UNIVERSITA' DEGLI STUDI DI SIENA
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SETTORE SCIENTIFICO-DISCIPLINARE
Biotecnologie dei vaccini

CICLO XXIX

COORDINATORE Annalisa Santucci

"Dissecting the role of Neisseria Heparin Binding Antigen cleavage during adaptation of *Neisseria meningitidis* to mucosal surface"

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To my mom,

who lives her everyday life as a scientist

This study was sponsored by Novartis Vaccines, now acquired by the GSK group of companies

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Ph.D. research activities

During the first year of my Ph.D., I focused my research activities on a project that took advantage of SAM vaccine platform to express candidate antigens and develop a new RNA based vaccine. I was responsible for the production of reagents necessary for investigating the immunogenicity of a selected antigen, including self-amplifying RNA encoding for the selected vaccine candidate, Virus-like Replicon particles and recombinant protein. In addition, I was involved in performing several immunological assays, such as Intracellular Cytokine Staining, T-cell ELISPOT and T cell proliferation assay, and in analyzing data derived from these assays, in order to evaluate T-cell immune response against the selected antigen.

After the closure of the deal between Novartis and GSK, the project in which I was involved was discontinued; thus, in the second and third year of my Ph.D. I focused my research activities on a different project.

The second project focused at investigating the role of the cleavage of Neisseria Heparin Binding Antigen (NHBA), one of the three main protein antigens of the Bexsero™ vaccine (a trademark of the GSK group of companies), during meningococcal interaction with epithelial cells. I was responsible for the preparation of reagents necessary for the study, including the production of recombinant proteins, the maintenance and differentiation of several human cell lines. With the supervision and the guidance of my GSK vaccine supervisor, I designed and performed most of the experiments. The results obtained during these two years are described in my Ph.D. thesis, and they will be part of the following scientific publication, which is in preparation:

“Identification of a novel human protease responsible for Neisseria Heparin Binding Antigen (NHBA) cleavage during meningococcal infection”. Di Fede M., Biagini M., Cartocci E., Parillo C., Martinelli M., Marchi S., Pezzicoli A., Delany I., Rossi Paccani S.

During my Ph.D. I was also involved in two different studies focused at characterizing the physiological role of NHBA during meningococcal infection. The results obtained are and will be part of the following scientific publications:

Neisserial Heparin Binding Antigen (NHBA) Contributes to the Adhesion of Neisseria meningitidis to Human Epithelial Cells. Vacca I., Del Tordello E., Gasperini G., Pezzicoli A., Di Fede M., Rossi Paccani S., Marchi S., Mubaiwa T.D., Hartley-Tassell L.E., Jennings M.P., Seib K.L., Masignani V., Pizza M., Serruto D., Aricò B., Delany I. PLoS One. 2016 Oct 25; 11(10):e0162878. doi: 10.1371/journal.pone.0162878

URL: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0162878

"Neisseria Heparin Binging Antigen (NHBA) processing by kallikrein from saliva". Pantano E., Marchi S., Nardi Dei V., Grassi E., Biagini M., Di Fede M., Rossi Paccani S., Savino S., Cartocci E. (In preparation)

Abstract:

Neisserial Heparin Binding Antigen (NHBA) is a surface-exposed lipoprotein specific for *Neisseria* (*N.*) and constitutes one of the three main protein antigens present in the Bexsero vaccine. NHBA can be structurally divided into an N-terminal and a C-terminal domain separated by an Arginine (Arg)-rich motif responsible for the protein binding to heparin and heparan sulfate proteoglycans. This protein is very susceptible to protease cleavage, such human proteases, including lactoferrin and kallikrein which cleave the protein downstream the Arg-rich motif releasing a C-terminal fragment that does not contain the Arg-rich motif. In a subset of *N. meningitidis* hyper virulent strains, meningococcal NalP protease cleaves NHBA protein upstream the Arg-rich motif instead, generating a C-terminal fragment that contains the Arg-rich motif; this fragment is referred as C2 fragment.

It has been demonstrated that C2 fragment exerts a toxic effect on endothelial cells, altering endothelial permeability. Since NHBA expression is upregulated at 32°C, a temperature encountered during initial *N. meningitidis* colonization of the nasopharynx, we hypothesized that the C2 fragment could also affect the human respiratory epithelium and facilitate the traversal of *N. meningitidis*.

To verify our hypothesis we assessed C2 fragment activity on polarized Calu-3 cells, a cell line that develops morphological features of a differentiated human airway epithelium. In line with previous studies showing that *N. meningitidis* traverses the epithelial barrier without disrupting the junctional structures, we observed no influence of C2 fragment on the cellular integrity. Unexpectedly, we discovered that epithelial cells rapidly processed the C2 fragment converting it into a shorter fragment that no longer contains the Arg-rich epitope. On the contrary, endothelial cells processed the C2 fragment with a slower kinetics, and this slow cleavage rate could be compatible with the C2 fragment-induced toxic effect previously reported.

Overall, these findings provide evidences for a new host defense mechanism against C2 fragment-induced toxic effect. C2 fragment proteolysis by host cells could eliminate the putative docking domain responsible for the interaction of the C2 fragment with the host cells, thus preventing its biological activity.

The cleavage of C2 fragment and NHBA full-length protein by epithelial cells was further characterized using several epithelial cell models as well as verifying the cleavage on live bacteria. We found out that epithelial cells from the upper respiratory tract constantly secreted proteases responsible for the cleavage of both proteins. The cleavage site of epithelial cell proteases were located upstream the Arg-rich motif within both proteins. Moreover, we demonstrated that NHBA processing by epithelial cell proteases occurred on live bacteria during meningococcal interaction

with epithelial cells. Finally, looking for the epithelial cell protease responsible for the cleavage of NHBA protein, we identified a novel human protease responsible for NHBA cleavage during meningococcal infection: the C3-convertase of alternative complement pathway.

Introduction:

1. *Neisseria meningitidis* epidemiology and disease

Neisseria meningitidis (N. meningitidis), a gram-negative bacterium with a coccoid shape, is an obligate human commensal typically residing in nasopharyngeal mucosa. It is a pathogenic member of the *Neisseria* family and it is the leading cause of bacterial meningitis worldwide. Globally the annual number of meningococcal invasive disease cases is estimated to be 1.2 million with 135,000 deaths, although the incidence of the disease varies from <1 case/100 000 population, in Europe and North America, to 10-1,000 cases/100,000 population, in the "meningitis belt" of sub-Saharan Africa (Jafri et al., 2013). Based on the immunologic reactivity of the capsular polysaccharides, 12 distinct serogroups have been defined (A, B, C, E, H, I, K, L, W135, X, Y and Z), six of which cause life-threatening disease (A, B, C, W135, X and Y) (Crum-Cianflone and Sullivan, 2016). Geographical distribution of pathogenic meningococcal serogroups is shown in Figure 1.

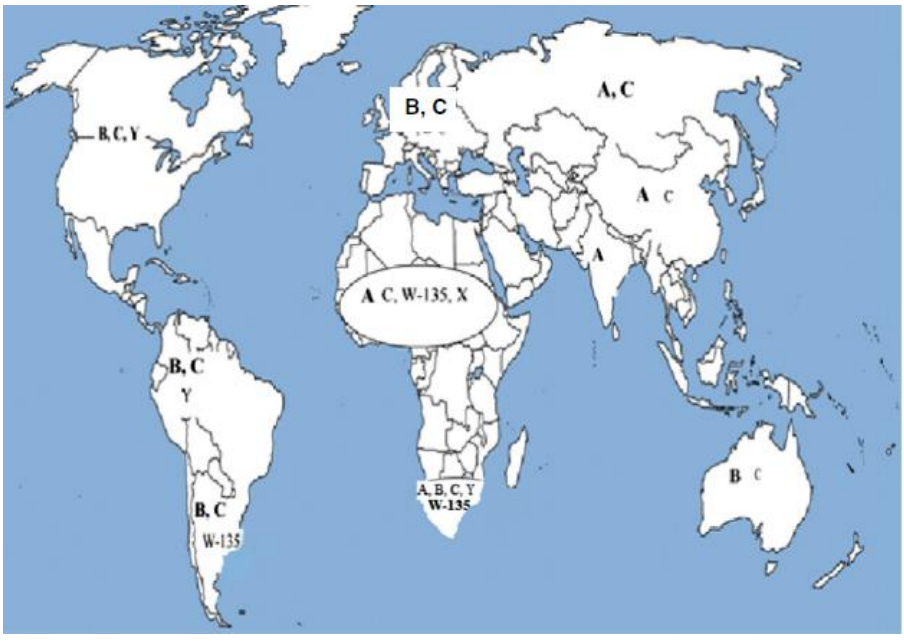


Figure 1 - Distribution of common and predominant meningococcal serogroups by region.
Figure adapted from Jafri et al. (2013). Predominant strains are highlighted in bold text.

In nonepidemic settings, approximately 10% of healthy individuals at any time carry *N. meningitidis* in the upper airways. Acquisition of the bacteria from a healthy carrier or an infected person occurs through close direct contact with respiratory droplets or secretions. For the majority of people, carriage can be an immunizing process that results in a systemic, serogroup-specific protective antibody response (Yazdankhah and Caugant, 2004). However, a subset of individuals exposed to the bacterium develops invasive diseases. Diagnosis of meningococcal disease is often challenging

since initials symptoms mimic the symptoms of common viral infection, such coryza, pharyngitis, fever, loss of appetite, nausea, vomiting and headache. Without an appropriate and urgent treatment, the infection can progress rapidly and result in death or permanent disability in as little as 24 hours from the first symptoms. Mortality rate associated with invasive disease still stands at 8-14%. The most common clinical presentations of invasive infections include meningitis (30-60%) and septicaemia (20-30%) often manifesting with petechial or purpuric rash (Pace and Pollard, 2011). All age groups are at risk for invasive meningococcal disease, but infants and adolescents are particularly susceptible. Disappearance of maternal antibodies early in life and the high rate of nasopharyngeal colonization in adolescence, respectively, increase the risk of meningococcal invasive infections in these population groups (Kovarik and Siegrist, 1998; Christensen et al., 2010; Soriano-Gabarró et al., 2011). An epidemiological study performed by the Italian national health agency "Istituto Superiore di Sanità" (ISS, 2014) looking at the number of hospitalization cases per age groups due to invasive meningococcal disease in Italy from 2001 and 2012 is reported in Figure 2 as an example of the different susceptibly to meningococcal disease depending on the age. This study shows that the highest incidence of the disease is observed in infants under 1 year of age. The rate of disease declines rapidly thereafter. A second, but smaller peak of disease occurs in adolescents and young adults between the ages of 15 and 19 years-old and the predominant serogroup responsible for invasive meningococcal disease in new-born babies is serogroup B (ISS, 2014; ISS, 2016).

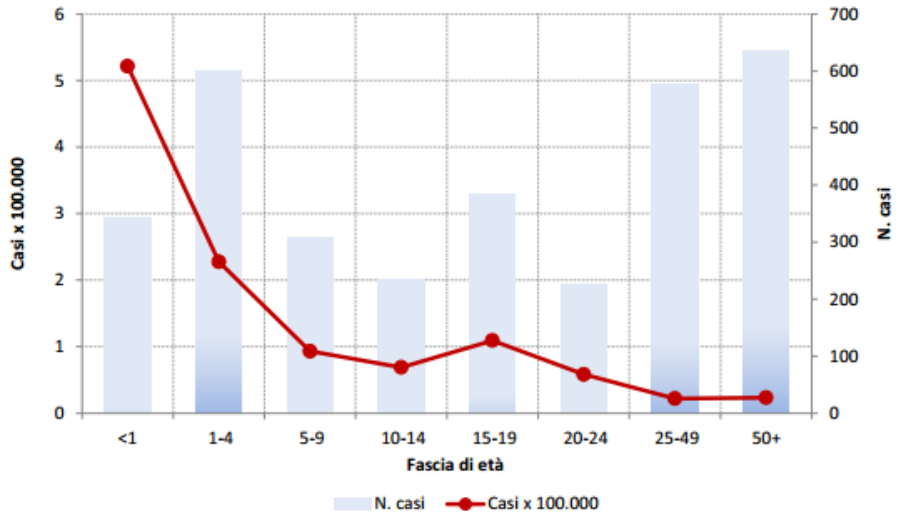


Figure 2 - Incidence of meningococcal invasive diseases in Italy

Figure adapted from ISS report (2014). Number of hospitalization cases per age groups due to invasive meningococcal disease in Italy from 2001 and 2012

Other risk factors for invasive meningococcal disease include some sceneries, which increase social interactions and can facilitate respiratory droplet transmission of *N. meningitidis*, such as schools,

university dormitories, military recruit camps and households (Brundage et al., 2002), as well as hereditary or acquired deficiencies of complement and aspleenia, that predispose individuals to invasive meningococcal infections (reviewed in Ram et al., 2010).

2. Pathogenesis of meningococcal infection

Colonization of human nasopharyngeal mucosa by *N. meningitidis* is the first step in establishment of both carrier state and invasive meningococcal disease. Bacteria attach selectively to host cells and enter certain non-ciliated columnar cells of the nasopharyngeal mucosa (Stephens et al., 1983).

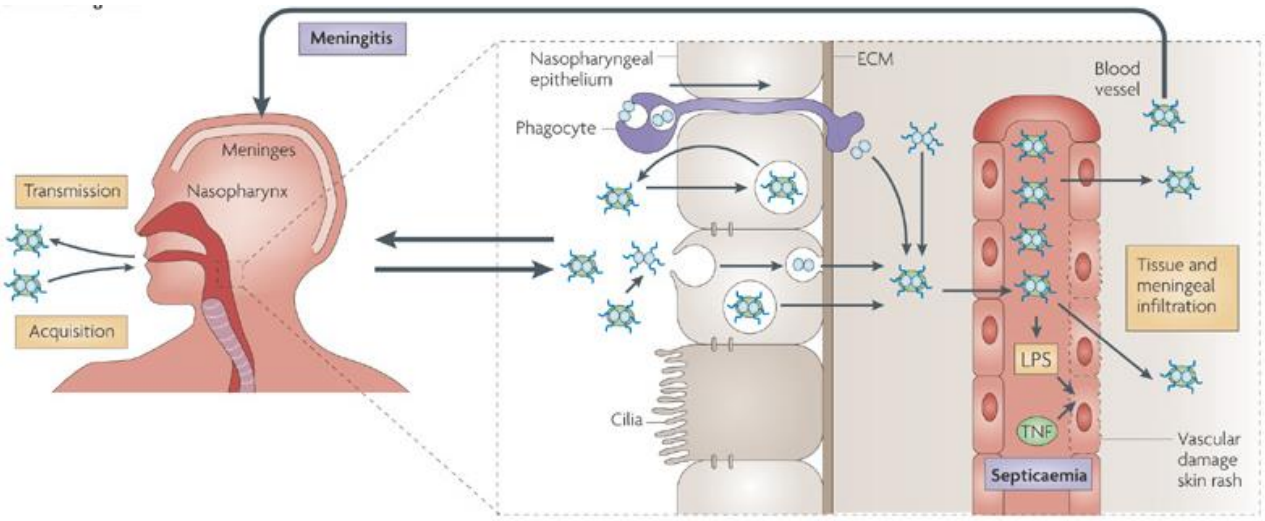


Figure 3 – Schematic representation of the pathogenesis of meningococcal infection

Figure and legend are adapted from Virji (2009). *N. meningitidis* can be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the nasopharynx and it may enter the cells. In asymptomatic carriage, bacteria that enter the body by crossing the epithelial barrier are eliminated; while, in susceptible individuals, bacteria survive and reach the bloodstream. Once in the blood, *N. meningitidis* multiply rapidly and disseminate throughout the body producing a systemic infection. Eventually, the bacterium can crosses the blood-brain barrier and infect the meninges and the cerebrospinal fluid.

Type IV pili and the outer membrane proteins Opa and Opc are the main responsible for attachment of *N. meningitidis* to epithelial cells. While type IV pili mediate the primary interaction with epithelial cells by binding to CD46 (Källström et al., 1997; Sjölinder and Jonsson et al. 2007); Opa and Opc proteins promote intimate contact and the entry of meningococci into epithelial cells. These latter interactions require down-modulation of both capsule and pili structures (de Vries et al., 1996; Deghmane et al., 2002). Opa protein binds to several members of carcinoembryonic antigen-related cell-adhesion molecule (CAECAM) family, such as CAECAM1, CAECAM3 and CAECAM6 on epithelial surface. Cell-surface-associated heparan sulfate proteoglycans (HSPGs) can also be used as receptors by Opa protein as well as by Opc protein (reviewd in Hill et al. 2010). In addition, meningococcal attachment to epithelial cells is supported by several minor adhesins,

including Neisserial adhesin A (Nad A), Neisseria hia homologue A (NhhA), adhesion and penetration protein (App) and Neisseria Heparin Binding Antigen (NHBA) (Serruto et al., 2003; Capecchi et al., 2005; Scarselli et al., 2006; Vacca et al., 2016).

After adhesion, interaction of *N. meningitidis* with epithelial cell induces the reconstruction of the cell surface. Microvilli of epithelial cells elongate surrounding the bacteria and eventually engulf meningococci by invagination of the cell membrane and vacuole formation (Stephens et al., 1983).

