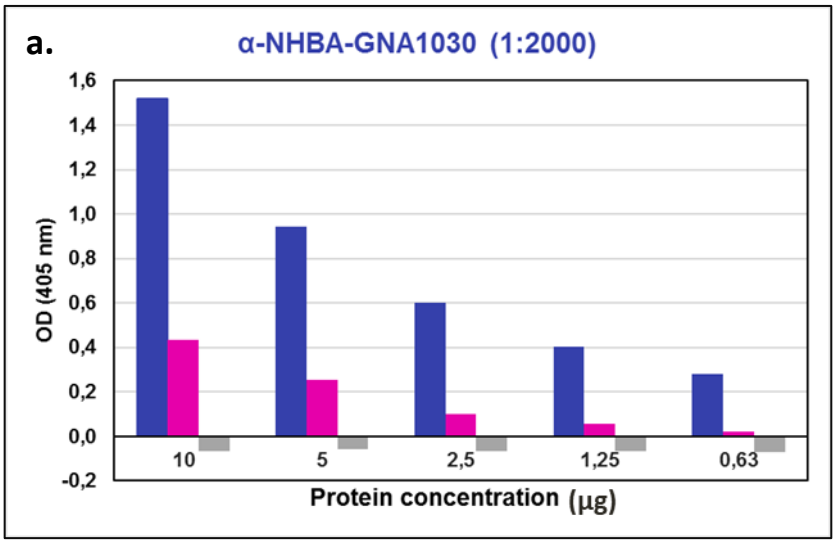
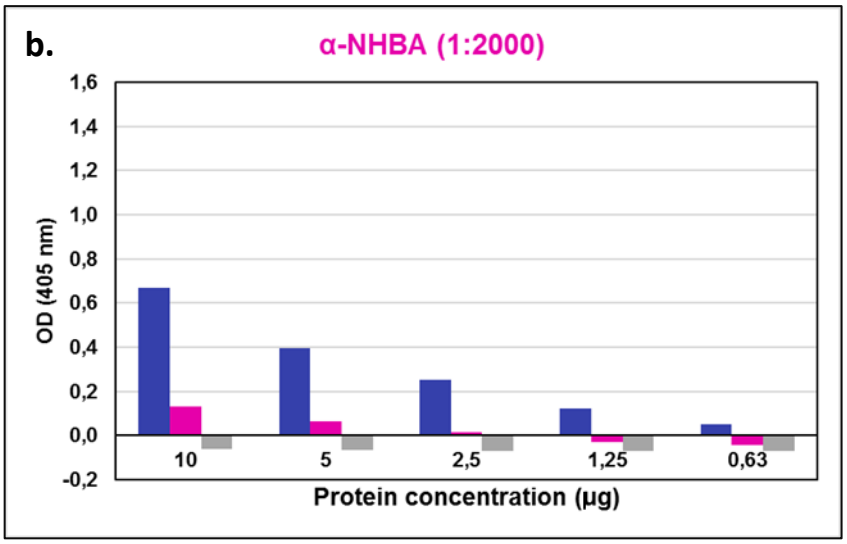


Figure 15. Schematic representation of the ELISA-based assay. Vitronectin coated on the plate in light blue; NHBA-GNA1030 in blue; NHBA in purple; GNA1030 in grey; Primary antibodies are shown in grey; the asterisk represents an ALP-conjugated secondary antibody.

The results showed a specific and dose dependent NHBA-vitronectin binding: anti-GNA1030 serum gave negative results; a low signal was detected with a serum directed to NHBA alone, and the highest signal was observed when a serum to NHBA-GNA1030 was incubated (Figure 16).





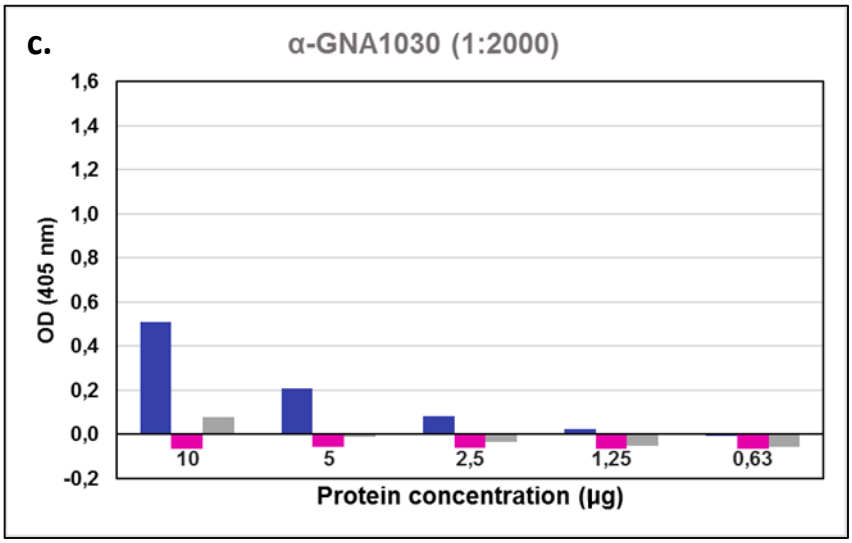


Figure 16. Signals detected at OD_{405nm} after incubation of NHBA-GNA1030 (blue), NHBA (purple) and GNA1030 (grey) scalar concentrations on Vitronectin coated plates, in presence of different antisera. **a.** anti-NHBA-GNA1030 serum diluted at 1:2000; **b**. anti-NHBA serum diluted at 1:2000; **c.** anti-GNA1030 serum diluted at 1:2000.

To characterize protein–protein interactions, Surface Plasmon Resonance analysis was performed with NHBA and vitronectin. This technique, measuring real-time quantitative binding affinities and kinetics, confirmed the NHBA-vitronectin binding previously observed in ELISA-based assay. Human Vitronectin was immobilized on the surface of the CM5 sensor chip

(Figure 8) using an amine coupling procedure. The positive control PE (light blue) with an RU of 75.85 and the negative control PilA (yellow) with RU of 0.65 are shown, a low binding between NHBA and Vitronectin was detected with an RU of 12.25 (Figure 17 and Table 18).

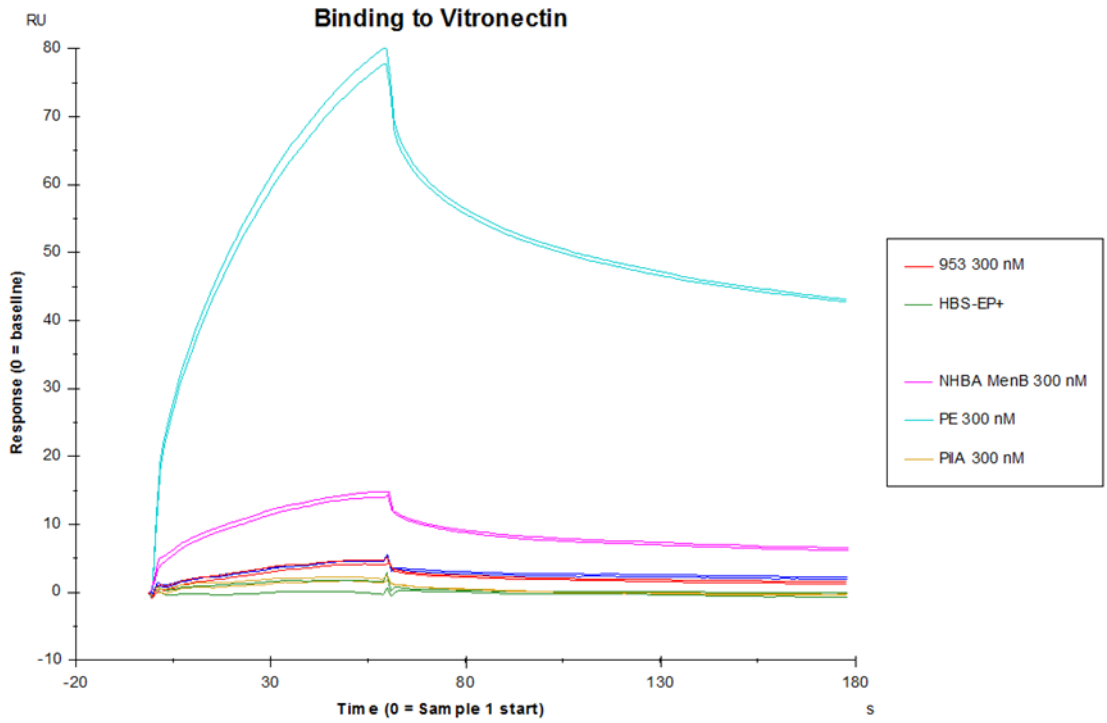


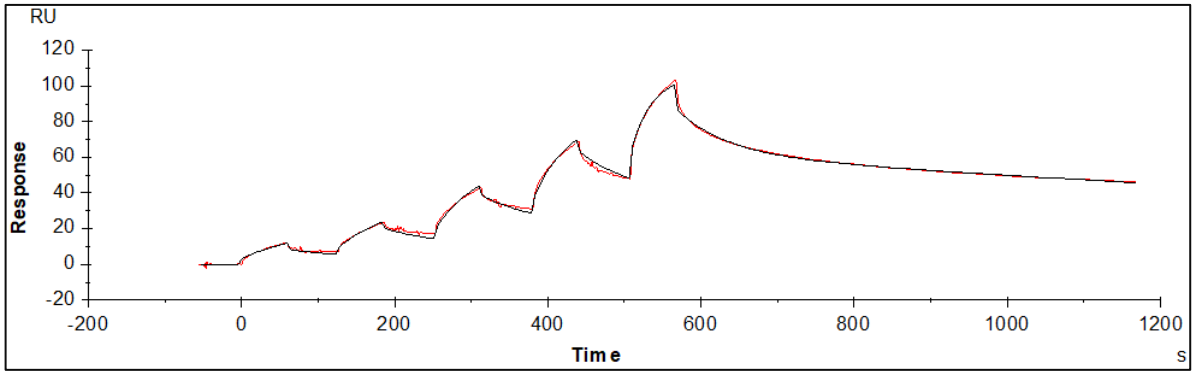
Figure 17. Time dependent SPR signal (RU) of PE (light blue), PilA (yellow), NHBA (pink), GNA1030 (red) and HBS (green).

