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CICLO XXV

C2 Fragment from *Neisseria meningitidis* Antigen NHBA Disassembles Adherence Junctions of Brain Microvascular Endothelial Cells

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Summary

Neisseria meningitidis is the major cause of meningitis and sepsis, two kind of diseases that can affect children and young adults within a few hours, unless a rapid antibiotic therapy is provided. The meningococcal disease dates back to the 16th century. The first description of the disease caused by this pathogen was stated by Viesseux in 1805 as 33 deaths occurred in Geneva, Switzerland [1].

It took about seventy years before two Italians (Marchiafava and Celli) in 1884 identified micrococcal infiltrates within the cerebrospinal fluid [2].

The worldwide presence of meningococcal serogroups may vary within regions and countries.

With the coming of antimicrobial agents, like sulphonamides, and with the development of an appropriate health care and prevention programme, the fatality rate cases has dropped from 14% to 9%, although 11% to 19% of patients continued to have post-infection issues such as neurological disorders, hearing or limb loss [3].

The bacteria can be divided into 13 different serogroups and, among these, up to 99% of infection is ascribed to the serogroups named A, B, C, 29E, W-135 and Y (Fig. 2). All the serogroups have been listed in 20 serotypes on the presence of PorB antigen, 10 serotypes on the presence of PorA antigen, and in other immunotypes on the presence of other bacterial proteins and on the presence of a characteristic lipopolysaccharide called LOS (lipooligosaccharide) [4].

The transmission from a carrier to an other person occurs by liquid droplet and the natural reservoir of *Neisseria meningitidis* is the human throat, in particular it usually invades the human nasopharynx where it can survive asymptomatically.

The reported annual incidence goes from 1 to 5 cases per 100000 inhabitants in industrialized countries, while in non developed-countries the incidence goes up to 50 cases per 100000 inhabitants. More then 50% of cases occur within children below 5 years of age, and the peak regards those under the first year of age. This fact is due to the loss of maternal antibodies by the newborn. In non-epidemic period, the percentage of healthy carriers range from 10 to 20%, and notably the condition of chronic carrier is not so uncommon [5, 6]. Only in a small percentage of cases the colonization progresses until the insurgence of the pathogenesis. This happens because in the majority of cases specific antibodies or the human complement system are able to destroy the pathogens in the blood flow allowing a powerful impairment of the dissemination.

In a small group of population the colonization of the upper respiratory tract is followed by a rapid invasion of the epithelial cells, and from there bacteria can reach the blood flow and invade the central nervous system (CNS), inducing the establishment of an acute inflammatory response.

How the balance between being an healthy carrier or a infected patient can change so rapidly it is still unknown. Some factors that can play a role in this switch could be the virulence of the bacterial strain, the responsiveness of the host immune system, the mucosal integrity, and some environmental factors [7].

Neisserial heparin binding antigen (NHBA) is a surface- exposed lipoprotein from *Neisseria meningitidis* that was originally identified by reverse vaccinology [8].

NHBA in Nm has a predicted molecular weight of 51 kDa. The protein contains an Arg-rich region (-RFRRSARSRRS-) located at position 296–305 that is highly conserved among different Nm strains. The protein is specific for *Neisseria*

species, as no homologous proteins were found in non redundant prokaryotic databases.

Full length NHBA can be cleaved by two different proteases in two different manners: NalP, a neisserial protein with serine protease activity cleaves the entire protein at its C-terminal producing a 22 kDa protein fragment (commonly named C2) which starts with Ser293 and hence comprises the highly conserved Arg-rich region. The human proteases lactoferrin (hLf) cleaves NHBA immediately downstream of the Arg-rich region releasing a shorter fragment of approximately 21 kDa (commonly named C1) [9] .

Although it is known that a crucial step in the pathogenesis of bacterial meningitidis is the disturbance of cerebral microvascular endothelial function, resulting in blood-brain barrier breakdown, the bacterial factor(s) produced by Nm responsible for this alteration remains to be established. The integrity of the endothelia is controlled by the protein VE-cadherin, mainly localized at cell-to-cell adherens junctions where it promotes cell adhesion and controls endothelial permeability [10]. It has been reported that alteration in the endothelial permeability can be ascribed to phosphorylation events induced by soluble factors such as VEGF or TGF- β [11] [12].

Our work demonstrates that the NHBA- derived fragment C2 (but not C1) increases the endothelial permeability of HBMEC (human brain microvasculature endothelial cells) grown as monolayer onto the membrane of a transwell system. Indeed, the exposure of the apical domain of the endothelium to C2 allows the passage of the fluorescent tracer BSA-FITC, from the apical side to the basal one, early after the treatment. Interestingly, the effect of C2 on the endothelium integrity is such to allow the passage of bacteria, *E. coli* but, notably, also *N.*

meningitidis MC58, from the apical to the basolateral side of the transwell and it depends on the production of mitochondrial ROS. Remarkably, we have found that the administration of C2 to endothelia results in a ROS-dependent reduction of the total VE-cadherin content. This event requires after VE-cadherin phosphorylation, the endocytosis and the subsequent degradation of the protein.

Collectively our data suggest the possibility that C2 might be involved in the pathogenesis of meningitis by permitting the passage of bacteria from the blood to the meninges.

Sommario

Neisseria meningitidis è uno dei patogeni in grado di causare meningite oltre che sepsi in soggetti infettati, due patologie che colpiscono maggiormente bambini e adolescenti entro poche ore dal contagio a meno di una tempestiva terapia antibiotica. La malattia meningococcica risale al sedicesimo secolo. La prima descrizione della malattia causata da questo agente patogeno avvenne ad opera di Viesseux nel 1805 come conseguenza di 33 decessi occorsi a Ginevra, Svizzera [1].

Circa 70 anni dopo, due italiani (Marchiafava e Celli) nel 1884 identificarono per la prima volta degli infiltrati meningococcichi nel fluido cerebrospinale [2].

La presenza di *Neisseria meningitidis* nel mondo varia in base a paesi e regioni e risulta essere ciclica. Grazie alla scoperta di agenti antimicrobicidi come i sulfonamidici e grazie alla diffusione di un adeguato protocollo di prevenzione sanitaria i casi di mortalita` dovuti a questo agente patogeno sono rapidamente diminuiti dal 14 al 9%. Ciò nonostante una percentuale compresa tra l'11 e il 19% dei soggetti ha continuato ad avere problemi post-infezione come disordini neurologici, o perdità dell'udito [3].

Esistono attualmente 13 sierogruppi e, di questi, il 99% delle infezioni è causato dai tipi A, B, C, 29E, W-135 e Y.

I sierogruppi sono stati a loro volta classificati in 20 sierotipi sulla base della presenza dell'antigene proteico PorB, in 10 sierotipi sulla base dell'antigene PorA e in altri immunotipi a seconda della loro capacita` di indurre una risposta immunitaria nell'ospite grazie alla presenza di altre proteine batteriche del patogeno, e per la presenza di un particolare lipopolisaccaride chiamato LOS (lipooligosaccaride) [4].

Neisseria meningitidis è in grado di colonizzare l'epitelio della mucosa orofaringea, dove vi può sopravvivere in maniera asintomatica per l'ospite.

La trasmissione inter-individuale avviene attraverso secrezioni dell'apparato respiratorio. L' incidenza annuale risulta essere di 1- 5 casi ogni 100000 abitanti nei paesi industrializzati, mentre nei paesi ancora in via di sviluppo questa sale a 50 casi per 100000 abitanti. Più del 50% dei casi riguarda bambini sotto i 5 anni d'età, con un'elevata incidenza per coloro che hanno meno di un anno di vita. Questo fatto dipende dall'emivita degli anticorpi materni solitamente in grado di proteggere il neonato per circa 3-4 mesi dopo la nascita. In periodi definiti non-epidemici la percentuale dei portatori sani varia tra il 10 e il 20% della popolazione, e per l'appunto la condizione di portatore asintomatico non è poi così infrequente [5, 6]. Soltanto in un numero ristretto di casi la colonizzazione del batterio progredisce manifestando la patogenesi meningococcica: ciò è per la maggior parte dovuto alla presenza di specifici anticorpi, o per l'attività del sistema del complemento dell'ospite che è in grado di controllare ed eliminare il patogeno impedendone così la sua disseminazione attraverso il flusso sanguigno.

Tuttavia, in un piccolo gruppo della popolazione, la colonizzazione del tratto respiratorio superiore è seguita da una rapida invasione delle cellule epiteliali della mucosa, da dove il batterio è in grado di entrare nel torrente ematico, e raggiungere il sistema nervoso centrale inducendo una forte risposta infiammatoria.

Quale sia l'evento che perturbi l'equilibrio tra essere portatore asintomatico e paziente infetto ancora non è noto. Alcuni fattori sembrano giocare un ruolo chiave in questo cambiamento come la virulenza del ceppo batterico, la capacità

della risposta immunitaria dell'ospite, l'integrità della mucosa e alcuni fattori ambientali [7].

La proteina NHBA, Neisserial Heparin Binding Antigen, è una lipoproteina esposta sulla superficie del batterio, originariamente identificata attraverso la tecnica della "reverse vaccinology" [8].

NHBA in Nm ha un peso molecolare predetto di 51 kDa. La proteina altresì contiene una regione ricca in Arginine (-RFRRSARSRRS-) localizzata in posizione 296 -305 ed altamente conservata in vari ceppi di Neisseria [9]. Tale proteina è altamente conservata in *Neisseria* e non ha omologie di sequenza con nessun'altra proteina registrata nei database procariotici.

Due diverse proteasi possono tagliare la proteina intera NHBA producendo due frammenti differenti: nel primo caso la proteasi batterica NalP taglia la proteina intera in posizione C-terminale producendo un frammento di 22 kDa (comunemente chiamato C2) che inzia con la Ser293 e quindi comprendendo lo stretch di Arginine. Invece, nel secondo caso, la lattoferrina umana (hLf) taglia NHBA immediatamente a monte della sequenza di Arginine, producendo un frammento più corto di circa 21 kDa (comunemente chiamato C1). Sebbene sia risaputo che un passaggio cruciale nella patogenesi mediata da *Neisseria meningitidis* sia l'alterazione della funzione di barriera della microvascolatura encefalica, che può dunque risultare in una rottura della barriera emato- encefalica stessa, non è ancora chiaro quali siano i fattori rilasciati o prodotti dal batterio in grado di indurre un simile effetto. L'integrità dell'endotelio è controllata dalla proteina VE-caderina, localizzata sulle giunzioni aderenti che regolano il contatto cellula- cellula. Tale proteina promuove e regola dunque la permeabilità endoteliale [10]. E' stato ben documentato che l'alterazione della permeabilità

endoteliale può essere dovuta a processi di fosforilazione indotti da fattori solubili come VEGF o TGF-β [11] [12].

Il nostro lavoro documenta come, a differenza del frammento C1, il frammento C2 prodotto dal taglio della proteina intera NHBA, sia in grado di aumentare la permeabilità delle cellule endoteliali HBMEC (human brain microvasculature endothelial cells) fatte crescere a monostrato sulla membrana di un sistema di transwell. L'esposizione della porzione apicale dell'endotelio polarizzato al frammento C2 consente il passaggio di un tracciante fluorescente, BSA-FITC, dal lato superiore a quello inferiore del transwell, in tempi rapidi a seguito del trattamento. E' interessante notare che l'effetto di C2 sull'endotelio è tale da permettere il passaggio dal lato superiore a quello inferiore del transwell non solo di *E. coli*, usato come modello batterico preliminare, ma anche dello stesso *Neisseria meningitidis MC58*, in maniera ROS dipendente. Degno di nota è il fatto che abbiamo osservato che la somministrazione di C2 alle cellule endoteliali provoca una riduzione ROS dipendente del contenuto totale di VE-caderina. A seguito della sua fosforilazione, infatti, VE-caderina viene endocitata all'interno della cellula per poi essere degradata probabilmente attraverso il trasporto di essa verso il proteasoma.

I nostri dati suggeriscono pertanto che C2 sia coinvolto nella patogenesi della meningite favorendo il passaggio di Nm attraverso il torrente ematico dell'ospite verso le meningi.

1. Introduction

1.1 *Neisseria meningitidis*

1.1.1 Features

*Neisseria meningitidis*is the major cause of meningitis and sepsis, two kind of diseases that can affect children and young adults within some hours, except for the availability of a rapid antibiotic therapy. The meningococcal disease dates back to the 16th century. The first description of the disease caused by this pathogen was mentioned by Viesseux in 1805 as 33 deaths occurred in Geneva, Switzerland [1].

It took about seventy year before two Italians (Marchiafava and Celli) in 1884 identified micrococcal infiltrates into the cerebrospinal fluid [2]. *Neisseria intracellularis* was the first name attributed to this bacterium by Anton Weichselbaum in 1887 after the identification of meningococcal infiltrates into the cerebrospinal fluid (CSF) of six patients who died of meningitis [13]. Around the beginning of the former century the morbidity caused by this bacteria was up to 70% of cases. The extreme heterogeneous epidemiology of the agent, being able to be sporadic as well as very fast in its occurrence of outbreaks and epidemics, worsened the situation. Moreover, the worldwide presence of meningococcal serogroups is very different between regions and countries, and cyclical. With the coming of antimicrobial agents, like sulphonamides, the fatality rate cases drop to values from14% to 9% together with appropriate health care and prevention programmes even though 11% to 19% of patients continued to have post-infection issues such as neurological disorders, hearing or limb loss.

The genre *Neisseria* includes two species pathogenic for humans: *Neisseria meningitidis* and *Neisseria gonorrhoeae*.

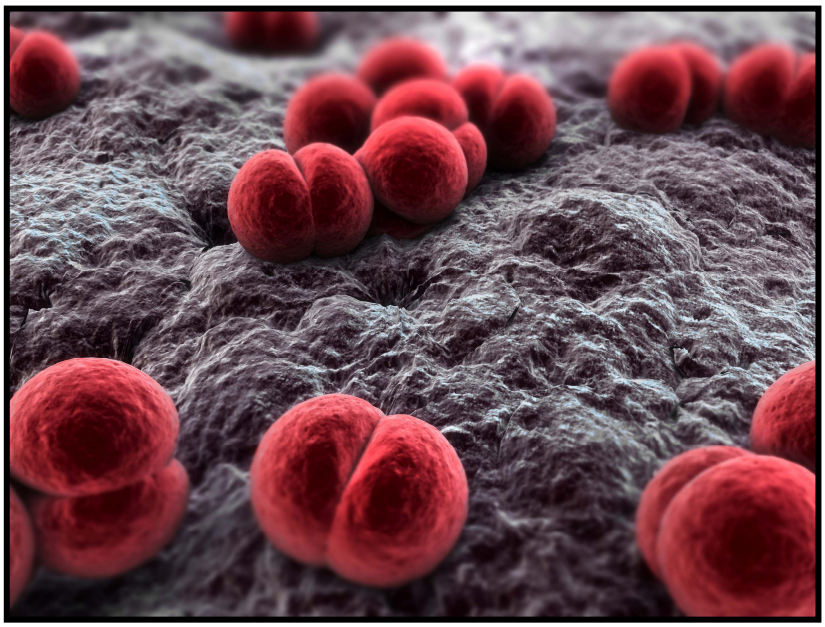

Fig. 1. Neisseria meningitidis is a Gram-negative diplococcus that is one of the most common causes of bacterial meningitis.

Neisseria meningitidis is a capsulated Gram- negative diplococcum with a diameter of 0.6-1.0 μm/coccus (Fig. 1). The best condition for its growth requires an aerobic microenvironment, with low oxygen concentration, 5% CO_2 and a temperature of 35° - 37° C.

The bacterium can be divided into 13 different serogroups, and, among these, up to 99% of infection is ascribed to the serogroups named A, B, C, 29E, W-135 and Y (Fig. 2).

All the serogroups are listed in 20 serotypes on the basis of proteic antigen (PorB), 10 serotypes for the presence of PorA antigens, and in other immunotypes for the capability to mount and drive an immunological response thanks to the outer membrane proteins localized on the membrane of the bacterium, and to the presence of a particular lipopolysaccharide called LOS (lipooligosaccharide) [4].