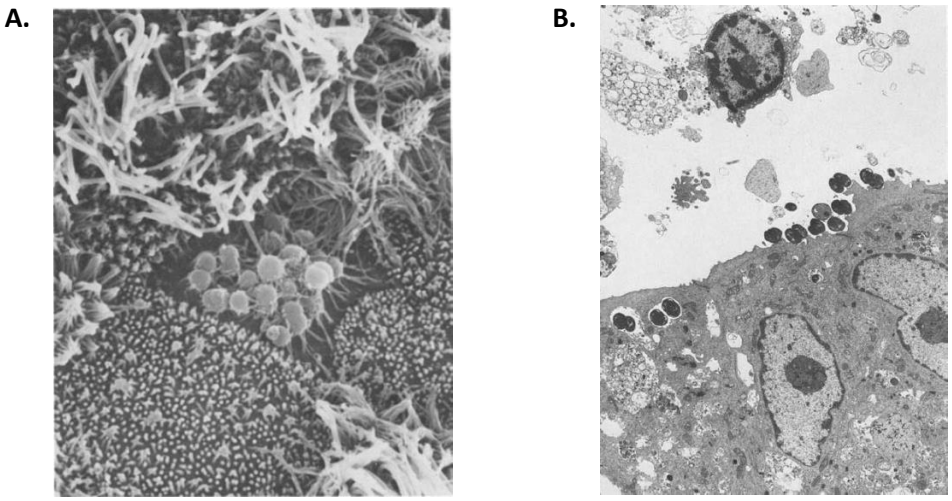


Figure 4 – Attachment of *N. meningitidis* to nasopharyngeal mucosa

Figures and the legend were adapted from Stephens et al. (1983). A) Scanning electron micrograph showing the interaction of piliated transparent *N. meningitidis* with human nasopharyngeal mucosa 6-8 hours after infection. Meningococci attached to only some of the nonciliated cells. The attachment appeared to stimulate interaction of the meningococci with the microvilli, events that sometimes created membrane folds and invaginations on the epithelial cell surface. B) Transmission electron micrograph of phagocytosis of meningococci by certain nonciliated nasopharyngeal cells. Micrograph shows nasopharyngeal mucosa 12-24 h after infection with piliated transparent *N. meningitidis*. Certain nonciliated epithelial cells appear to be engulfing meningococci by invagination of the cell membrane and vacuole formation.

Penetration of meningococci through the epithelial layer occurs by a transcellular route without disrupting the epithelium integrity (Sutherland et al., 2010), and this is not an unusual event since bacteria are found in subepithelial tissue of healthy individuals (Sim et al., 2000). While in asymptomatic carriers, bacteria that cross the epithelial barrier are eliminated; in susceptible individuals, bacteria survive and enter the bloodstream resulting in a systemic infection.

Manifestations of invasive meningococcal disease only appear when *N. meningitis* has already reached the bloodstream. Within the bloodstream, meningococci produce a strong inflammatory response and activate the complement and the coagulation cascades (reviewed in Lewis and Ram, 2014). Lipid A moiety of lipopolysaccharide is the bacterial component responsible for eliciting the inflammatory response associated with meningococcal sepsis (Pridmore et al., 2001; Zughaier et al.,

2004). It stimulates the overproduction of various cytokines (IL-1, IL-6 and TNFα) and chemokines (monocyte chemoattractant protein (MCP)–1, macrophage inflammatory protein (MIP)-1 α, and IL-8) within the vasculature (Waage et al., 1989; Pridmore et al., 2001; Zughaier et al., 2004; Møller et al., 2005) that ultimately leads to endothelial damage and capillary leakage, leading to necrosis of peripheral tissues and multiple organ failure. The high levels of circulating lipopolysaccharide during meningococcal infection are the result of a large numbers of bacteria in the bloodstream.

Yet in the blood, *N. meningitidis* encounters numerous host killing mechanisms, including complement-mediated antibody-dependent killing and opsonophagocytic killing (Granoff, 2009); however the pathogen has developed several strategies to overcome to those mechanisms and survive. Polysaccharide capsule is the most important bacterial component in preventing direct killing by the complement. It is present in all invasive clinical isolates and capsule negative mutants are highly sensitive to killing by human sera (Vogel et al., 1997). Recruitment of negative complement regulators to the bacterial surface, such as factor H, vitronectin and C4 binding protein (C4bp), is another strategy used by the bacterium to subvert the complement system (Lewis and Ram, 2014). Several meningococcal components have redundant function in recruiting complement regulators. For example, fHbp, NspA, PorB2 and to sialyated lipopolysacaride are all able to bind human factor H, and inhibit the alternative complement pathway (Madico et al., 2006; Lewis et al., 2010; Lewis et al., 2012; Lewis et al., 2013). Moreover, other membrane vesicles released from bacterial surface during the infection are involved in meningococcal serum resistance. They are able to initiate complement activation in the human blood (Bjerre et al., 2002) likely diverting complement activation and antibody binding away from live intact bacteria. Recently, an additional mechanism of immune evasion has been reported: meningococcal NalP protease cleaves complement component C3 at four amino acids upstream from the natural C3 cleavage site generating a longer C3b-like fragment. This C3b-like fragment is degraded in the presence of the complement regulators (factors H and I), limiting the deposition of C3b on the bacterial surface (Del Tordello et al., 2013).

N. meningiditis has the ability to form microcolonies on the apical surface of capillary endothelial cells of various origins, including spleen, skin, liver, kidney, heart and brain (Coureuil et al., 2014). Only low blood flow conditions, such as existing in capillaries and microvessels, allow the adhesion of the bacterium to the endothelial cells. However, following the initial attachment, bacteria can resist to high shear stress levels and remain attached over long periods of time. Dividing cells remain attached to the colony (Mairey et al., 2006). As introduced before Type IV pili and Opc are the most important adhesins for *N. meningitidis* to interact with endothelial cells (Virji, et al., 1991; Virji et al., 1992; Virji et al., 1993-A; Virji et al., 1993-B). Pili promote the initial adhesion onto epithelial cells and also the formation of aggregates, which prevent the detachment of bacteria

from the colony (Hélaine et al., 2005; Mairey et al., 2006). Opc protein binds to a activated vitronectin facilitating the attachment and the entry into brain endothelial cells (Sa E Cunha et al., 2010). In addition, Opa protein is probably involved in bacterial adhesion to endothelial cells, since CEACAM1 protein is expressed on endothelial cells and its expression increased in inflammatory conditions (Muenzner et al., 2000). *N. meningitidis* is found both in intracellular vacuoles and in the intercellular space between adjacent retracted endothelial cells (Dupin et al., 2012). Frequently, meningococci translocate from the bloodstream across the blood-brain barrier, proliferate in the cerebrospinal fluid, and cause meningitis.

3. Control and prevention of meningococcal disease

Parental administration of antibiotics is the primary therapeutic treatment for a patient whom has been diagnosed an invasive meningococcal infection as well as for a suspected case. Multiple antimicrobial agents are effective against *N. meningitidis*, including cefotaxime, benzylpenicillin, ceftriaxone and chloramphenicol. The widespread use of antibiotics has dramatically reduced the mortality for invasive meningococcal disease from 70-85% to 8-14% (MacNeil and Cohn, 2011); however, some cases of antibiotic resistant strains have been reported (Yagupsky et al., 1993; Fermer et al., 1995; Latorre et al., 2000; Jorgensen et al., 2005; Ibarz-Pavón et al., 2011). After a case of invasive meningococcal disease, chemoprophylaxis of close contacts is offered by health care institutions in order to eradicate carriage of invasive strains and prevent secondary cases. Persons who have had close contact with the patient are at greatly increased risk for contracting the disease, indeed (Cartwright et al., 1991). Rifampicin, ceftriaxone and ciprofloxacin are recommended antibiotics for chemoprophylaxis (ECDC GUIDANCE, 2010; MacNeil and Cohn, 2011; Guidance for public health management of meningococcal disease in the UK, 2012). Carriage eradication is also recommended in convalescent patients prior to discharge from the hospital, in particular for patients who received penicillin as therapeutic treatment. A secondary antibiotic administration is given to these patients for ensuring carriage eradication (ECDC GUIDANCE, 2010; Guidance for public health management of meningococcal disease in the UK, 2012). Since meningococcal invasive disease has been recognized as a vaccine-preventable disease, vaccination is also a recommended option for prophylaxis and it has been used in response to outbreaks (ECDC GUIDANCE, 2010; Guidance for public health management of meningococcal disease in the UK, 2012). In addition, some countries already include meningococcal vaccines in their routine immunization programs in order to prevent and reduce the incidence of invasive meningococcal diseases. Several vaccines are currently available against serogroups A, B, C, W135 and Y. Vaccines consisting of purified polysaccharides or polysaccharides conjugated to a carrier protein have been developed for serogroups A, C, W135 and Y, taking advantage of the protective immunity conferred

by capsular polysaccharide antigens (Ali et al., 2014). Both monovalent and multivalent compositions are available in the market (Ali et al., 2014). Vaccine development for serogroup B has been more challenging, since its capsular polysaccharide is structurally identical with a component of human fetal NCAM (neural cell-adhesion molecule), crucial for functional plasticity of the central and peripheral nervous systems (Rouphael and Stephens, 2012). Being a self-antigen, it is poor immunogenic (Bruge et al., 2004) and it poses safety concerns regarding the possibility to elicit autoantibody. For these reasons, different strategies have been used for vaccine development, and recently two protein-based vaccines for serogroups B have been licensure: Bexsero and Trumenba. Introduction of meningococcal vaccines worldwide has been resulted in a reduction of the overall burden of the disease. An example in which immunization with meningococcal vaccine has been effective in prevention of disease caused by vaccine-homologous serogroups of *N. meningitidis* is reported in Figure 5.

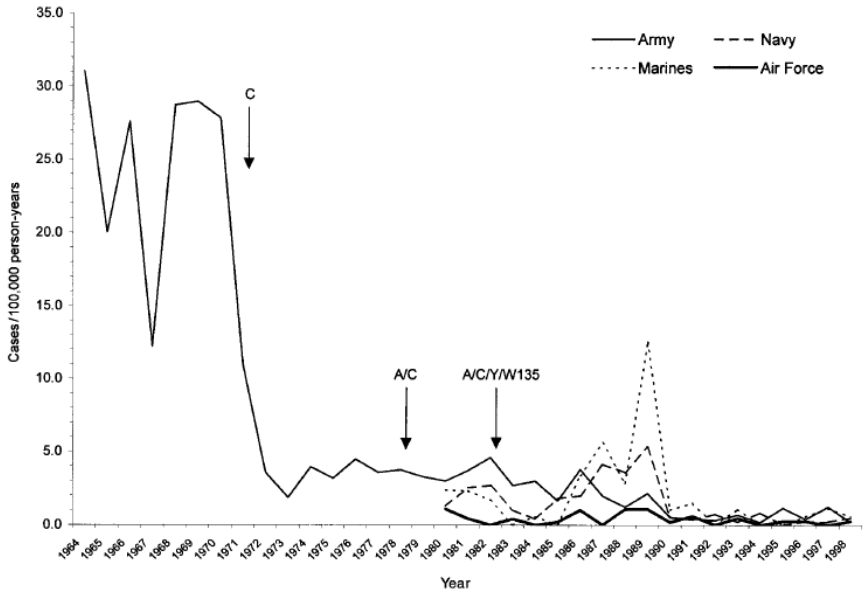


Figure 5 –US military: a successful example of immunization program in prevention of meningococcal disease

Incidence rates of meningococcal disease among active-duty members of the US Armed Forces in relation to routine uses of meningococcal vaccines with various serogroup-specific components, 1964–1998. Arrows indicate dates of initiation of routine use of various vaccines. Figure and legend adapted from Brundage et al. (2002)

Historically, military recruits have been at high risk of acquiring meningococcal disease. Since 1971, all new recruits in the US military have been immunized against *N. meningitidis* during their first days of service. In 1978 and 1982, respectively, bivalent (serogroups A and C) and quadrivalent (serogroups A, C, Y, and W135) meningococcal vaccines have been incorporated into routine recruit immunization schedules. The incidence rate of meningococcal disease in the US military decreased from 23.6 case/100 000 population to 1.4 case/100 000 population in this period of time (1971-1998), and it further decreased at >1 case/100 000 population in the following years (Brundage et al., 2002; Broderick et al., 2015).

4. Bexsero vaccine

The Bexsero vaccine, also known as 4CMenB vaccine, has been approved in Europe, Canada, Australia, the USA, and some Latin American countries for active immunization to prevent invasive disease caused by *N. meningitidis* serogroup B. It is a multicomponent vaccine containing four main immunogenic components: three recombinant protein antigens: fHbp (factor H binding protein), NadA (Neisserial adhesin A), NHBA (Neisserial heparin binding antigen), and the outer membrane vesicles derived from meningococcal NZ98/254 strain carrying PorA (Porin A) antigen. PorA is the major immunogenic component able to induce protective antibodies against *N. meningitidis*. However, this antigen confers protection only against homologous PorA serosubtype-expressing strains since primary epitopes of PorA antigen are highly variable among meningococci (Bart et al., 1999). On the contrary, antibodies arise against fHbp, NadA and NHBA antigens have been shown to be cross-reactive to different peptide variants from those contained in the vaccine formulation allowing to extend the coverage of the Bexsero vaccine (Welsch et al., 2003). fHbp, NadA and NHBA antigens have been discovered using the innovative approach of the reverse vaccinology (Pizza et al., 2000). Briefly, during the sequencing process to determine the complete genome of the MC58, unassembled DNA fragment have been analyzed to identify Open Reading Frames (ORFs) that potentially encode novel-surface exposed proteins. DNA fragments of hypothetical genes have been cloned in *E. coli*, and the expressed proteins have been purified and used to immunized mice. Then, sera of immunized mice have been analyzed with several techniques in order to test the suitability of these proteins as candidate antigens for conferring protection against *N. meningitidis* serogroup B. Candidate antigens have been selected for being actually exposed on bacterial surface and, most importantly, for their ability to elicit functional antibody response able to induce complement-mediated antibody-dependent killing of *N. meningitidis*.

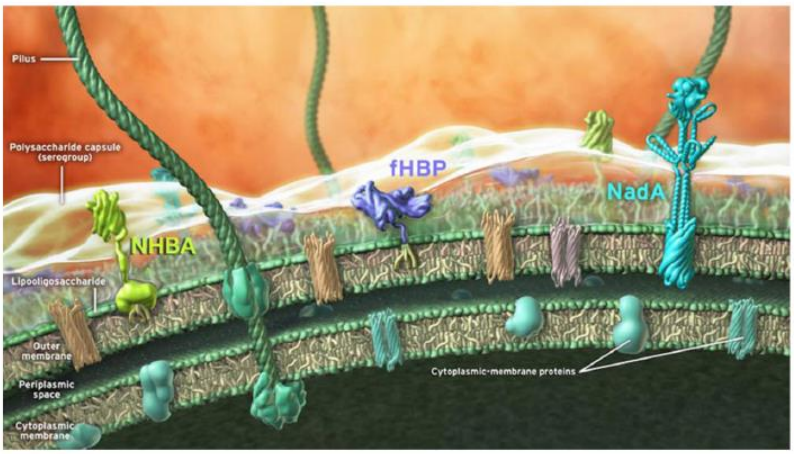
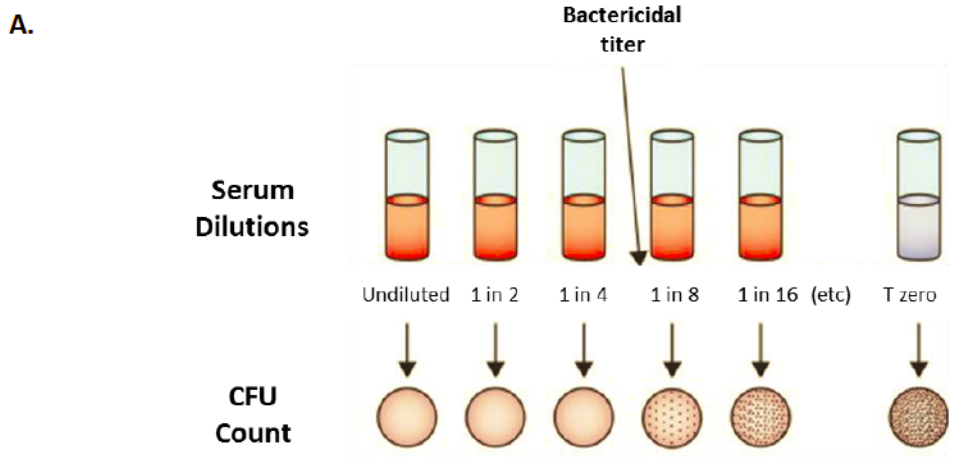


Figure 6 - Schematic representation of the Bexsero vaccine antigens on the surface of *N.meningitidis*

Figure adapted from Serruto et al. (2012). The different bacterial compartments (outer membrane, periplasmic space, cytoplasmic membrane) and the main antigens identified through reverse vaccinology approach (NHBA, fHbp and NadA) are depicted. Other components of the meningococcal membranes are also shown (pilus, polysaccharide capsule, lipooligosaccharide and integral inner and outer membrane proteins).

fHbp, NadA and NHBA antigens are proteins that contribute to the ability of the bacterium to cause disease. In details, fHbp is a surface lipoprotein that binds to human factor H, the major inhibitor of the alternative complement pathway, and by recruiting this inhibitor on the bacterial surface down regulates the complement cascade favoring the survival of *N. meningitidis* in human blood (Madico et al., 2006). NadA is a surface protein that mediates the adhesion and the entrance of the bacterium in epithelial cells and also likely in endothelial cells (Capecchi et al., 2005; Scetti et al., 2016). NHBA is a surface lipoprotein able to bind heparin and heparan sulfate proteoglycans, and it is involved in different steps of meningococcal pathogenesis, including bacterial adhesion to epithelial cells, biofilm formation, bacterial survival in the blood and vascular leakage (Serruto et al., 2010; Arenas et al. 2013; Casellato et al., 2014; Vacca et al., 2016).

Effectiveness of the Bexsero vaccine against diverse serogroup B strains is evaluated using a serological surrogate marker for protection: the serum bactericidal antibody (SBA) assay (Figure 7). This assay measures the concentration of functional antibodies induced by the vaccination that are able to lyse meningococci in the presence of complement, otherwise known as complement mediated killing via the classical pathway. This is the primary mechanism of protection against invasive meningococcal disease applied by the natural immunity (Goldschneider et al., 1969).