Ligand	Samples	Mean of Resp (RU)
Vitronectin 20 µg/ml pH 4.5	PE 300 nM	75.85
Vitronectin 20 µg/ml pH 4.5	PilA 300 nM	0.65
Vitronectin 20 µg/ml pH 4.5	NHBA 300 nM	12.25
Vitronectin 20 µg/ml pH 4.5	GNA1030 300 nM	3.50
Vitronectin 20 µg/ml pH 4.5	HBS-EP+	-0.80

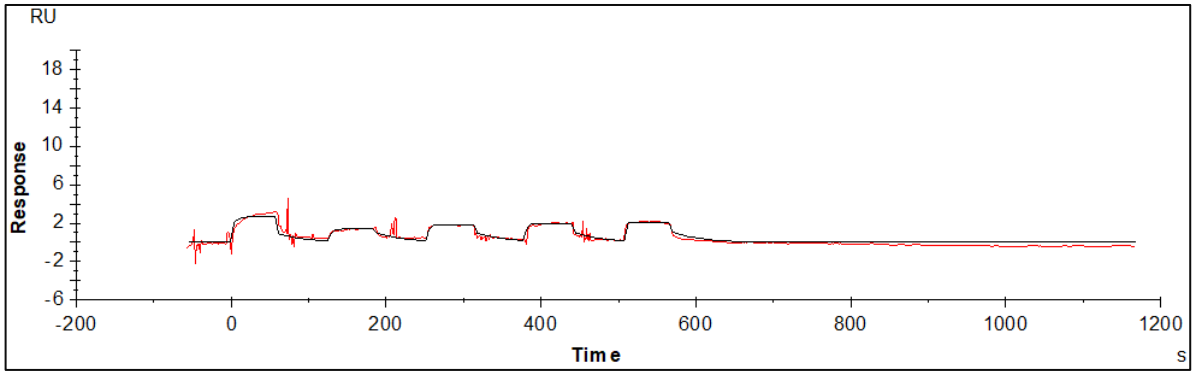
Table 18. Mean of reponses (RU) of the tested samples (at 300 nM concentration) on the ligand Vitronectin (20 µg/ml at pH 4.5). The RU considered negative are shown in grey and those considered positive in green.

Rmax is the maximum binding capacity of the immobilized ligand and depends directly on the molecular weight of the protein-protein complex that is formed on the surface of the sensor chip after the analyte molecule injection. The binding of the positive control PE to vitronectin showed a Rmax of 108.7 RU with a measured affinity of 1.3e-8 M. The binding between Vitronectin and NHBA was of a lower magnitude with a Rmax value of 19.5 RU, but the affinity was comparable to that of PE ($3.6e^{-8}$ M) (Figure 19 and Table 20).

PE



PilA



NHBA

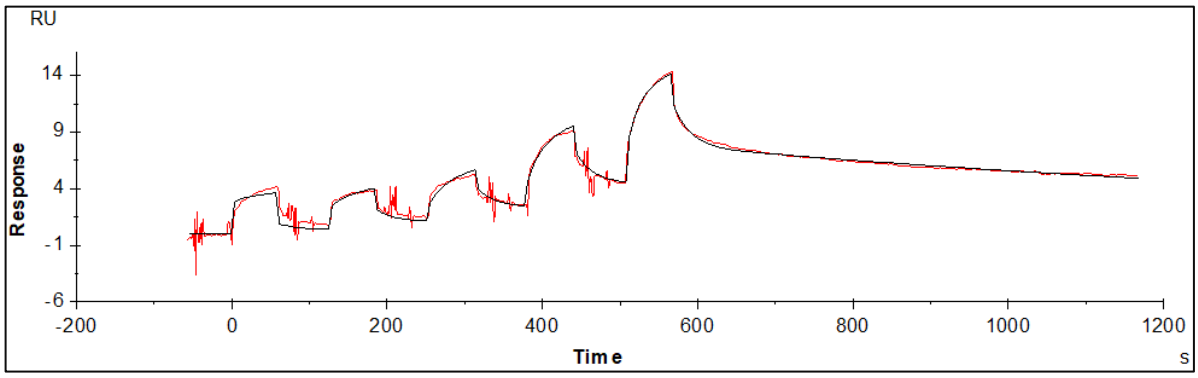


Figure 19. The time dependence of vitronectin binding to PE, PilA and NHBA.

Ligand	Sample	Rmax (RU)	KD (M)	Model
Vitronectin	PE	108.7	1.319e-8	2 state
Vitronectin	NHBA	19.5	3.612e-8	2 state
Vitronectin	PilA	1.1	n/a	1:1

Table 20. Affinity constants (Kd) measured for PE, PilA and NHBA on SPR vitronectin chip. The values considered negative are shown in grey and those considered positive in green.

A comparison of NHBA binding to vitronectin from different origins (mouse, rabbit and human) by SPR revealed an increased binding for human vitronectin (Figure 21). Although the SPR GNA1030 signal was negative, the signal of NHBA-GNA1030 was higher with respect to NHBA alone, suggesting that the conformation of NHBA in the context of the fusion could expose crucial epitopes relevant for the binding.

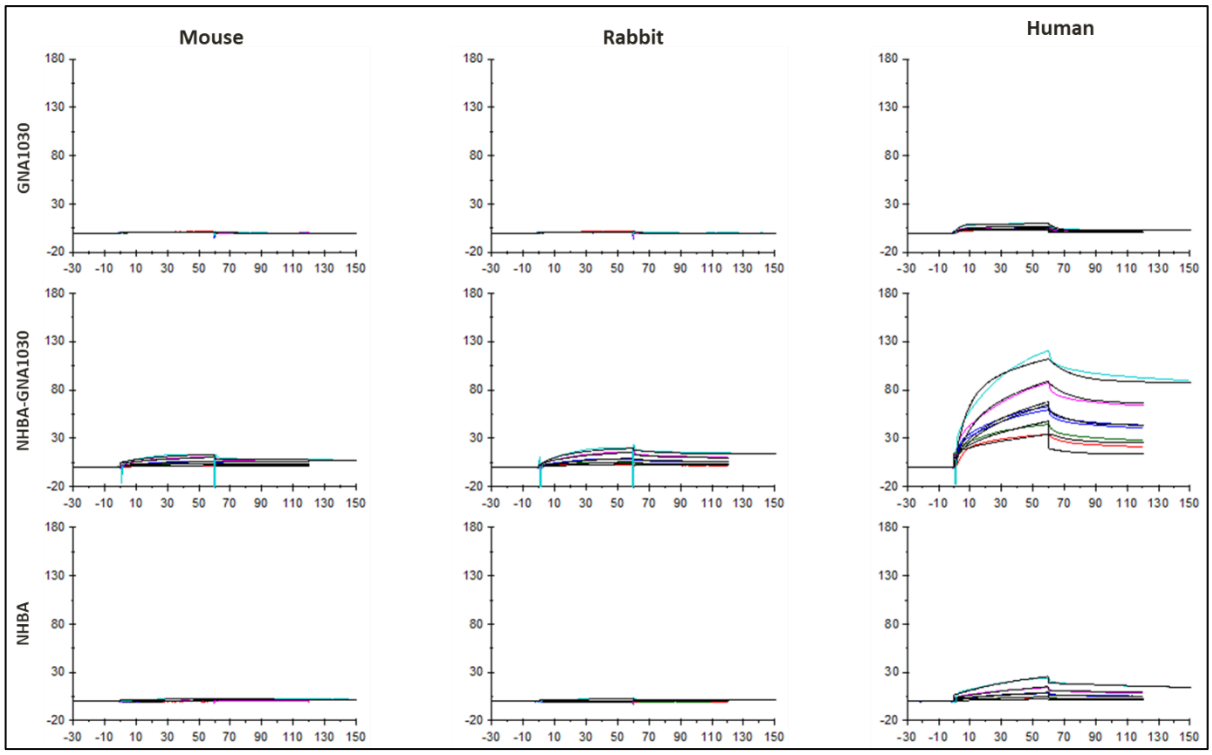


Figure 21. SPR signals (RU) of GNA1030, NHBA-GNA1030 and NHBA binding to vitronectin from mouse, rabbit and human origin.

6.4. Antigen Density

6.4.1. Strain generation

Given the scarce distribution of NHBA on the bacterial surface, an NHBA overexpressing strain was generated in the NGH38 WT strain background with the aim of studying the mechanism of protection mediated by antibodies raised against this antigen. A stabilized version of the PorA promoter was fused with the Contact Regulatory Element of Neisseria and used for constitutive overexpression of NHBA (Figure 22).

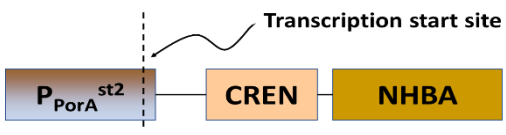


Figure 22. Schematic representation of the construct used for overexpression of NHBA.

6.4.2. Strain characterization

Growth kinetic evaluation showed no major differences between the NGH38 WT and NGH38OENHBA strains (Figure 23).

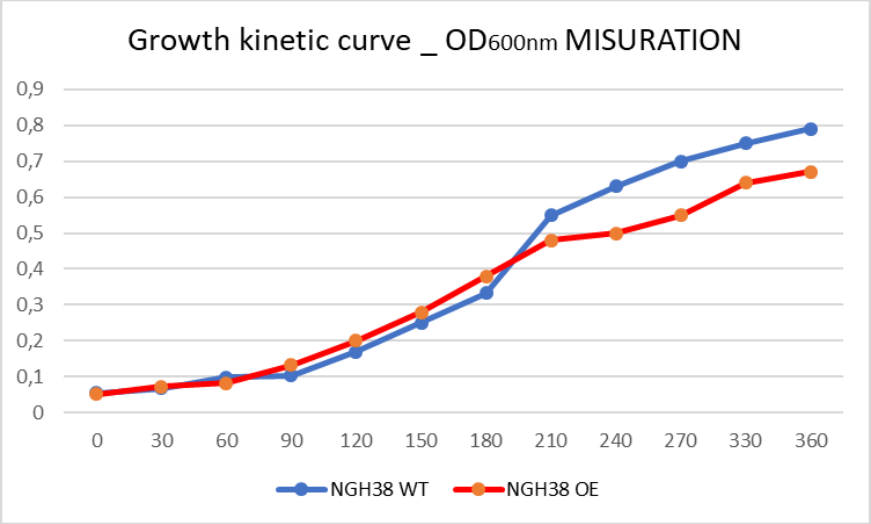


Figure 23. The OD_{600nm} dependence for NGH38 WT (blue) and NGH38OE NHBA (red) on time of growth.

Characterization of the strains by FACS analysis showed no differences among NGH38 WT, NGH38 OE and NGH38KONHBA regard to the capsule level of expression. However, a slight decrease of the fHbp signal was detected for the NHBAOE strain. The analysis of NHBA protein expression showed a clear increase on the intact bacterial surface of NGH38OE (Figure 24).

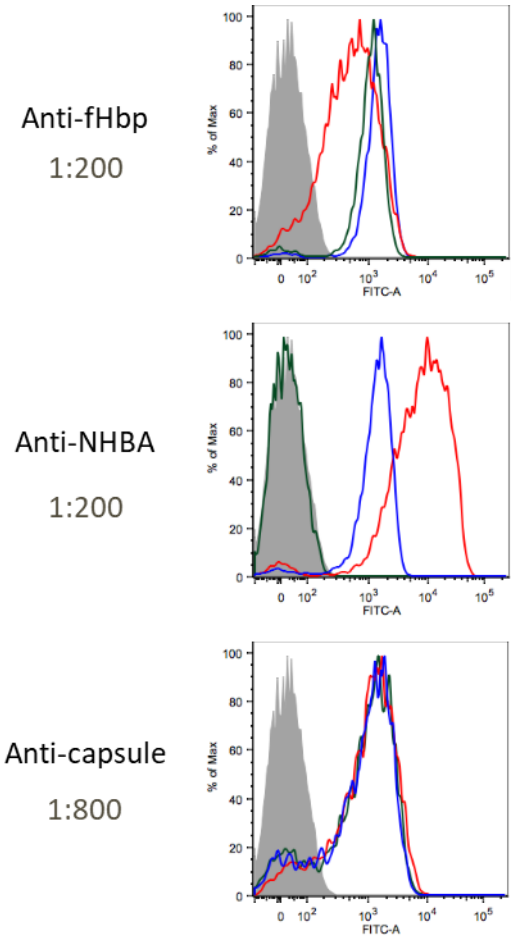


Figure 24. Flow cytometry analysis of anti-fHbp (dilution 1:200), anti-NHBA (dilution 1:200) and anti-capsule (dilution 1:800) sera are reported for NGH38 WT (blue), NGH38 OE NHBA (red) and NGH38KO NHBA (green). The negative control of only bacteria detected with the FITC-conjugated secondary antibody is shown in grey.