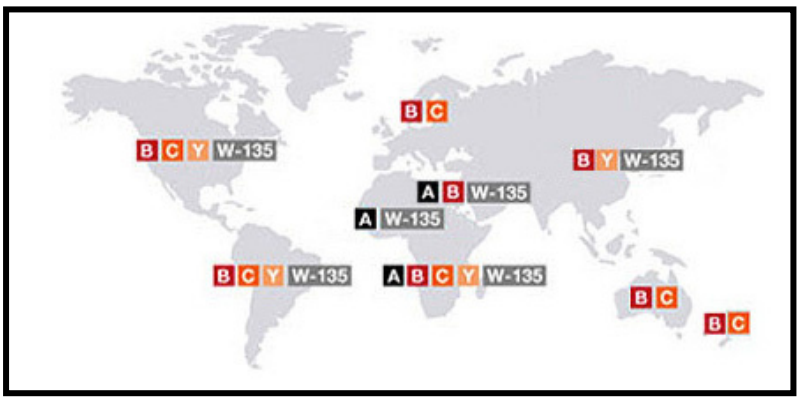

Fig. 2. Distribution of the 5 main disease-causing serogroups of meningococcal bacteria differs from place to place worldwide.

1.1.2 Virulence Factors

The presence of a capsule is fundamental for the survival of the bacteria in the environment before the colonization of the host mucosa, and for the dissemination of the bacteria into the blood flow and the cerebrospinal fluid.

A capsule which contains the sialic acid is specific for serogroups B, C, W-135 and Y.

The *cps* genic complex express the fundamental enzymes for the capsule biosynthesis. *SiaA*, *siaB*, *siaC* and *siaD* are the genes involved in this process.

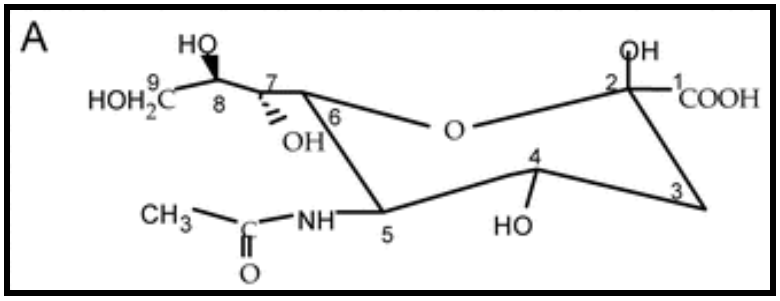

Fig. 3. N-acetylneuraminic acid (Neu5Ac) (present in neuroinvasive bacteria, human tissues, and foods).

The most important feature of the serogroup type B is that the polysaccharide mimics the composition of the sialic acid of several eukaryotic cells, thus impairing the humoral response of the host (Fig. 3). Moreover, the

presence of this polymer protects the bacterium to the action of the C3b complement factor.

The most important proteins localized within the outer membrane of the bacterium are the so-called opacity proteins (Opa and Opc) and the porins (PorA and PorB). The first ones are able to bind the host CD66, in the case of Opa, or the heparan-sulfate proteoglycans mainly exposed on host epithelial and endothelial cells, in the case of Opc. The family gene *opa* codifies for these proteins. The meningococcal strain has 4- 5 different *opa* loci [14]. A typical 5' tandem repeat unit [CTCTT]n All of these genes is responsible for the phase variation.

The phase variation is an efficient tool possessed by bacteria to evade the host immune response, and it relies on the random switching of phenotype at frequencies that are much higher (sometimes >1%) than classical mutation rates. Hence, phase variation contributes to virulence by generating heterogeneity; certain environmental or host pressures select those bacteria that express the best adapted phenotype.

Opa proteins are made of 8 transmembrane β-sheets and 4 highly variable loops exposed [15].

Different *N. meningitidis* strains could be serologically differentiated by Por proteins; both PorA and PorB have been demonstrated to be able to translocate from the bacterial outer membrane to the host plasma membrane creating high-voltage channels which destabilize the transmembrane potential of the host cell, altering many eukaryotic signalling pathways [16].

PorA belongs to the class 1 OMPs (outer membrane proteins) that are different from the OMPs class 2 and 3 because they have more marked loops which

facilitates the bactericidal activity of antibodies directed against them [17]. Moreover, they possess highly variable regions VR1, VR2 and VR3. Of these, the most important one is the VR2 region responsible for evading the host immune system response [18]. It is widely known, in fact, that this variability is largely due to insertions, deletions or amino acidic substitutions in the VR2 or VR1 regions, leading to antigenic variation of the protein.

On the other hand, the class 2 and 3 OMPs are codified by the *porB* gene and they can be considered as two alleles. Bacteria have only one of these two alleles, but the protein of this type they express, is the most abundant on the membrane.

Other major components of the outer membrane involved in the virulence against the host are pili. These structures allow the bacterial adhesion to the host cells and the movement of the cocci along the epithelial surface during the colonization process. They are helicoidal structures composed by pilin, a polypeptide of 18- 22 kDa synthesized as precursor with a non-conventional signal sequence that is subsequently processed by the prepilin peptidase/transmetilase PilD owned by bacteria to form the mature form of the protein [19]. After the maturation process, other post- trasductional events take place, such as phosphorylations and glycosylations [20, 21]. The pilar subunits polymerize inserting the hydrophobic tails inside the core of the main cylindrical helix to form a *coiled- coil* structure, whereas the globular hydrophilic heads are exposed outside to render the cylindrical surface of the filament [22].

The canonical host- pathogen interaction is driven by the pilC protein, a 110KDa protein which is bound to the distal tail of the pili, responsible for the adhesion process. In *Neisseria meningitidis*, there are two kinds of pilC, pilC1 and pilC2,

which both have adhesion properties even if the pilC1 protein is essential for the pili- mediated adhesion [23].

Such adhesion process is an important event that induces a rearrangement of the cellular cytoskeleton leading to plasma membrane alteration and, as a consequence, to the formation of the so- called cortical- plaque, by which the bacterium is able to enter the cells.

When the colonization of the host mucosa process is established, the immune response of the host can be triggered to counteract the infection. One of the very first steps in this defence mechanism is the production of IgA within the host mucosa. The protective role of IgA is particularly relevant if we consider that, in the sub-Saharan zone, the onset of the *Neisseria*- mediated pathogenesis occurs together with the peak of the dry- season. The high concentration of dust, due to the lack of heavy rains, could interfere with the local secretion of IgA thus avoiding the correct establishment of the immune response.

Neisseria meningitidis itself can impair this humoral response producing and secreting IgA proteases. These proteases includes several endopeptidases that directly target and degrade the human IgA. *iga* genes of different *Neisseria* strains can be subject of phase variation in order to be antigenically not targetable by the host response [24].

In *Neisseria gonorrhoeae*, IgA proteases, apart from their role in neutralizing the immunoglobulins secreted by the host, seem to be required for the degradation of LAMP1 (Lysosome Associated Membrane Protein), a protein that regulates the lysosomal biogenesis. The degradation of this protein enhances the survival rate of the bacteria inside the host epithelial cells [25, 26].

Lypooligosaccharide is one of the major components of the outer membrane of Nm. It is composed of the 3-Deoxy-D-manno-oct-2-ulosonic acid

bound to the lipid A and to two internal eptoses. For this reason it is named LOS (lipooligosaccharide). The N-acetylneuramic acid (NANA) constitutes the variable region together with glucose and galactose. LOS is fundamental for the prevention of the bactericidal activity of the host serum as well as for the epithelial cells and the host phagocytes. The prevention system relies on static repulsion due to the high negative charges of sialic acid. It is well documented that LOS decreases the activity of the complement system and, afterwards, it interferes with the polymorphonuclear cells (PMNs) activation, thus limiting the host immune response [27]. This molecule is also fundamental for the survival and replication of the bacteria within the blood flow or the CSF, as well as in the enviroNment during the aerial transmission of the pathogen.

1.2 Meningococcal disease

1.2.1 Epidemiology

The transmission from a carrier to another person occurs by liquid droplet, and the natural reservoir of *Neisseria meningitidis* is the human throat, in particular it is able to colonize the human nasopharynx where it can survive asymptomatically.

The reported annual incidence goes from 1 to 5 cases per 100000 inhabitants in industrialized countries, while in non- developed countries the incidence goes up to 50 cases per 100,000 inhabitants. More then 50% of cases occur among children below the age of 5, and the peak regards those under their first year of age. This fact is due to the loss of maternal antibodies by the newborn. In a non-epidemic period, the percentage of healthy carriers range from 10 to 20%, and notably the condition of chronic carrier is not so uncommon [5, 6]. Only in a small percentage of cases does the colonization progress until the insurgence of

the pathogenesis. This happens because in the majority of cases specific antibodies or the human complement system are able to destroy the pathogens in the blood flow allowing a powerful impairment of the dissemination.

Many studies conducted on the insurgence of epidemic events testify how the meningococcal disease mostly occurs within a few days after the infection, hence when still no specific antibodies have yet been produced.

Neisseria meningitidis A strain is known for its epidemic capacity in still non developed countries; it is, in fact, very rare in North America and in Europe. The most lethal epidemic spreading is localized in Africa and, in particular, in the so-called meningitis- belt, from Ethiopia to Senegal (Fig. 4).

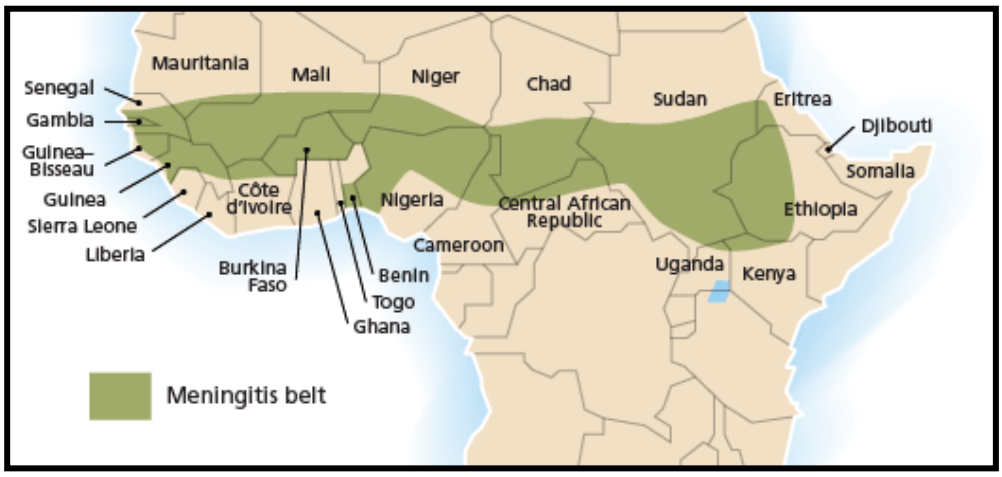

Fig. 4. The African meningitis belt. Source: Control of epidemic meningococcal disease, WHO practical guidelines, World Health Organization, 1998, 2nd edition, WHO/EMC/BAC/98.3.

In developed countries, instead, the most common strain is *Neisseria meningitidis* type C, found in Spain, Italy, Greece, Canada and UK.

Nevertheless, *Neisseria meningitidis* strain B is the most important cause of endemic meningitis in developed countries, and it is responsible for 30- 40% of cases in North America and for the most of 80% in Europe.

The majority of *Neisseria meningitidis* strain B infections show a high seasonal incidence, with its peak during the winter, affecting mostly children below the

first year of age. In high contrast to epidemic events that characterize the serogroups A and C, those caused by Nm type B are known for their slow onset, as well as for their long duration, which can persist for over 10 years. This epidemic has already affected in past years Latin Americas, Norway and since 1991 New Zealand, countries in which the epidemic showed a 10 time greater incidence than the “normal” ones, prevalently in the Pacific Islands and among the Maori population [28, 29].

Since 1990, in the U.S. a high incidence of cases has been identified for what concerns the Y strain of Nm; this pathogenesis has been associated to patients with a defiance in the complement system functionality, aged-persons, and afro- American people.

Globally, *Neisseria meningitidis* affects 1.2 million people per year and, in particular, 3000 cases are reported in the U.S and 7000 in Europe, where the bacterium causes the majority of bacterial meningitis among toddlers and children. Despite several steps forward in prevention, diagnosis, and health-care programmes for the disease associated to Nm, the fatality remains at high levels, like 5-15%, and in about 30-50% of survived persons, permanent neurological disorders are reported [30].

1.2.2 Clinical manifestations

Despite the high pathogenicity, *N. meningitidis* is a human common commensal, found in 10% of adults in the nasopharyngeal mucosa (Fig. 5).

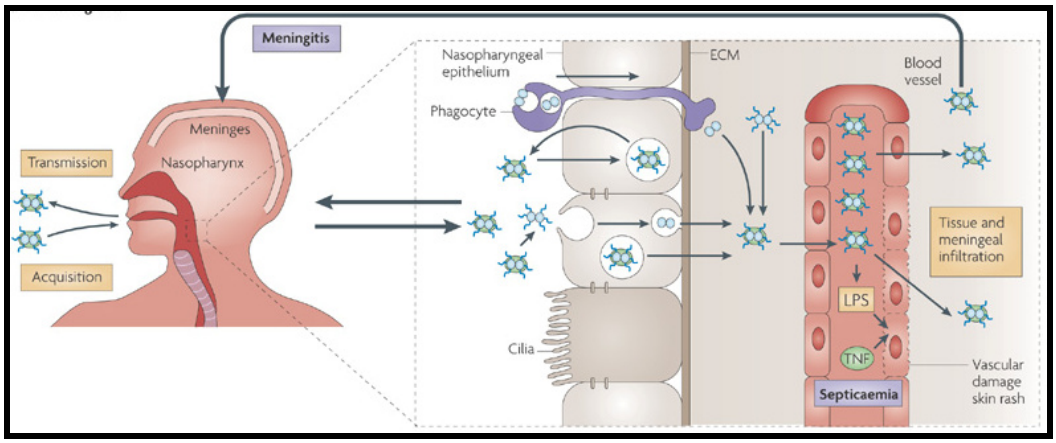

Fig. 5. Neisseria meningitidis may be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells. N. meningitidis can cross the epithelium either directly following damage to the monolayer integrity or through phagocytes in a 'Trojan horse' manner. In susceptible individuals, once inside the blood, N. meningitidis may survive, multiply rapidly and disseminate throughout the body and the brain. Meningococcal passage across the brain vascular endothelium (or the epithelium of the choroid plexus) may then occur, resulting in infection of the meningis and the cerebrospinal fluid. Source: Nature Reviews Microbiology 7, 274-286 (April 2009).

In a small group of the population, the colonization of the upper respiratory tract is followed by a rapid invasion of the epithelial cells, and from this site bacteria can reach the blood flow and invade the central nervous system (CNS), inducing the establishment of an acute inflammatory response.

Children and infants are the main target of the pathogen, while only 10-20% of adults develop immunodeficiency correlated with the pathogenesis.

It is a matter of fact that some hyper virulent strains can cross the nasopharyngeal mucosa disseminating in the blood flow leading to meningococcemia. How the balance between being an healthy carrier or a infected patient can change so rapidly is still unknown. Some candidate factors that could play a role in this switch are the virulence of the bacterial strain, the responsiveness of the host immune system, mucosal integrity, and other environmental factors [7].

The host immune system responds to a *Neisseria* infection by both innate and adaptive immunity. Moreover the rate and efficacy of the host immune response could depend on the age of the patient, as well as on the virulence of the strain, as already previously discussed.

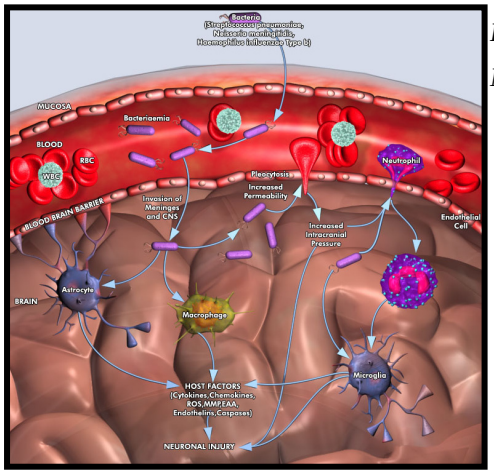

Fig. 6. Mechanism of possible brain invasion by Neisseria meningitidis. Source: Qiagen web page, https://www.qiagen.com/geneglobe/pathwayview.asp x ?pathwayID=50

If bacteria are able to reach the flow, the disease associated to Nm infections are FMS (fatal meningococcal sepsis) and meningococcal meningitis (Fig. 6). The first one is characterized by the insurgence, in a very short time (6-12 hours), of high fever, lack of consciousness, and disseminated rush that depends on the intravascular coagulation and thrombotic events in small vessels. This could lead to a micro vascular failure that can damage host tissues (Waterhouse-Friderichsen syndrome) until necrosis of the limbs occurs. In this case amputation is required [31, 32].