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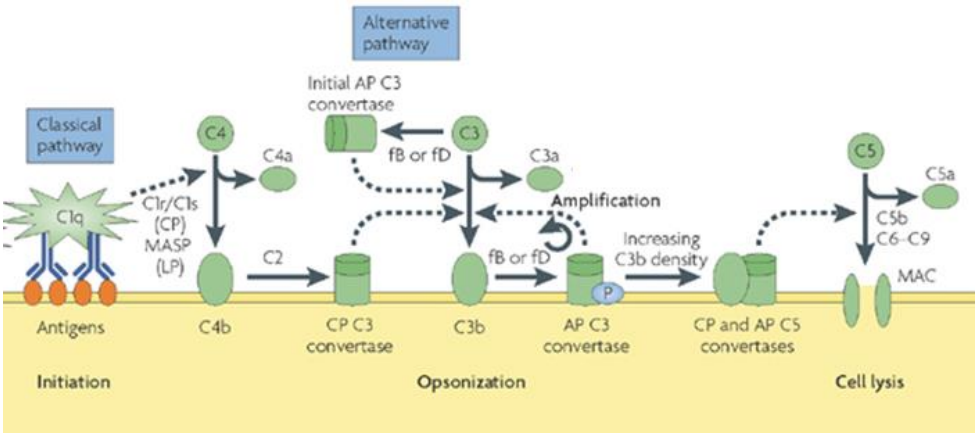


Figure 7 - Schematic representations of the molecular mechanism involved in the killing of bacteria during SBA assay

A) Schematic representation of SBA assay. Serial dilutions of the test serum are incubated with appropriate target strain in presence of a source of active complement. Antigen-specific antibodies contained in the tested serum bind to the target cell surface and lead to the activation of the classical pathway of complement, which ultimately results in the lysis of the target cell. After the incubation, survived bacteria are plated, and colony forming units (CFUs) are counted in the next day. SBA titer is defined as the dilution of the test serum that results in at least a 50% decrease in colony forming units (CFUs) at 60 minutes, compared with the number of CFUs at time zero. It is expressed as a reciprocal number.

B) Overview of complement cascade activation via the classical pathway. The classical pathway is activated when the recognition molecules C1q of C1 complex senses antibody/antigen clusters and activate associated serine proteases (C1r, C1s) that cleave C4 and C2 to form the C3 convertase (C4b2b) of the Classical Pathway (CP) on the target cell. The C3 convertase cleaves C3 into the anaphylatoxin C3a and C3b. C3b molecules covalently bind to the bacterial surface (opsonization), and promote the formation C3 convertase of the Alternative Pathway (AP). Factor B (FB) binds to C3b and gets activated by Factor D (FD) to form the final Alternative Pathway C3 convertase (C3bBb), which activates more C3 to C3b and fuel an amplification loop. Increasing densities of C3b deposition favour the generation of C5 convertases, which activate C5 to release the anaphylatoxin C5a and C5b, which, after tiered interactions with C6–C9 and membrane insertion, in turn forms membrane attack complexes (MACs), which leads to lysis, damage, or activation of target cells. Figure adapted from Lambris et al.(2009).

Different sources of active complement can be used for performing SBA assay, either intrinsic from the serum being tested or the addition of exogenous complement, either from a human or from another species such as rabbit. An SBA titre of ≥1:4 using human serum as the exogenous complement source (hSBA) is established to correlate with protection against invasive meningococcal disease. This threshold has been defined on the basis of the observation that there is an inverse correlation between SBA titre of ≥1:4 and incidence of invasive meningococcal disease (Goldschneider et al., 1969). Being the Bexsero vaccine composed of 4 main antigens, effectiveness is evaluated performing four distinct SBA assays with 4 different reference strains, one specific for each antigen, in order to measure the contribution of each component in conferring protection against the disease (Vesikari et al., 2013).

5. Neisseria Heparin Binding Antigen

Neisseria Heparin Binding Antigen (NHBA), also known as GNA2132 (Genome-derived *Neisseria* antigen 2132), is a surface-exposed lipoprotein specific for *Neisseria* and is one of the three main protein antigens of the Bexsero vaccine. This antigen is recognized by sera of convalescent patients after meningococcal disease and it is able to elicit antibodies in animal and humans that cross-react with diverse NHBA subvariants (Welsch et al., 2003). Antibodies against NHBA are bactericidal whereas SBA assay is performed using rabbit complement as a source of complement (Pizza et al., 2000; Giuliani et al. 2006, Serruto et al., 2010). With human complement, conflicting data are reported in literature (Welsch et al., 2003; Donnelly et al., 2010; EMA, 2012; Vesikari et al., 2013; Partridge et al., 2016). For example, anti-NHBA antibodies have bactericidal activity on M10713 strain, the reference strain that has been used in clinical trials for evaluating the contribution of this antigen in the protection against meningococcal disease (Vesikari et al., 2013). However, it has been reported by Welch and colleagues (2003) that anti-NHBA sera do not have any bactericidal activity on other meningococcal strains in hSBA assay. In spite of this, these sera passively protected infant rats against meningococcal bacteremia after challenge with all hSBA resistant strains (Welsch et al., 2003). In addition, antibodies against NHBA are functional in inhibiting meningococcal adhesion to epithelial cells (Vacca et al., 2016).

The gene encoding for NHBA protein is present in all *N. meningitidis* strains, and it is also found in *N. gonorrhoeae* and the commensal species *N. lactamica*, *N. polysaccharea*, and *N. flavescens* (Pizza et al., 2000; Jacobsson et al., 2006; Bambini et al., 2009; Lucidarme et al., 2009; Lucidarme et al., 2010, Muzzi et al., 2013). Gene sequences from genetically diverse group B strains reveal the existence of more than 400 protein subvariants that have some association with clonal complexes and sequence types (Muzzi et al., 2013; Bambini et al., 2009). Hyper variable regions within the *nhba* gene are located mostly in the amino-terminal half of the protein; while the sequence corresponding to the C-terminal region of the protein is highly conserved in all strains (Pizza et al., 2000; Esposito et al., 2011). In addition, sequences of the lipobox motif (-LXXC-) and the central part of the Arg-rich motif (-RSARSRRS-), two important functional motifs described for NHBA protein (see below), has been shown to be present in all of *Neisseria* species harboring *nhba* gene (Muzzi et al., 2013).

Levels of *nhba* expression are temperature dependent; *nhba* gene is strongly upregulated at 32°C (Lappann et al. 2016), a temperature encountered by the bacterium during transmission and initial colonization of the nasopharynx (Keck et al., 2000). Moreover, transcription of *nhba* gene is induced upon contact with the epithelial cells whereas the strain harbors the Contact Regulatory

Element of Neisseria (CREN) regulatory element in the promoter region of the gene, as it has been shown for MC58 strain (Deghmane et al., 2003).

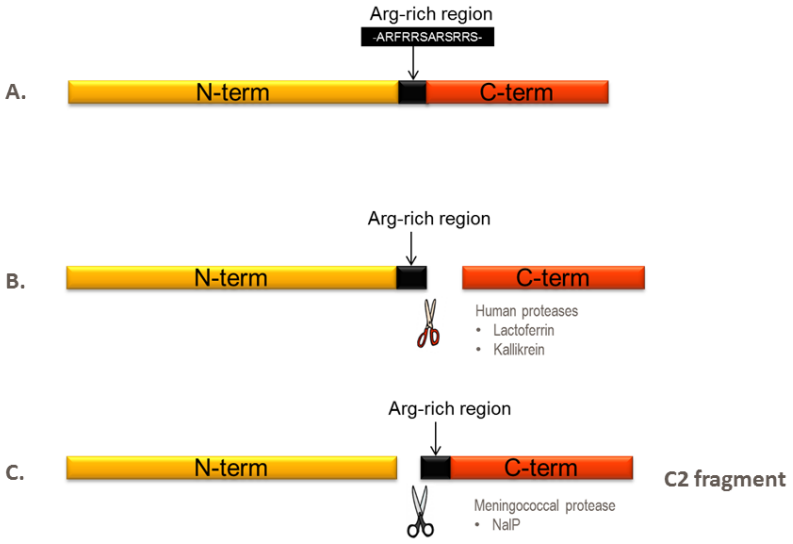


Figure 8 – Schematic structural representation of NHBA full-length protein and its digested fragments

NHBA protein can be structurally divided into an N-terminal and a C-terminal domain separated by an Arginine (Arg)-rich motif (Figure 8) (Serruto et al., 2010). While the N-terminal domain (residues 1 to ~230, in strain 2996) is annotated as an intrinsically unfolded region by commonly used structure prediction algorithms, the structure of the C-terminal domain of NHBA has been resolved by NMR spectroscopy. NHBA C-terminal region (residues 246 to 428, in strain 2996) is an independently folded globular domain with high thermal stability, and its structure consists of a β-barrel with 8 anti-parallel strands. Detergents are able to interact with the surface of the β-barrel, and this observation leads to hypothesize that C-terminal domain may have a co-factor on bacterial surface to who interact with, and this interaction may have a role in stabilizing the disordered N-terminal region in the full-length protein. NHBA C-terminal domain shows structural similarity to transferrin binding protein B (TbpB) and to the C-terminal domain of fHbp, even if these proteins share very low sequence similarity (Esposito et al., 2011). Functional characterization of NHBA protein reveals that the Arg-rich motif is responsible for the protein binding *in vitro* to heprin and heparan sulfate proteoglycans (Serruto et al., 2010; Vacca et al., 2016). In addition, in the first part of N-terminal region, it is present a lipobox motif (-LXXC-), which likely anchors the protein on bacterial surface (Serruto et al., 2010).

NHBA protein is very susceptible to protease cleavage; it can be cleaved upstream or downstream the Arg-rich motif (Figure 8). The cleavage results in the release of a C-fragment into the supernatant; while the N-fragment remains anchored to the bacterial surface (Serruto et al., 2010). Human proteases, including lactoferrin and kallikrein, cleave NHBA protein downstream the Arg-

rich motif generating a C-terminal fragment that does not contain the Arg-rich motif (Serruto et al., 2010; Pantano MSc thesis). Meningococcal NalP protease cleaves NHBA protein upstream the Arg-rich motif, instead, generating a C-terminal fragment that contains the Arg-rich motif. This fragment is referred as C2 fragment (Serruto et al., 2010). The cleavage site of human proteases are present in all NHBA subvariants; on the contrary, cleavage site of NalP protease is less conserved (Muzzi et al., 2013; Vacca PhD thesis).

During meningococcal infection, *nhba* expression has been shown to promote bacterial adhesion to epithelial cells, and this propriety is strongly evident at lower temperature than 37 °C (Vacca et al., 2016; Lappann et al. 2016). In addition, it contributes to initial biofilm formation (Arenas et al., 2013; Lappann et al. 2016). These findings highlighted the importance of this antigen in the colonization step of meningococcal infection. Moreover, it has been reported that, in presence of heparin, *nhba* expression increase the survival of unencapsulated *N. meningitidis* in human sera (Serruto et al., 2010). Lastly, recombinant C2 fragment has been shown to enter endothelial cells, and to alter the endothelial permeability *in vitro*. ROS production and phosphorylation/degradation of the adherens-junction protein VE-cadherin are involved in this latter process (Casellato et al., 2014).

Materials and Methods:

1. Cell culture and cell supernatant preparation

1.1. Calu-3 cell culture

Calu-3 epithelial cells, derived from lung adenocarcinoma (ATCC® HTB-55™), were cultured in T75 flasks, with DMEM:F12 supplemented with L-Glutamine (Gibco™-Themo Fischer Scientific), 10% fetal bovine serum (Gibco™-Themo Fischer Scientific) and Penicillin-Streptomycin (Gibco™-Themo Fischer Scientific), at 37°C in 5% CO_2. For cleavage assay using recombinant proteins, cells were seeded on 24-well plate at a density of 0.1-0.15 × 10^6 cells per well. Cells were grown for 2-3 days until ~80-90% confluence before performing the assay.

Polarization of Calu-3 cells was performed according to the method developed by Sutherland et al. (2010). Briefly, cell monolayers were grown on 0.33 cm^2, 1µm-pore-size, BD Falcon cell culture inserts (BD Bioscience) containing polyethylene terephthalate membranes in 24-well plate. Calu-3 cells, between passages 4 and 8, were seeded at a density of 0.15 × 10^6 cells per transwell, onto the apical side of membranes that were previously coated with a solution of collagen type I from rat tail (10ug/cm^2; Sigma). Cell monolayers were maintained with 0.8 mL of culture medium in the apical chamber and 0.5 mL in the basolateral chamber. Cells were allowed to grow and differentiate for 5-6 days, with the media changed every second day. Cell polarity and tight junction barrier function were verified by measuring trans-epithelial electrical resistance (TEER) using an epithelial volt ohmmeter (EVOM2; World Precision Instruments) attached to STX chopstick electrodes (World Precision Instruments). Cultures with TEER values of more than 1,400 Ω were retained for experimentation.

1.2. NHBE cell culture

Primary normal human bronchial epithelial (NHBE) cells, isolated from a single healthy donor (Lonza CC-2540S), were differentiated according to the manufacturer's instructions. Briefly, cells were expanded in T75 flasks, using bronchial epithelial basal growth medium (BEBM; Lonza) supplemented with the BEGM (bronchial epithelial cell growth medium) BulletKit (Lonza), as recommended by the supplier, at 37°C in 5% CO2 until ~80% confluence and used between passages 1 and 3. Then, cells were dissociated using StemPro Accutase cell dissociation reagent (Life Technologies) and were seeded onto semipermeable membrane supports (12-mm diameter, 0.4-mpore size; Costar) that were previously coated with a solution of collagen type I from rat tail (Gibco) at a concentration of 0.03 mg/mL. Cells were seeded at a density of 0.1 x 10^6 cells per well using bronchial air-liquid interface (B-ALI) medium (Lonza) supplemented with the B-ALI BulletKit

(Lonza). When confluence was reached, the apical medium was removed, and an air-liquid interface was established to trigger differentiation. Cells were maintained at the ALI for at least 28 days prior to use in biological assays, being the basolateral medium changed every second day, to ensure a differentiated cell population with a mucociliary phenotype. The apical side was rinsed with phosphate-buffered saline (PBS) every week to remove excess mucus production. Cell polarity and tight junction barrier function were verified by measuring trans-epithelial electrical resistance (TEER) using an epithelial volt ohmmeter (EVOM2; World Precision Instruments) attached to STX chopstick electrodes (World Precision Instruments). Cultures with TEER values of more than 1,200 Ω were retained for experimentation.

1.3. HBEC cell culture

Simian virus 40 large T antigen-transformed Human Brain microvascular Endothelial Cells (HBEC) from ATCC (ATCC® CRL-3245™) were cultured in T75 flasks that were previously coated with 2% gelatin solution (Sigma), in DMEM:F12 supplemented with L-Glutamine (Gibco™-Themo Fischer Scientific), 10% fetal bovine serum (Gibco™-Themo Fischer Scientific), Penicillin-Streptomycin (Gibco™-Themo Fischer Scientific), and Endothelial Cell Growth Supplement (Sigma) at the concentration of 40 µg/mL. For cleavage assay using recombinant proteins, cells were seeded on gelatine-coated 24-well plate at a density of 0.01×10^6 cells per well. Cells were grown until ~80-90% confluence before performing the assay.

On the previous day of each experiment performed with Calu-3 or polarized Calu-3 or HBEC cells or cell derivate of these cells, serum concentration in the culture medium was decreased to 1%. On the experimental day, cell monolayers were abundantly washed with DMEM:F12 supplemented with L-Glutamine (Gibco™-Themo Fischer Scientific), from now referred as DMEM/F12 medium in order to eliminate any trace of serum prior to perform the experiment.

Cell supernatants were recovered from Calu-3, or polarized Calu-3, or differentiated NHBE cells. For Calu-3 and polarized Calu-3 cells, cell supernatant were recovered after incubation of cells in DMEM/F12 medium during at least 8h or overnight, at 37°C and 5% CO_2. Cell supernatant were then collected and stored at 4°C or -20°C until use. For differentiated NHBE cells, PBS washes of the apical side routinely performed for removing excess of mucus were collected and stored at 4°C or -20°C until use.

2. Bacterial strains, growth conditions and working seed preparation

2.1. N. meningitidis

N. meningitidis strain M2934 is a clinical isolate from the United States (Giuliani et al., 2005), and it belongs to cc32 (ST-32) (Budroni et al., 2011).

M2934 strain was cultured on GC agar plates (Difco) or DMEM/F12 medium at 37 °C plus 5% CO_2. Working seeds were prepared inoculating overnight colonies grown on GC agar plate in 25ml of DMEM/F12 medium contained in 125mL- Corning® Erlenmeyer cell culture flask (Sigma). OD_{600nm} ~ 0.1 was used as start culture density. Liquid culture was grown at 37 °C plus 5% CO_2, under aerobic condition (180 rpm) until OD_{600nm} ~ 1. After adding glycerol (final concentration of 15%), liquid culture was aliquoted in 1mL criovials and stored at -80 °C until use.
For cleavage assay on live bacteria, 4 working seeds (OD_{600nm} ~ 0.6) were used to inoculate ~20 ml DMEM/F12 medium contained in 125mL- Corning® Erlenmeyer cell culture flasks (Sigma). Liquid cultures were grown at 37 °C plus 5% CO_2, under aerobic condition (180 rpm). OD_{600nm} ~ 0.1 was used as start culture density. Liquid culture was grown at 37 °C plus 5% CO_2, under aerobic condition (180 rpm) until OD_{600nm} ~ 0.5, and then used for experimentation.

2.2. E. coli

E. coli strains BL21 (DE3) (Invitrogen) were cultured at 37 °C in Luria Bertani (LB) agar plates, or LB broth, or using EnPresso® B-biosilta growth kit (Sigma); when required ampicillin was added in the medium at a final concentrations of 100 µg/mL. Liquid cultures prepared with LB broth were grown at 37 °C, under aerobic condition (180 rpm); while liquid cultures prepared using EnPresso® B-biosilta kit were grown according to the manufacturer's instructions. Details of the strains used in this work are reported in the table below.

Strain	**Carried plasmid**	**Relevant characteristics**	**Reference**
BL21 (DE3)	pET-GNA2132-MC58-his	pET21b derivative for expression of recombinant GNA2132 protein (MC58 strain - rGNA2132MC58-his)	(Serruto et al., 2010)
BL21 (DE3)	pET-GNA2132-C-his	pET21b derivative for expression of recombinant C-terminal region (293-488) of GNA2132 protein (MC58 strain - C-his)	(Serruto et al., 2010)

3. Expression and purification of recombinant proteins

EnPresso® B growth system (Sigma) was used for expression of recombinants proteins. Single colony of BL21(DE3) *E. coli* strain expressing either NHBA full-length protein or C2 fragment (Serruto et al., 2010) was inoculated in 5 mL of LB supplemented with ampicillin (final concentration 100 ug/mL) and grown over day at 37 °C, under aeration (180 rpm). Over day culture was subsequently diluted 1:100 in 75 mL of expression medium contained in Corning® Erlenmeyer baffled cell culture flasks (Sigma), and grown overnight at 30°C, under aeration (160 rpm). Expression medium consisted of 3 medium tablets (EnPresso® B kit – biosilta) dissolved in H_2O, and it was supplemented with amylase (EnPresso® B kit – biosilta) and ampicillin (final concentration 100 ug/mL). On the second day, 1.5 booster tables and amylase (EnPresso® B kit – biosilta) were added to the bacterial culture in order to maintain cell viability, and protein expression was induced by the addition of 1 mM IPTG (Sigma). Bacteria culture was grown at 25 °C, under aeration (160 rpm). On the third day, bacterial culture was centrifuge in order to collect bacteria; the pellet was store at -20 °C until protein purification.