Given the slight decrease of fHbp exposure on the overexpressing strain, factor H deposition on the bacterial surface was investigated to evaluate if NHBA overexpression could have some way impacted Factor H biding on the bacterial surface.

Purified human Factor H and human complement were used to investigate the ability of NGH38 WT, NGH39OE and NGH38KO NHBA to bind Factor H, showing no major differences between the two sources used. Overlays of signals showed no major differences between the strains in the ability of binding human Factor H (Figure 25 and 26).

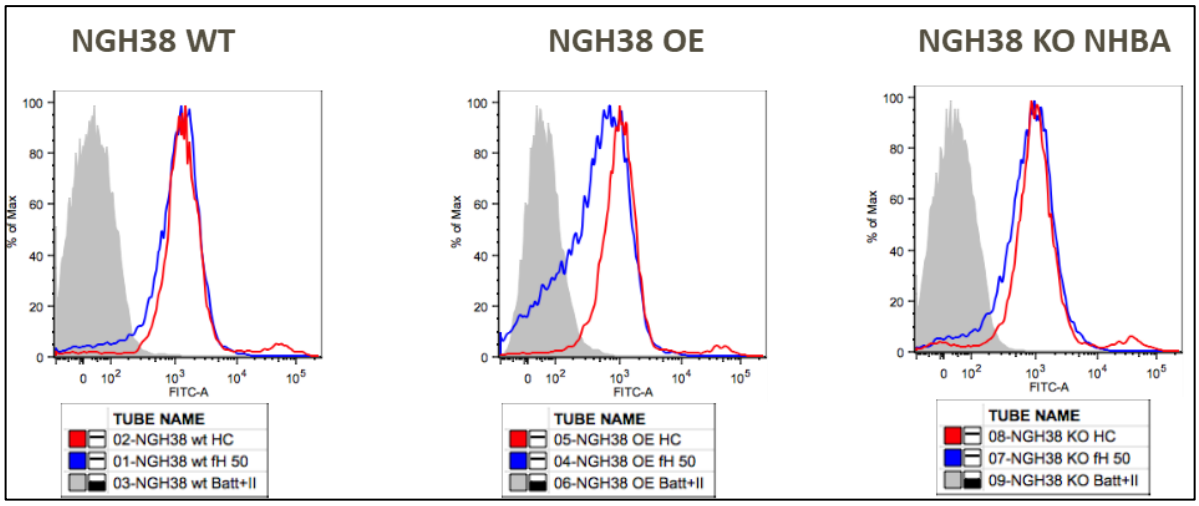


Figure 25. Factor H deposition flow cytometer analysis performed on NGH38 WT, NGH38 OE NHBA and NGH38KO NHBA. A comparison of bacteria incubated with human complement (in red) and purified human Facotr H (in blue). In grey the negative control of only bacteria detected with the FITC-conjugated secondary antibody.

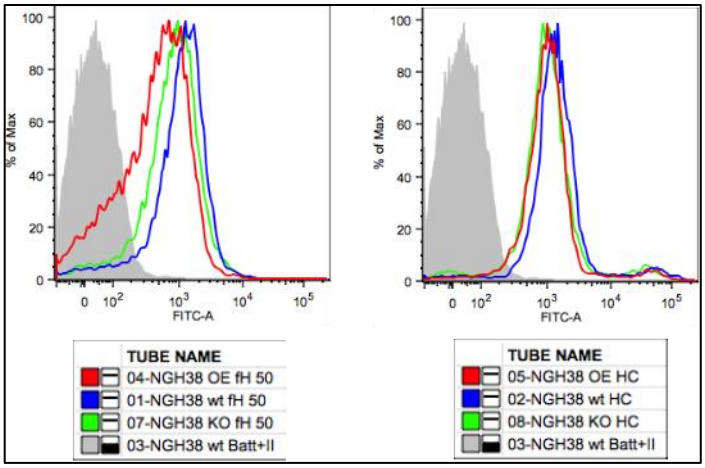


Figure 26. Factor H deposition flow cytometer analysis performed on NGH38 WT (blue), NGH38 OE NHBA (red) and NGH38KO NHBA (green). A comparison of bacteria incubated with purified human Facotr H (left figure) and human complement (right figure). In grey the negative control of only bacteria detected with the FITC-conjugated secondary antibody.

Complement C3 deposition assay was performed to detect C3b deposition on bacterial cell surfaces, so activation of the complement cascade. This ability was evaluated for NGH38 WT,

NGH39OE and NGH38KO NHBA, showing a great increase of complement deposition for the NGH38OE strain mediated by anti-NHBA antibodies, but no differences were detected if anti-fHbp serum was used (Figure 27).

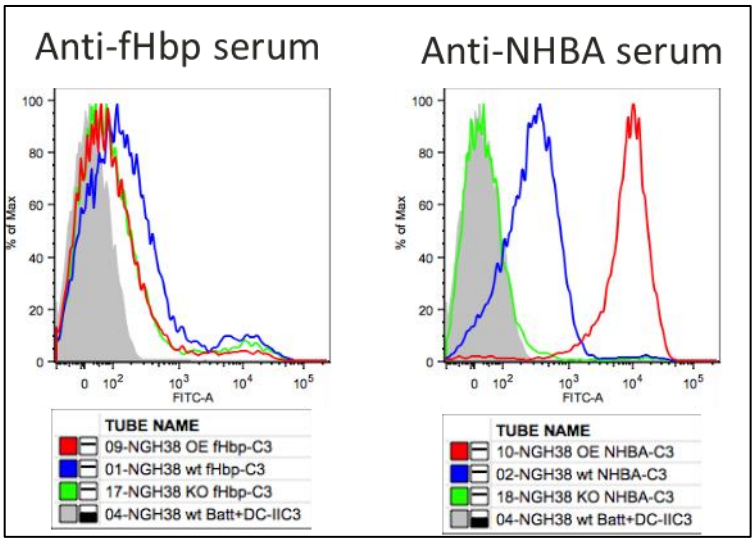


Figure 27. C3 deposition flow cytometer analysis performed on NGH38 WT (blue), NGH38 OE NHBA (red) and NGH38KO NHBA (green). A comparison of bacteria incubated with purified anti-fHbp serum (left panel) and anti-NHBA serum (right panel). In grey the negative control of only bacteria detected with the FITC-conjugated secondary antibody.

Furthermore, Complement C9 deposition assay was performed to detect C9 deposition on bacterial cell surfaces, with the aim of studying the end of the complement cascade, thereby the formation of the Membrane Attack Complement (MAC) for bacterial lysis. This ability was evaluated for NGH38 WT, NGH39OE and NGH38KO NHBA, showing great increase of complement deposition for the NGH38OE strain mediated by anti-NHBA antibodies, but no differences were detected if anti-fHbp serum was used (Figure 28).

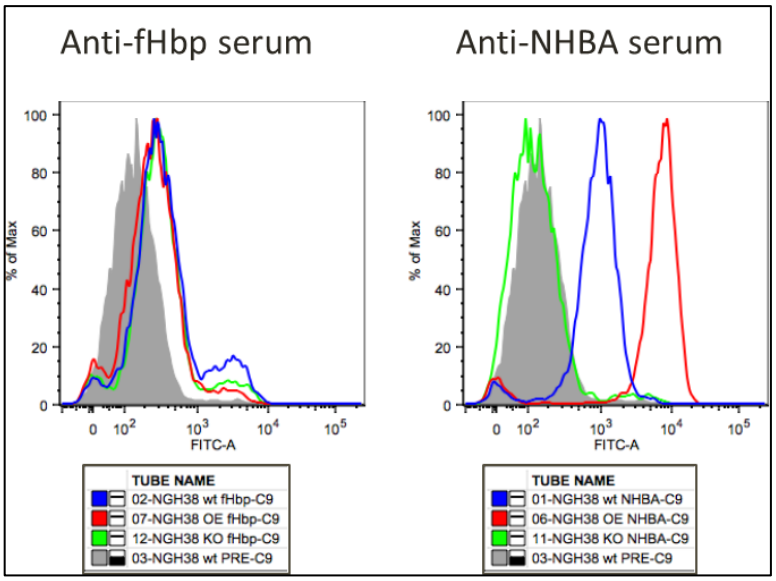


Figure 28. C9 deposition flow cytometer analysis of NGH38 WT (blue), NGH38 OE NHBA (red) and NGH38KO NHBA (green). A comparison of bacteria incubated with purified anti-fHbp serum (left figure) and anti-NHBA serum (right figure). In grey the negative control of only bacteria detected with the FITC-conjugated secondary antibody.

To evaluate the distribution of NHBA on the bacterial surface a confocal microscopy analysis was performed, comparing the NGH38 WT and NHBA OE strains. The scarce and punctiform distribution of NHBA on the bacterial surface was confirmed for the WT strain, being slightly more intense in the septum of the diplococcus (Figure 29).