At these stages, LOS can have a fundamental role in inducing a shock syndrome much more severe than its vascular concentration. This kind of infection leads to the release of lytic proteins or inflammatory cytokines that, instead of being useful for the clearance of the pathogen, worsen the situation by highly damaging the already compromised tissues with bleeding events and, in up to 80% of cases,

result in the death of the host. The majority of patients die after 24 hours of insurgence of the primary symptoms.

Meningitis is led by high fever, headache, photophobia, altered state of consciousness, nape and neck stiffness. The purulent infection of the meningis occurs when, for some still unknown reasons, the bacteria from the blood flow cross the blood brain barrier (BBB) reaching this tissue where the most important humoral and cellular immune response systems cannot access. In this scenario the bacterium can freely proliferate leading to a critical inflammation of the CNS. The fatality rate is not so high, but in 8-20% of patients there could be permanent neurological disorders, like mental retardation, spasticity and loss of sensitivity.
Despite the availability of antibiotics, the mortality rate remains between 5-10% in industrialized countries, but it can double in developing countries, and for these reasons it is extremely important to have a quick early diagnosis and an effective highly-specific antimicrobial therapy.

1.2.3 Vaccines

Over the last century, many vaccines have been found and developed to counteract Neisserial infection, with various results.
In many cases, diseases are vaccine-preventable; the first vaccine against serogroups A and C, was around since the 1960s [33].

A quadrivalent purified polysaccharide vaccine against serogroups A, C, W-135 and Y was licensed in the U.S. in 1981 [34]. Except for type A, this vaccine was poorly immunogenic in children below 2 years of age. Another negative aspect of this vaccine was the short-lived immunity, mainly because it was raised against capsular polysaccharides, known to be T-cell independent

antigens, and then, unable to elicit a long term humoral response. Repeated administrations (every 3-5 years) were then required; moreover these repeated immunizations could induce antibody hypo- responsiveness because of mechanisms of tolerance instauration.

From that time on, efforts to develop vaccines to circumvent the limitation of capsular vaccines were carried on, until the introduction of conjugate vaccine against type C strain in UK in 1999 in response to an epidemic event. This vaccine, administered at 2, 3 and 4 months of age, was protective up to the first year of age, but not extended beyond the year [35]. In year 2000 a new tetravalent vaccine (Menveo, or MCV4) conjugated to diphtheria toxoid was licensed in U.S. for people between 2 and 55 years of age. This vaccine is now recommended for all those people that travel in *Neisseria* endemic areas (like the meningitis belt), military recruits or immunocompromised subjects. But, again, the immunogenicity of this vaccine for infants is extremely low. A second generation vaccine conjugated with a mutant diphtheria toxoid was recently licensed by Novartis in U.S.

Moreover, another combined vaccine with *H. influenzae* type B and meningococcal C and Y capsules, each conjugated to tetanus toxoid, is undergoing clinical trials [36].

There is still no licensed polysaccharide based vaccine against *Neisseria* serogroup type B because of the low immunogenicity of the type B strain capsule, mimicking sialic residues of mammalian cells and tissues. Of course, alternative strategies have been investigated.

A polysaccharide-tetanus toxoid conjugate was developed, substituting the sialic acid of type B strain with an N-propyonil group, to avoid self tolerance.

Despite being highly immunogenic, no bactericidal activity was found in mice. Moreover the concern that auto- reactive antibodies could be formed against the remaining portion of polysialic acid residues was high. However, Granoff et al. have shown that antibodies raised against epitopes of this vaccine components do not cross- react with human sialic residues, thus extending the case for further considerations for the use of this vaccine strategy [37].

OMV (outer membrane vesicle) based vaccines were generated from culture supernatants of *Neisseria* by detergent extraction of these vesicles. These kind of vaccines were delivered to different countries such as Chile, Brazil, Cuba, Norway, and most importantly New Zealand to counteract a huge epidemic. The main issue for these preparations is that the majority of antibodies are directed against the protein PorA, which is highly variable among different meningococcal strains. It is then evident that these vaccines give protection against only a particular strain, but the induction of any antigenic shift in PorA or mutations in *porA* gene would render the vaccine ineffective. A possible idea to take into consideration, is the production of OMVs vaccines based on several PorA variants to confer wide protection from different circulating type B strains.

In the year 2000 the discovery of the “Reverse Vaccinology” technique may have overturned the common lines of thought for the development of vaccines. By genome sequencing it has been possible to identify novel potential surface exposed protein antigens in *Neisseria meningitidis B* [38, 39].

Among all the protein candidates, 350 were expressed in *E. coli*, purified and used to immunize mice. The collected sera allowed the identification of those surface exposed proteins that were highly conserved among several strains, and that were able to induce a bactericidal antibody response. Five promising antigens, NadA

(*Neisseria* adhesion A), fHbp (factor H binding protein), NHBA (Neisserial Heparin Binding Antigen), GNA2091 and GNA1030 (Genome-derived *Neisseria* Antigen) were identified, characterized and combined with OMVs to create a meningococcus recombinant vaccine, called 4MenB. Immunized mice showed bactericidal antibodies directed against a panel of selected serogroup B strains [40-42]

4MenB vaccine, at the end of November 2012, received a positive opinion from the Committee for Medicinal Products for Human Use (CHMP) of the European Medicines Agency (EMA) for the use in individuals from 2 months of age and older.

Functional characterization of MenB antigens has been described for NadA, fhbp and NHBA. Neisseria adhesin A(NadA) is a pathogenicity factor involved in host cell adhesion and invasion and is reported to be present in less than 50% of isolated strains tested; it has a low level of representation among carriage isolates and up to 100% coverage in some hyper virulent lineages [43]. fHBP is a virulence factor that specifically binds to the human complement-regulating protein factor H, thereby enhancing serum resistance [44, 45]. So far, all isolates have been shown to harbour an fHbp allele, and the antigen falls into one of three major variant groups: variant 1 and variants 2 and 3 [46].

All isolates possess an *nhba* allele. The protein binds heparin *in vitro* through an Arg-rich region and this property correlates with increased survival of the un-encapsulated bacterium in human serum [9].

The investigation of the role in pathogenesis of the NHBA cleaved fragments will be subject of my thesis.

1.3 NHBA

1.3.1 Features

Neisserial heparin binding antigen (NHBA) is a surface-exposed lipoprotein from *Neisseria meningitidis* that was originally identified by reverse vaccinology [8].

All isolates possess an *nhba* allele. The protein binds heparin *in vitro* through an Arg-rich region and this property correlates with increased survival of the un- encapsulated bacterium in human serum.

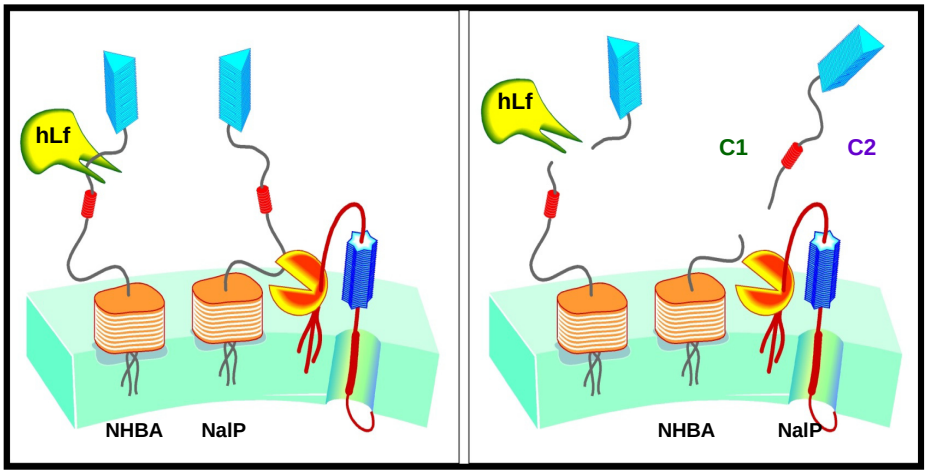

Fig. 7. Mechanism of cleavage of full length NHBA. The hLf cleaves the full length protein downstream an Arg- rich region (red box motif in the picture) mediating the release of a fragment called C1. The NalP protease cleaves NHBA protein mediating the release of a longer fragment called C2, which comprises the Arg-stretch. In both cases the N- fragment remains anchored to the bacterial surface.

Furthermore, two proteases, the meningococcal NalP and human lactoferrin (hLf), cleave the protein upstream and downstream from the Arg-rich region, respectively (Fig. 7). Moreover, anti-NHBA antibody elicited deposition of human C3b on the bacterial surface and passively protected infant rats against meningococcal bacteraemia after challenge with Nm strains [47]. NHBA was thus considered a promising candidate for prevention of meningococcal disease.

The predicted molecular weight of NHBA is 50,553 Da. The protein has a signal peptide with a typical lipobox motif (-LXXC-) and in the Nm MC58 strain it has an Arg-rich region (-RFRRSARSRRS-) located at position 296–305, highly conserved among different Nm strains [9]. The protein is specific for *Neisseria* species, as no homologous proteins can be found in non- redundant prokaryotic databases.

Arg and Lys residues are present in the heparin-binding sites of different proteins [48], where they are able to interact with negatively charged residues of proteoglycans. By affinity chromatography with heparin as ligand it was demonstrated that the full length protein bounds to heparin [9, 49]. To define the role of the Arg-rich region in the interaction, a deletion mutant of the Arg-rich region and another mutant wherein all Arg residues were substituted with a Gly were generated. Neither of these mutants were able to bound heparin confirming the fundamental role of the Arg- stretch for the binding.

Moreover, western blot analysis performed on outer membrane proteins (OMPs) showed the presence of two NHBA -specific bands in strain MC58, which were absent in the mutant strain (MCΔ2132). The first band, relative to the full leght NHBA, had a molecular weight of approximately 60 kDa, and a second band at approximately 22 kDa was identified in the supernatant, suggesting the processing of the protein and the release of a fragment. Purification and N-terminal sequencing of the 22-kDa protein fragment showed that this fragment started with Ser293 and hence corresponded to the C-terminal region of NHBA.

A panel of different meningococcal strains were tested to screen the specificity of this band pattern. Western blot analysis revealed that NHBA was expressed by all strains tested. However, the protein was cleaved and the C-fragment released in

the supernatant in only five of 20 strains tested, which belongs to the hyper-virulent clonal complex 32. The presence of the NalP protease, a phase variable auto- transporter protein with serine protease activity, was considered to be a strong candidate for the processing of NHBA because NalP has been shown to process many other surface exposed Nm proteins [50, 51].

A NalP deletion mutant was generated in strain MC58 to test NHBA expression and processing by immunoblotting of OMP and supernatants. In the NalP- deleted strain, a higher amount of the NHBA full-length protein was detected, whereas the N- and C-fragments were not detectable. The point that NHBA could be processed in only some Nm strains, might correlate with the finding that the *nalP* gene is prone to phase variation. Together with this evidence, it was also demonstrated that human lactoferrin (hLf), could recognize and cleave NHBA[52, 53]. Full length NHBA was incubated with hLf purified from human milk and by western blot analysis it has been showed that NHBA was cleaved into two fragments of approximately 43 kDa (N1) and approximately 21 kDa (C1). The 21-kDa fragment was subjected to N-terminal sequence analysis. The sequence analysis from the 21 kDa fragment obtained (245-SLPAEMPL-252) showed that the cleavage mediated by hLf occurs immediately downstream of the Arg-rich region. Other experiments performed by Esposito and colleagues demonstrated that the recombinant C-his fragment containing the Arg-rich region is also a target of hLf and suggests that hLf can act on the full-length NHBA as well as on the secreted C fragment [49].

Moreover in that manuscript, his-tagged forms of the N-terminal and the C-terminal regions generated by the NalP protease and by the hLf cleavage were used to evaluate their ability to bind heparin. Only the fragment containing the

Arg-rich region was able to bind heparin, confirming the key role of the region in this interaction [9, 49, 54].

1.4 VE-cadherin and the regulation of endothelial permeability

1.4.1 Features

The endothelium is located on the inner side of all vessel types and is constituted by a monolayer of endothelial cells [55, 56].

Interendothelial junctions contain complex junctional structures, namely adherens junctions (AJ), tight junctions (TJ) and gap junctions (GJ), playing pivotal roles in tissue integrity, barrier function and cell–cell communication, respectively (Fig. 8).

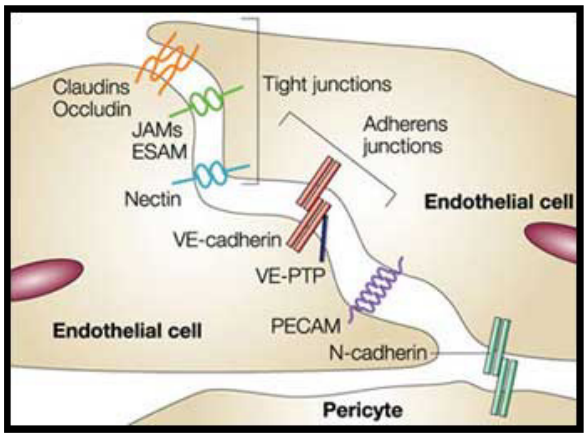

Fig. 8. Transmembrane adhesive proteins at endothelial junctions. At tight junctions, adhesion is mediated by claudins, occludin, members of the junctional adhesion molecule (JAM) family and endothelial cell selective adhesion molecule (ESAM). At adherens junctions, adhesion is mostly promoted by vascular endothelial cadherin (VE-cadherin), which, through its extracellular domain, is associated with vascular endothelial protein tyrosine phosphatase (VE-PTP). Source: Dejana E,Nat Rev Mol Cell Biol. 2004

The endothelium constitutes a barrier for the vascular system by controlling and regulating permeability properties between the blood and the underlying tissues.

As well established, endothelial permeability is mediated by the so-called transcellular and paracellular pathways by which, solutes and cells can pass through (transcellular) or between (paracellular) endothelial cells [10]. Transcellular passage occurs via specialized pore-like fenestrae that can control cellular permeability to water and solutes, or via a complex system of transport vesicles [57-61]. The paracellular pathway, by contrast, is mediated by the tightly regulated and coordinated opening and closure of endothelial cell-cell junctions. This is of particular importance to maintain endothelial integrity and to prevent exposure of the subendothelial matrix of blood vessels [62-64]. Many soluble factors can increase permeability, such as histamine, thrombin and vascular endothelial growth factors (VEGFs). The process is reversible, then not necessarily affecting endothelial-cell viability or functional responses for long periods [11, 65, 66].

The junctional structures located at the endothelial intercellular cleft are similar to the epithelial ones with some exceptions: their organization is more variable and, in general AJ, TJ and GJ are often intermingled and form a complex zonular system with variations in depth and thickness [67-72].

AJs are formed by members of the cadherin family of adhesion proteins. Two types of cadherins are the main components localized on the apical domain of endothelial cells: a cell-type-specific cadherin (VE-cadherin) and neuronal cadherin (N- Cadherin), which is also present in other cell types such as neural cells and smooth muscle cells [73]. Other non-cell-type-specific cadherins can be variably expressed in different types of endothelial cells [74].

VE-cadherin is the major determinant of endothelial cell and the regulation of its activity or its presence is essential to control the permeability of the blood vessels [64].

Cadherins are defined by the typical extracellular cadherin domains (EC-domain) and mediate adhesion via homophilic, Ca^{2+}- dependent interactions.