After resuspending the pellet in NaH_2PO_4 50 mM, NaCl 300 mM, Imidazole 10 mM, pH=8, supplemented with protease inhibitors (cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche), bacteria cells were lysed by sonication. Cell lysate was centrifuge at 15,000 g, for 1 h, at 4 °C in order to collect the soluble fraction containing the expressed protein. Recombinant proteins were purified by affinity chromatography. Soluble fraction was filtered using 0.22 µm filter, and then loaded on 5 mL HisTrap FF Crude column (GE Healthcare) using a peristaltic pump (flow rate 0.5 mL/minute). After an extensive column washing with NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 30 mM, pH=8, buffer supplemented with protease inhibitors (Roche), the protein was eluted with NaH_2PO_4 50 mM, NaCl 300 mM, imidazole 250 mM, pH=8, buffer supplemented with protease inhibitors (Roche), increasing the imidazole concentration. The elution product was then dialyzed at 4 °C overnight against NaH_2PO_4 10 mM, pH=7, buffer using Snake Skin Dialysis Tubing, 10,000 MWCO, 22 mm (Thermo Scientific). A second step of purification was performed to obtain a high pure product taking advantage of the protein ability to bind heparin. Dialyzed sample was load on 1 mL HiTrap heparin HP column (GE Healthcare) using a peristaltic pump (flow rate 0.5 mL/minute). After an extensive column washing with NaH_2PO_4 10 mM, NaCl 30 mM, pH=7, buffer supplemented with protease inhibitors (Roche), the protein was eluted with NaH2PO4 10 mM, NaCl 350 mM, pH=7, supplemented with protease inhibitors (Roche), increasing the salt concentration. PD-10 Desalting column (GE Healthcare) was used for buffer exchange and protein was eluted in PBS. For each purification, flow through, washes and elution fractions were visualized by SDS-PAGE. Protein content was quantified using the BCA Kit (Themo Fischer Scientific). Purity was checked by SDS-

PAGE analysis, as well as by SEC-UPLC loading the sample on BEH200 4,6x300mm column (Waters). Size exclusion chromatography was performed using 10 mM NaH_2PO_4, 400 mM $(NH_4)_2PO_4$, pH=6, buffer. Endotoxin content was quantified by LAL assay using ENDOSAFE instrument (Charles River).

4. BSA-FITC/ Dextran-Texas Red permeability assay

The formation of intact monolayers on transwell inserts was evaluated by adding BSA- FITC (Thermo Scientific) or 10,000 MW Dextran-Texas Red (Thermo Scientific) at the concentration of 1 mg/mL to the upper chamber and measuring after 1 h the amount of labeled probe passed into the lower chamber by a fluorescence microplate reader (TECAN). Transwell inserts were used only when the intensity of fluorescence in the lower chamber was negligible. 5 µM of recombinant C2 fragment or PBS, as negative control, were added to the upper chamber of selected transwell inserts, and the fluorescence was evaluated in the lower chamber at various time intervals (45 min, 2 h, 4 h, and 24 h). The permeability of cell monolayers for a probe was quantified calculating the Δ mean of fluorescent intensity normalized versus pre-treatment value.

5. Immunofluorescence analysis

Cell monolayers were washed with PBS and fixed for 20 min in 2 % formaldehyde (Carlo Erba Reagents) in PBS. After several washing steps using PBS, cell monolayers were removed from transwell inserts by cutting the membranes. Samples were permeabilized with 0.1 % Triton X-100 in PBS for 10 min and blocked for 10 min with 10 % di goat Serum (Invitrogen). Incubation with the primary rabbit anti-ZO 1 antibody (working dilution 1: 200, REF. 402200, Lot. QA213066, Life Technologies) was performed for 1 h at room temperature (RT), under gently agitation. After several washing steps, samples were incubated for 45 min, at RT, under gently agitation, with Alexa flour 568-conjugated goat anti rabbit IgG secondary antibody (working dilution 1: 1,000, Life Technologies). All washing steps and antibodies dilutions were performed using 0.01 % Triton X-100, 3 % BSA in PBS. Lastly, after washing, labeled preparations were mounted using prolong gold antifade reagent with DAPI (Molecular probes-Life Technologies) and analyzed with a confocal microscope (Zeiss). Cell nuclei and tight junction structures were examined using the UV and red filter sets, respectively.

6. SDS-PAGE and Western Blot analysis

Proteins were separated by SDS-PAGE electrophoresis using 4-12 % or 12 % Acrylamide NuPAGE Bis-Tris Precast Gels (Invitrogen). For SDS-PAGE analysis, gels were stained with SimplyBlue Safe Stain (Invitrogen) in order to visualize proteins. For Western Blot (WB) analysis, proteins contained

in the gels were transferred onto nitrocellulose membranes. Western blots were performed according to standard procedures. NHBA full-length protein and its C-terminal fragments were identified with polyclonal mouse antisera raised against the recombinant NHBA full-length protein (working dilution 1:1,000) or against the recombinant C2 fragment (working dilution 1:1,000), respectively. An anti-mouse antiserum conjugated to horseradish peroxidase (Dako) was used as secondary antibody. Bands were visualized with Super Signal West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's instructions.

7. NHBA cleavage assay using recombinant protein

Both recombinant C2 fragment and NHBA full-length protein was used as substrates for the assay. Cell monolayers, cell supernatants, fractions of cell supernatants, plasma-purified kallikrein (Sigma), human sera (Complement Technology) were used as protease sources for the assay. 0.5 µM or 5µM of recombinant protein was incubated at 37 °C with protease source or with DMEM/12 medium, as negative control, for various time intervals (45 min, 1 h, 2 h, 4 h, 24 h). At each time point, samples were collected and the cleavage of substrates was evaluated by SDS-PAGE of WB. When required, protease sources were pre-treated with protease inhibitors 30 min before adding the substrate. Protease inhibitors used in this work were the followings: EDTA (Sigma), Leupeptin (Sigma), Pepstatin A (Sigma), GI254023X (Sigma), E-64 (sigma).

8. NHBA cleavage assay using live bacteria

Cell monolayers, grown both in T75 flasks and in transwell inserts, or cells supernatants, or DMEM/12 medium, as negative control, were incubated for 2 h, at 37 °C, 5 % CO_2, with *N. meningitidis* strain M2934. For each condition tested, 2 mL of liquid culture of M2934 strain grown until OD_{600nm} ~ 0.5 was used to prepare the inoculum. For cell monolayers, 2 ml of bacterial culture was inoculated in 5 ml DMEM/12 medium (final volume=7 mL) and used for the infection. The inoculum was added directly to the T75 flask for infecting Calu-3 cells; while it was divided equality to all the transwell inserts for infecting polarized Calu-3 cells. For cell supernatants and the negative control, 2 ml of bacterial culture was inoculated in 5 ml of cell supernatants or DMEM/12 medium. After 2 h of incubation, cell supernatants containing bacteria was centrifuge at 4,000 rpm, for 10 min in order to collect both bacterial pellet and cell supernatants. Bacteria were immediately processed for FACS analysis. Cell supernatants were filtered using 0.22 µm filter, concentrated using 10,000 MW Amicon® Ultra Centrifugal Filters (Millipore), and then processed for WB analysis.

9. FACS analysis

Bacteria were washed and then incubated for 1 h, at RT, with polyclonal mouse antisera raised against the recombinant C2 fragment (working dilution 1:500). After several washing steps, samples were incubated for 30 min, at RT, with Alexa flour 488-conjugated goat anti mouse IgG secondary antibody (working dilution 1: 1,000, Life Technologies). All washing steps and antibodies dilutions were performed using 1 % BSA in PBS. Labeled bacteria were washed and fixed for 1 h at RT, using 2 % formaldehyde (Carlo Erba Reagents) in PBS. Samples were analyzed with BD FACSCanto™ II system (BD Bioscience).

10. Fractionation of cell supernatants

Pooled supernatants prepared from polarized Calu-3 cells were fractionated by ion exchange chromatography. Sample was dialyzed at 4 °C overnight against 50 mM Tris-HCl, pH=8, using Snake Skin Dialysis Tubing, 3,500 MWCO, 22 mm (Thermo Scientific). Dialyzed sample was load on 1 mL HiTrap Q HP column (Ge Healthcare). Fractions were eluted increasing the ionic strength; a one-step NaCl gradient was performed using 50 mM Tris-HCl, 1 M NaCl, pH=8. The AKTA FPLC system was used to control the NaCl gradient (from 0 M to 1 M NaCl).

11. Mass spectrometry analysis

Mass spectrometry analysis of cell supernatants as well as intact mass measurements of NHBA fragments derived from epithelial cell processing were performed by the mass spectrometry core facility of GSK Vaccines, Siena.

12. RNA extraction and Semi-quantitative PCR

Calu-3 and polarized Calu-3 cells were used for RNA isolation. Total RNA was extracted using the TRIzol reagent (Ambion) according to the manufacturer's instructions, precipitated and resuspended in RNase free water (Ambion). The yields of total RNA obtained were quantified against water at 260 nm using Nanodrop spectropotometer. cDNA was prepared from 2 µg of total RNA by using GoScript™ Reverse transcription system with oligo(dT) primers (Promega) according to the manufacturer's instruction. The presence of cDNA for GADPH, Factor B and C3 were determined by PCR amplification. After an initial denaturation step at 94 °C for 5 min, temperature cycling was initiated. Each cycle consisted of 30 s at 98 °C, 30 s at 59 °C and 1 min at 72 °C; in total 35 cycles were performed. Primers used for PCR amplification are listed in the table below:

Primer ID	Primer sequence
GADPH forward	5’- TCGGAGTCAACGGATTTGGTCG -3’
GADPH reverse	5‘- GACTGTGGTCATGAGTCCTTCCA-3’
Factor B forward	5’- CAACAGAAGCGGAAGATCGTC -3’
Factor B reverse	5’- TATCTCCAGGTCCCGCTTCTC -3’
C3 forward	5’- TCGGATGACAAGGTCACCCT-3’
C3 reverse	5’- GACAACCATGCTCTCGGTGA -3’

Amplification products were separated by electrophoresis using 1.5 % agarose gels, and stained with SYBR Safe DNA Gel Stain (Themo Fischer Scientific) in order to be visualized.

Aims:

This study aimed at investigating the role of NHBA cleavage during meningococcal interaction with epithelial cells. In the first part of the study, we aimed to assess if NHBA-derived C2 fragment could alter the epithelial permeability and facilitate the traversal of *N. meningitidis*. However, during our investigations we found out that epithelial cells were able to process both C2 fragment and NHBA full-length protein. Thus, we subsequently aimed to investigate deeper on this cleavage using several epithelial cell models of study as well as verifying the cleavage on live bacteria. In addition, we aimed to identify the cleavage site of epithelial cell proteases within both proteins and the epithelial cell protease responsible for the cleavage.

Results:

1. NHBA-derived C2 fragment does not alter the integrity of the epithelium

It has been demonstrated that recombinant NHBA-derived C2 fragment (C2 fragment), containing the Arg-rich motif, exerts a toxic effect on endothelial cells by altering the endothelial permeability (Casellato et al., 2014). In addition, Delany and collaborators recently observed that NHBA expression and its cleavage is upregulated at temperatures lower than 37°C, which are representative temperatures encountered during initial *N. meningitidis* colonization of the nasopharynx (unpublished data; Lappann et al. 2016). At these temperatures, likely more C2 fragment would be produced. These two experimental evidences, led us to hypothesize that recombinant C2 fragment could also affect the human respiratory epithelium and facilitate the traversal of *N. meningitidis*.

We first assessed if recombinant C2 fragment was able to destabilize the integrity of epithelial cell monolayers. Since it was previously observed that serum proteases processed NHBA protein, every experiment in this study was performed using serum–free medium. Serum is a key component for cell culture maintenance, so in order to remove any traces of serum, the day before of the experiment serum concentration was decreased to 1%; and on the experiment day, cells were abundantly washed before starting the experiment. As a model of study, we selected polarized Calu-3 epithelial cells (ATCC®HTB-55™), a human tumor lung cell line widely used in meningococcal pathogenesis studies (Sutherland et al., 2010; Barrile et al., 2015; Vacca et al. 2016). Cells grew on collagen-coated permeable membranes in cell culture inserts developing morphological features of a polarized respiratory epithelium within 6 days of seeding. In details, they formed a continuous monolayer of columnar cells linked by tight junctions, with nuclei located toward the basolateral side of cells and with microvilli on the apical surface (Sutherland et al., 2010). Since polarized Calu-3 cells formed an effective barrier between the apical and basal chamber of cell culture inserts, it was possible to evaluate the integrity of cell monolayers after treatment with recombinant C2 fragment by monitoring the permeability of a fluorescently labelled probe. Briefly, following the addition of a fluorescent probe in the apical chamber, cells were treated with 5µM of C2 fragment. At each time point, media were collected from the basal chamber and the fluorescent was measured. Fluorescence of the basal chamber corresponded to the amount of probe that has been passing thought cell monolayers. *Clostridium difficile* toxin (TcdA) was included as positive control in the assay because of its well-known ability to disrupt tight junctions of epithelial barriers (Voth and Ballard, 2005).

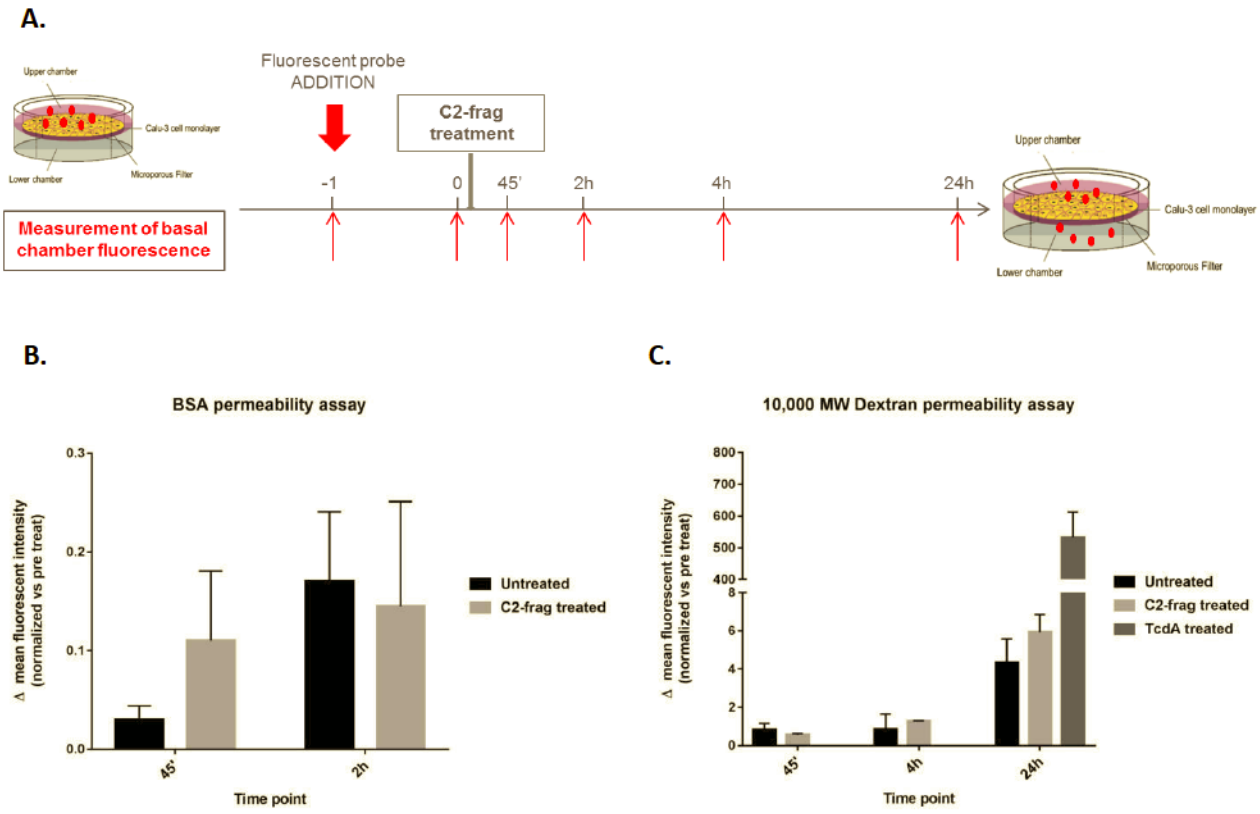


Figure 1 – Recombinant C2 fragment does not alter the permeability of the epithelium

A) Schematic representation of the experimental procedure; B) and C) Permeability assay of polarized Calu-3 cell monolayer treated with 5 µM of C2 fragment or untreated, using BSA-FITC (B) or 10,000MW Dextran-Texas Red (C) as probes. TcdA, *Clostridium difficile* toxin, was included in the assay as positive control. The permeability of cell monolayers for a probe was quantified calculating the Δ mean of fluorescent intensity normalized versus pre-treatment value.

At all time points tested (45', 2h, 4h, 24h), treatment with 5µM of recombinant C2 fragment did not alter the permeability of epithelial cell monolayers neither when we used BSA (Figure 1, B) nor smaller molecule such as 10,000MW Dextran (Figure 1, C) as probes. In addition, organization of tight junction structures of epithelial cell monolayers after treatment with C2 fragment was evaluated by confocal analysis. We looked at the distribution of Zona Occuldens protein 1 (ZO-1), an intracellular tight junction-associated protein which anchors transmembrane adhesion proteins to the actin cytoskeleton; and, we did not observed any difference comparing C2 fragment-treated cell monolayers with untreated cell monolayers (Figure 2, red staining).