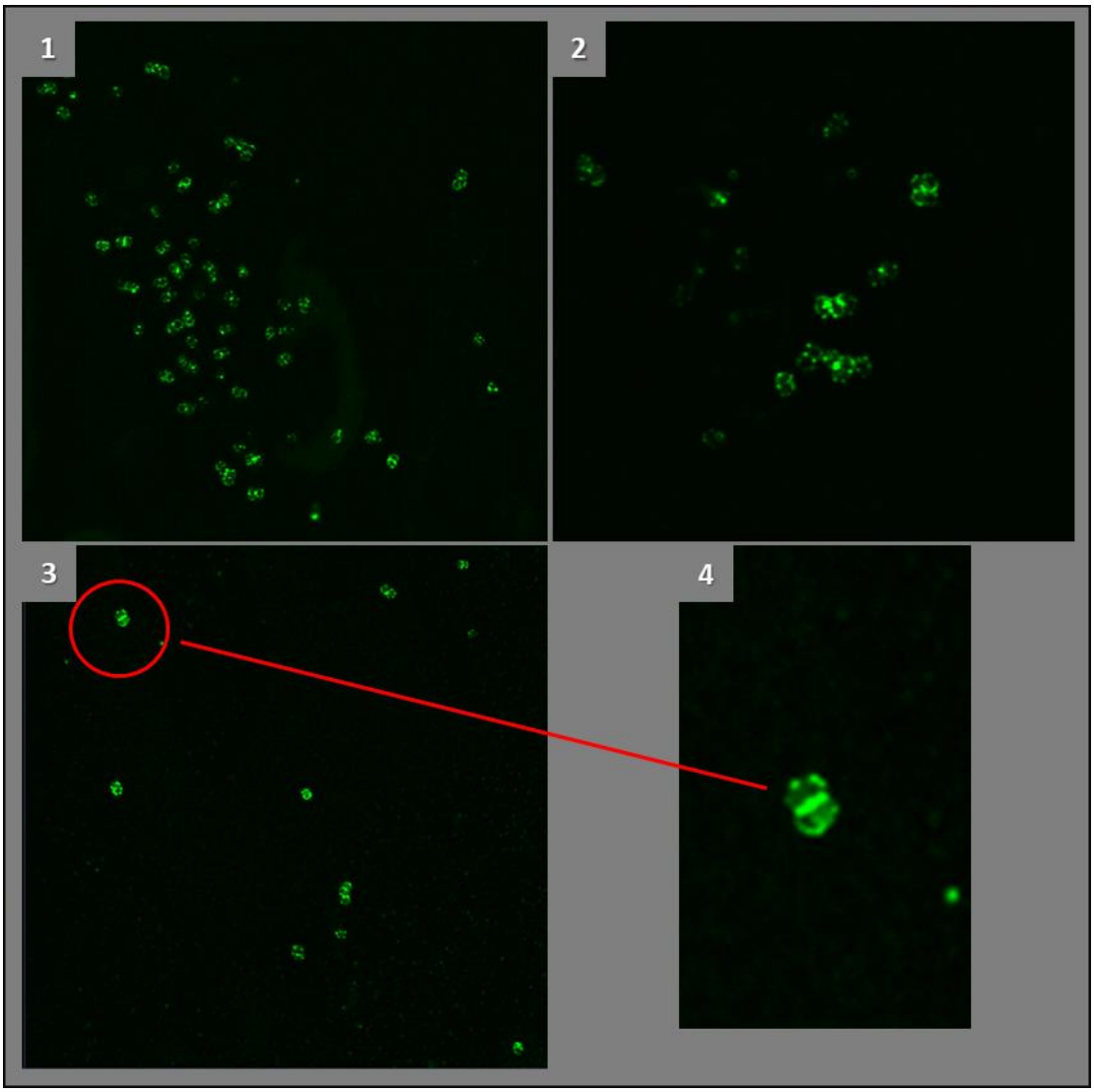


Figure 29. Confocal microscopy images of the NGH38 WT strain incubated with anti-NHBA serum and the secondary antibody Alexa fluor 488 conjugated.

For the Over Expressing strain, the distribution was found to be more homogeneous confirming that the amount of NHBA expressed on the bacterial surface is, as expected, high (Figure 30).

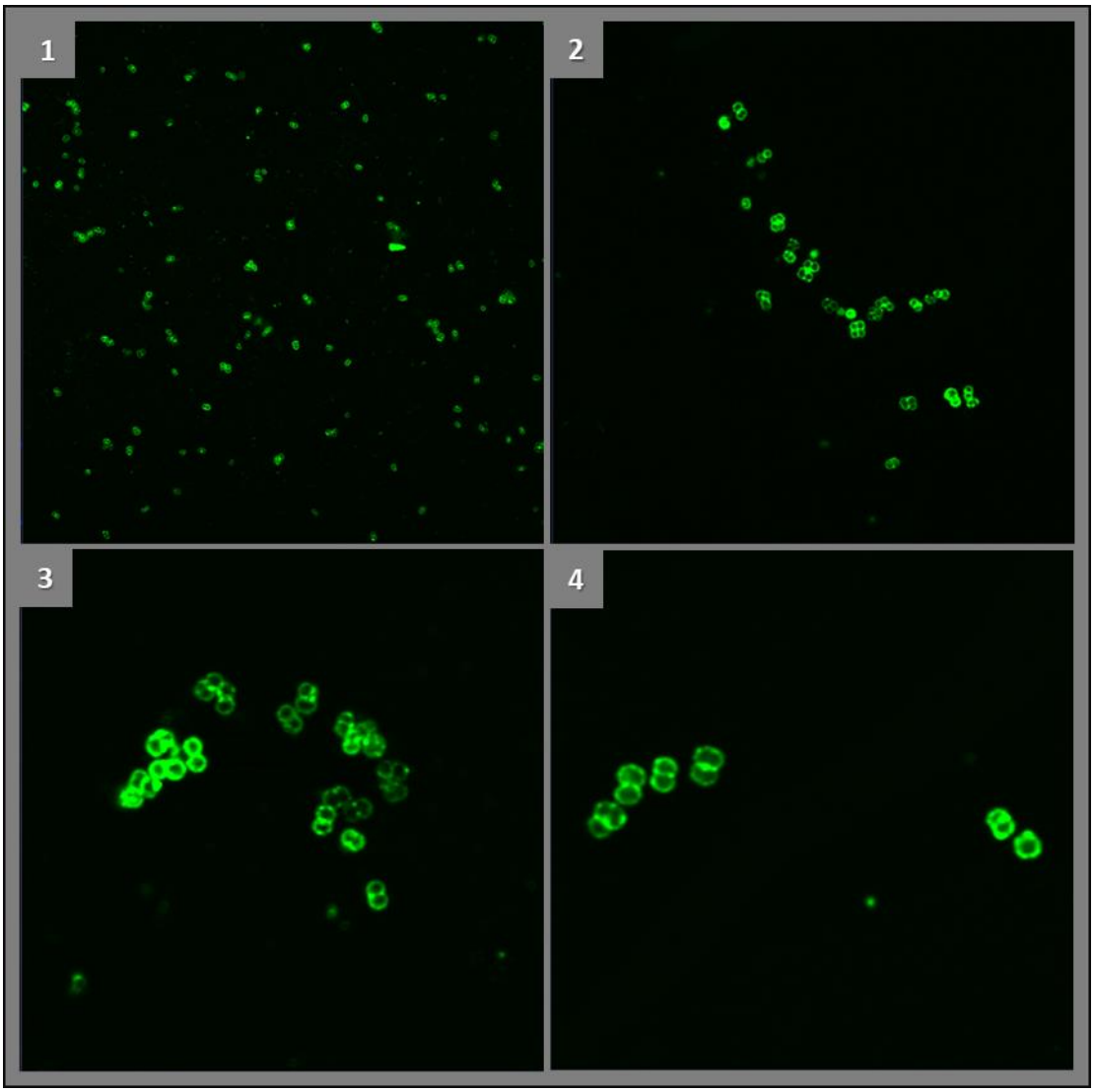


Figure 30. Confocal microscopy images of the NGH38 OE NHBA strain incubated with anti-NHBA serum and the secondary antibody Alexa fluor 488 conjugated.

To evaluate if NHBA expression changes during bacterial growth, a time dependence study was undertaken. No major differences were observed for NHBA distribution on NGH38 WT and NGH38OE for the three time points evaluated (Early log phase OD_{600} = 0.25; Mid log phase OD_{600} = 0.5; Stationary phase OD_{600} = 1) (Figures 31 and 32).

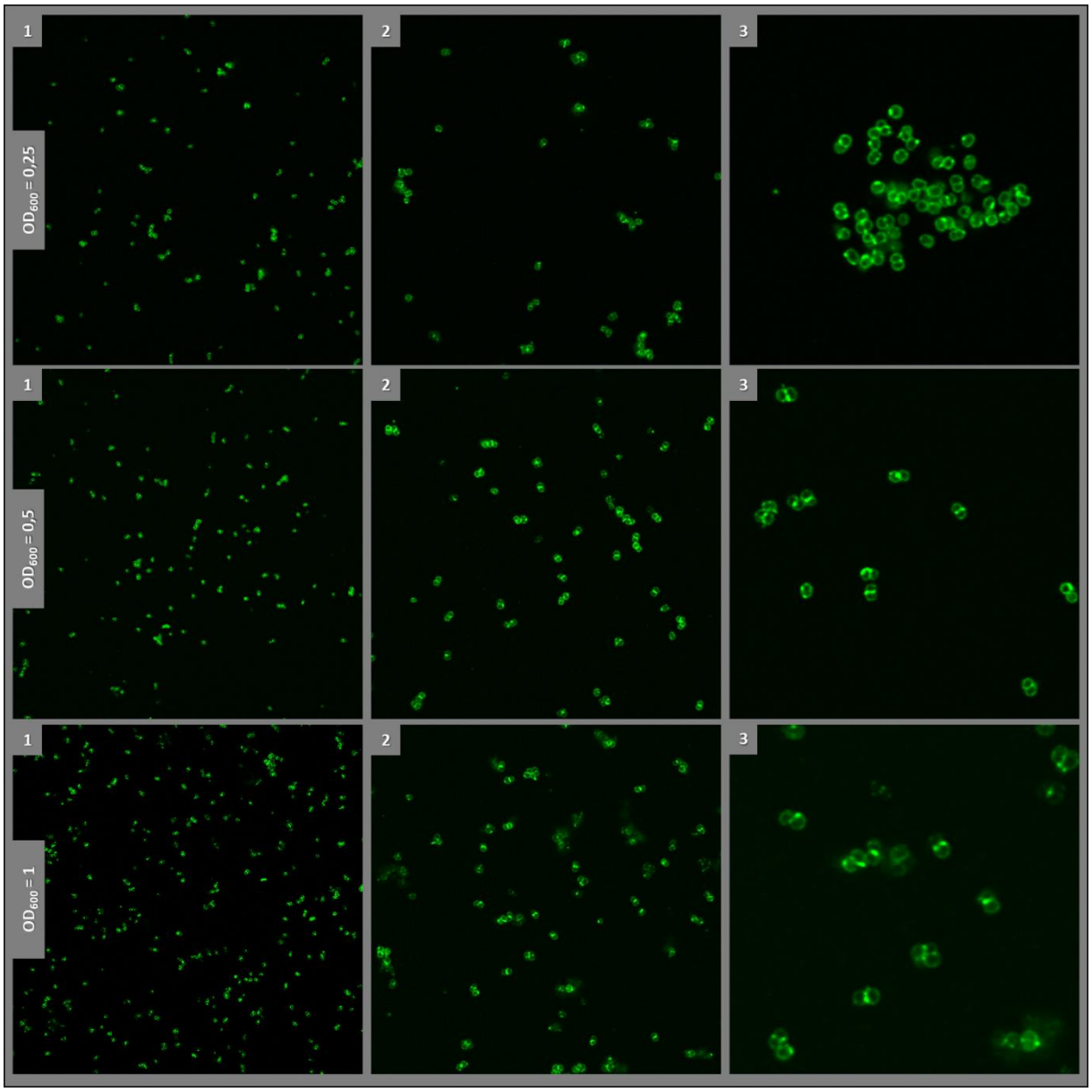


Figure 31. Confocal microscopy images of the NGH38 WT strain (at OD_{600} = 0,25-0,1-1) incubated with anti-NHBA serum and the secondary antibody Alexa fluor 488 conjugated.