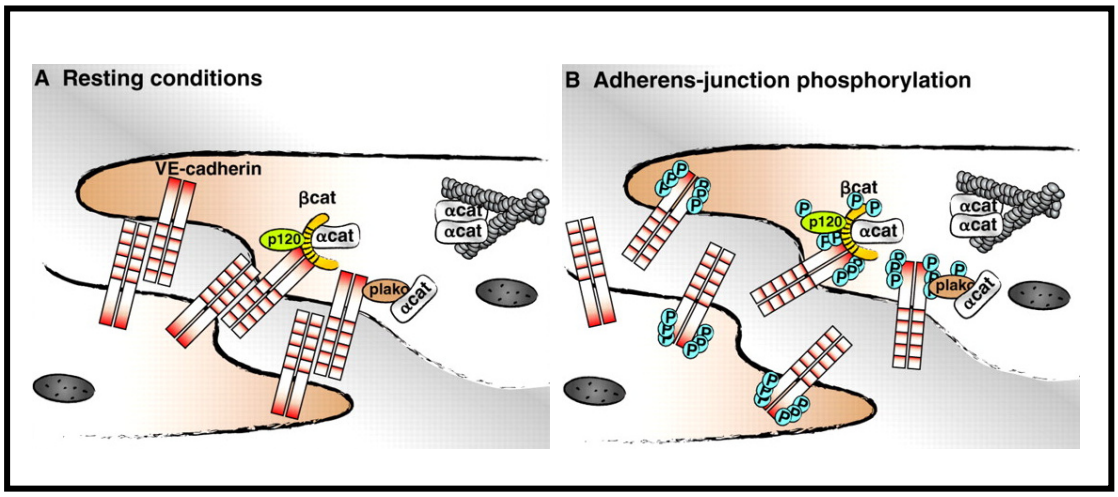

Fig. 9. Functional modifications of endothelial AJs (A) Under resting conditions, VE-cadherin clusters at junctions in zipper-like structures; p120, β- catenin (βcat) and plakoglobin (plako) bind directly to VE-cadherin, whereas α- catenin (αcat) binds indirectly through its association with β-catenin or plakoglobin. (B) Phosphorylation (P) of tyrosine residues of VE-cadherin, β- catenin, plakoglobin and p120 reduces AJ strength. The VE-cadherin complex might become partially disorganized without any evidence of cell retraction. Phosphorylation of VE-cadherin at Ser665 has also been reported. This process is thought to mediate VE-cadherin internalization and increase vascular permeability. Source: E. Dejana, et al. (2008). J Cell Sci, 2115–2122.

Optimal adhesive function of cadherins requires association of their C terminus with cytoplasmic proteins: the catenins (Fig. 9). Cadherins bind directly to β-catenin (alternatively to plakoglobin) and to p120. β- catenin and plakoglobin can bind to α- catenin, an actin binding protein. For many years, it has been generally accepted that linkage of the cadherins via the catenins to the actin cytoskeleton is the mechanism by which catenins strengthen cadherin-mediated adhesion. The lack of catenin association with cadherin is commonly accepted as a destabilizing event for the endothelial integrity. Various intracellular signalling molecules, as

well as phosphorylation of tyrosine and serine residues of catenins or cadherins, have been reported to play a role in cadherin regulation.

Several studies focus on the effect of agents that increase vascular permeability on the organization of endothelial cell-cell junctions [66, 75-79]. Some agents, such as histamine or thrombin, act very rapidly, and the effect is quickly reversible once they are removed. By contrast, inflammatory cytokines increase vascular permeability if the effect is sustained up to 24 and 48 hours. Thus, it is clear that the mechanism of action might vary depending on the factor(s) released or produced to modify the endothelial permeability. However, in many reported cases, the junctional weakness did not reflect morphological alteration of endothelial monolayers; for instance, the internalization of VE-cadherin or the phosphorylation of AJ proteins reduces junctional strength without necessarily opening intercellular gaps [65, 76].

1.4.2 Tyrosine phosphorylation of AJ components

Endothelial permeability can be modulated in several molecular mechanisms; for instance, the phosphorylation, cleavage and internalization of VE-cadherin are all thought to affect endothelial permeability (Fig. 10). It has been reported that the tyrosine phosphorylation of VE-cadherin and other components of AJs is associated with weak junctions and impaired barrier function. Agents such as histamine, tumour necrosis factor-α (TNFα), platelet-activating factor (PAF) and VEGF induce tyrosine phosphorylation of VE-cadherin and its binding partners β-catenin, plakoglobin and p120[65, 80].

The mechanism of VE-cadherin phosphorylation has not yet been fully clarified. In some manuscripts it is declared that tyrosine kinase Src is probably

implicated, being directly associated with VE –Cadherin. Moreover, VEGF-induced phosphorylation of VE-cadherin is inhibited in Src-deficient mice or in wild-type mice treated with Src inhibitors [66]. In addition to Src, other kinases are thought to associate with the VE-cadherin–β- catenin complex and to modulate endothelial permeability [81].

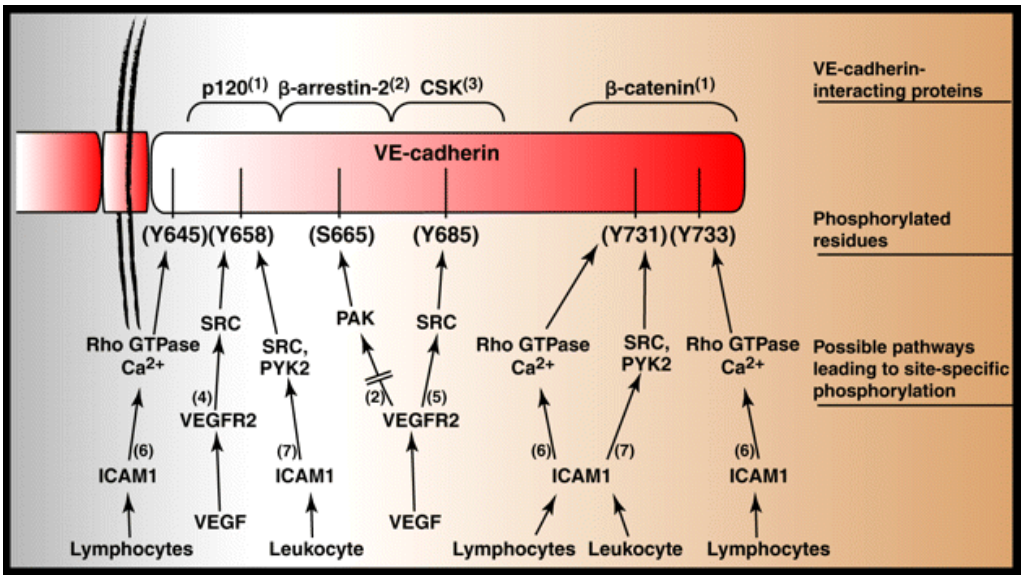

Fig. 10. Phosphorylation of VE-cadherin. The sites of tyrosine (Y) and serine (S) phosphorylation are shown. The interaction of VE-cadherin with individual proteins can be positively (CSK, β-arrestin-2) or negatively (p120, β-catenin) regulated by its phosphorylation at specific amino acid residues. Source: E. Dejana, et al. (2008). J Cell Sci, 2115–2122.

Several publications report on correlations between changes in the stability of VE-cadherin adhesion and changes in the tyrosine phosphorylation of the VE-cadherin catenin complex. It has been suggested that tyrosine phosphorylation of VE-cadherin itself might affect VE-cadherin functions. Based on permeability studies of transfected CHO cells, expressing point mutated forms of VE-cadherin with tyrosine residues replaced by either glutamate or phenylalanine, tyrosine residues 731 and 658 were suggested to participate in the regulation of the adhesive function of VE-cadherin [82].

VEGF was found to enhance the permeability of HUVEC monolayers and to increase tyrosine phosphorylation of VE-cadherin, β-catenin, and plakoglobin [76]. Intravenous injection of mice with VEGF was reported to lead within 2 to 5 minutes to the dissociation of a pre-existing complex of the VEGF-receptor 2 with VE-cadherin and β- catenin, as well as Src- dependent tyrosine phosphorylation of VE-cadherin and β- catenin [83].

This complex is most likely important for the regulation of VE-cadherin mediated adhesion [84-86]. An alternative mechanism for the down regulation was proposed for VE-cadherin function during VEGF-induced permeability. This process could be based on the phosphorylation of serine 665 in the cytoplasmic tail of VE-cadherin, leading to endocytosis [87].

VE-cadherin seems to be internalized through a process regulated by a clathrin-dependent endocytosis [88]. Interestingly, the binding of p120 to VE-cadherin prevents its internalization, introducing the concept that p120 might act as a plasma-membrane-retention signal.

VE-cadherin is an important determinant of the barrier function of the vascular endothelium. From the knowledge of how the expression and function of this protein are regulated, it should be possible to design specific agents that can increase or decrease vascular permeability. Further work is required, however, to address important issues such as the relationship between the transcellular and paracellular permeability pathways and their specific biological roles in different regions of the vascular tree.

2. Materials and Methods

2.1 Reagents

Phosphate-buffered saline (PBS), D-MEM High Glucose and Foetal bovine serum (FBS) were purchased from Euroclone (Siziano, IT). Gentamicin and Hepes were purchased from Gibco (Scotland ,UK). Endothelial cells growth supplement (ECGS), BSA-FITC, Red Ponceau, tetramethylbenzidine (TMB) and TMB Stop Solution (0.16 M sulphuric acid), MEM non essential aminoacids, MEM vitamins, BSA, gelatine type B, N-acetylcysteine (NAC) , DTT and Tween-20 were obtained from Sigma-Aldrich (St Louis, MO). 5ml His Trap HPcolumn, Nitrocellulose membrane, X-ray film and ECL (enhanced chemiluminescence system) were purchased from GE Healthcare (Buckinghamshire, UK). BCA protein assay reagent was purchased from Pierce (Rockford, IL). Mitosox Red, α-mouse Alexa Fluor 488 and α-rabbit Alexa Fluor 594, 4-12% and 10% SDS-PAGE gels, LDS 4X sample buffer, NuPAGE antioxidant, NuPAGE MES 20X Running Buffer, NuPAGE 20x Transfer Buffer were obtained from Invitrogen (San Diego, CA). VEGF was obtained from Immunological Sciences (Rome, Italy). Mitochondria Isolation kit and QiAMP mini-prep Kit were purchased from Qiagen (Hilden, Germany). SU6656 was purchased from Merck-Millipore (Darmstadt, Germany). Goat polyclonal and monoclonal anti-total VE-cadherin antibodies and agarose-coupled Protein G were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against EEA1 was from Abcam (Cambridge, UK) and monoclonal antibody against phosphotyrosine (clone G410) was obtained from Upstate Biotechonolgy. Monoclonal anti complex II antibody was purchased form Mitoscience (Eugene, OR). 8-well chambers slide, NU-serum

IV and monoclonal anti-beta catenin was obtained from BD Bioscences (Franklin Lakes, NJ).

2.2 Bacterial strains and cell culture

Escherichia coli strain DH5α and *Neisseria meningitidis* strain MC58 were used in trans-endothelial migration assays. *Neisseria meningitidis* strain was a serogroup B isolate (United Kingdom 1983) of the ST-32 complex characterized as serotype B:15:P1.7,16. Simian virus 40 large T antigen-transformed human brain microvascular endothelial cells (HBMEC) were kindly provided by Novartis Vaccines and Diagnostics s.r.l (Siena, Italy) and were cultured in T75 flasks, in FBS/NU-serum IV-supplemented DMEM high glucose plus non-essential aminoacids and vitamins, to a confluent monolayer. For *in vitro* permeability assays, cells were split and seeded on gelatine-coated Trans-well cell culture chambers (polycarbonate filters, 0.3 μm or 3 μm pore size; Corning Costar Corporation, Cambridge, MA, USA) at a density of 7×10^4 cells per well. Cells were grown for 5 days before performing permeability assays.

VEC+ endothelial cells derived from murine embryonic stem cells with homozygous null mutation of the VE-cadherin gene and overexpressing wild-type human VE-cadherin [89, 90] were kindly provided by E. Dejana (IFOM, Milan, Italy). Cells were maintained in culture in T75 flasks in FBS-supplemented DMEM high glucose plus heparin and ECGS.

Mouse embryonic fibroblast (MEFs) were maintained in culture in T75 flasks in FBS-supplemented DMEM high glucose.

2.3 Construction of plasmids

For the expression of all the recombinant proteins considered in this study, the specific DNA fragments were amplified by PCR from N. meningitidis MC58 genomic DNA and cloned into the pET-21b+ expression vector (Invitrogen), as detailed in Serruto et al., 2010. Briefly, to obtain a recombinant full-length protein rGNA2132MC58-his, the nmb2132 gene was amplified from the MC58 genome using the oligonucleotides 2132-dG-FOR and 2132-REV, digested with NdeI and XhoI restriction enzymes and cloned into the NdeI/XhoI sites of the pET-21b+ vector, generating pET-GNA2132-MC58-his. The constructs for the expression of C-terminal domains of GNA2132 were prepared by ligating PCR products, digested with NdeI and XhoI restriction enzymes, into the pET21b+ expression vector. For pETGNA2132-C2-his (recombinant C2-terminal region, aa 293–488), the PCR fragment was obtained using the 2132-C–FOR and 2132–REV primers. Finally, for pET-GNA2132-C1-his (recombinant C1-terminal region, aa 307–488), the PCR fragment was obtained using the 2132-C1–FOR and 2132–REV primers.

2.4 Transformation of competent *Escherichia coli*

E. coli BL21(DE3) chemically competent cells which have been kept on -80°C storage were thawed on ice. 100-200 ng of plasmid DNA were added to the competent cells and the transformation mix was kept on ice for 30 min. Cells were heat-shocked for 30- 40 sec at 42°C and the cooled on ice for 2-3 min. The cells were incubated for 45 min at 37°C in 500 µl of Luria-Bertani (LB) broth (10 g/l Bacto Tryptone, 5 g/l Bacto yeast extract, 10 g/l NaCl) in agitation. The mix was plated on LB agar plates which contained the antibiotics ampicillin and

chloramphenicol that select for transformants. The plates were incubated overnight at 37°C. Bacterial colonies were colony-PCR analyzed.

2.5 Plasmid DNA isolation from bacteria (Miniprep)

E. Coli cells carrying the plasmid of interest were incubated overnight at 37°C at constant shaking (200-220 rpm) in 5 ml of LB broth supplemented with the appropriate antibiotic (chloramphenicol 20 μg/ml). The cells were harvested by centrifugation at 13,000 x g (microcentrifuge Biofuge, Haeraeus) for 3 min, and the plasmid DNA was isolated using the QIAprep Spin miniprep kit (Qiagen) following the manufacturer's instruction. Briefly, cellular pellet was resuspended in 250 μl of buffer P1 (Qiagen), then were added 250 μl of buffer P2 (Qiagen) and the suspension was gently inverted 2-3 times; 350 μl of neutralizing buffer N3 (Qiagen) were added, the suspension was gently inverted and centrifuged 10 min at 13,000 x g. Supernatants were applied in the Qiaprep spin column and centrifuged 1 minute at 13,000 x g; the column was washed two times by adding 750 μl of buffer PE (Qiagen) and centrifuged 1 min at 13,000 x g. The purified plasmid DNA was eluted from the column with 50 μl of sterile water. The concentration and quality of the purified DNA was measured with a UV spectrophotometer at OD 260-280.

2.6 NHBA, C1 and C2 expression and purification

E. coli transformation was carried out according to standard protocols. *Escherichia coli* strain BL21(DE3)-pLysS containing the expression vectors were grown overnight at 37°C in 500 ml of LB medium supplemented with ampicillin (20 μg/ml) to an OD600 of 0.6. NHBA, C1 and C2 expression was induced by 1

mM IPTG. After 3 h, bacteria were pelletted by centrifugation at 8000g for 10 min and resuspended in 10 ml of lysis buffer (50 mM Na-Phosphate (pH 8.0), 300mM NaCl, 20 mM Imidazole, plus protease inhibitors). After 5 sonication passages, for 1 min at 20 mA amplitude, debris were removed by centrifugation at 32000g for 30 min at 4°C. Supernatant was filtered through a 0.2 μm syringe filter and the proteins were eluted from affinity chromatography His Trap HP column by applying 150mM imidazole. Purity of the proteins was checked by SDS/PAGE. Protein was concentrated using the ultrafiltration system Centricon® (Millipore) and the content was quantified using the BCA assay.

2.7 Permeability assays

HBMECs were seeded onto 2% gelatin-coated Transwell filters (0.3 μm pore size) at the density of 7×10^4 cells per well in a 24-well plate. Cells were used 5 days after seeding onto filters. The formation of intact monolayer on the insert was evaluated by adding FITC-BSA (1 mg/ml) to the upper chamber and measuring after 5 min the amount of labeled BSA passed into the lower chamber by a Fluostar microplate reader (SLT Labinstruments). Transwells were used only when the intensity of fluorescence in the lower chamber was negligible.