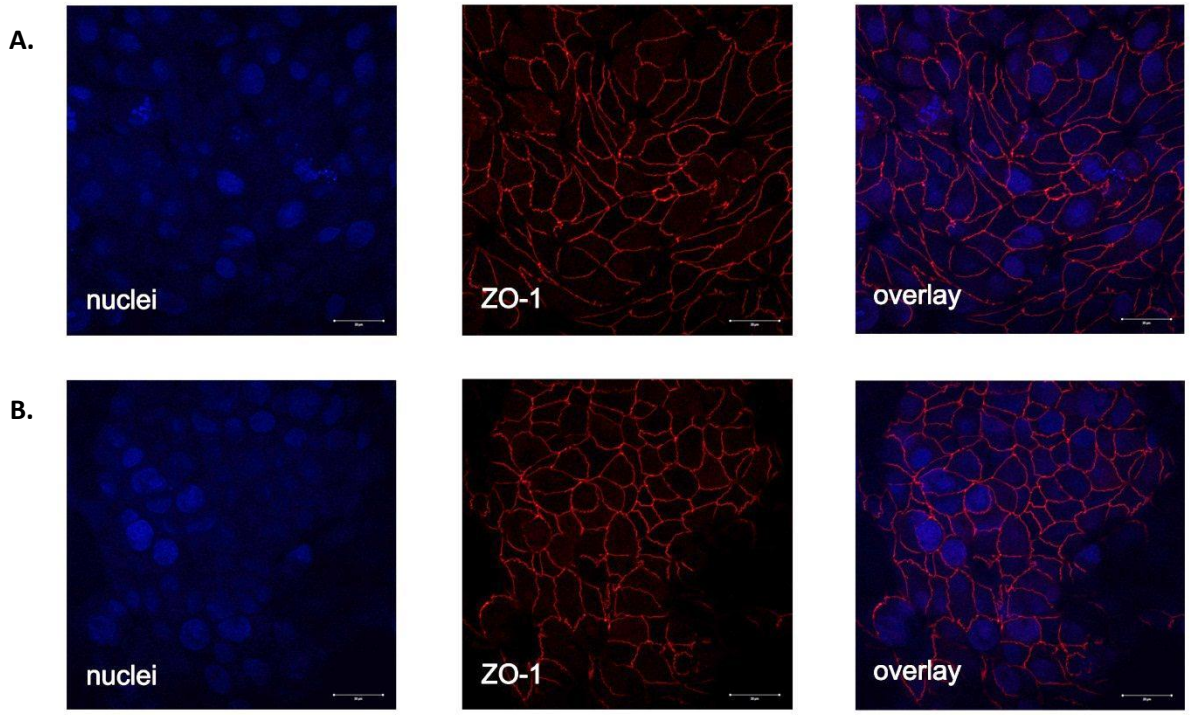


Figure 2 – Recombinant C2 fragment does not alter the organization of tight junction structures

Immunofluorescence analysis of ZO-1 distribution (red staining) in C2-fragment treated (B) or untreated (A) polarized Calu-3 cells. Cell nuclei were detected with DAPI (blue staining).

2. C2 fragment is processed by epithelial cells eliminating the Arg-rich motif

To check the stability of C2 fragment during permeability assays, we collected supernatants of treated cells at each time point, and western blot analysis was performed. Using anti NHBA C-terminal domain antibody, recombinant C2 fragment was detected on the blot as a single band with a molecular weight of 22 kDa (Figure 3A, lane 7; Figure 3B, lane 8). Surprisingly, a second shorter band below the expected one was observed in supernatants of treated cells at each time point (Figure 3A, lane 1-6), indicating that epithelial cells were able to process the C2 fragment during the assay. This cleavage increased in a time-dependent manner. Using a different experimental condition for resolving proteins, we could appreciate better the second shorter band that appeared on the blot when the C2 fragment was incubated with epithelial cells (Figure 3C, lane 1-6). Moreover, we assessed if the Arg-rich region was still present on the shorter fragment, and Western blot analysis of treated cell supernatants was repeated using anti Arg-rich motif antibody. At 24 hours, when C2 fragment is completely processed, no band was detected on the blot (Figure 3B, lane 6), indicating that the shorter fragment no longer contained the epitope of the Arg-rich motif.

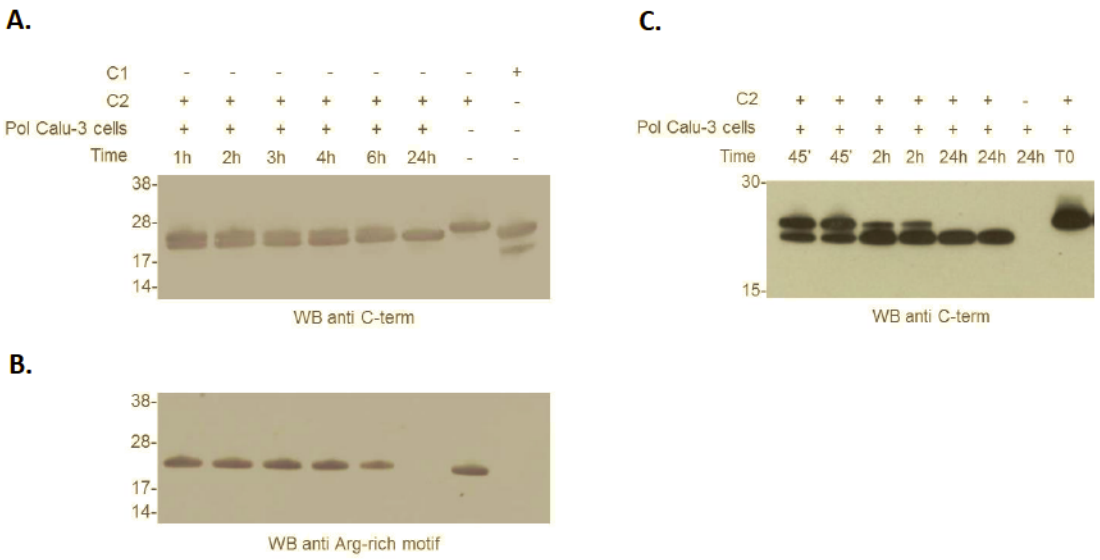


Figure 3 – Recombinant C2 fragment is processed by epithelial cells eliminating the Arg-rich motif

Western Blot analysis of supernatants of polarized Calu-3 cells treated with 5µM of recombinant C2 fragment. Analyzed samples derived from the permeability assay and they were collected at different time point (45', 1h, 2h, 3h, 4h, 5h, 6h and 24h). Polyclonal mouse sera against NHBA C-terminal domain (A and C) or polyclonal rabbit sera against the Arg-rich domain (B) were used for blotting the membranes.

3. C2 fragment is processed by endothelial cells with a slow kinetics

Subsequently, we assessed if endothelial cells, for which it has been reported that the C2 fragment exerted a toxic effect on endothelial permeability (Casellato et al., 2014), have the ability to process the C2 fragment or if this ability was exclusively possessed by epithelial cells, which are not susceptible to C2 fragment treatment. To this end, HBEC cells were treated with 5µM of recombinant C2 fragment, and cell supernatants collected at different time intervals (45', 2h, 3h, 4h, 8h and 24h) was analyzed by Western blot.

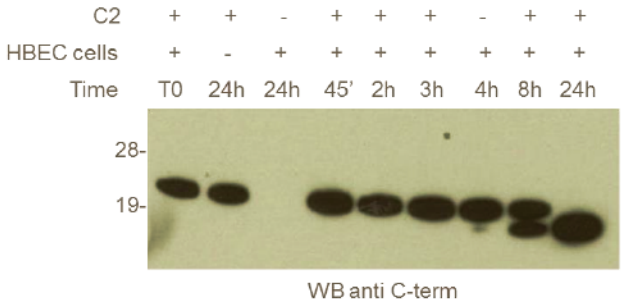


Figure 4- Recombinant C2 fragment is processed by endothelial cells with a slow kinetics

Western blot analysis of supernatants of HBEC cells treated with 5µM of recombinant C2 fragment. Cells supernatants were collected at different time point (45', 2h, 3h, 4h, 8h and 24'). Polyclonal mouse sera against NHBA C-terminal domain were used for blotting the membrane.

HBEC endothelial cells were able to process the C2 fragment, but the cleavage was detected only after 4h of incubation (Figure 4), indicating a slower cleavage kinetics of endothelial cells compared to that of polarized Calu-3 epithelial cells.

4. Characterization of NHBA cleavage by epithelial cells

4.1 Characterization of NHBA cleavage by epithelial cells using recombinant proteins

As a next step, we characterized further the cleavage of C2 fragment and NHBA full-length protein by epithelial cells using several human epithelial cell models; this allowed us to evaluate if the ability of processing the C2 fragment was a peculiar feature of polarized Calu-3 epithelial cells or it could be extended to the other epithelial cell models giving a higher physiological relevance to our observations. Time course experiments were performed treating epithelial cells with recombinant proteins and evaluating the cleavage by SDS-PAGE or Western blot analysis of treated-cell supernatants. First, we tested Calu-3 cells in order to assess if the protease responsible for the cleavage was constitutively expressed by Calu-3 cell line or if it was presented only in polarized cells. As it is shown in Figure 5, at all time points (45', 2h, 4h, 24h), Calu-3 cells were able to process both C2-fragment and NHBA full-length protein and the cleavage increased in a time-dependent manner.

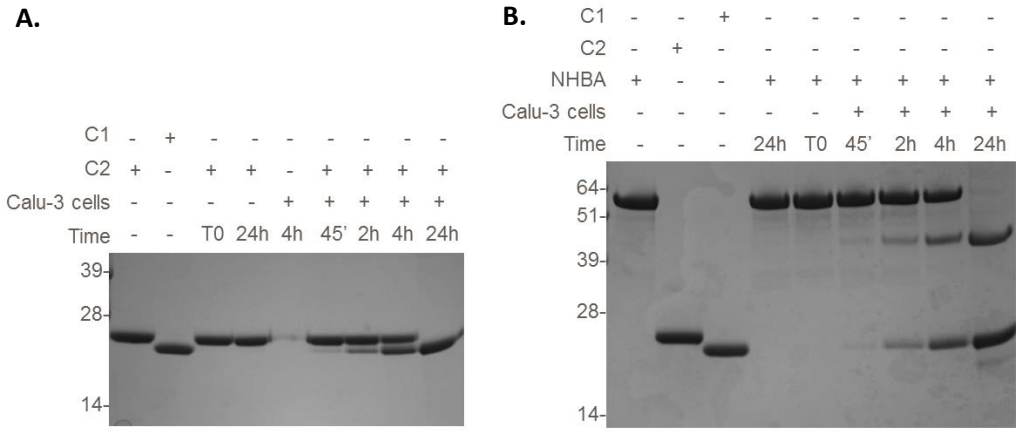


Figure 5 – Calu-3 cells process both recombinant C2 fragment and NHBA full-length protein

SDS-page analysis of supernatants of Calu-3 cells treated with 5 µM of recombinant C2-fragment (A) or with 5 µM of recombinant NHBA full length protein (B). Cell supernatants were collected at different time point (45', 2h, 4h and 24h). Proteins were stained with blue coomassie.

Then, to validate our data generated from a tumor cell line on a more physiological system, we assessed the cleavage of both C2 fragment and NHBA protein using differentiated primary Normal Human Bronchial Epithelial (NHBE) cells. This primary cell line, grown on collagen-coated permeable membranes in cell culture inserts, developed a pseudo stratified epithelium composed

of basal, ciliated and non-ciliated goblet cells (Davis et al., 2015). In addition, it allowed us to exclude the possibility that the cleavage we detected was the result of contamination by serum proteases, since NHBE cells were cultivated and differentiated with serum-free medium. Similarly to Calu-3 cells, we observed the cleavage when either C2 fragment or NHBA protein was incubated with differentiated NHBE cells (Figure 6). In both cases, it increased overtime and resulted in the generation of a C-terminal fragment that did not contain the Arg-rich motif epitope. For NHBA protein, the Arg-rich motif epitope was retained in the N-terminal part of the protein after the processing (Figure 6D, lane 7-9).

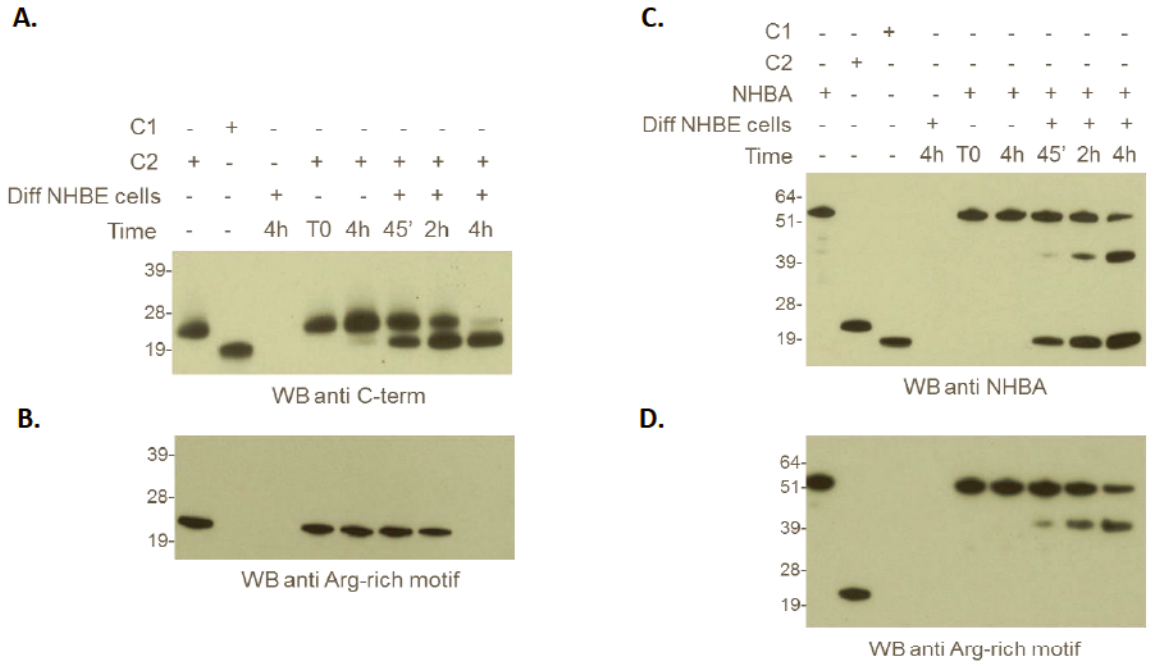


Figure 6 – Differentiated primary NHBE cells process both recombinant C2 fragment and NHBA full-length protein

Western blot analysis of supernatants of differentiated NHBE cells treated with 5 µM of recombinant C2-fragment (A and B) or with 5 µM of recombinant NHBA full length protein (C and D). Cell supernatants were collected at different time point (45', 2h, 4h and 24h). Polyclonal mouse sera against NHBA C-terminal domain (A) or polyclonal mouse sera against NHBA full-length protein (C) or polyclonal rabbit sera against the Arg-rich domain (B and D) were used for blotting the membranes.

Finally, we assessed if the protease responsible for the cleavage could be secreted or not by epithelial cells into the supernatant. To this end, serum-free medium was incubated for at least 8 hours with epithelial cells in order to produce cell supernatant. Then, cell supernatants were collected and used for the cleavage assay. All epithelial cell models, which have been tested before, were used for preparing cell supernatants.

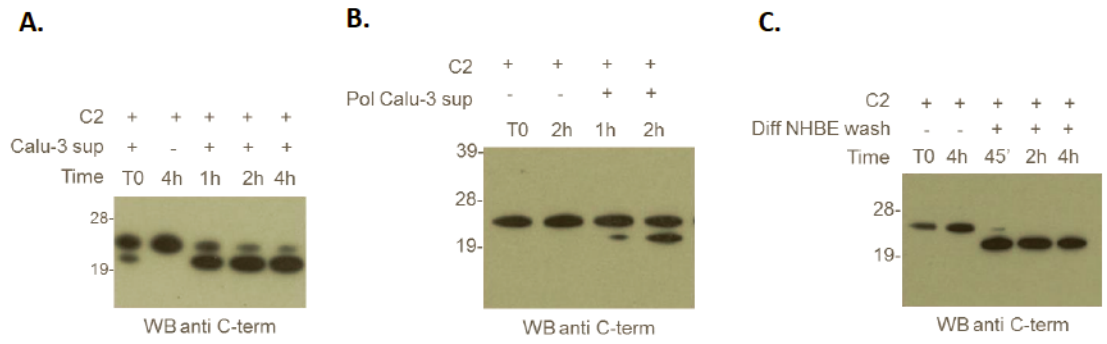


Figure 7 – Epithelial cell supernatants cleave recombinant C2 fragment

Western blot analysis of recombinant C2 fragment incubated with cell supernatants prepared from Calu-3 cells (A) or polarized Calu-3 cells (B) or differentiated NHBE cells (C). Samples were analyzed at different time point (45', 1h, 2h and 4h). Polyclonal mouse sera against NHBA C-terminal domain were used for blotting the membranes.

Western blot analysis of cell supernatants incubated with C2 fragment revealed that all the cell lines used are able to cleavage the C2 fragment (Figure 7). Thus, these results indicate that, the protease responsible for the cleavage was secreted by epithelial cells into the supernatant.

4.2 Characterization of NHBA cleavage by epithelial cells on live bacteria

To verify our *in vitro* observations on live bacteria, we decided to use natural strain M2934 expressing NHBA variant 5 in which NHBA protein was not cleaved by meningococcal proteases. NHBA variant 5 presented a deletion of five amino acids on the NalP cleavage site indeed, and it was not cleaved even if NalP protease was expressed in the strain. Thus, M2934 strain allowed us to focus exclusively on NHBA cleavage by epithelial cell proteases. For a first analysis, supernatant of Calu-3 cells was used as source of epithelial cell proteases. This cell supernatant showed a faster NHBA cleavage rate and its production was less laborious compared to supernatant prepared from polarized Calu-3 cells. Bacteria were incubated with cell supernatants or with DMEM/F12 medium, as negative control. After two hours, samples were stained with anti NHBA C-terminal – FITC antibody and the percentage of bacterial cells exposing the C-terminal domain of NHBA on its surface was measured by flow cytometry analysis (Figure 8).