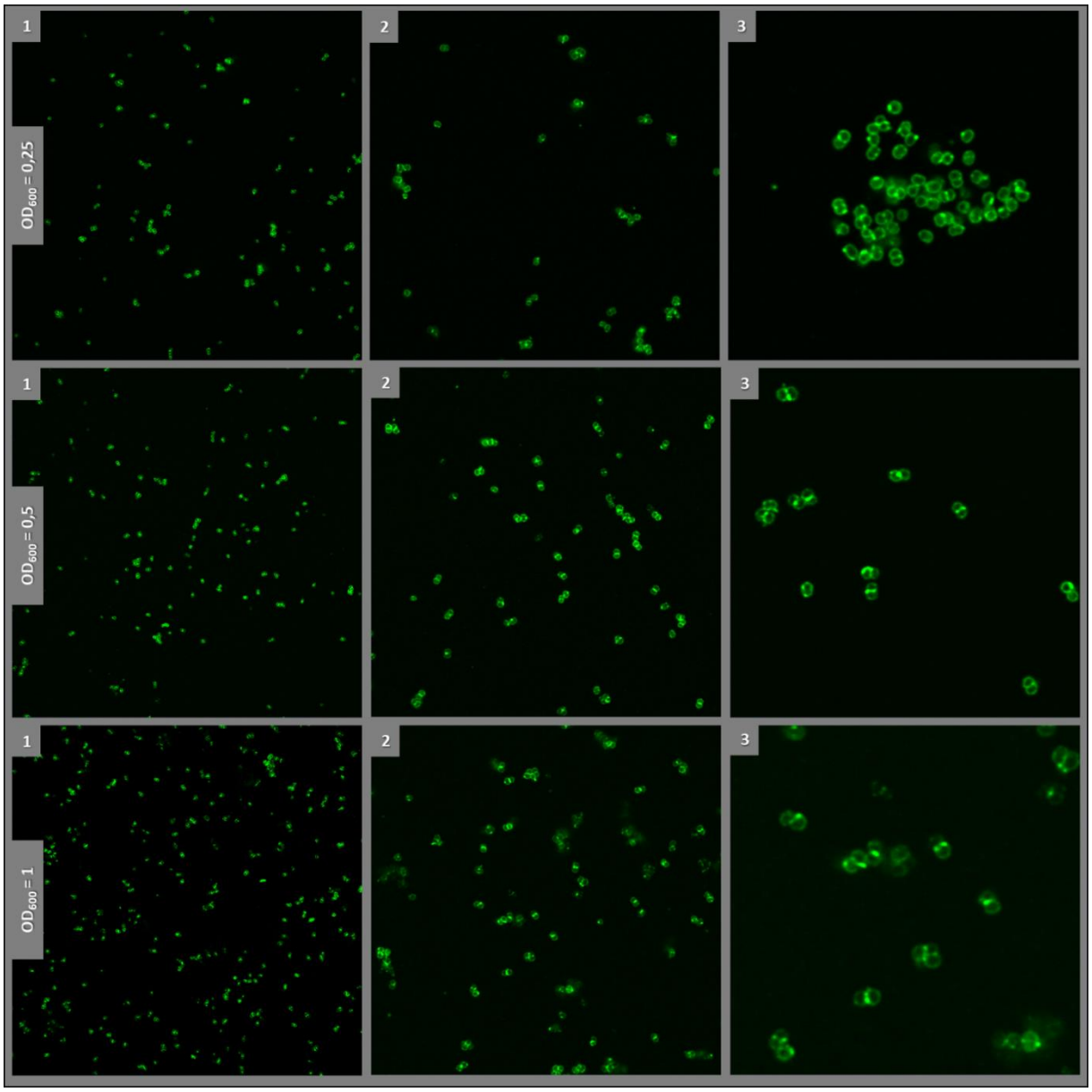


Figure 32. Confocal microscopy images of the NGH38 OE NHBA strain (at OD_{600} = 0.25-0.1-1) incubated with anti-NHBA serum and the secondary antibody Alexa fluor 488 conjugated.

The impact of antigen density on bactericidal activity of anti-NHBA antibodies was evaluated by Serum Bactericidal Assay. Rabbit and mouse anti-NHBA sera were tested in presence of human complement against NGH38 WT showing negative titers, comparable to the negative control of Pre-Immune serum. However, they showed high hSBA titers when tested against

the NGH38OE strain, highlighting the importance of the antigen expression during the *in vitro* testing (Table 33).

Animal serum	Immunization	NGH38 hSBA	NGH38 OE hSBA
Rabbit	Pre-immune	<16	<16
Rabbit	NHBA-GNA1030	<16	4096
Mouse	NHBA-GNA1030	<16	≥32768

Table 33. The hSBA titers of rabbit and mouse anti-NHBA-GNA1030 sera against NGH38 WT and NGH38OE NHBA are reported. Preimmune serum was used as a negative control. The values considered negative are shown in grey and those considered positive in green.

To explore the ability of NHBA present in Bexsero to induce functional antibodies in humans, a panel of anti-NHBA monoclonal antibodies, isolated from Bexsero vaccinees (Giuliani et al. 2018) mapping different regions of NHBA and with different range of affinity, was selected to verify their ability to induce bactericidal killing in the functional assay. Although no bactericidal activity was detected in the presence of human complement against the NGH38 WT strain, high hSBA titers were detected when mAbs were tested against the NGH38OE strain. It is noteworthy that the mAb 12E1, with non-detectable affinity, also showed lower bactericidal titers when compared with the other mAbs (Table 34).

HumAb	Binding region	Affinity	NGH38 OE hSBA
12E1	N-term	n.d.	256
10C3	N-term	Low	8192
4D11	C-term	High	8192
5H2	C-term	High-medium	16384

Table 34. The hSBA titers of anti-NHBA human monoclonal antibodies against the NGH38OE NHBA strain are reported. The values in green are considered positive.

6.5. Antibody with enhanced C1q deposition

Three of the four humAbs (10C3, 4D11 and 5H2) were selected, with respect for their affinity and results in hSBA against NGH38OE, to be cloned in an IgG scaffold with two point-mutations in the fc region of the heavy chain, resulting in an enhanced hexamerization activity leading to an increased C1q recruitment (Figure 35). This approach was used to verify if enhanced ability of complement activation could be driven also in presence of low Antigen copies (NGH38 WT strain).

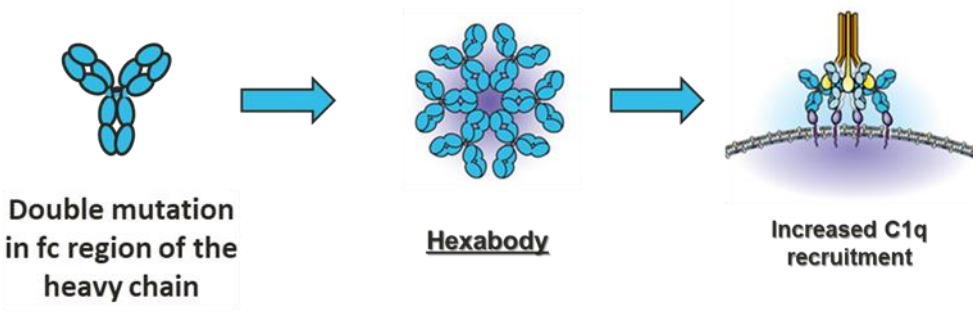


Figure 35. Double point mutations in the fc region of the antibody result in an increased ability to hexamerize and recruit C1q.

The testing of this "hexabody" in hSBA functional assay against the NGH38 WT strain (NHBA low expression scenario) demonstrated a significant enhancement of titers with respect to the corresponding Wild-Type mAb (Figure 36).

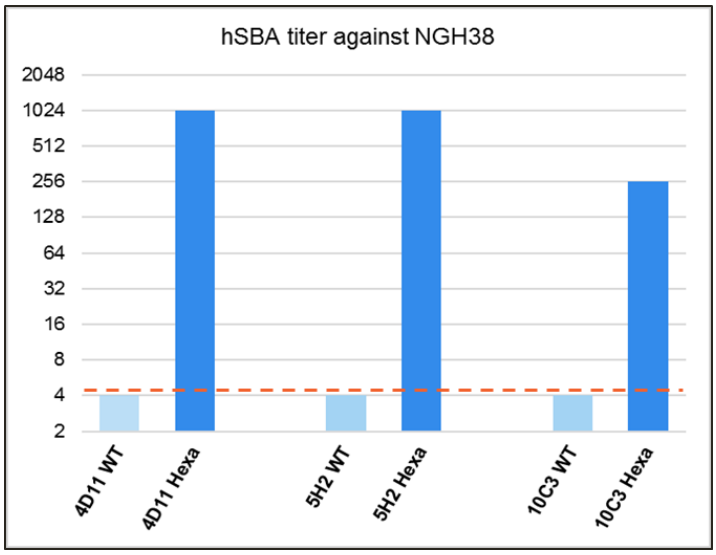


Figure 36. The hSBA titers of anti-NHBA human monoclonal antibodies against the NGH38OE NHBA strain are reported. The WT mAb (light blue) and hexabody mAb (dark blue) values are shown.

The two anti-C term mAbs (4D11 and 5H2) were used for Immunolocalization and Electron Microscopy visualization on both NGH38 WT and NGH38OE bacterial surface, in both their forms (WT and Hexabody).

When both 4D11 and 5H2 were tested on the NGH38 WT surface, the secondary antibody was scarcely localized in singlet or doublets.

Conversely, when both 4D11 and 5H2 were tested on the NGH38 OE surface, the secondary antibody was highly localized, with no specific distribution pattern.

It is noteworthy that when both 4D11 Hexabody and 5H2 Hexabody were tested on the NGH38 WT surface, despite the low expression level of NHBA, the secondary antibodies were often localized as a group of three or more, displaying the pattern of hexamerization that IgGs usually exploit for C1q recruitment. Interestingly, the distribution pattern of mAbs correlates with the functional activity detected in hSBA (Figure 37).