Permeability assays were performed after administrating, in the lower or upper chamber, the following stimuli: 5 μM C1, 5 μM C2 or NHBA or 1 μM bradikinin (BK). When required, cells were exposed to 1mM N-acetylcysteine (NAC) 30 min before adding the stimuli. FITC-BSA fluorescence was evaluated in the lower chamber at various time intervals. Calibration curves were set up measuring the fluorescence intensity of increasing concentrations of FITC-BSA.

2.8 Evaluation of *E. coli* crossing through the endothelium

HBMECs were seeded onto 2% gelatin-coated Transwell filters (3 μm pore size) at the density of 7×10^4 cells per well in a 24-well plate. Cells were used 5 days after seeding onto filters. Each Transwell was checked for the formation of intact monolayer by adding FITC-BSA to the upper chamber, as described above. *E. coli* strain DH5α , together with the stimuli, 5 μM C2 or C1 or NHBA or 1 μM BK, was added to the upper chamber (10^6 bacteria/well, MOI: 15). When required, cells were exposed to 1mM NAC 30 min before adding the stimuli. After 1 and 2 h-incubation, bacteria-containing medium from the lower chamber was collected and plated onto LB agar plate at 37°C. After 18 h, colony-forming units (CFU) were counted.

2.9 Evaluation of *N. meningitidis* crossing through the endothelium

Monolayers of HBMEC, prepared as above, were infected for 4 hours with 10×10^6 bacteria/well, strain MC58 (MOI: 30). Infections were carried out in the presence of NHBA or one of the two recombinant fragments (C1; C2). After 30 min, 1 h, 2 h, 3 h and 4 h the medium of the lower chamber was collected and plated on Mueller Hinton Medium (MHM) plates for further colony counting.

2.10 Mitochondria isolation

HBMECs were seeded onto T75 flasks and, once confluent, were exposed to 5 μM C1 or C2 for 5, 15 and 30 min. Cells were collected, washed in ice-cold PBS and processed by Qiagen Mitochondria Isolation Kit. Protein content of isolated fractions, corresponding to mitochondria, cytosol and microsomal fraction, was determined by BCA assay. 10 μg of each fraction were loaded on SDS-PAGE 4-12% and analyzed by western blot.

2.11 SDS-PAGE (PolyAcrilamide Gel Electrophoresis)

Cell extracts as well as isolated fraction or immunoprecipitated samples were diluted in Loading buffer which was prepared as follows:

- 1X NuPAGE® LDS Sample Buffer
- DTT 50 mM

The volume of each sample was brought to 15 µl. The samples were denaturated at 99 °C for 10 min. Samples were loaded on SDS 4-12% or 10% precast polyacrylamide gels. The electrophoresis was run in 1X MES Running buffer containing the antioxidant at 110 mA and 200 V constant for 45 min.

2.12 Western Blot

After electrophoretic run, proteins were transferred from gel to nitrocellulose membranes. The gel and the membrane were equilibrated in Transfer Buffer. The Transfer Buffer was prepared as follows:

- 20X NuPAGE® Transfer buffer
- 10X NuPAGE® Antioxidant
- 10% Methanol

The volume was brought to 1 l with distilled water.

The transfer was obtained by applying a current of 170 mA and 30 V constant for 1 h. To evaluate the efficiency of the transfer, proteins were stained with Red Ponceau 1X. The staining was easily reversed by washing with distilled water. Once the proteins were transferred on nitrocellulose membranes, the membranes were saturated with Blocking Buffer (5% no fat milk powder solubilizated in PBS with 0.2% TWEEN-20, or 5% BSA powder solubilizated in TBS with 0.1% TWEEN-20) for 1 h at room temperature, and then incubated overnight with the primary antibody of interest at 4°C. The membranes were then washed 3 times

with PBS with 0.2% TWEEN-20 (or TBS with 0.1% TWEEN-20) at room temperature and incubated with secondary antibody-HRP Conjugate, for 1 h at room temperature. Immunoreaction was revealed by ECL PRIME and followed by exposure to X- ray film.

2.13 Immunoprecipitation

Murine endothelial cells overexpressing wild-type human VE-cadherin were grown in T25 flask. Cell layers were serum-starved for 3 days before the application of stimuli. 5 μM C2 or 10 ng/ml VEGF were added for 15, 30 and 45 minutes. When required, cells were pre-incubated for 30 min with1 mM NAC. Cells, detached by scraping, were collected, washed in ice-cold PBS and lysed in RIPA buffer, supplemented with protease and phosphatase inhibitors. Lysates were centrifuged at 12000 g for 20 min at 4°C. Supernatants were collected and their protein content determined by BCA assay. 500 μg cell extract for each sample was immunoprecipitated with 2 μg goat polyclonal anti-VE-cadherin conjugated to 20 μl protein G agarose. The immunoprecipitates finally recovered were run in SDS-PAGE (10% polyacrylamide) for blot with anti-phosphotyrosine antibody.

The total content of VE-cadherin was assayed using a goat polyclonal antibody anti-total VE cadherin and the total content of beta-catenin was revealed by a specific monoclonal antibody. Western blots were developed with HRP-conjugated anti-IgG followed by ECL.

2.14 Measurement of changes in mitochondrial ROS production in HBMECs

HBMECs were grown on 24 mm diameter glass dishes till confluence; medium was removed and replaced with HBSS buffer plus Ca^{2+} and Mg2+, 10 mM

Glucose and 4 mM Hepes. Cells were incubated for 30 min with 1 μM Mitosox Red before starting the live imaging recording of fluorescence (10 sec intervals), at 580 Nm, by Olympus IX81 microscope. Stimuli added were 5 μM C1 or C2; when required, cells were pre-treated 30 min with 1 mM NAC.

Mitochondrial H_2O_2 generation in confluent HBMECs was also evaluated in cells transfected with 2 μg mitochondria-targeted HyPer-Mito (Evrogen, www.evrogen.com), which is a fully genetically encoded fluorescent sensor capable for highly specific detection of mitochondrial H_2O_2 [91]. Following the application of stimuli (5 μM C1 or C2), in HBSS buffer plus Ca^{2+} and Mg^{2+}, 10 mM glucose, 4 mM Hepes, fluorescence emission at 530 Nm was recorded (10 sec intervals) by Olympus IX81 microscope following excitation at 430 Nm and 480 Nm. When required, cells were pre-treated 30 min with 1 mM NAC.

2.15 Immunofluorescence

Murine endothelial cells overexpressing wild-type human VE-cadherin seeded (0.5×10^4/ml) on 8 wells chamber slides (BD Biosciences) were pre- treated with 100 μM chloroquine before to be exposed to C2 fragment or pre-treated for 30 min with NAC before the addiction of C2 fragment. After 45 min, cells were fixed with 3.7% formaldehyde in PBS for 30 min, permeabilized with 0.01% Nonidet P40 for 20 min at RT and blocked with PBS 0.5% BSA. VE-cadherin was stained with a monoclonal anti-VE-cadherin followed by an ALEXA 488-conjugated anti-mouse secondary antibody. EEA1 was stained with a polyclonal anti-EEA1 antibody followed by a ALEXA 594-conjugated anti-rabbit secondary antibody. Cells were visualized with a 63× oil immersion objective on a laser-scanning confocal microscope and images were acquired using a LAS-AF software (Leica TCS-SP5, Leica Microsystems, Wetzlar, Germany). Images were then processed

using ImageJ software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). Mander's coefficient for colocalization analysis was calculated using Mander's coefficient plug-in of ImageJ. The quantification by Mander's colocalization coefficient was performed in a blinded manner.

2.16 Cell-based ELISA for VE-cadherin expression

Cell-based ELISA was performed as previously reported with minor modifications [12]. Briefly, murine endothelial cells overexpressing wild-type human VE-cadherin were seeded onto 96-well plates precoated with 2% gelatin. Three days after all cells reached confluence and formed a contact-inhibited monolayer, cells were treated with 5 μM C2 or 10 ng/ml VEGF for 45 min or 3 hours. When required cells were pre-treated for 30 min with 1 mM NAC or 280 Nm SU6656. Cells were fixed with 3.7% formaldehyde for 10 min at RT and incubated with blocking buffer (PBS with 10% FBS) for 60 min at 37°C. After washing with 0.1% Triton X-100 in PBS, cells were incubated with a goat polyclonal antibody against VE-cadherin (1:500) overnight at 4°C. After washing with PBS, a secondary HRP-conjugated antibody was added and incubated for 1 h at room temperature. After washing again, TMB solution was added, incubated for 15 min followed by the stop solution for 5 min. The optical density of each well was read at 450 nm using a plate reader (Tecan, Infinite 200 pro, Salzburg, Austria). Results were expressed as % of the control group (cells exposed to vehicle).

2.17 Statistical analysis

Statistical significance was calculated by unpaired Student's t-test. Data, reported as the mean ± S.D., were considered significant if p-values ≤ 0.05.

Results

3.1 C2 fragment increases brain microvasculature endothelial permeability

Once *Neisseria meningitidis* crosses human epithelial cell, can spread within the vasculature, and from there it can escapes towards the host tissue in a mechanism still not fully understood [92]. To verify whether the two fragments, C1 and C2, produced upon the cleavage of the full length protein NHBA, are involved in the alteration of endothelial permeability to allow the passage of bacteria, or of some bacterial factors, from one side to the other of a vessel, we seeded human brain microvasculature endothelial cells (HBMEC) onto a polycarbonate membrane of a transwell system. This system is composed by two chambers: the apical one is separated from the basolateral one by a filter. On the latter the cells were seeded and left to grow until they became confluent. Immediately before the experiment the tracer FITC- BSA was added in the apical chambers together with NHBA, C1, C2 or bradikine (BK), as positive control, and the passage of the tracer in the lower chambers was monitored at different time points. Our results, depicted in Figure 11 A, revealed that, similarly to BK, C2 fragment induced an increase of endothelial permeability already after 15 min, an effect that become stronger after 30 min and even more after 45 min. Notably, neither the C1 fragment nor the full-length protein NHBA were able to produce a similar effect. Moreover, the alteration of the monolayer integrity occurred only if C2 fragment was administrated to the apical side of endothelia, whereas nothing occurred if the exposure of the endothelium to the fragment was carried-on at the baso-lateral side (data not shown).

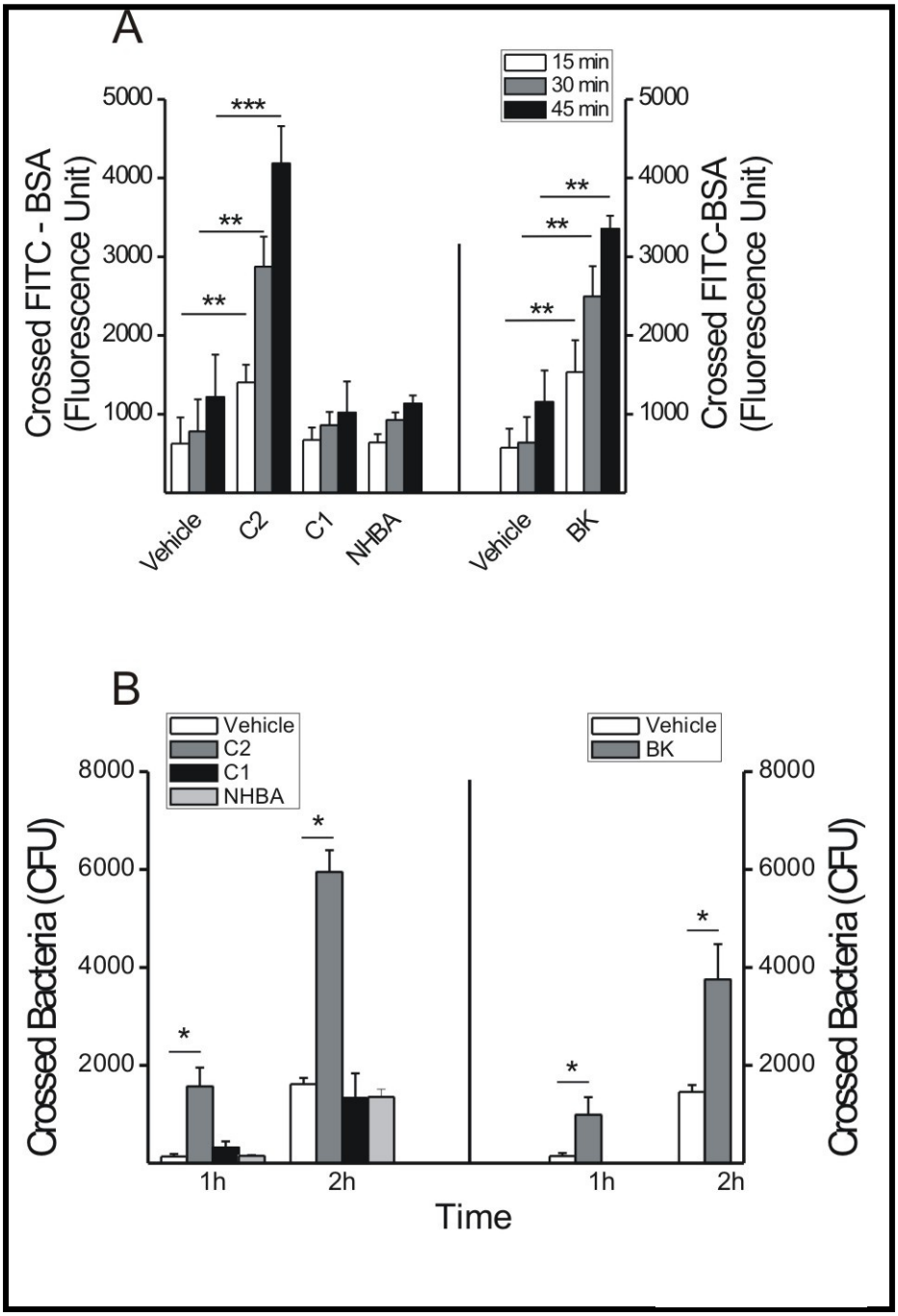

*Figure 11. C2 fragment induces the leakage of HBMECs-formed endothelia. A) HBMECs, grown as monolayer onto the membrane of a Transwell system, were stimulated with 5 μM C2, C1 NHBA, or left untreated (vehicle). 1 μM Bradikinin (BK) was used as positive control. The passage of BSA the lower chamber at various time intervals was evaluated. B) HBMECs grown as monolayer onto the membrane of a Transwell system, were exposed to 10^6 E. coli bacteria in the presence of C2, C1 or NHBA (5 μM). 1 μM BK was used as positive control. At the indicated time points, medium of the lower chamber was collected and plated onto agar plates. After 18 h colony-forming units (CFU) were counted. Values are expressed as means ± SD of duplicate determinations of four separate experiments. *, p< 0.05; **, p < 0.01; ***, p < 0.001 vs vehicle.*

Looking at the *in vivo* situation, this evidence suggests that C2 has to be within the vasculature to exert its perturbing activity on the endothelium.

Next, we moved to evaluate whether the increased permeability induced by C2 could allow the passage of bacteria; to address this possibility, we repeated the previous experiment applying, instead of the tracer BSA-FITC, *E. coli* as bacterial model. 10^6 *E. coli* were added to the upper chamber of a transwell apparatus together with the single fragments or the full length protein; after 1 or 2 h, the entire medium of the lower chamber was collected and plated on LB agar plates to permit the bacteria to multiply. After 18 h, colony-forming units (CFU) were counted. Figure 11 B shows that the extent of endothelia permeabilization induced by C2 allowed the passage of bacteria, in a time dependent manner. According to the results of the previous experiment, neither C1 nor NHBA application resulted in any appreciable bacteria movement. Remarkably, as for the passage of the tracer, bacteria did not cross endothelium in case C2 was applied at the baso-lateral side of the endothelium (data not shown), thus confirming the previous evidence that the fragment has to be present into the vascular lumen of a vessel to trigger the alteration event.