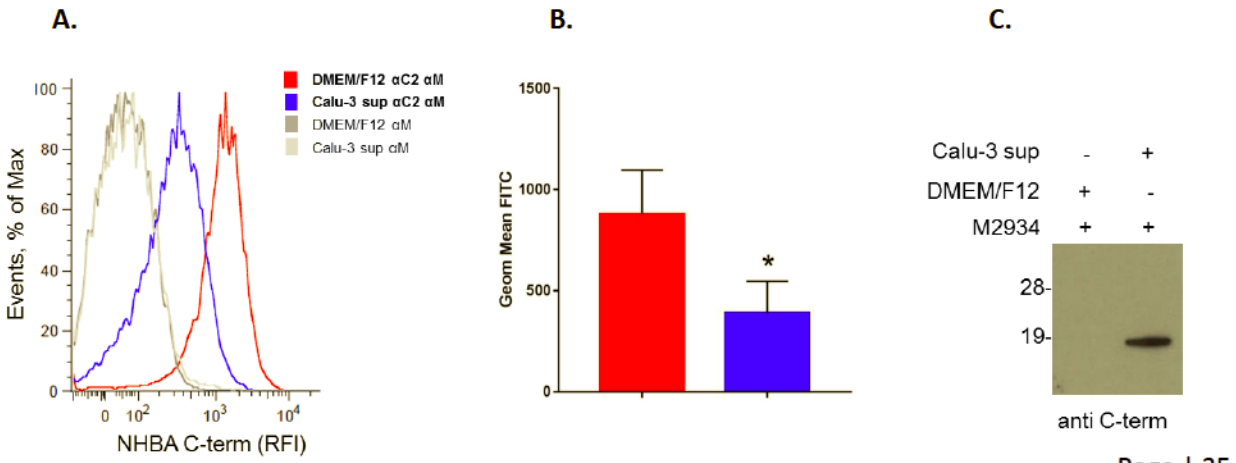


Figure 8 – NHBA cleavage by epithelial cell protease occurs on live bacteria

A) and B) FACS analysis of M2934 strain stained with anti NHBA C-terminal- FITC antibody. Bacteria were incubated for two hours with epithelial cell supernatant (blue) or medium (red); A) Histograms plot fluorescent intensity versus number of events; B) Histograms plot the geometric mean of Fluorescence Intensity (FITC). Data are means of results from three independent experiments. Error bars denote standard deviation; C) Western blot analysis of concentrated supernatants derived from M2934 bacteria incubated for 2 hours with epithelial cell supernatant or medium. Polyclonal mouse sera against NHBA C-terminal domain were used for blotting the membranes.

As it is shown in Figure 8A, we observed a negative shift in fluorescence intensity when we compared bacteria incubated with epithelial cell supernatant (blue line) to bacteria incubated with medium (red line), indicating that treatment with cell supernatants led to a decrease of C-terminal domain on surface-exposed NHBA proteins in M2934 strain. To increase the statistical significance of this observation, three repetitions of FACS analysis were performed. One-fold change reduction of fluorescence intensity was observed comparing the geometric mean of bacteria treated with epithelial cells supernatant to untreated bacteria, and this reduction was statistically significant (t-value= 0.035; Figure 8B). The evidence that epithelial cell supernatants cleaved NHBA protein on live bacteria was further demonstrated by Western blot analysis. Supernatants derived from bacteria incubated for 2 hours with epithelial cell supernatants or medium were collected, concentrated and tested for the presence of NHBA C-terminal fragment. As Figure 8C show, NHBA C-terminal fragment was only detected in the concentrated supernatant of bacteria treated with epithelial cell supernatants, corroborating that the removal of NHBA C-terminal domain from bacterial surface by epithelial cells proteases resulted in the accumulation of C-terminal fragment into the supernatant.

Subsequently, we assessed NHBA cleavage by epithelial cell proteases on live bacteria during meningococcal infection of epithelial cells. Both Calu-3 cells and polarized Calu-3 cells were infected with M2934 strain. M2934 bacteria were also incubated with medium, as negative control. After 2 hours, both not-adherent bacteria and infected-cell supernatants were collected, and we evaluated the presence of NHBA C-terminal domain on bacterial surface by FACS analysis or its accumulation into infected-cell supernatant by Western blot analysis, as described above. Unexpectedly, for both cell models, we did not detect any significant change in surface exposed C-terminal domain by FACS analysis on non-adherent bacteria with respect to bacteria incubated with medium (Figure 9A); even if NHBA C-terminal fragment was strongly accumulated into infected-cell supernatants (Figure 9B).

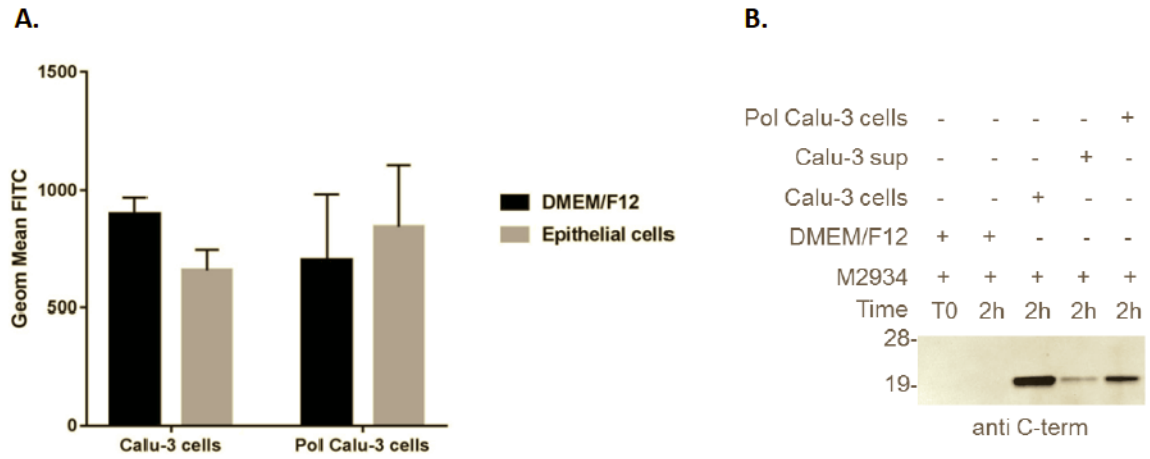


Figure 9 – NHBA cleavage by epithelial cell protease occurs during meningococcal infection of epithelial cells

A) FACS analysis of M2934 strain stained with anti NHBA C-terminal- FITC antibody. Bacteria were incubated for 2 hours with Calu-3 cells or polarized Calu-3 cells or with medium (DMEM/F12), as negative control. Histograms plot the geometric mean of Fluorescence Intensity (FITC). B) Western blot analysis of concentrated supernatants derived from M2934 bacteria incubated for 2 hours with epithelial cells or medium. Polyclonal mouse sera against NHBA C-terminal domain were used for blotting the membranes.

Since it has been reported in literature that some pathogenic meningococcal strains harbor a regulatory element in the promoter region of *nhba* gene, named Contact Regulatory Element of *Neisseria* (CRE*N*), which is involved in the induction of *nhba* transcription upon contact with the target cells (Deghmane et al., 2003), we hypothesized that M2934 strain might have this regulatory element in its *nhba* locus and thus, during bacterial interaction with epithelial cells, the expression of NHBA full-length protein might increase on bacterial surface masking the NHBA cleavage by epithelial cell proteases. To test this hypothesis, the presence of CREN element in *nhba* locus of M2934 strain was evaluated *in silico*. Genome of M2934 strain was previously sequenced and assembled internally. The sequences of *nhba* promoter region of M2934 strain was compared to the sequence of MC58 strain for which it has been described to possess a CREN regulatory element (Deghmane et al., 2003). BLAST alignment revealed an identity of 100% between the two nucleotide sequences (Figure 10A). Moreover, the expression of NHBA full-length protein during meningococcal infection of epithelial cells was assessed by performing a Western blot analysis of total protein extracts prepared from bacterial samples used in the infection assays. Using an anti NHBA antibody, a band of 64KDa corresponding to the NHBA full-length protein and a non-specific band of 97KDa were detected on the blot. The non-specific band was used as an internal loading control for normalization. As it shown in Figure 10B, expression of NHBA full-length protein strongly increased in non-adherent bacteria (lane 3 and 5) with respect to bacteria incubated with medium (lane 2). These experimental evidences supported our hypothesis. Thus, the increase of NHBA

protein production in not-adherent bacteria, due to the induction of *nhba* gene expression driven by CREN regulatory element upon the bacterial interaction with epithelial cells, likely led to an increased exposition of NHBA full-length protein on bacterial cell surface that hindered the detection by FACS analysis of NHBA processing by epithelial cell proteases.

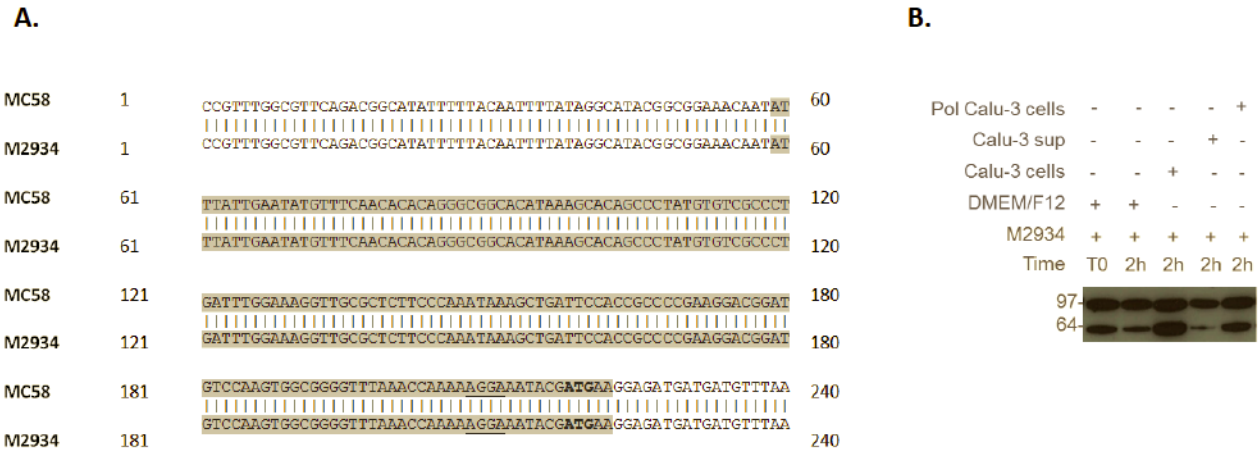


Figure 10 – Expression of NHBA protein increases during meningococcal infection of epithelial cells

A) Nucleotide sequence alignment of *nhba* promoter region containing the CREN element of M2934 and MC58 strain. Gray background highlights CREN element. Shine-Dalgarno sequence and **start codons of the ORFs** are indicated. B) Western blot analysis of protein total extract of M2934 bacteria incubated for 2 hours with Calu-3 cells or polarized Calu-3 cells or with medium, as negative control. Polyclonal rabbit sera against NHBA full-length protein were used for blotting the membranes.

5. Identification of the cleavage site of epithelial cell proteases in C2 fragment and NHBA full-length protein

In a first effort to define the site within C2 fragment or NHBA protein where epithelial cell proteases cleave, we examined if the presence of the Arg-rich motif was necessary for the cleavage. To this end, we made use of recombinant mRR NHBA mutant protein (Serruto et al., 2010), a mutant protein wherein all arginine of the Arg-rich motif were substitute with glycine. A time course experiment was performed incubating mRR NHBA recombinant protein with polarized Calu-3 cells. At each time point, cell supernatant was collected and analyzed by Western blot with an anti NHBA antibody. As it is shown in Figure 11, mRR mutant protein was not cleaved by polarized Calu-3 cells proteases, indicating that the presence of Arg-rich motif was necessary for the processing of NHBA by epithelial cell proteases. This result suggested also that the cleavage by epithelial cells proteases occurred somewhere in the arginine-rich motif.

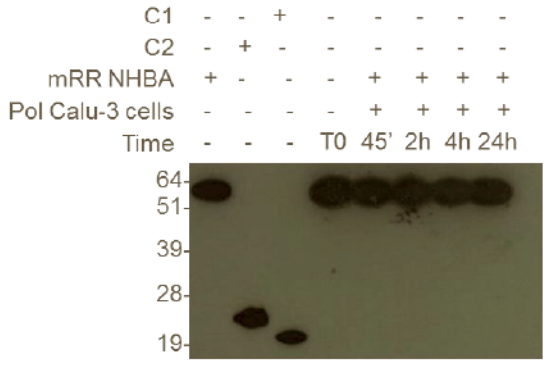


Figure 11 – Recombinant mRR NHBA mutant protein is not cleaved by epithelial cell proteases

Western blot analysis of supernatants of polarized Calu-3 cells treated with 5 µM of recombinant mRR NHBA mutant protein. Samples were collected at different time point (45', 2h, 4h and 24h). Polyclonal mouse sera against NHBA full-length protein were used for blotting the membranes.

Subsequently, to further define the cleavage site of epithelial cell proteases, the intact mass of C-terminal fragments generated by either the cleavage of C2 fragment or the cleavage of NHBA protein was measured by mass spectrometry. If it is known the sequence of a protein, with this technique it is possible to determine with accuracy the sequence of a fragment of this protein, since this fragment will have a univocal experimental mass. In both case, a mass of 20.3 kDa was detected corresponding to a C-terminal fragment that started at Ser14 or Ser281, respectively (Figure 12). Thus, we concluded that the cleavage site of epithelial cell was located immediately downstream the Arg-rich motif.

A.

Protein	Expected Mass (Da)	Experimental Mass (Da)	Sequence
C2-fragment	22014.3	22014.05 ± 0.21	1-204
C2-fragment + Pol Calu-3 cells	-	20379.32 ± 0.18	14-204
NHBA + Pol Calu-3 cells (His-tag purified C-term frag)	-	20379.73 ± 0.36	281-472

B.

C2 fragment

SFARFRRSARSRR⬇SLPAEMPLIPVNQADTLIVDGEA
VSLTGHSGNIFAPEGNYRYLTYGAEKLPGGSYALRVQ
GEPAKGEMLAGAAVYNGEVLHFHTENGRPYPTRGRF
AAKVDFGSKSVDGIIDSGDDLHMGTQKFKAAIDGNG
FKGTWTENGSGDVSGKFYGPAGEEVAGKYSYRPTD
AEKGGFGVFAGKKEQDLEHHHHHH

C.

NHBA protein

MPDVKSADTLSKPAAPVVSEKETEAKEDAPQAGSQGQ
GAPSAQGSQDMAAVSEENTGNGGAVTADNPKNEDEV
AQNDMPQNAAGTDSSTPNHTPDPNMLAGNMENQATD
AGESSQPANQPDMANAADGMQGDDPSAGGQNAGNTA
AQGANQAGNNQAAGSSDPIPASNPAPANGGSNFGRVD
LANGVLIDGPSQNITLTHCKGDSCSGNNFLDEEVQLKS
EFEKLSDADKISNYKKDGKNDKFVGLVADSVQMKGIN
QYIIFYKPKPTSFARFRRSARSRR↓SLPAEMPLIPVNQA
DTLIVDGEAVSLTGHSGNIFAPEGNYRYLTYGAEKLPG
GSYALRVQGEPAKGEMLAGAAVYNGEVLHFHTENGRP
YPTRGRFAAKVDFGSKSVDGIIDSGDDLHMGTQKFKAA
IDGNGFKGTWTENGSGDVSGKFYGPAGEEVAGKYSYR
PTDAEKGGFGVFAGKKEQDLEHHHHHH

Figure 12 – The cleavage site of epithelial cell proteases is located immediately downstream the Arg-rich motif

A) Mass spectrometry analysis of integral mass of C-terminal fragments generated by the cleavage of epithelial cells using C2 fragment or NHBA full-length protein as substrates. Table indicated the expected mass of recombinant C2 fragment, the experimental mass values measured and the corresponding sequence lengths expressed in term of amino acid position in the sequence. B and C) Amino acid sequence of recombinant C2 fragment (B) and NHBA full-length protein (C); Arg-rich motif is highlighted in blue and the cleavage site of epithelial cells protease is indicated with the blue arrow.

6. Identification of a novel human protease responsible for NHBA proteolysis

Finally, we wanted to identify the epithelial cell protease responsible for NHBA cleavage. To this end, we performed an ion exchange chromatography of polarized Calu-3 cell supernatant in order to enrich a fraction of cell supernatant with the protease of our interest. With this type of chromatography, proteins were separated according to their total charge. Fractions were eluted increasing the ionic strength and then examined for their ability to cleave both recombinant C2 fragment and NHBA full-length protein. Therefore, recombinant proteins were incubated overnight with each eluted fraction and the cleavage was assessed by SDS-PAGE analysis. As it is shown in Figure 13, cleavage was observed with fractions 16-19; however, only fraction 17 and 18 were selected for further analysis since they showed a greater cleavage efficiency and likely contained more protease molecules of our interest. These fractions were pooled, subjected to trypsin digestion and analyzed by mass spectrometry.