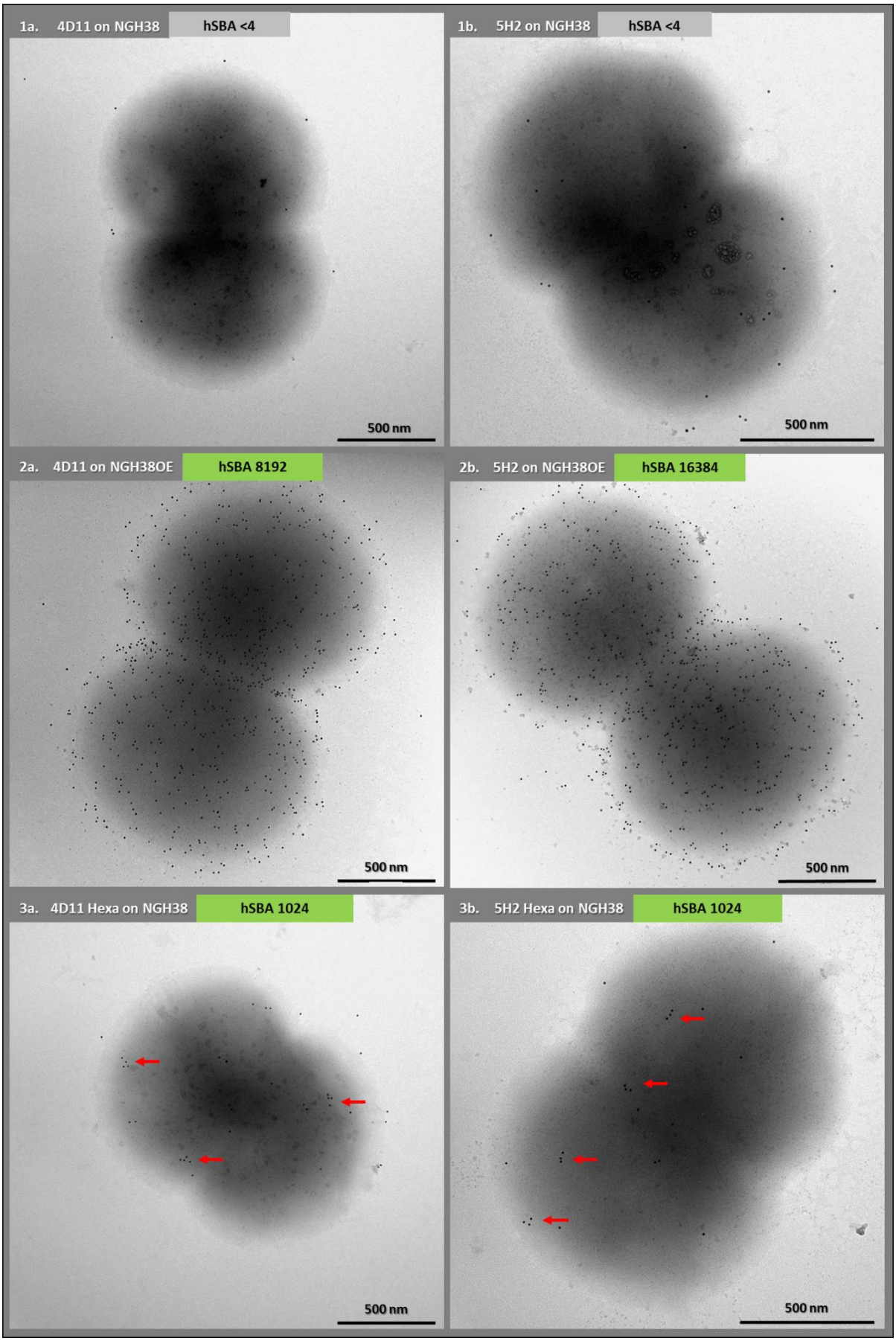

1a. 4D11 on NGH38
hSBA <4
500 nm
1b. 5H2 on NGH38
hSBA <4
500 nm
2a. 4D11 on NGH38OE
hSBA 8192
500 nm
2b. 5H2 on NGH38OE
hSBA 16384
500 nm
3a. 4D11 Hexa on NGH38
hSBA 1024
500 nm
3b. 5H2 Hexa on NGH38
hSBA 1024
500 nm

Figure 37. Immuno-gold labelling and transmission electron microscopy of the NGH38 WT strain (1a, 1b, 3a and 3b) and NGH38OE NHBA (2a and 2b). Analysis of the strains was performed with anti-NHBA monoclonal antibody (4D11 and 5H2, both WT and Hexabody). Scale bar: 500nm. The red arrows identify the pattern of secondary antibodies in groups of three of more.

6.6. Meningococcal infection *in vivo* model

Furthermore, the antibodies ability to mediate passive protection against meningococcal infection was investigated in the Infant Rat challenge model. A naïve serum, PBS and an unrelated mAb were used as controls in experiments and no differences were detectable among them in the lack of ability to mediate protection, given the high number of CFUs recovered (Figure 38).

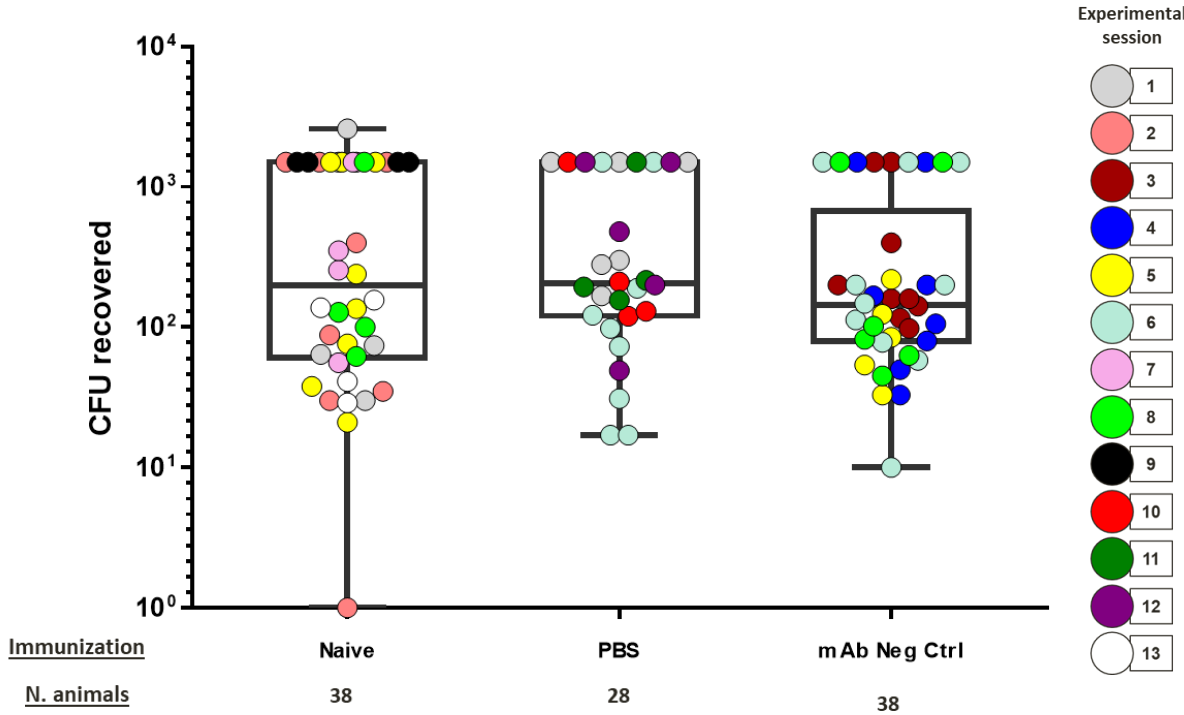


Figure 38. Comparison of different negative controls used for the Infant Rat challenge model. Lack of passive protection is clearly evident. Dots represent the individual number of CFU recovered. For each group, the box

extends from the 25th to 75th percentiles and the line inside the box represents the median. The dot colors represent different experimental sessions.

Anti-Bexsero, anti-NHBA-GNA1030 and anti-NHBA polyclonal mouse sera were used for immunization of pups. Complete passive protection was recovered after infection in the case of anti-Bexsero and anti-NHBA-GNA1030 sera. Partial but still significant protection was mediated by the anti-NHBA serum (Figure 39).

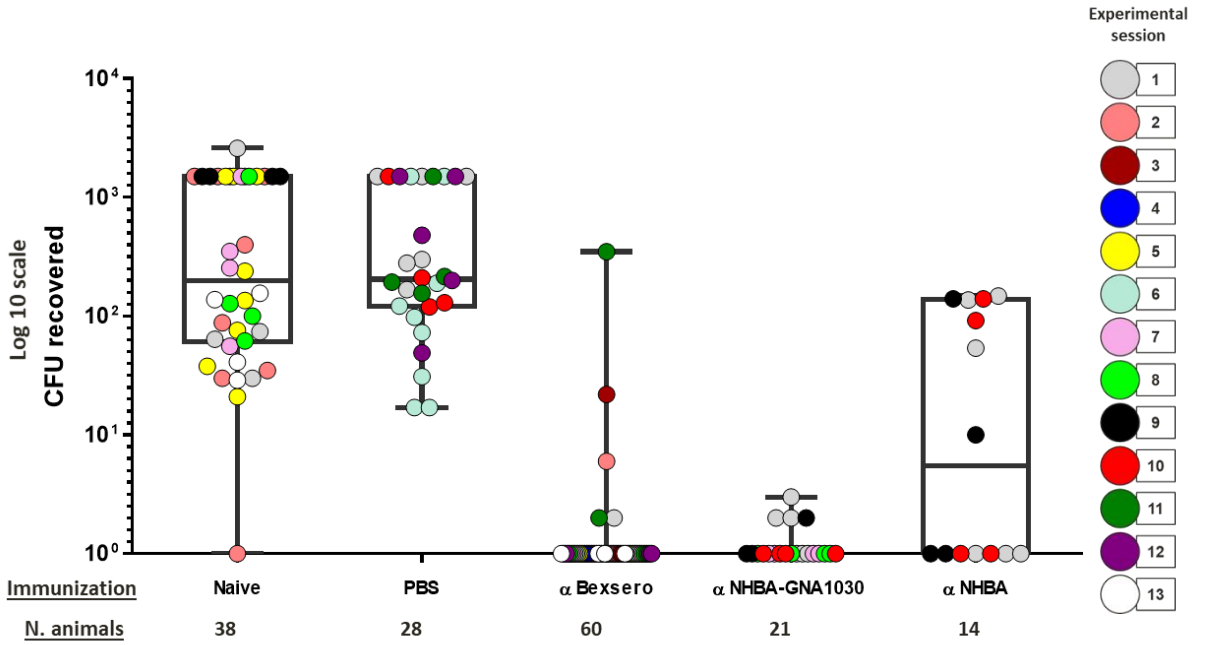


Figure 39. Passive protection induced by Bexsero, NHBA-GNA1030 or NHBA specific polyclonal antisera in the Infant Rata challenge model. Dots represent the individual number of CFU recovered. For each group, the box extends from the 25th to 75th percentiles and the line inside the box represents the median. Dot colors represent different experimental sessions.

Anti-NHBA human mAbs (12E1, 5H2 and 4D11) were tested in the model at three different concentrations (10, 50 and 100 µg in 100 µl of immunization volume). A slight decrease of

CFUs was detected for 12E1 at 100 µg and 5H2 at 100 µg. No significant decrease was detected for the other samples tested (Figure 40).

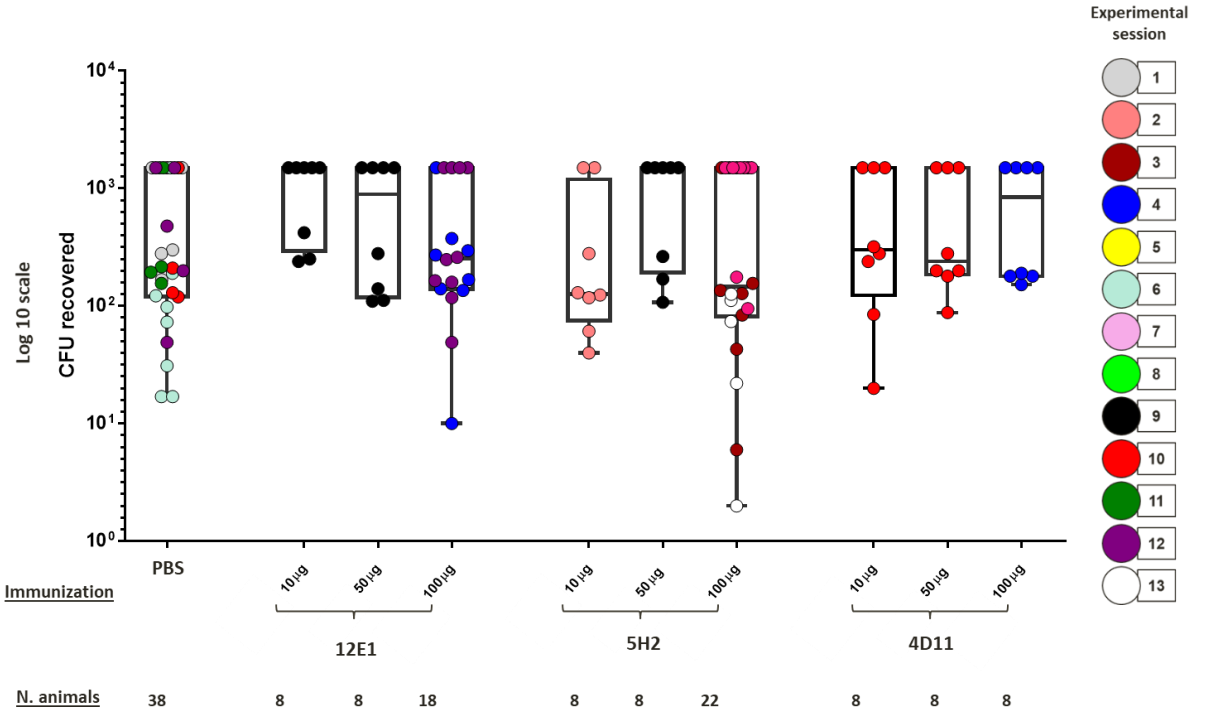


Figure 40. Passive protection induced by anti-NHBA humAbs in the Infant Rata challenge model. Dots represent the individual number of CFU recovered. For each group, the box extends from the 25th to 75th percentiles and the line inside the box represents the median. Dot colors represent different experimental sessions.