3.2 C2 localizes within mitochondria

In order to address how C2 elicited endothelia perturbation, we moved to investigate its subcellular localization within host endothelial cells. As first, we took advantage of informatic softwares capable to predict a possible localization of a peptide within cells. Among them, we used MitoProt software (http://ihg.gsf.de/ihg/mitoprot.html), which defines whether an N-terminal protein region contains a mitochondrial targeting sequence. The software attributes a

score value ranging from 1 to 0, depending on whether the sequence analyzed is more or less compatible to a mitochondria localization. In case of C2, MitoProt scored a value of 0.7152 which strongly suggested a mitochondrial localization. In accordance with the prediction, the N-terminal domain of C2 is enriched in basic residues (arginine), that usually confer to a protein the ability of targeting mitochondria [93]. On the contrary, for NHBA and C1 MitoProt gave a score of 0.205 and 0.0199 respectively.

In order to verify whether the bioinformatic prediction was exact, we incubated HBMECs with C2, before proceeding with the isolation of mitochondrial, microsomal and cytosolic fractions, at different time points. The protein content of all the fractions was analyzed by western blot for evaluating the presence of C2 and possibly defining its intracellular trafficking. Figure 12 shows that, after 5 min, C2 was detectable both in microsomes and in the cytosol, while, 15 min after its administration, a quote of C2 accumulated in mitochondria. After 45 min, C2 was entirely confined in the latter organelle. These observations, which confirm the bioinformatic prediction, suggest that C2 probably in virtue of the arginine-rich domain behaves as a trojan peptide. Notably, independently from the subcellular localization, C2 always maintained the N-terminal domain, as demonstrated by the fact that the protein was revealed by a policlonal antibody raised specifically against the arginine-rich peptide.

Trojan peptides are cell-permeable peptides able to translocate into cells without deleterious effects and polycationic homopolymers, such as short oligomers of arginine, effectively enter cells [94-96].

To further support the specific localization of C2 within mitochondria, the same subcellular fractionation and analysis was carried on HBMECs exposed to C1; in

this case, not only the peptide did not show any accumulation in mitochondria, but it was undetectable also in the other two fractions (Figure 12). This result, which probably reflects a week ability of C1 to interact with the cells, confirms once again the crucial role of the arginine domain of C2 for its biological activity.

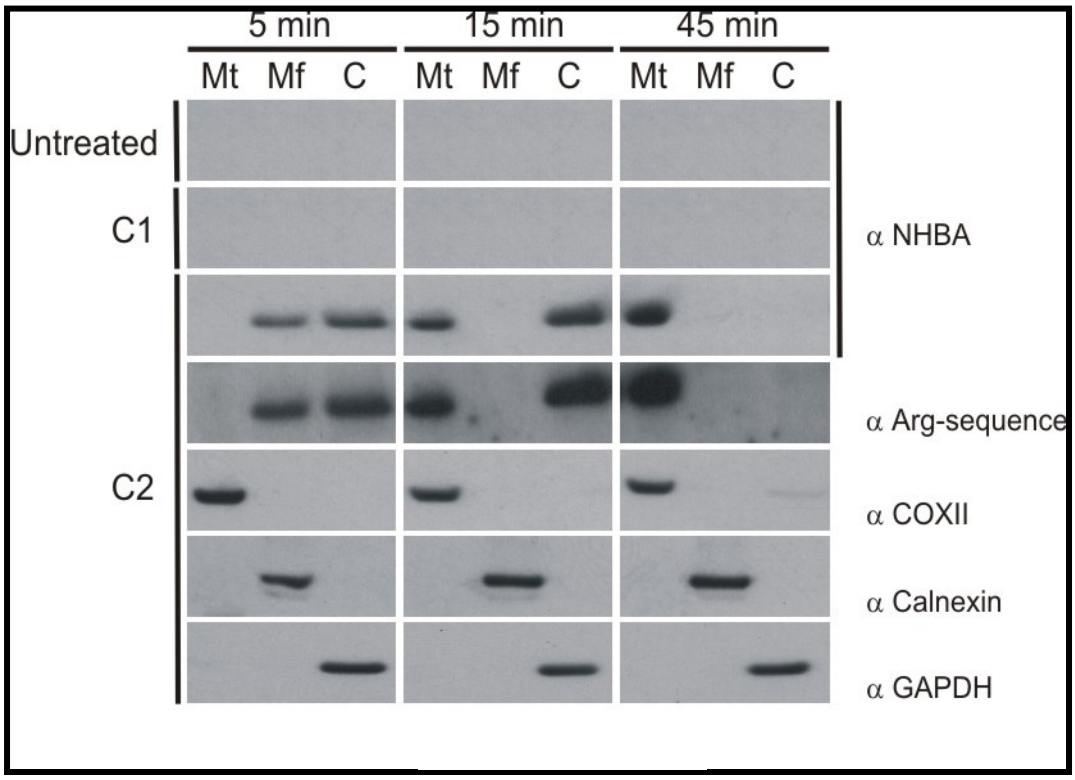

Figure 12. C2 fragment accumulates in mitochondria. HBMECs, grown to confluence in a T75 flask, were exposed to 5 μM C2 or C1. After 5, 15 and 45 min mitochondrial (Mt), microsomal (Mf) and cytosolic (C) fractions were isolated and processed for Western blot analysis. A mouse polyclonal anti-NHBA antibody was used to reveal both C1 and C2 peptides. The latter was also revealed by a polyclonal antibody specific for the arginine-rich domain. Monoclonal antibodies anti-COXII, anti-calnexin and anti-GAPDH were used to check the purity of each fraction. HRP-conjugated secondary antibodies were used before developing in chemiluminescence.

3.3 Mitochondrial ROS production

It is established that the integrity of endothelial permeability can be perturbed by ROS [11]. This has been demonstrated to occur for example when endothelia are exposed to VEGF, which, in fact, induces ROS production [11]. Although the relationship between ROS and endothelial permeability remains to be precisely

defined, it is known that an event of phosporylation of VE-caherin and β-catenin, both proteins of the adherence junctions, follows the production of ROS.

On the basis of the localization of C2 in mitochondria, we moved to verify whether the increased endothelial permeability caused by the fragment could follow ROS production within the organelle: indeed, the mitochondrial electron transport chain has been recognized as one of the major cellular generators of reactive oxygen species, which include superoxide, hydrogen peroxide and the hydroxyl free radical [97-99]. Moreover, the production of ROS can be further enhanced by toxins, such as rotenone, that inhibit complex I and antimycin A that inhibits complex III [100, 101].

Microscopic imaging applied to HBMECs, loaded with the fluoroprobe MitoSOX Red [102], that permits quantitative detection of mitochondrial superoxide production, demonstrated significant increase in mitochondrial fluorescence intensity in the cells, following the administration of C2, but not of C1 (Figure 14A). Notably, ROS started to increase after 3 min, a time interval that is apparently in contrast to the fact that C2 was undetectable in mitochondria 5 min after its administration (Figure 12). However, considering the sensitivity limitation of the western blot technique, we cannot exclude that a small amount of C2, sufficient to promote the initial ROS production, reached mitochondria in the first minutes. As expected, pre-treatment of the cells with the ROS scavenger N-acetylcysteine (NAC) fully prevented the C2-induced fluorescence increase, confirming that it was due to an increase in ROS generation (Figure 13 B).

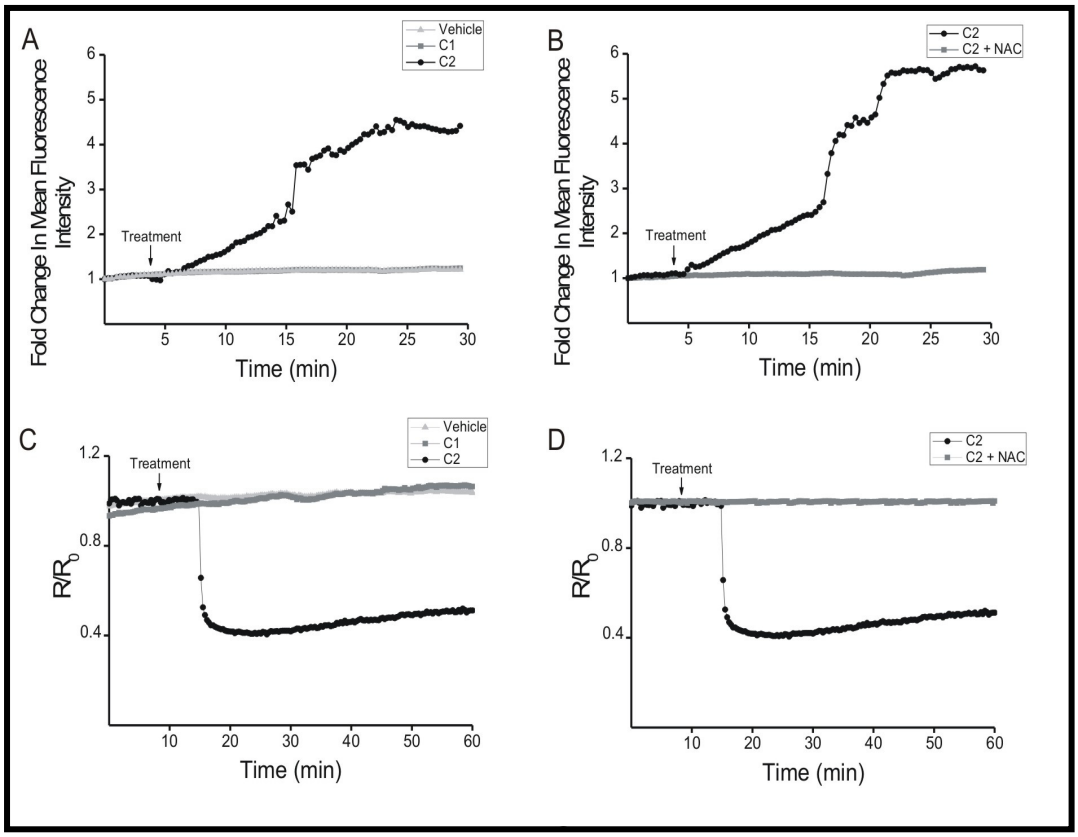

Figure 13. C2 induces mitochondrial ROS formation in HBMECs. A) HBMECs, grown as monolayer, were loaded with 1 μM MitoSOX Red, just before live-imaging recording. At the indicated time point (arrow) cells were exposed to 5 μM C1, C2, or saline (vehicle) as negative control. B) Cells were pre-treated or not with 1 mM NAC before being exposed to C2 as in A). Cells were excited by laser at 514 nm and fluorescence emitted was recorder at 560 nm every 10 sec for 30 min. Data are expressed as fold change in mean fluorescence intensity compared to cells exposed to saline (vehicle). N= 3 for each condition.C) HBMECs, grown as monolayer, were transfected with the Hyper-dMito vector. After 24 h of expression, cells were exposed to 5 μM C1, C2, or saline (vehicle). D) Cells were pre-treated or not with 1 mM NAC before being exposed to C2 as in C). Fluorescence intensities were recorded every 10 sec for 1 h. Normalized fluorescence ratio changes (430/480 nm) was calculated as measure of H_2O_2 production. N= 3 for each condition.

Mitochondrial H_2O_2 generation in confluent HBMECs was also evaluated in cells transfected with mitochondria-targeted HyPer-dMito, which is a fully genetically encoded fluorescent sensor capable for highly specific detection of mitochondrial H_2O_2 [91]. In the presence of a basal level of ROS, the probe has two excitation peaks at 430 nm and 480 nm and one emission peak at 516 nm. Upon ROS

induction, the excitation peak at 430 nm decreases proportionally to the increase of the peak at 480 nm, allowing ratiometric measurement.

As shown in Figure 13 C, cell exposure to C2 fragment was characterized by a decrease of the ratio between the two excitation peaks, while on the contrary, it remained constant following C1 administration as well as in case of vehicle-exposed cells.

As before, pre-incubation of HBMECs with NAC prevented H_2O_2 induction: accordingly, the ratio remained constant (Figure 13 D).

3.4 Reactive oxygen species are fundamental in the alteration of the integrity of endothelial monolayers induced by C2

Next we moved to verify whether ROS induced by C2 were involved in the alteration of endothelial integrity; to this aim we repeated the experiments with HBMECs seeded on transwells and we evaluated both the passage of BSA and that of bacteria, but in presence or absence of NAC.

Results shown in Figure 14 A and 14 B reveal that at any time point considered, ROS scavenger significantly reduced the accumulation of BSA and bacteria in the lower chamber of the apparatus: both return to levels similar to C1 which, as before, did not affect endothelial permeability and remained insensitive to the presence of NAC.

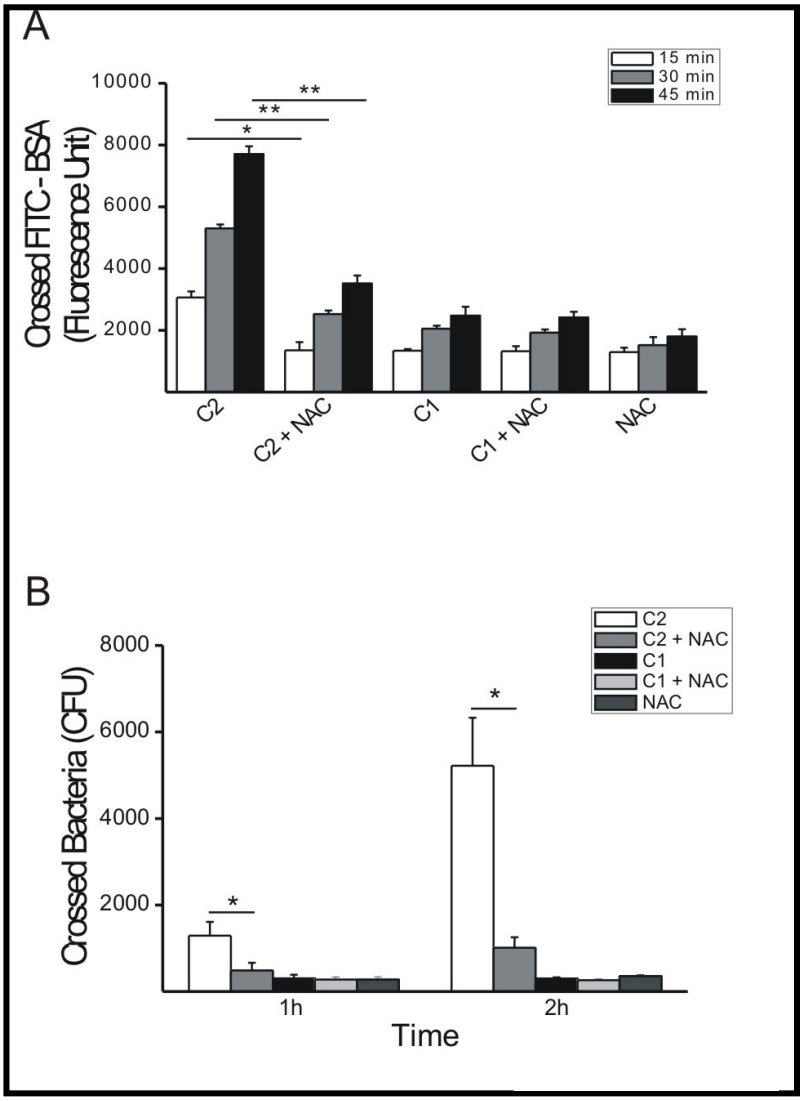

*Figure 14. ROS-induced by C2 are essential for the alteration of endothelial permeability. HBMECs, seeded onto the membrane of a Transwell system, were exposed to 5 μM C2 or C1 fragment together with BSA-FITC, A) or E. coli, B). When required, HBMECs were pre-treated with 1 mM NAC for 30 min. The accumulation of BSA and bacteria in the lower chamber was performed as detailed in Figure 1. Values are expressed as means ± SD of duplicate determinations of four separate experiments. *, p< 0.05; **, p < 0.01 vs C2 + NAC.*

Collectively, these data support the conclusion that perturbation of endothelial cells due to C2 requires intracellular accumulation of ROS; the latter are mainly produced within mitochondria where C2, after entering the cells probably directly through the plasma membrane, localizes.

3.5 C2 inducesVE-cadherin phosphorylation in a ROS-dependent manner

It has been reported that VEGF leads to the generation of ROS which, in turn, elevates the tyrosine phosphorylation of VE-cadherin, ultimately regulating adherence junction integrity [63]. Phosphorylation of VE-cadherin and of its partners β-catenin, plakoglobin and p120 is linked to the alteration of endothelial permeability in case of other mediators than VEGF, such as such as histamine, tumour necrosis factor-α (TNFα) and platelet-activating factor (PAF) [65, 80, 103, 104].