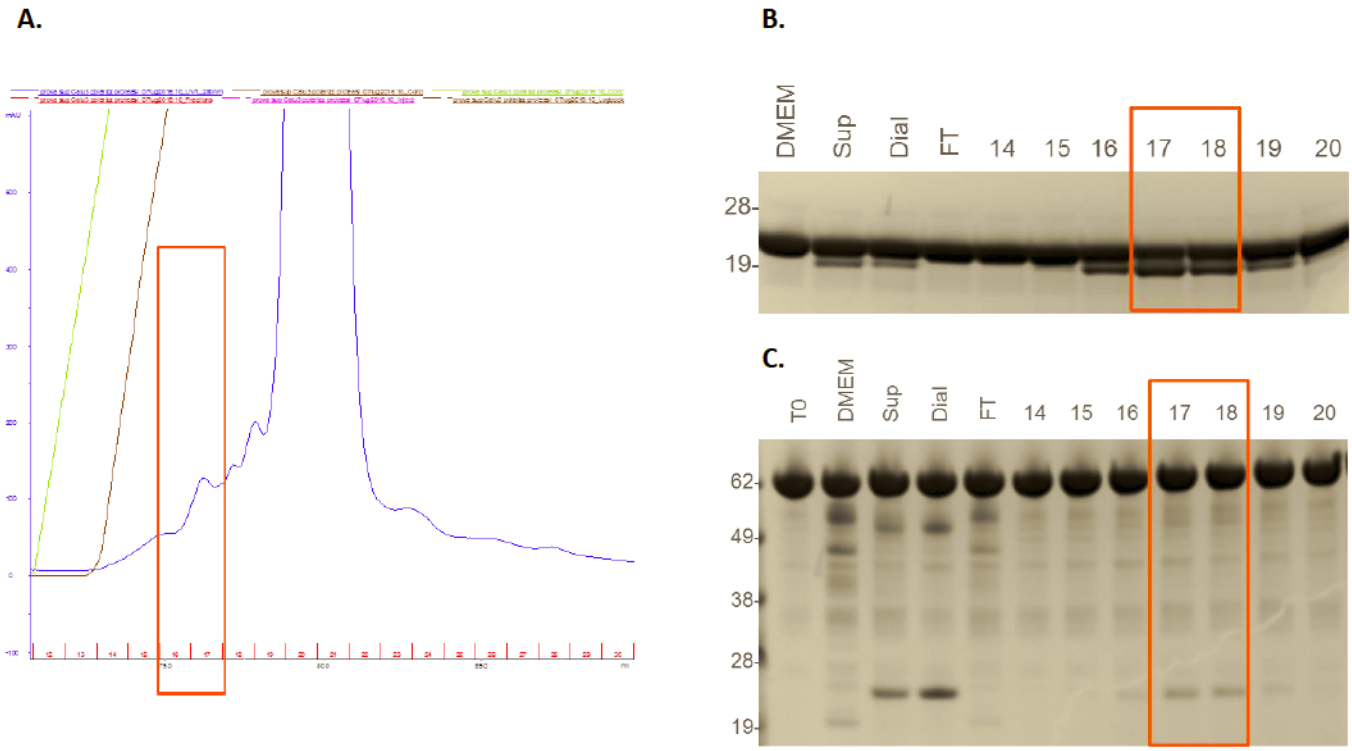


Figure 13 – Identification of cell supernatant fractions enriched with the epithelial cell protease responsible for NHBA cleavage

Ion exchange chromatography of polarized Calu-3 cell supernatant A) Chromatogram shows elution of fractions (x axis) and corresponding amount of proteins contained in the fractions (y axis). B and C) SDS-PAGE analysis of each eluted fraction incubated with 5 µM of recombinant C2-fragment (B) or with 5 µM of recombinant NHBA full length protein (C). Proteins were stained with blue coomassie. Orange selection indicated eluted fractions that processed C2 fragment and NHBA protein; these fractions were selected for mass spectrometry analysis.

Peptide sequences, detected by mass spectrometry, were then launched on a human protein database in order to identify all the proteins contained in selected fractions. From a list of 500 proteins, we selected proteins annotated as proteases and secreted by cells. Six protease candidates were identified and they are listed in figure 14.

Protease	Activity	Co-factor	Localization	Inhibitor
ADAM 9	*metalloendopeptidase activity	Zn^{++}	Isoform 2 secreted	GI254023X
Cathepsin D	*aspartic endopeptidase activity *cysteine endopeptidase activity *serine endopeptidase activity		Secreted and Lysosome	Pepstatin A
Cathepsin L1	*cysteine endopeptidase activity *serine endopeptidase activity		Secreted	E64
Complement factor B	*serine endopeptidase activity	Mg^{++} In the presence of Mg^{++}, factor B binds to C3b (*) and the C3bB complex can be activated by factor D or kallikrein	Secreted	
Lactoferrin	*serine endopeptidase activity		Secreted	
Prostasin	*serine endopeptidase activity		Light chain secreted	

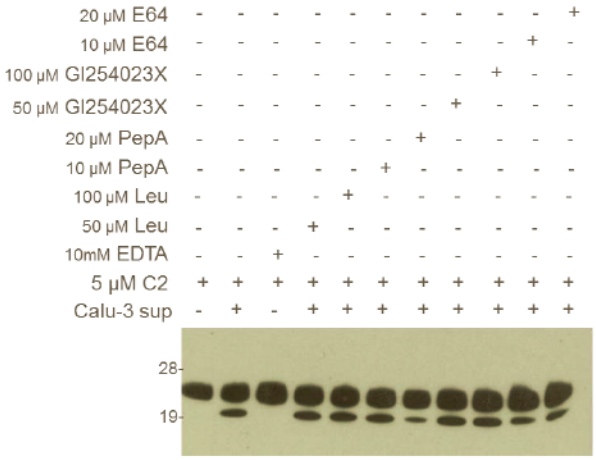
(*) C3 complement component was also identify by mass spectrometry analysis

Figure 14 – List of protease candidates identified by mass spectrometry analysis

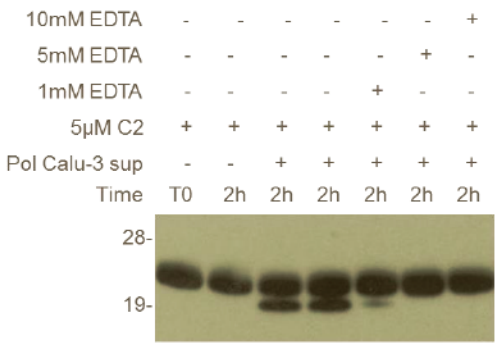
For each protease candidate, the following information was listed in the table: any protease activity annotated in literature, co-factor, cellular localization and specific protease inhibitor that blocks its activity.

Specific protease inhibitors were used to assess if a candidate protease was responsible for the cleavage of C2 fragment or NHBA full-length protein. If the inhibitor did not abolish the cleavage of epithelial cell supernatants, we removed that candidate from our list. Looking in the literature, the following specific inhibitors were chosen and tested at several concentrations: GI254023X (Moss et al., 2010), Pepstatin A and E-64. With this approach, ADAM9, Cathepsin D and L1 were removed from the list (Figure 15A).

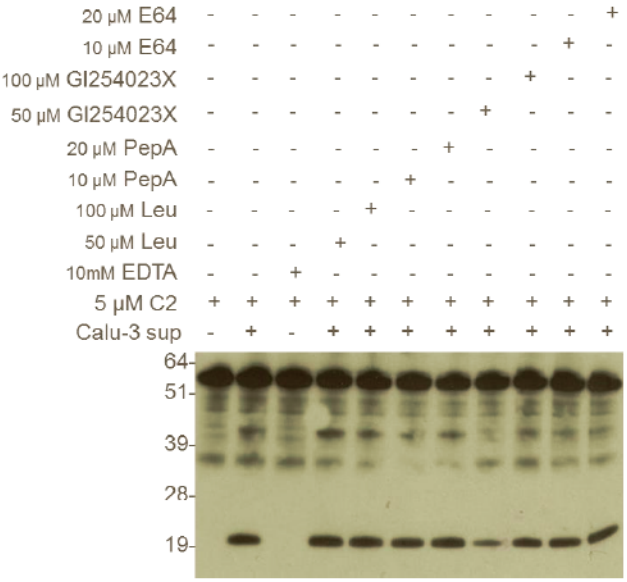
A.



B.



C.



D.

Protease inhibitors:

- **EDTA** → chelator of divalent and trivalent cations
- **Leupeptin** → cysteine and serine protease inhibitor
- **Pepstatin A** → aspartyl protease inhibitor
- **GI254023X** → ADAM10 and ADAM9 inhibitor
- **E64** → cysteine protease inhibitor

Figure 15 – EDTA completely inhibits NHBA cleavage by epithelial cells proteases

Western blot analysis of recombinant C2 fragment (A and C) or NHBA full-length protein incubated for 2 hours with Calu-3 cell supernatants that were pre-treated or not with protease inhibitors for 30 minutes. Protease inhibitors tested: EDTA, Leupeptin (leu), Pepstatine A (PepA), GI254023X and E-64. Polyclonal mouse sera against NHBA C-terminal domain (A and C) or against NHBA full-length protein (B) were used for blotting the membranes. D) The inhibitory property of each protease inhibitor tested is described in the table.

EDTA, a chelate agent of divalent and trivalent positive ions (i.e. Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}), was the only inhibitor able to block the activity of the epithelial cell protease of our interest (Figure 15). A concentration range of 1-10mM of EDTA was tested, and at 5mM the cleavage of epithelial cell supernatant was completely inhibited (Figure 14C). From the list of candidates, the protease activity dependent on positive ions, and that could be affected by EDTA treatment, was the human complement factor B. Complement factor B in presence of Mg^{2+} interacts with the hydrolyzed form of complement C3 (C3(H_2O)) or C3b. This complex is then recognized by the complement factor D that cleaves and activates factor B, and thus generates the C3-convertase of the alternative complement pathway (Ricklin et al., 2016). Human complement component C3 was also identify by mass spectrometry in the selected fraction of polarized Calu-3 cell supernatant. Since we were surprised to find some complement components into epithelial cell supernatant, we verified their expression by epithelial cells at the mRNA level. Total mRNA of Calu-3 cells and polarized Calu-3 cells was extracted, reverse transcribed and cDNA was used for semi-quantitative PCR analysis. Specific primer pairs were designed for C3 and factor B mRNA (see Material and Methods) and used for PCR amplification. GADPH mRNA was included in the analysis as internal positive control. Amplification products of C3 and factor B mRNA were observed by agarose gel electrophoresis (Figure 16), confirming that human epithelial cells produced and secreted these complement components.

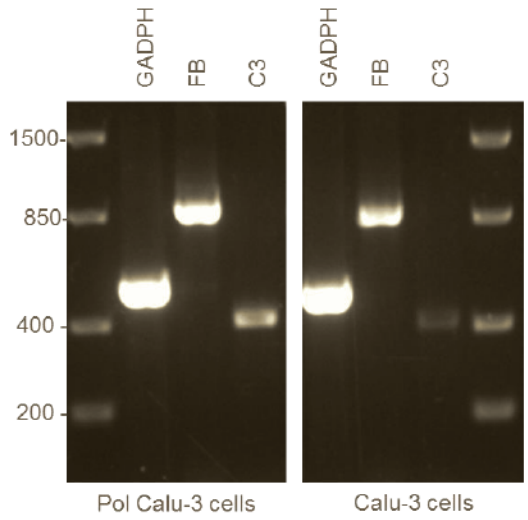


Figure 16 – Calu-3 epithelial cells express complement component C3 and factor B

Electrophoresis analysis of expression of human *CFB* and *C3* genes in Calu-3 cells and polarized Calu-3 cells. GADPH was used as internal positive control. Total RNA was isolated from epithelial cells, retro transcribed with oligo(dT) and cDNA were used as templates for PCR amplification. For each gene analyzed, oligonucleotides used amplified part of the mRNA (GADPH: 518nt; CFB: 885 nt; C3: 408nt). Size markers are indicated on the left.

To exclude the possibility that kallikrein, a recently identified human protease able to process NHBA protein (Pantano MSc thesis), was the protease responsible for NHBA cleavage in epithelial cell supernatants even it was not detected by mass spectrometry, we tested if EDTA was able to inhibit kallikrein activity. Plasma-purified kallikrein was incubated overnight with recombinant C2 fragment and NHBA full-length protein in presence or in absence of 10 -20mM of EDTA. As it is shown in Figure 17, we still observed the cleavage by kallikrein in presence of EDTA, ruling out the possibility that this protease could be accountable for the cleavage of NHBA protein in epithelial cell supernatants. In addition, we noticed that kallikrein processing of C2 fragment or NHBA full-length protein resulted in the generation of two distinct smaller C-terminal fragments.

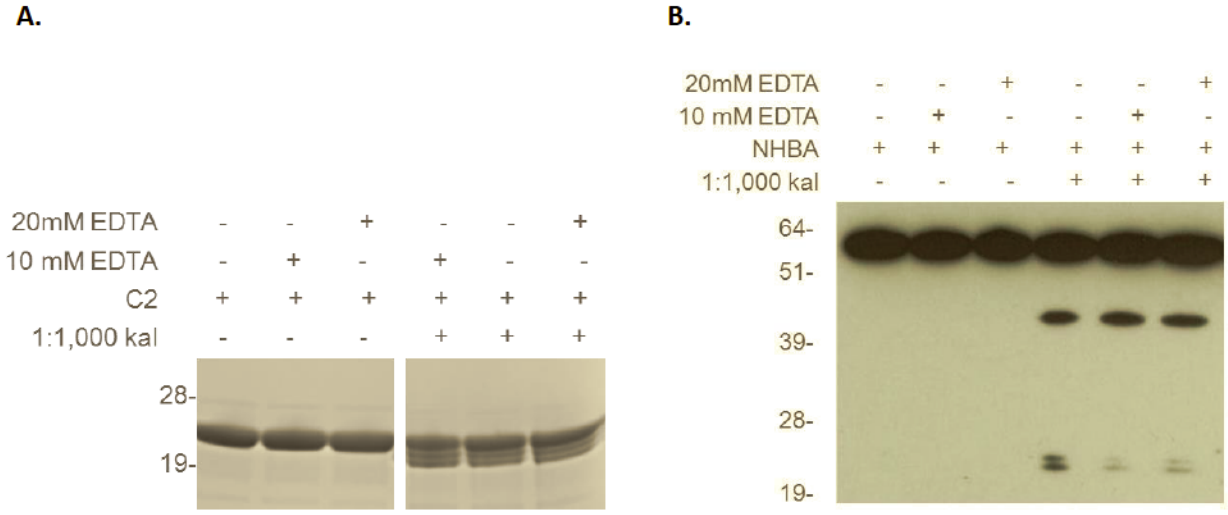


Figure 17 – EDTA does not inhibit the activity of kallikrein

A) SDS-PAGE analysis of recombinant C2 fragment incubated overnight with plasma-purified pre-treated or not with EDTA. Proteins were stained with blue coomassie. B) Western blot analysis of recombinant NHBA full-length protein incubated overnight with plasma-purified pre-treated or not with EDTA. Polyclonal mouse sera against NHBA full-length protein were used for blotting the membranes.

Then, to evaluate if C3-convertase of the alternative complement pathway was a proteases able to process C2 fragment and NHBA full-length protein, we decided to use Normal Human Serum (NHS) since in NHS complement components are highly concentrated and it is where they play their main physiological role. The cleavage was tested in three different conditions for which the C3-convertase of alternative complement pathway could not be formed: (i) NHS was treated with EDTA in order to sequestrate Mg^{2+} and prevent the interaction between factor B and $C3(H_2O)$; (ii) NHS was depleted of factor B; (iii) NHS was depleted of factor D in order to prevent the activation of the $C3(H_2O)B$ complex.

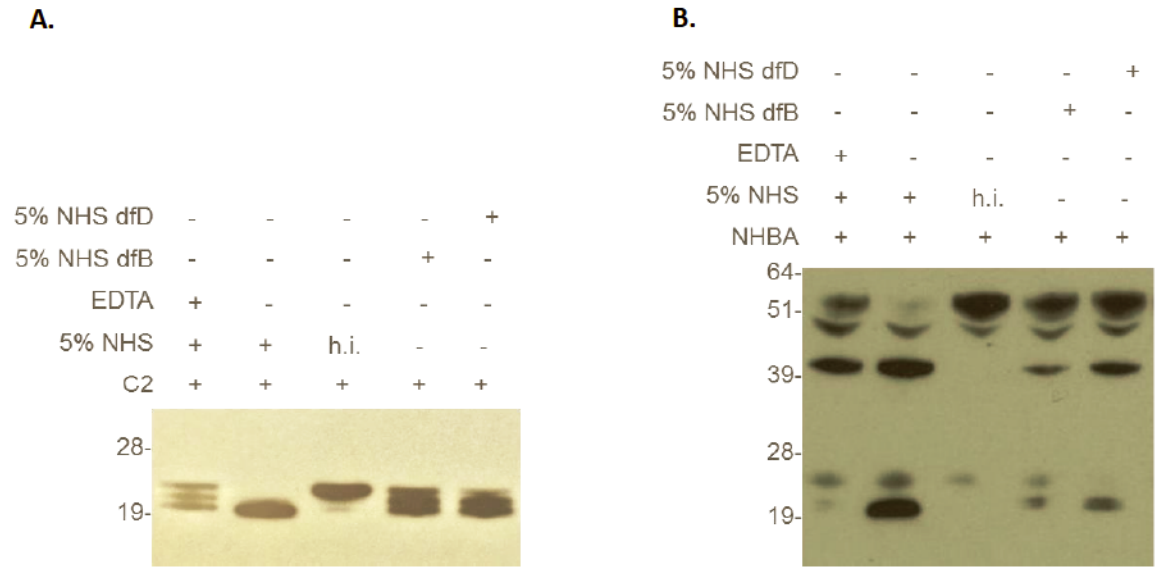


Figure 18 – C3 convertase of alternative complement pathways: a novel human protease responsible for NHBA cleavage

Western blot analysis of recombinant C2 fragment (A) or NHBA full-length protein (B) incubated for 1 hour with NHS (lane 2), NHS pre-treated with 10mM EDTA (lane1), NHS depleted of factor B (lane 4), NHS depleted of factor D (lane5) and NHS heated inactivated (h.i.) as negative control (lane 3). Polyclonal mouse sera against NHBA C-terminal domain (A) or against NHBA C-full-length protein (B) were used for blotting the membranes.

Regarding to NHS, in all the three conditions tested, in which the formation of C3-convertase was inhibited, we observed a reduction of the cleavage of both substrate proteins and the generations of two distinct smaller C-terminal fragments, similarly to kallikrein cleavage pattern (Figure 18). These results indicated that C3-convertase of the alternative complement pathway is another human protease responsible for the cleavage of C2 fragment and NHBA protein during

meningococcal infection; whereas it cannot be assembled in NHS, plasma kallikrein processes both proteins generating two smaller C-terminal fragments.