The combinations of two mAbs were tested immunizing with 50 µg of each selected mAbs. No significant effect was generally observed, but for the 5H2 – 12E1 combination a slightly decreased CFU with respect to the mAbs alone was observed (Figure 41).

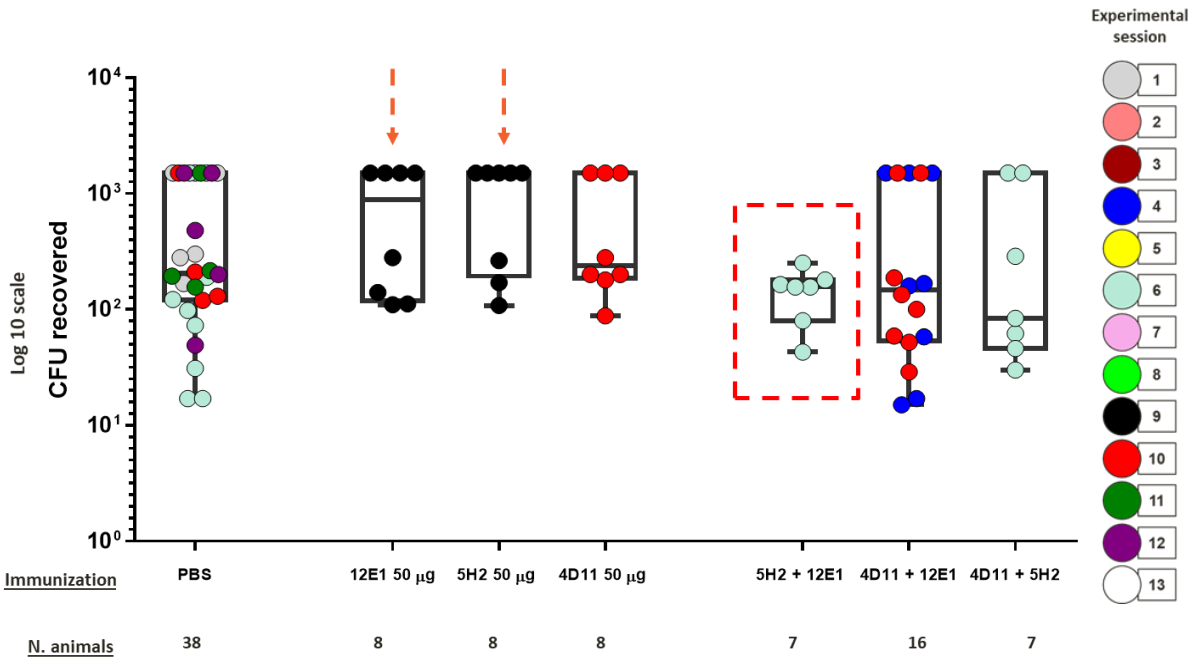


Figure 41. Comparison of passive protection induced by single anti-NHBA humAbs or their combinations in the Infant Rat challenge model. Dots represent the individual number of CFU recovered. For each group, the box extends from the 25th to 75th percentiles and the line inside the box represents the median. Dot colors represent different experimental sessions.

For the initial set up of the bioluminescent assay, infection dose was evaluated for the following strains: 2996_LUX (negative control for luciferase), 2996_NHBA_LUX (luciferase under the control of the NHBA promoter), 2996_NadA_LUX (luciferase under the control of the NadA promoter) and the non-bioluminescent 2996 strain; infection was performed with both 10^4 and 10^6 CFU/100 µl of infection volume and images were acquired at t0, t3 hours and t20 hours before bleed outsacrifice. Blood samples were plated and the CFUs were recovered. No variation in the signal was detected at T0 and T3. However, a strong signal was detected for the 2996_NHBA_LUX strain at T20 at both 10^4 and 10^6 CFU/100 µl. The results obtained at T20 were not strictly comparable with the read-out of a canonical Infant Rat experiment (Figure 42).

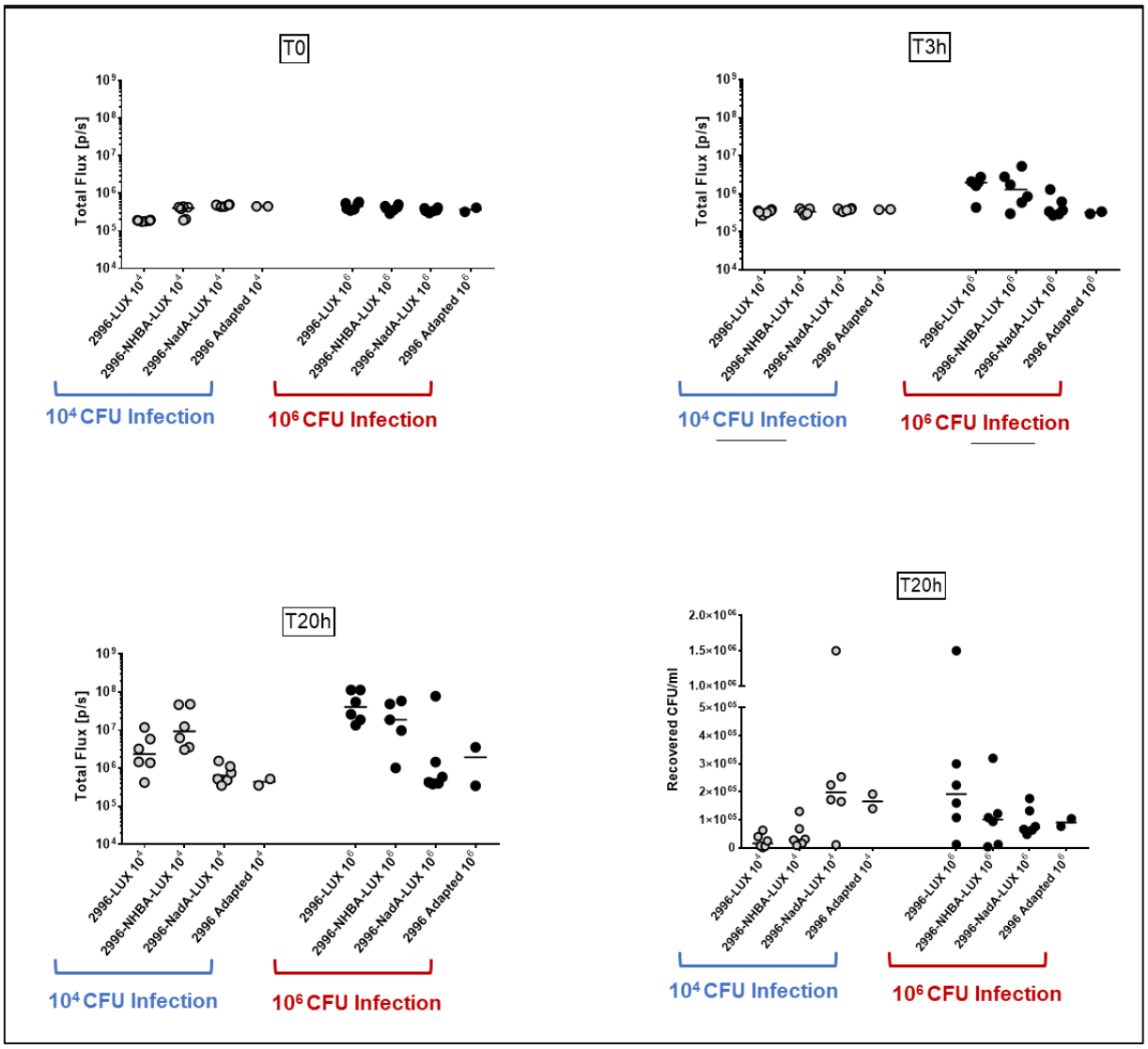


Figure 42. Set up of infection dose. Dots represent individual photon/second or number of CFU recovered. The line represents the median. To = time zero; T3 = 3 hours; T20 = 20 hours. T0, T3 and T20 reported as total flux signals (photon/secon).

Passive protection with IVIS analysis after infection with 2996_NHBA_LUX strain confirmed what previously shown with classical read-out: complete passive protection was mediated by polyclonal anti-Bexsero, anti-NHBA-GNA1030 and surprisingly also with anti-NHBA serum. In this experimental session, the IVIS read-out at T20 was comparable with the CFUs recovered after Bleed out (Figure 43).