We sought to determine if the C2-induced increase in microvascular permeability was also associated to some change in the tyrosine phosphorylation of VE-caderin. To this aim, we used endothelial cells derived from murine embryonic stem cells with homozygous null mutation of the VE-cadherin gene and overexpressing wild-type human VE-cadherin [90]. Cells were serum-starved for 72 h, in order to abolish the basal phosphorylation rate that commonly occurs in cells as part of the turnover that involves senescent or partially damaged proteins [105], before being exposed to C2. After VE-cadherin immunoprecipitation the phosphorylation state of the protein tyrosines was evaluated by Western blot.

Fig. 15 A shows that, already after 15 min of treatment, VE-cadherin was highly phosphorylated in cells exposed to C2 fragment, and the phosphorylation level increased further up to 30 min, before decreasing at 45 min.

To determine if ROS were required for C2-induced VE-cadherin phosphorylation, HBMECs were pre-treated with NAC before exposing the cells to C2 and evaluating the level of VE-cadherin phoshorylation. C2 was no longer able to

cause phosphorylation of VE-cadherin after treatment with NAC (Figure 15 B), suggesting that ROS are required for the phosphorylation of the protein.

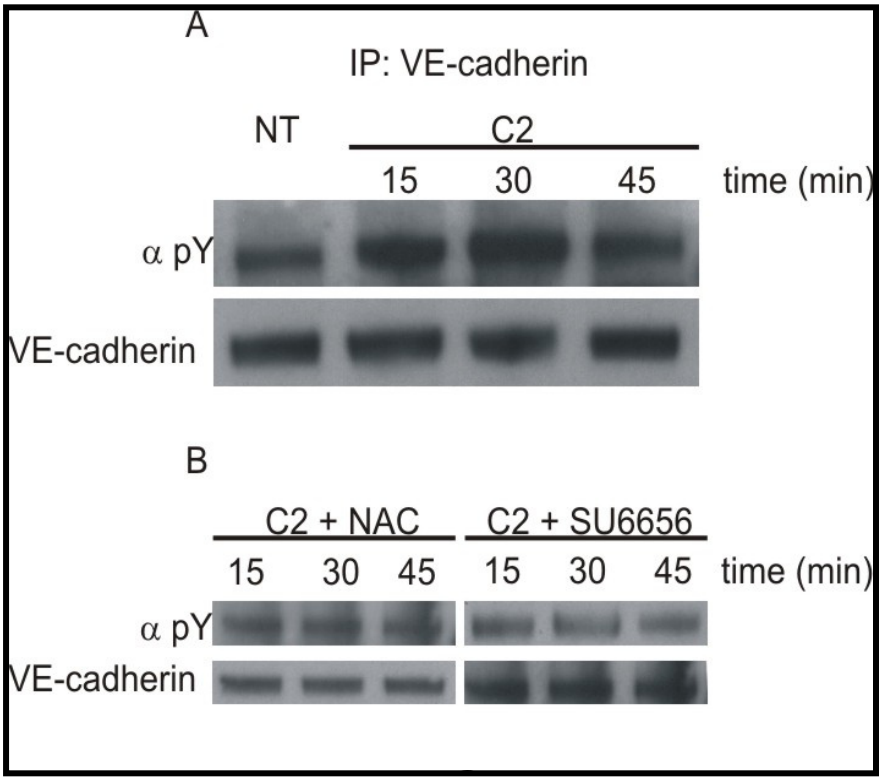

Figure 15. C2 induces VE-cadherin phosphorylation. A) Murine endothelial cells overexpressing wild-type human VE-cadherin were grown to confluence before being serum-starved for 72 h and exposed to 5 μM C2. After 15, 30 and 45 min cells were harvested and processed for immunoprecipitation with a polyclonal antibody anti-VE-cadherin. Phosphorylation state of the protein was determined in western blot by developing with anti-phosphotyrosine antibody. Total VE-cadherin was used as loading control. NT, not-treated cells: cells exposed to saline. B) Cells were pre-treated with NAC for 30 min (left panel) or with the Src kinase inhibitor SU6656 for 2 h (right panel), before the administration of C2.

Notably, pre-treatment of cells with the Src kinase inhibitor SU6656 also fully prevented the phosphorylation of VE-cadherin (Figure 15 B), as already reported for VEGF that, similarly to C2, increases endothelial permeability [66].

3.6 C2 decreases VE-cadherin intracellular content

It has been recently reported for TGF-β, another cytokine that increases paracellular permeability of endothelia, that its effect results from a change in the total cell content of VE-cadherin [12].

In a cell-based ELISA on post-confluent murine endothelial cells overexpressing wild-type human VE-cadherin, we found that exposure to C2 for 45 min resulted in reduced levels of VE-cadherin, of about 20%, that were maintained similar at 3 h. Instead, NAC pre-treatment almost fully prevented the C2-induced protein disappearance (Figure 16 A).

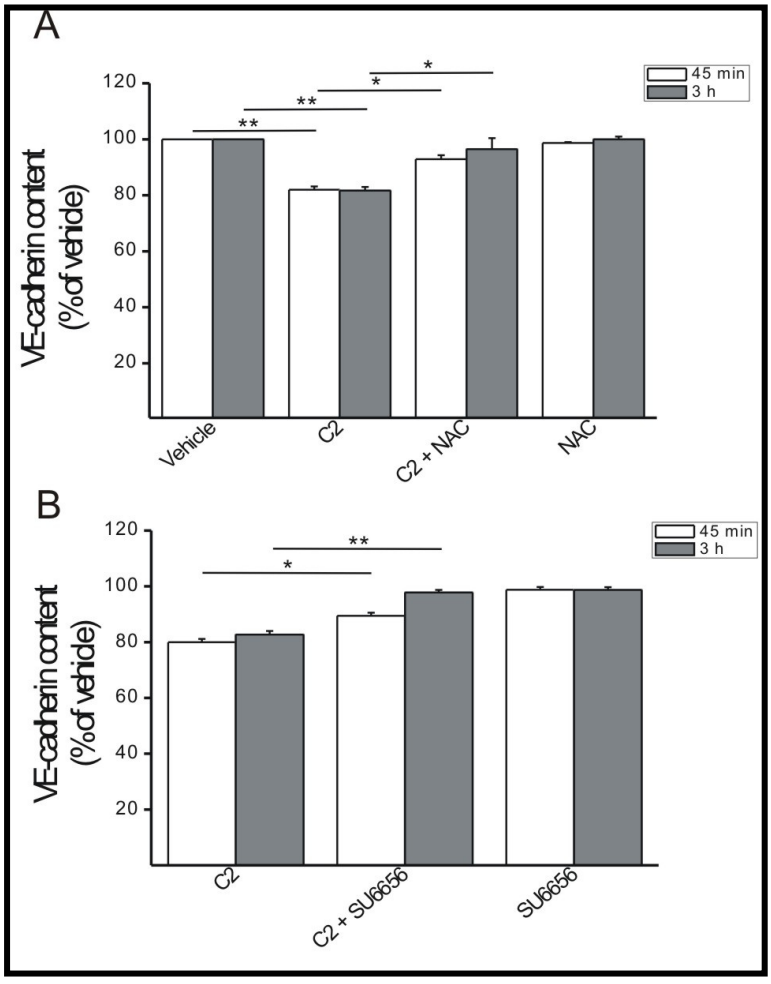

Figure 16. C2 lowers cellular VE-cadherin content. Murine endothelial cells overexpressing wild-type human VE-cadherin were grown to confluence before being exposed to 5 μM C2. After 45 min and 3 h a cell-based ELISA was performed for evaluating the total cell content of VE-cadherin. Where indicated, cells were pre-treated with 1 mM NAC for 30 min, A) or with SU6656 for 3 h, B).

*Values are expressed as mean percentage (± SD) of the absorbance readings at 450 nm in groups exposed to saline (vehicle). *, $p < 0.05$; ** , $p < 0.01$, for C2 vs vehicle and for C2 vs C2 + NAC.*

As we demonstrated that C2 induced phosphorylation of VE-cadherin and that the latter was abolished when Src kinase was inhibited, we wondered whether the phosphorylation of the junctional protein was required for reducing its cellular content in cells exposed to the fragment. To determine this, we pre-treated cells with SU6656; as shown in Figure 16 B, the blockage of Src significantly prevented the reduction of VE-cadherin.

Collectively, our data suggest that C2 leads to the generation of ROS which, in turn, elevates the tyrosine phosphorylation of the junctional protein VE-cadherin; the latter event, probably mediated at least in part, by Src kinase, culminates in the reduction of the VE-cadherin cell content.

3.7 C2 promotes VE-cadherin endocytosis

VE-cadherin endocytosis is part of a normal regulatory mechanism that endothelial cells utilize to control the adhesive properties of the plasma membrane. The internalized VE-cadherin is processed through the endosome-lysosome pathway and requires an active proteasome system for its degradation [106].

Based on the reduction of VE-cadherin content in cells exposed to C2, we addressed whether this resulted from the internalization of the junctional protein. To this aim we performed an immunofluorescence analysis on the cells overexpressing human VE-cadherin that were pre-treated with cloroquine before the exposure to C2: this drug prevents the fusion of endosomes and lysosomes,

therefore it permits to visualize endocytosed proteins, avoiding their final degradation.

Figure 17 shows that in cells exposed to saline (vehicle), VE-cadherin was mainly concentrated at the plasma membrane, with only a small proportion of protein localized within endocytic compartments, reflecting the basal cellular turnover process.

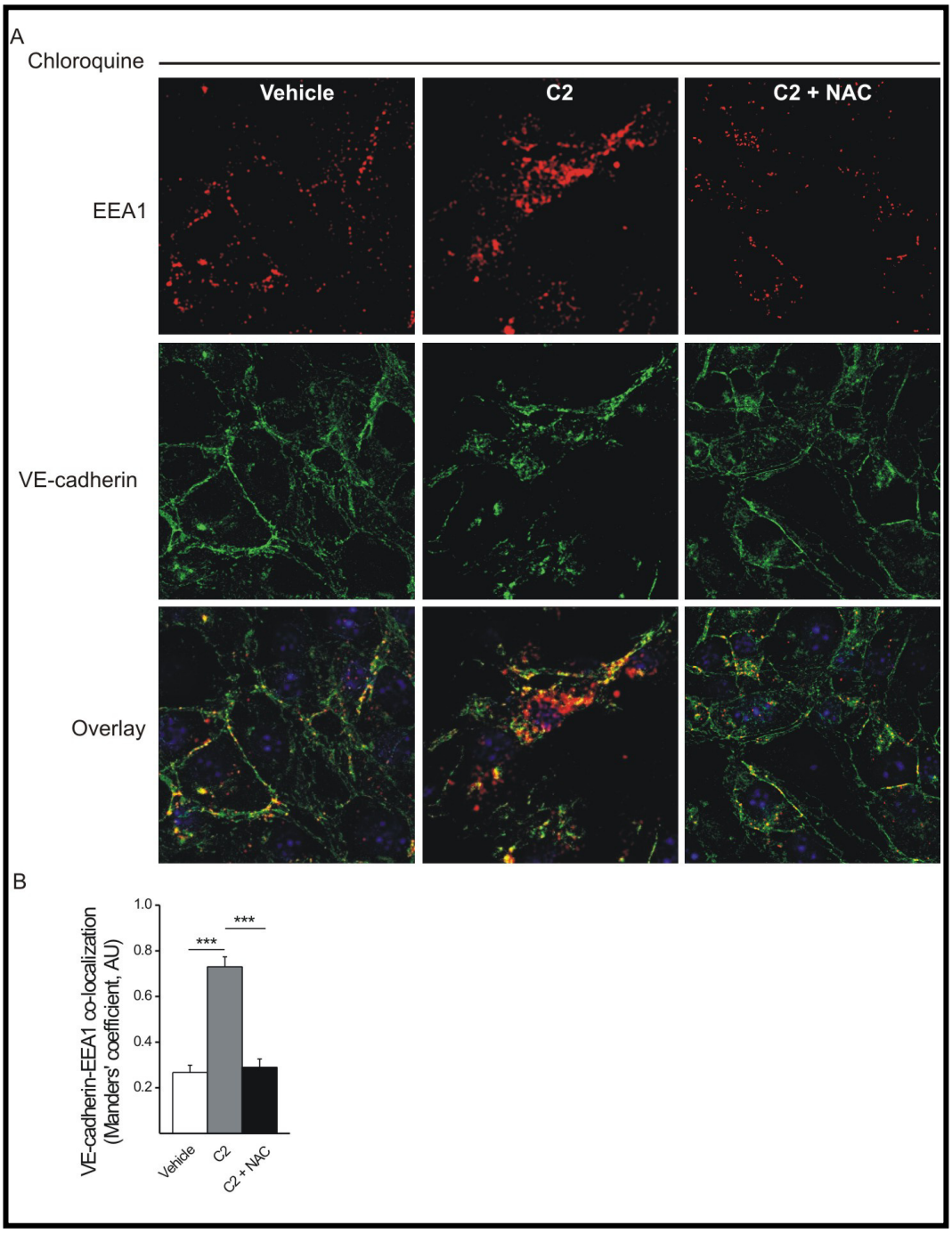

*Figure 17. C2 induces VE-cadherin internalization. A) Murine endothelial cells overexpressing wild-type human VE-cadherin were grown to confluence. Cells were pre-treated for 2 h with 100 μM chloroquine and, when required, with 1 mM NAC for 30 min. Cells were exposed to 5 μM C2 fragment for 45 min, before being fixed and permeabilized. VE-cadherin was labelled with a monoclonal anti-VE-cadherin antibody while early endosomes were labelled with polyclonal anti-EEA1 antibody. A secondary anti-mouse Alexa488-conjugated antibody and an anti-rabbit Alexa 594-conjugated antibody were used. Confocal images were acquired using LEICA microscope with a 63X oil immersion objective. Images were merged using ImageJ software. B) Mean ± S.D. (n = 3, 10 cells per independent experiment) of colocalization data from A). ***, p <0.001, for C2 vs vehicle and for C2 vs C2 + NAC.*

Interestingly, in C2-exposed cells there was a significant redistribution of VE-cadherin, which was massively present within endosomes and only in a minimal amount at the plasma membrane as suggested by its co-localization with the endosomal specific marker EEA1. On the contrary, in NAC pre-treated cells VE-cadherin distribution was comparable to that of control cells, supporting the result showed above on the role of ROS in the C2-induced decrease of cellular VE-cadherin content.

A quantitative analysis of the re-distribution of VE-cadherin within endosomes, was performed using the Mander's co- localization index. According to the immunofluorescence analysis the Mander's index revealed that the co-localization of VE-cadherin and early endosomes was significantly higher compared to saline-treated cells and NAC plus C2 treated-cells (Fig. 17 B).

3.8 C2 allows *Neisseria meningitidis* MC58 endothelial crossing

To verify whether the enhanced endothelial permeability induced by the rC2 could affect the *N.meningitidis* translocation capacity, we infected the HBMEC intact monolayer with Mc58 [41, 107], a virulent and well-charaterized

encapsulated serogroup B strain, in the presence or absence of the rC1, rC2 or rNHBA. The recombinant proteins were maintained during all the infection experiment while the medium in the lower chamber was collected every 30 minutes and further plated for colony counting. As shown in Figure 18, Mc58 was able to significantly cross the cellular monolayer in 4 hours while in control cell medium.

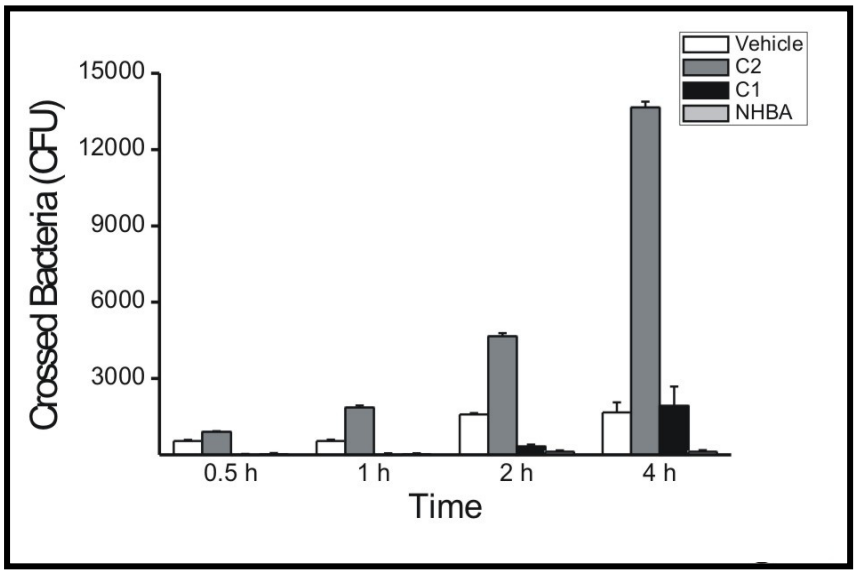

Figure 18. C2 improves N. meningitidis translocation through the endothelium. HBMECs, grown as monolayer onto the membrane of a Transwell system, were apically infected with N. meningitidis strain MC58 (MOI of 30). At the indicated time points, medium of the lower chamber was collected and plated onto agar plates. After 18 h colony-forming units (CFU) were counted. Graph shows mean values ± S.D. of one representative experiment done in triplicates.