Discussion:

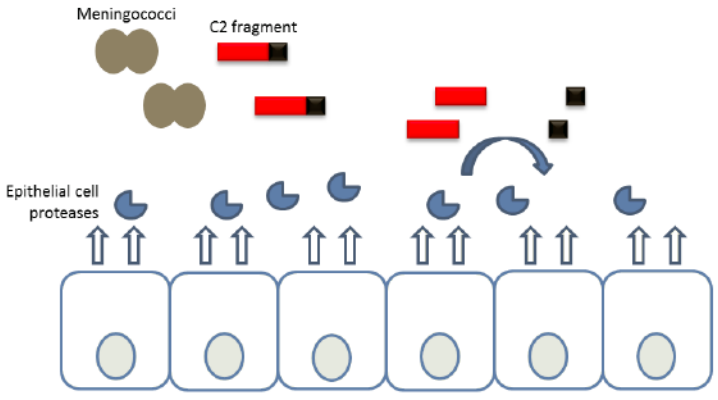
N. meningitidis infection remains an important cause of globally illness. The high associated risk of death and the substantial long-term disability among survivors makes the control of invasive meningococcal disease a public health priority. In addition, the rapidly progressive nature of invasive infections and the emergence of antibiotic resistant bacteria raised concerns regarding the therapy of invasive disease case and the prophylaxis of close contacts. Taken together, these considerations point out that the most effective strategy to control meningococcal disease is prevention. Since it is well established the importance of complement-mediated antibody-dependent killing of *N. meningitidis* in protection against invasive meningococcal disease (reviewed in Lewis and Ram, 2014), prevention can be achieved building a protective antibody response through vaccination. Vaccines currently available for invasive meningococcal diseases include polysaccharide-protein conjugate vaccines for serogroups A, C, W135 and Y, and protein-based vaccines for serogroups B (Bexsero and Trumenba).

In vaccinology field, the initial priority of pre-clinical research during the development of a new vaccine is to identify candidate antigens and test their safety and immunogenicity in animal models (Leroux-Roels et al., 2011), leaving aside the understanding of the biological role of those antigens during the disease whereas it is not already known. However, information around selected antigens is extremely important for supporting the vaccine during the licensure step as well as after the vaccine release. Thus, further pre-clinical studies are needed to be carried out. The urgency to have a vaccine against the serogroups B directed the development process of the Bexsero vaccine exactly through this path. NHBA, fHbp and NadA proteins, identified with the innovative reverse vaccinology approach, were selected as main protein antigens for their propriety of being immunogenic and able to elicit bactericidal antibody against *N. meningitidis* (Pizza et al., 2000). How these surface antigens contribute to the ability of the bacterium to cause disease have been discovered afterwards(NHBA: Serruto et al., 2010; Arenas et al. 2013; Casellato et al.,2014; Vacca et al., 2016. fHbp: Madico et al., 2006; NadA: Comanducci et al., 2002; Capecchi et al., 2005; Scetti et al., 2016), and there are still research activities ongoing in order to clarify mechanisms involved in this process. Focusing on NHBA antigen, recent publications showed that this protein has distinct roles in different steps of meningococcal pathogenesis. NHBA has been reported to contribute to host-pathogen interaction via the ability to bind to heparan sulfate proteoglycans on epithelial cells (Vacca et al., 2016), to contribute to biofilm formation by binding to extracellular DNA (Arenas et al. 2013) and to influence bacterial survival in blood through binding to heparin (Serruto et al., 2010). In addition, NHBA-derived fragment C2, originating from the cleavage of NHBA protein by meningococcal proteases, has been shown to contribute to vascular leakage by altering endothelial

permeability (Casellato et al., 2014). Since Delany and collaborators previously observed that NHBA expression and its cleavage by meningococcal proteases is upregulated at temperatures lower than 37°C (Delany unpublished data; Lappann et al., 2016), we focalized our attention in investigating the effects of C2 fragment on the airway epithelium, where the temperature fluctuates around 34°C (Keck et al., 2000), in order to assess if the release of C2 fragment could facilitate the traversal of *N. meningitidis*.

In the present study, using an *in vitro* model of human airway epithelium, we demonstrated that C2 fragment did not alter the integrity of epithelial monolayers, and this was in agreement with previous findings showing that *N. meningitidis* traverses the epithelial barrier without disrupting the junctional structures (Stephens et al., 1983; Pujol et al., 1997; Sutherland et al., 2010). Unexpectedly, we discovered that host cells were able to process the C2 fragment converting it into a shorter fragment. Cleavage kinetics depended on cell types; epithelial cells rapidly cleaved the protein while endothelial cells cleaved it with a slower rate. The slow cleavage rate of endothelial cells, detectable after 4 hours, could be timing compatible with the C2 fragment-induced toxic effect on endothelial integrity that has been reported to occur in only 45 minutes (Casellato et al. 2014). Proteolysis of C2 fragment by host cells eliminated the Arg-rich motif, the putative docking domain responsible for the interaction of C2 fragment with host cells (Casellato et al. 2014), likely preventing its biological activity. Therefore, this cleavage could represent a host defense mechanism against C2 fragment-induced toxic effect. Based on these findings we propose a model, depicted in Figure 1, to explain different outcomes as a result of the interaction between C2 fragment and host cells depending on the cell type. In a subset of hyper virulent strains, NHBA protein is cleaved by meningococcal proteases and the C2 fragment, a potential virulence factor, is released from bacterial surface. Acting as a first line of defense against meningococcal invasion, the airway epithelium constantly produces proteases that inactivate the C2 fragment activity. Contrariwise, under *in vitro* conditions endothelial cells do not express proteases that are promptly able to process the C2 fragment, allowing the C2 fragment to exert its toxic effect that results in the perturbation of endothelial integrity.

A.



B.

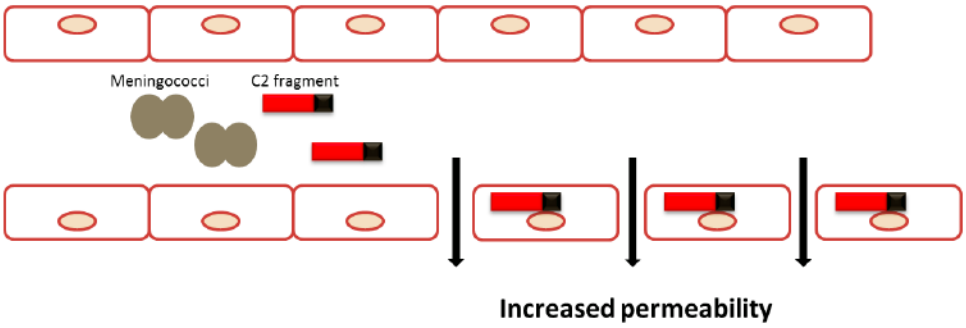


Figure 1 – Differential outcomes during C2 fragment interaction with host cells depending on cell type

A) Epithelial cells constantly produce proteases that are able to process C2 fragment released by meningococci. This cleavage prevents C2 fragment-induced toxic effect by eliminating the docking domain, and thus the integrity of the epithelium is maintained. B) Under *in vitro* condition, endothelial cells do not express proteases that process C2 fragment. Thus, C2 fragment produced by meningococci interacts with endothelial cells and induces the alteration of endothelial integrity.

It is still to be clarified if the cleavage of C2 fragment by endothelial cells observed after a long exposition to C2 fragment was due to a cellular defense response induced by the protein which resulted in the production of protease able to process the C2 fragment, or it depended on the slow cleavage rate of endothelial proteases which made the cleavage visible only after several hours of incubation.

Investigating further the cleavage by epithelial cells using different models of study, we found that epithelial cells from the upper respiratory tract constantly secreted proteases responsible for a rapid cleavage of both C2 fragment and NHBA full-length protein. We demonstrated that processing of NHBA protein occurred on live bacteria during meningococcal interaction with epithelial cells. Removal of NHBA C-terminal domain from bacterial surface resulted in the generation of an N-terminal fragment containing the Arg-rich motif disclosed as new C-terminal domain, which might remain exposed on bacterial surface. In non-adherent bacteria, new synthesis of NHBA proteins, likely induced by the presence of CREN regulatory element in *nhba* locus (Deghmane et al., 2003), was concurrent to the cleavage of surface-exposed NHBA proteins by epithelial cell proteases. Exposure of newly synthetized NHBA full-length protein on bacterial surface might favor the attachment of *N. meningitidis* to epithelial cells, and this would be in agreement with e recent report showing that an increased expression of NHBA protein, occurring at 32°C, correlated to an increased adhesion to epithelial cells (Lappann et al., 2016). On the other hand, removal of NHBA C-terminal domain from bacterial surface by epithelial cell proteases might hinder meningococcal contact to epithelial cells. In fact, Vacca et al. (2016), using recombinant

proteins, showed that only NHBA full-length protein bind to epithelial cells through the Arg-rich motif via a direct interaction with heparan sulfate proteoglycans (HSPGs) on epithelial cell surface, while this is not the case for its digested fragments containing the Arg-rich motif. Likely, a peculiar spatial orientation of the Arg-rich motif within the protein is required in order to mediate the interaction with HSPGs. Thus, by decreasing the amount of NHBA full-length protein exposed on bacterial surface through the processing of epithelial cell proteases, this host-pathogen interaction could be impaired.

Recombinant proteins not always mirror the real folding of a protein, especially if that protein required a lipid environment or a co-factor to be correctly folded. In the case of the N-terminal region of NHBA protein, that has been predicted to be mostly unstructured (Esposito et al., 2011), it could be important to be exposed on bacterial surface for acquiring its correct folding. Therefore, further investigations using live bacteria need to be carried out to clarify if on bacterial surface the Arg-rich motif is displayed in the correct orientation for the interaction with HSPGs only when is contained in NHBA full-length protein, or also when is disclosed after the processing of epithelial cell proteases. This would allow us to confirm that the NHBA cleavage by epithelial cell protease is a host defense mechanism against *N. meningitidis* adhesion.

Finally we demonstrated that, in addition to kallikrein and lactoferrin (Serruto et al., 2010; Pantano MSc thesis), C3-convertase of the alternative complement pathway is a human protease able to process both C2 fragment and NHBA full-length protein. Formation of alternative pathway C3-convertase on the surface of *Neisseria meningitidis* certainly occurred in human blood, and it might also occur during meningococcal colonization of the nasopharynx. In agreement with previous studies on expression profiling of human epithelial cells of the upper respiratory tract (Varsano et al., 2000; Ali et al., 2011; Martinez-Anton et al., 2013; Klijn et al., 2014), we reported, indeed, that Calu-3 epithelial cells expressed and secreted complement component C3 and Factor B. As a results of tick-over mechanism of C3 molecule (reviewed in Ricklin et al., 2016), C3(H_2O) can interact with factor B in presence of Mg^{2+} and forms the C3-convertase of the alternative complement pathway. This complex needs to be activated by the cleavage of Factor D or kallikrein (DiScipio, 1982; Hiemstra et al., 1985). In this study, neither Factor D nor kallikrein has been detected in the fractions of epithelial cell supernatants responsible for NHBA cleavage by mass spectrometry analysis. However, it is well known that Tissue kallikrein 1 is expressed by tracheobronchial submucosal glands in human airways (Proud and Vio, 1993) and its production increases under inflammatory conditions, such as asthmas and viral infection (Christiansen et al., 1987; Forteza et al., 1999; Leu et al., 2015). Thus, C3-convertase of alternative pathway could be activated on mucosal surface at least under these conditions. With *Neisseria meningitidis*, a

concomitant inflammation of the airway epithelium, might lead to the formation of alternative pathway C3-convertase on bacterial surface. Once activated, it might cleave surface-exposed NHBA protein.

Removal of NHBA C-terminal domain from bacterial surface by C3-convertase of alternative complement pathway might affect the complement activation acting on two different steps of the complement cascade: (i) the antibody-mediated activation of the classical pathway and (ii) the amplification loop of the alternative pathway (red selections, Figure 2).

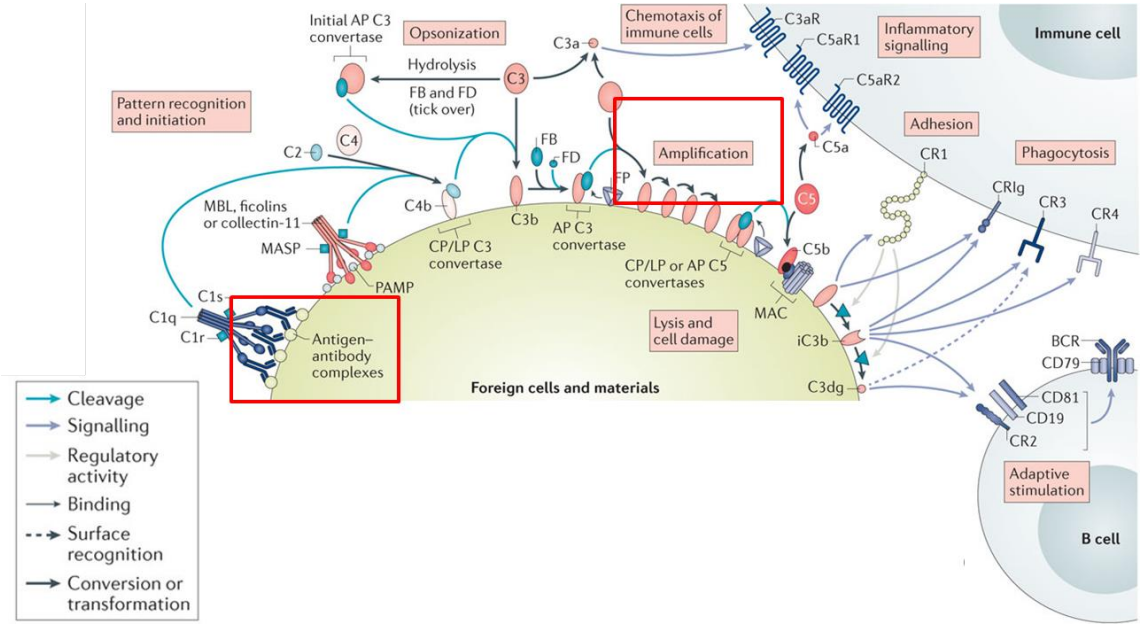


Figure 2 – Overview of the complement cascade

"When complement encounters a foreign cell, the recognition molecules C1q, mannose-binding lectin (MBL), ficolins, and collectin-11 sense antibody clusters or pathogen-associated molecular patterns (PAMPs) and activate associated serine proteases (C1r, C1s or MBL-associated serine proteases (MASPs)) that cleave C4 and C2 to form the C3 convertase (C4b2b) of the classical pathway (CP) and lectin pathway (LP) on the triggering cell. In addition, tick-over or surface interactions of C3 with certain cell surfaces leads to C3 hydrolysis and generation of initial alternative pathway (AP) C3 convertases. All C3 convertases cleave C3 into the anaphylatoxin C3a and C3b, the latter of which is deposited on activating surfaces (opsonization). Factor B (FB) binds to C3b and gets activated by Factor D (FD) to form the final AP convertases (C3bBb), which activate more C3 to C3b and fuel an amplification loop. Increasing densities of C3b deposition favour the generation of C5 convertases, which activate C5 to release the anaphylatoxin C5a and C5b, which, after tiered interactions with C6–C9 and membrane insertion, in turn forms membrane attack complexes (MACs), which leads to lysis, damage, or activation of target cells. The released anaphylatoxins act as immune mediators and, particularly in the case of C5a, attract and prime immune cells. Interaction of the C3b opsonin and its degradation products iC3b and C3dg with complement receptors (CRs) mediates cell adhesion (via CR1 (also known as CD35)) and/or phagocytic uptake (via CR3, CR4, and CRIg). Concurrently, iC3b and C3dg modulate adaptive immune responses by binding to CR2 on the B cell co‑receptor, thereby lowering the threshold of B‑cell stimulation. Properdin (FP) stabilizes the C3 and C5 convertases and increases complement-mediated opsonization and effector generation." Figure and legend were adapted from Ricklin et al. (2016). The two steps of

complement cascade at which complement activation can be dampened by removal of NHBA C-terminal domain from bacterial surface by alternative complement C3-convertase are highlighted with a red selection.

First of all, if C-terminal domain of NHBA protein is removed from the bacterial surface, antibody raised against the C-terminal domain will not be able to initiate the complement cascade. This mechanism could be extended to all proteases that process surface-exposed NHBA protein, meningococcal and host proteases. In spite of this, antibody directed to the N-terminal domain will be functional also after the processing of NHBA protein, since N-terminal fragment remains anchored to the bacterial surface. Using rabbit complement as source of complement, it has been shown that NHBA cleavage, by either meningococcal NalP protease or by human lactoferrin, does not significantly affect the NHBA-mediated bactericidal activity, since bactericidal titers varied within one dilution (Serruto et al., 2010). However, further confirmations using human complement as source of complement need to be carried out, in order to exclude the possibility that removal of NHBA C-terminal domain is a human species-specific mechanism to avoid complement killing, as it has been discovered to be for fHbp and NspA proteins (Granoff et al., 2009; Lewis et al., 2010). Secondly, involvement of alternative pathway C3-convertase in the cleavage of NHBA protein from bacterial surface might interfere with the amplification loop of the alternative pathway. The amplification loop is the balance between two separate competing cycles: C3b–C3 convertase formation and C3 breakdown, and it contributes to the overall complement response pushing the complement cascade to the formation of C5-convertase that finally will lead to the lyses of bacteria trough the formation of MAC complex on bacterial surface (reviewed in Ricklin et al., 2016). Since the Alternative Complement C3-convertase has a very short half-life (90 seconds), if it is occupied with the processing of NHBA protein on bacterial surface instead of activate more C3 to C3b, the balance will likely shift in favor of the C3 breakdown cycle dampen the complement cascade. This might represent an additional mechanism used by *Neisseria meningitidis* to evade the complement system and survive in human blood.

Overall, these conclusive speculations raise the question whether the direct interaction between NHBA protein and alternative pathway C3-convertase may limit the functionality of SBA assay, which is used for measuring the concentration of functional antibodies induced by NHBA antigen, leading to an underestimation of the real functionality of these antibodies. Since antibodies against NHBA have been shown to inhibit adhesion of *N. meningitidis* to epithelial cells (Vacca et al. 2016), inhibition of meningococcal adhesion to epithelial cells by anti-NHBA sera can be taken in consideration as an additional assay for measuring their functionality. A recent report, showing that vaccination with Bexsero reduced meningococcal carriage rates of diverse circulating strains during 12 months after vaccination (Read et al., 2014), highlights the fact that the Bexsero

vaccine likely confers protection against meningococcal disease acting at different stages of the infection, including colonization. Antibodies induced by NHBA antigen may contribute to protection mainly at this initial stage of the infection by inhibiting colonization of NHBA-expressing bacteria (Vacca et al., 2016). Therefore, it will be important to evaluate the contribution of NHBA antigen in vaccine efficacy at the level of meningococcal colonization, and not only for its ability to induce bactericidal antibody.

In conclusions, this study has provided us with new insights on the cleavage of NHBA protein during meningococcal infection. This cleavage occurs at different stages of the infection, and likely has a different role depending on the environment with whom the bacteria is interacting with.

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