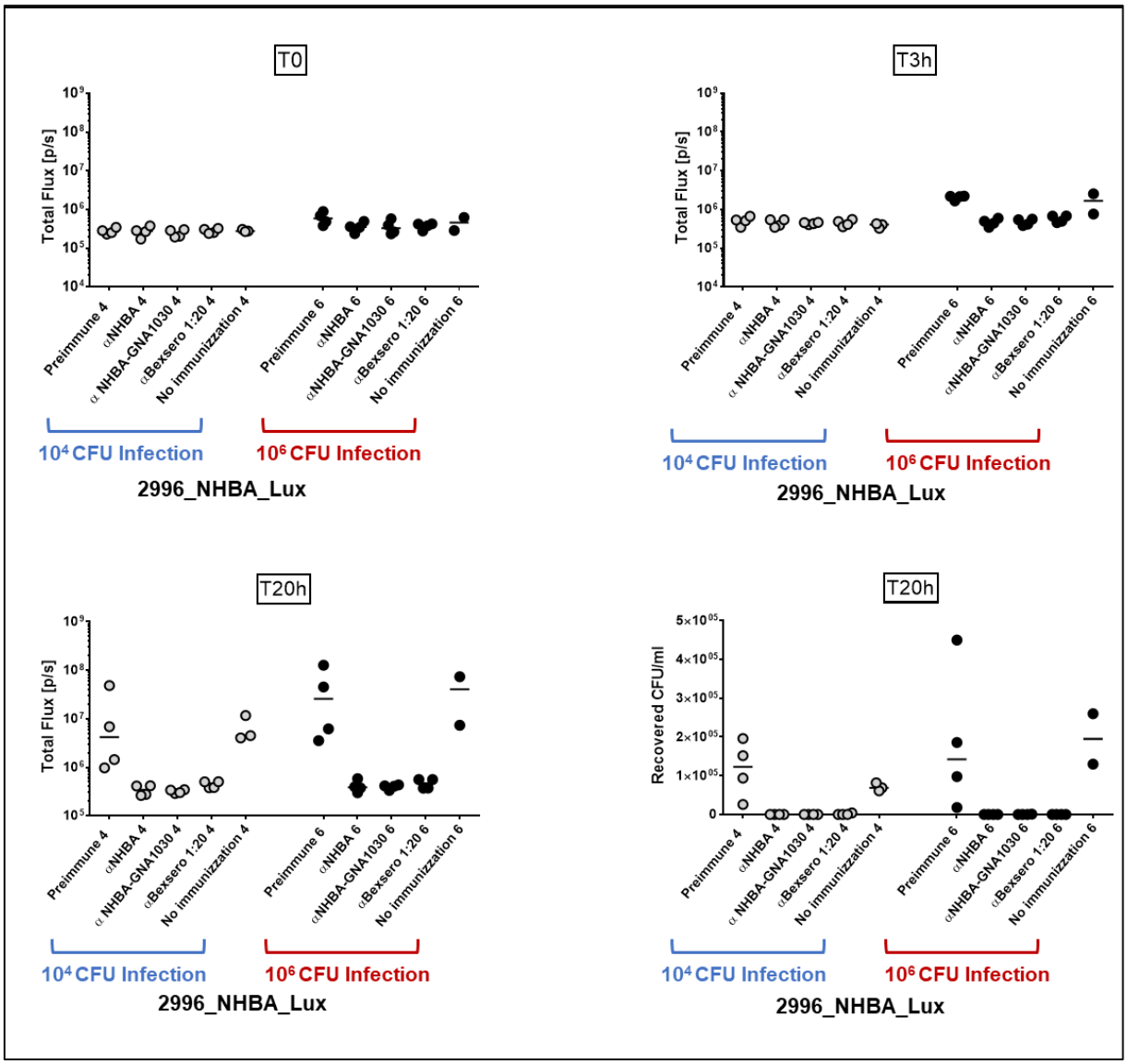


Figure 43. Time Course of passive protection experiment with IVIS technology and comparison with classical read-out method. Dots represent individual photon/second or number of CFU recovered. The line represents the median. To = time zero; T3 = 3 hours; T20 = 20 hours. T0, T3 and T20 are reported as total flux signals (photon/second).

7. DISCUSSION AND CONCLUSIONS

Neisserial Heparin Binding Antigen (NHBA) is one of the three protein antigens of the 4CMenB vaccine. It is strongly conserved among MenB strains and considered a key virulence factor, given its involvement in multiple steps of meningococcal colonization, infection and survival. In this work, I investigated the mechanism of action of the anti-NHBA antibodies-mediated protection.

Sensitivity of the read-out commonly used was firstly investigated. Serum Bactericidal Assay (SBA) is considered the gold standard for the evaluation of correlate of protection for Meningococcal vaccines. Results showed the high variability of the standard read out (7 µl reaction-drop on MHA), confirming the **potential underestimation** of the anti-NHBA bactericidal activity. This impact should be considered also for other antigens displaying low expression, with strong rabbit serum bactericidal activity but undetectable human bactericidal titres.

We also investigated the impact of Factor H down-regulation. Factor H is a negative regulator of the alternative pathway and a very abundant protein in human plasma, preventing complement attack against host tissues. This glycoprotein of about 150 kD is the major negative regulator of the alternative pathway (AP) of the complement system, acting as cofactor for factor I and triggering irreversible degradation of C3 convertase (permanent dissociation of C3b,Bb) (Welsch and Ram 2008). Downregulation of the AP can facilitate the survival of meningococci in the human host by increasing serum resistance (Madico et al. 2006, Welsch et al. 2008). In fact, inhibition of the AP through binding of fH to microbial surfaces is used by several bacterial species to evade the complement system activity (Kraiczy and Wurzner 2006).

Herein the **significant impact of Factor H binding** to the meningococcal surface in the SBA *in vitro* testing has been confirmed. By sequestering human Factor H from the hSBA reaction it was possible to obtain positive titres for anti-NHBA Abs, mimicking the results usually obtained in rabbit SBA. Conversely, human Factor H addition to the rabbit SBA reaction resulted in a non-specific effect of titre negativization, affecting sera raised against different meningococcal antigens, which has a greater impact for a poorly expressed antigen as NHBA.

Interaction of NHBA with the negative regulator of the complement system vitronectin was also investigated. This multifunctional glycoprotein of about 75 kDa is present in blood and in the extracellular matrix, acting as a key regulatory factor for cell adhesion and physiological proteolysis (Schvartz et al. 1999). Several pathogens are known to bind vitronectin (VN), to interact with human epithelial cells or to elude complement deposition effects by exploiting structural similarities with this membrane-attack-complex inhibitor (Podack and Müller-Eberhard 1979). Nontypeable *Haemophilus influenzae* interacts with vitronectin on bronchial cells through its protein-E (Schvarts et al. 1999, Ikeda et al. 2015); whereas the meningococcal outer membrane protein Opc binds to vitronectin to enhance cellular entry through human brain endothelial cells barrier (Sa et al. 2010). In addition, the meningococcal surface fibril (Msf, previously called Neisseria hia homologue A (NhhA)) was described to bind activated vitronectin enhancing pathogen survival in human serum and evasion of the membrane attack complex (Sjölinder et al. 2008). Recent studies reported a low residual binding of vitronectin to meningococcal surface of a double Knock-Out strain (Opc-/Msf-) suggesting possible contribution of an additional meningococcal antigen. Driven by its heparin binding activity and the similarity with NhhA, we decided to investigate the ability of NHBA to interact with vitronectin.

This work has described and highlighted the **novel binding of NHBA with vitronectin**.

Both in ELISA and SPR assays, the NHBA-VN interaction was found to be much more evident when the NHBA-GNA1030 fusion was used as a probe compared to NHBA alone. These observations suggest that the conformation acquired by NHBA when fused to GNA1030 could play a crucial role in the interaction, contributing to the exposure of relevant binding epitopes. It is noteworthy that the antibodies induced by the fusion proteins of 4CMenB were generally more effective than those induced by the individual antigens (Giuliani et al. 2006).

Remarkably, increased binding activity was observed for human vitronectin compared to that of rabbit or mouse, a situation resembling the selective affinity of meningococcal fHbp for human factor H (Granoff et al. 2009), and emphasising the species specificity of meningococcus as a human pathogen.

Another key aspect for effective bactericidal activity that has been analyzed is the **antigen density**. Due to the sparse NHBA distribution on the bacterial surface, an NHBA over-expressing strain was used to confirm that, in the presence of multiple copies of the antigen, anti-NHBA antibodies (both polyclonal and a monoclonal) are perfectly able to elicit C1q deposition and trigger high bactericidal activity. This conclusion is in agreement with previous studies demonstrating that the spatial proximity of a single antigen or cooperative antigens, as in case of multicomponent vaccine-elicited antibodies repertoire, is a key condition for activation of the complement cascade (Natali et al. 2020). This also follows the fact that C1q is physiologically able to activate the complement cascade in the presence of clusters of cell-bound IgG. In structural terms, it has been suggested that for an effective activation of C1q an optimal relative orientation and distance between the two mAbs are necessary (Peschiera et al. 2019). The six-globular head of C1q requires, for proper activation, IgG hexamers

assembled at the cell surface through Fc:Fc interactions (Diebolder et al. 2014). Different antibodies simultaneously bound to the same antigen, or to cooperative antigens, increase the interaction of Fc regions facilitating the successful recruitment of C1q. For this reason, anti-NHBA human monoclonal antibodies from Bexsero vaccinees were engineered to increase hexamerization ability. These hexabodies were tested against the WT MenB strain, with low expression of NHBA, and were demonstrated to have high bactericidal activity despite the low amount of the antigen.

Since the early stages of meningococcal vaccine correlates studies, it was observed that, even in the absence of Serum Bactericidal Activity with human complement, anti-NHBA antibodies were able to passively protect infant rats against meningococcal bacteraemia (Welsch et al. 2003).

In this work the early indications have been confirmed, demonstrating that a strong **passive protection is mediated by anti-NHBA sera** and a complete protection comparable to Bexsero-induced passive protection, is obtained in the infant rat model when anti-NHBA-GNA1030 sera are passively administered. This observation is highly significant because it demonstrates that anti-GNA2132 antibodies are functional against active bacteria replicating in the blood stream, a condition probably distant from that of organisms grown *in vitro*.

With the aim of demonstrating possible differences between *in vitro* testing conditions and *in vivo* growth circumstances, a proteomic analysis highlighted significant differences in the protein expression profile between 37°C (temperature commonly used for *in vitro* experimental setting) and 32°C (physiological nasopharynx temperature). Among all the proteins evaluated, NHBA was one of the most upregulated at 32°C (9.47-fold upregulated,

P=0.0337) and, moreover, it was identified as a factor involved in enhanced self-aggregation, biofilm formation, and cellular adherence (Lappann et al. 2016).

These results, taken together with our findings on the interactions with vitronectin, reinforce the role of NHBA in meningococcal pathogenesis and complement evasion strategies. Increased NHBA expression in the nasopharyngeal mucosa can contribute to the colonization step and successively, interactions with heparin and vitronectin can contribute to increase pathogen survival in the host blood.

In conclusion, in this work considerable evidence has been presented on the effective functionality of anti-NHBA antibodies and insight gained into the possible interactions with the complement system.

The negative human Serum Bactericidal Activity has been clarified through multiple proofs of concept, highlighting the underestimation effect of the read-out commonly used for *in vitro* testing, the strong impact of human Factor H downregulation and describing a novel interaction with vitronectin.

By engineering a NHBAOE strain, it has been demonstrated that WT antibodies are effective in eliciting bacterial killing, while enhanced c1q-recruitment of hexabodies is sufficient to mediate killing even in a low-expression scenario. Moreover, the ability of anti-NHBA antibodies to passively protect infant rats from meningococcal bacteraemia has been confirmed, emphasising the function of these antibodies against active *in vivo*-replicating bacteria; hence, stressing the contribution of NHBA in the multicomponent vaccine 4CMenB.

Future studies will be aimed at achieving a better understanding of the variations of NHBA expression during the different steps of meningococcal colonization and pathogenesis. Better

insight into the interaction with vitronectin, could contribute to the evaluation of anti-NHBA antibody functionality, investigating their ability to inhibit this mechanism of complement evasion and their contribution in preventing meningococcal colonization and dissemination.

8. ETHICAL STATEMENTS

This work was sponsored by GlaxoSmithKline Biologicals SA.

Silvia Principato is a student of the University of Siena (Life Science Department) and participates in a PhD program at GSK. Silvia Principato is currently an employee of the GSK group of companies.

Bexsero is a trademark of the GSK group of companies.

Human monoclonal Antibodies were obtained from adults in a Phase I clinical study conducted in Krakow, Poland and sponsored by Novartis Vaccine, now part of the GSK group of Companies, using two doses of multicomponent serogroup B meningococcal vaccines. The Clinical trial protocol was approved by the Bioethics Committee of the District Medical Doctors' Chamber in Krakow and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each of the subjects.

All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EEC and the GSK policy on the Care, Welfare and Treatment of Animals.

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