When the rC2 was present in the apical medium, Mc58 needed only one hour to reach the same level of crossing rate while at the 4th hour of infection the bacterium shown 4 fold incremented crossing rate. When the infection was conducted in the presence of the other recombinant proteins we did not notice any significant variation. Additionally we tested the Mc58 KO mutant for NHBA (Mc58ΔNHBA) in a separate experiment (data not shown). The mutant strain showed very similar results when compared with the wild type Mc58. When the rC2 or the rNHBA were incubated with the Mc58ΔNHBA strain we did not

observe any significant difference. These results show that MenB do not require NHBA for moving across the endothelium and furthermore the contribution of the C2 fragment to the bacterial crossing does not depend on the presence of the membrane exposed full-length antigen.

Discussion

Neisseria meningitidis (Nm) is an encapsulated, Gram negative bacterium that colonizes the nasopharynx of 8-20% of healthy individuals. Occasionally, the bacterium, from its natural niche in the nasopharyngeal mucosa of healthy patients, crosses the epithelium and enters the bloodstream where it multiplies and causes a form of sepsis characterized by an impressive endothelial disruption responsible for the disseminated intravascular coagulation (DIC). From the blood, the bacterium can cross the blood-brain barrier and cause meningitis. Meningococcal sepsis is a devastating disease and, despite the availability of effective antibiotics, it can kill children and young adults within hours. Vaccines containing purified polysaccharide antigens are available against four of the five pathogenic serogroups. The capsular polysaccharide of Meningoccus serotype B (MenB) is widely expressed in humans, thus rendering the strategy adopted for the other strains not suitable for MenB. The obstacle was overcome by a new approach, adopted by Novartis Vaccines in the year 2000 and named reverse vaccinology. Such a bio-informatic method, combined with molecular biology and biochemical techniques, led to the discovery of several new conserved antigens of MenB potentially interesting as vaccine candidates.

Among these antigens, there was Neisserial Heparin Binding Antigen (NHBA elsewhere called GNA2132). NHBA stimulates the production of antibodies able to confer protection in humans and covering a wide spectrum of the MenB strains [108]. NHBA is a surface exposed protein characterized by an arginine-rich domain, responsible for the binding to heparin and heparansulfate [9]. NHBA can be cleaved upstream or downstream of the arginine-rich region by

bacterial or host proteases. When NHBA is cleaved by the surface exposed *Neisseria* peptidase NalP, it generates a fragment, called C2 that maintains the arginine-rich domain; alternatively, NHBA can be cleaved by the human lactoferrin, generating the fragment named C1, in which the arginine-rich domain is absent [9]. Since C2 fragment conserves the domain responsible for the binding to heparin [9, 49], it is plausible that this fragment might interact with secreted or cell-associated proteoglycans in order to exert its own biological role; if this was the case, lactoferrin would have a protective role for the host.

Till now, nothing was known about the biological role of these two different fragments in the pathogenesis of *Neisseria*-associated diseases.

The present study was aimed to verify whether C1 and/or C2 were involved in the escape of Nm from the blood lumen towards peripheral tissues including meninges.

To this end, we examined the ability of the fragments to increase the endothelial permeability, by an *in vitro* assay applied to human brain microvasculature endothelial cells grown as monolayer. We demonstrated that the C2 fragment, provided by the arginine-rich domain, but not C1, increases endothelial permeability in such a way to permit not only the movement of soluble factors across endothelia, but also that of Nm. Notably, the effect exerted by C2 requires its contact with the luminal side of the endothelium: accordingly, its application at a baso-lateral side of endothelium does not culminate in any alteration.

The effect of C2 on endothelial permeability relies on the induction of mitochondrial ROS production; indeed, pre-treatment of cells with the ROS scavenger, N-acetylcysteine (NAC), prevents the effect of C2. The involvement of

ROS in altering endothelial barrier is established: for example, the angiogenic factor VEGF actually promotes an increase of endothelial permeability via the induction of oxygen radicals' production [11].

Although it remains to be established the molecular mechanism by which ROS affect endothelial integrity, it has been demonstrated that the phosphorylation of VE-cadherin, a glycoprotein of the adherence junctions, follows and requires the production of oxygen radicals and results in an enhanced endothelial leakage [9, 10, 62, 64, 109]. According to this evidence, we found that also C2 fragment promotes VE-cadherin phosphorylation and the latter requires ROS and the activity of Src-kinase. Moreover, we demonstrated that, upon C2-induced VE-Cadherin phosphorylation, the glycoprotein is internalized via endocytic route and degraded. Accordingly, a complete redistribution of the junctional protein is observed in cells exposed to C2 fragment, with the protein confined within endosomes; internalization of VE-cadherin is fully prevented in the presence of the ROS scavenger NAC.

Collectively our data suggest the possibility that C2 might be involved in the pathogenesis of meningitis by permitting the passage of bacteria from the blood to the meninges and we hypothesize that the following scenario may occur: Nm colonizes human nasopharyngeal mucosa; for some reasons, that nowadays remain obscure, bacteria spread in the blood. In virtue of the entire NHBA protein, *Neisseria* would establish a close contact with the luminal membrane of the endothelium. The subsequent activity of the bacterial protease Nalp on NHBA would result in the release of C2, which could reach a high concentration in the areas where endothelial cells and bacteria are in closed proximity. C2, in turn, by causing the alteration of endothelial integrity, would help the bacteria in escaping

from the blood towards the brain. Once here, bacteria, together with a strong inflammatory process, would lead to the fatal outcome.

Another intriguing consideration emerges from our data: C2 fragment is the solely portion of NHBA with a biological activity functional to the spreading of *Neisseria*; C1 fragment, released from NHBA by lactoferrin and identical to C2 but lacking the N-terminal arginine-rich domain, does not exert the same activity. Therefore, it is possible to speculate that lactoferrin, abundant in all the mucosa secretions, may represent a host defence mechanism to prevent the generation of the virulent factor.

References

1. Vieusseux, G., *Mémoire sur la maladie qui a régné à Genève au printemps de 1805*. J Med Chir Pharm. Vol. XI. 1805.
2. Marchiafava E, *Sopra i micrococchi della meningite cerebrospinale epidemica*. Gazzetta degli Ospedali 1884.
3. Manchanda, V., S. Gupta, and P. Bhalla, *Meningococcal disease: history, epidemiology, pathogenesis, clinical manifestations, diagnosis, antimicrobial susceptibility and prevention.* Indian J Med Microbiol, 2006. **24**(1): p. 7-19.
4. Frasch, C.E., W.D. Zollinger, and J.T. Poolman, *Serotype antigens of Neisseria meningitidis and a proposed scheme for designation of serotypes.* Rev Infect Dis, 1985. **7**(4): p. 504-10.
5. Caugant, D.A., *Population genetics and molecular epidemiology of Neisseria meningitidis.* Apmis, 1998. **106**(5): p. 505-25.
6. Caugant, D.A., G. Tzanakaki, and P. Kriz, *Lessons from meningococcal carriage studies.* FEMS Microbiol Rev, 2007. **31**(1): p. 52-63.
7. Morley, S.L., et al., *Immunogenicity of a serogroup B meningococcal vaccine against multiple Neisseria meningitidis strains in infants.* Pediatr Infect Dis J, 2001. **20**(11): p. 1054-61.
8. Lucidarme, J., et al., *Characterization of fHbp, nhba (gna2132), nadA, porA, sequence type (ST), and genomic presence of IS1301 in group B meningococcal ST269 clonal complex isolates from England and Wales.* J Clin Microbiol, 2009. **47**(11): p. 3577-85.
9. Serruto, D., et al., *Neisseria meningitidis GNA2132, a heparin-binding protein that induces protective immunity in humans.* Proc Natl Acad Sci U S A. **107**(8): p. 3770-5.
10. Dejana, E., F. Orsenigo, and M.G. Lampugnani, *The role of adherens junctions and VE-cadherin in the control of vascular permeability.* J Cell Sci, 2008. **121**(Pt 13): p. 2115-22.
11. Monaghan-Benson, E. and K. Burridge, *The regulation of vascular endothelial growth factor-induced microvascular permeability requires Rac and reactive oxygen species.* J Biol Chem, 2009. **284**(38): p. 25602-11.
12. Shen, W., et al., *Tyrosine phosphorylation of VE-cadherin and claudin-5 is associated with TGF-beta1-induced permeability of centrally derived vascular endothelium.* Eur J Cell Biol. **90**(4): p. 323-32.
13. Weichselbaum A, *Ueber die Aetiologie der akuten Meningitis cerebrospinalis.* Fortschr Med 1887. **5**: p. 573–83.
14. Dehio, C., S.D. Gray-Owen, and T.F. Meyer, *Host cell invasion by pathogenic Neisseriae.* Subcell Biochem, 2000. **33**: p. 61-96.
15. Bhat, K.S., et al., *The opacity proteins of Neisseria gonorrhoeae strain MS11 are encoded by a family of 11 complete genes.* Mol Microbiol, 1991. **5**(8): p. 1889-901.
16. Ulmer, J.B., et al., *Pore formation and mitogenicity in blood cells by the class 2 protein of Neisseria meningitidis.* J Biol Chem, 1992. **267**(27): p. 19266-71.

17. McGuinness, B.T., P.R. Lambden, and J.E. Heckels, *Class 1 outer membrane protein of Neisseria meningitidis: epitope analysis of the antigenic diversity between strains, implications for subtype definition and molecular epidemiology.* Mol Microbiol, 1993. **7**(4): p. 505-14.
18. Woods, J.P. and J.G. Cannon, *Variation in expression of class 1 and class 5 outer membrane proteins during nasopharyngeal carriage of Neisseria meningitidis.* Infect Immun, 1990. **58**(2): p. 569-72.
19. Strom, M.S., D.N. Nunn, and S. Lory, *A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family.* Proc Natl Acad Sci U S A, 1993. **90**(6): p. 2404-8.
20. Forest, K.T., et al., *Crystallographic structure reveals phosphorylated pilin from Neisseria: phosphoserine sites modify type IV pilus surface chemistry and fibre morphology.* Mol Microbiol, 1999. **31**(3): p. 743-52.
21. Jennings, M.P., et al., *Identification of a novel gene involved in pilin glycosylation in Neisseria meningitidis.* Mol Microbiol, 1998. **29**(4): p. 975-84.
22. Scheuerpflug, I., et al., *Roles of PilC and PilE proteins in pilus-mediated adherence of Neisseria gonorrhoeae and Neisseria meningitidis to human erythrocytes and endothelial and epithelial cells.* Infect Immun, 1999. **67**(2): p. 834-43.
23. Nassif, X., et al., *Roles of pilin and PilC in adhesion of Neisseria meningitidis to human epithelial and endothelial cells.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3769-73.
24. Morelli, G., et al., *Immunogenicity and evolutionary variability of epitopes within IgA1 protease from serogroup A Neisseria meningitidis.* Mol Microbiol, 1994. **11**(1): p. 175-87.
25. Lin, L., et al., *The Neisseria type 2 IgA1 protease cleaves LAMP1 and promotes survival of bacteria within epithelial cells.* Mol Microbiol, 1997. **24**(5): p. 1083-94.
26. Ayala, P., et al., *Infection of epithelial cells by pathogenic neisseriae reduces the levels of multiple lysosomal constituents.* Infect Immun, 1998. **66**(10): p. 5001-7.
27. Plant, L., et al., *Lipooligosaccharide structure contributes to multiple steps in the virulence of Neisseria meningitidis.* Infect Immun, 2006. **74**(2): p. 1360-7.
28. Girard, M.P., et al., *A review of vaccine research and development: meningococcal disease.* Vaccine, 2006. **24**(22): p. 4692-700.
29. Lancellotti, M., et al., *Conserved virulence of C to B capsule switched Neisseria meningitidis clinical isolates belonging to ET-37/ST-11 clonal complex.* Microbes Infect, 2006. **8**(1): p. 191-6.
30. Kim, K.S., *Pathogenesis of bacterial meningitis: from bacteraemia to neuronal injury.* Nat Rev Neurosci, 2003. **4**(5): p. 376-85.
31. Arevalo, J.M., J.A. Lorente, and R. Fonseca, *Surgical treatment of extensive skin necrosis secondary to purpura fulminans in a patient with meningococcal sepsis.* Burns, 1998. **24**(3): p. 272-4.
32. van Deuren, M. and P. Brandtzaeg, *Parents' and GPs' key role in diagnosis of meningococcal septicaemia.* Lancet, 2000. **356**(9234): p. 954-5.
33. Gotschlich, E.C., I. Goldschneider, and M.S. Artenstein, *Human immunity to the meningococcus. IV. Immunogenicity of group A and group C*

meningococcal polysaccharides in human volunteers. J Exp Med, 1969. **129**(6): p. 1367-84.
34. Bilukha, O.O. and N. Rosenstein, *Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP).* MMWR Recomm Rep, 2005. **54**(RR-7): p. 1-21.
35. Trotter, C.L., et al., *Effectiveness of meningococcal serogroup C conjugate vaccine 4 years after introduction.* Lancet, 2004. **364**(9431): p. 365-7.
36. Nolan, T., et al., *A novel combined Haemophilus influenzae type b-Neisseria meningitidis serogroups C and Y-tetanus-toxoid conjugate vaccine is immunogenic and induces immune memory when co-administered with DTPa-HBV-IPV and conjugate pneumococcal vaccines in infants.* Vaccine, 2007. **25**(51): p. 8487-99.
37. Granoff, D.M., et al., *Bactericidal monoclonal antibodies that define unique meningococcal B polysaccharide epitopes that do not cross-react with human polysialic acid.* J Immunol, 1998. **160**(10): p. 5028-36.
38. Pizza, M., et al., *Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing.* Science, 2000. **287**(5459): p. 1816-20.
39. Rappuoli, R., *Reverse vaccinology.* Curr Opin Microbiol, 2000. **3**(5): p. 445-50.
40. Fletcher, L.D., et al., *Vaccine potential of the Neisseria meningitidis 2086 lipoprotein.* Infect Immun, 2004. **72**(4): p. 2088-100.
41. Tettelin, H., et al., *Complete genome sequence of Neisseria meningitidis serogroup B strain MC58.* Science, 2000. **287**(5459): p. 1809-15.
42. Giuliani, M.M., et al., *A universal vaccine for serogroup B meningococcus.* Proc Natl Acad Sci U S A, 2006. **103**(29): p. 10834-9.
43. Comanducci, M., et al., *NadA, a novel vaccine candidate of Neisseria meningitidis.* J Exp Med, 2002. **195**(11): p. 1445-54.
44. Madico, G., et al., *The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance.* J Immunol, 2006. **177**(1): p. 501-10.
45. Schneider, M.C., et al., *Functional significance of factor H binding to Neisseria meningitidis.* J Immunol, 2006. **176**(12): p. 7566-75.
46. Masignani, V., et al., *Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870.* J Exp Med, 2003. **197**(6): p. 789-99.
47. Welsch, J.A., et al., *Antibody to genome-derived neisserial antigen 2132, a Neisseria meningitidis candidate vaccine, confers protection against bacteremia in the absence of complement-mediated bactericidal activity.* J Infect Dis, 2003. **188**(11): p. 1730-40.
48. Rostand, K.S. and J.D. Esko, *Microbial adherence to and invasion through proteoglycans.* Infect Immun, 1997. **65**(1): p. 1-8.
49. Esposito, V., et al., *Structure of the C-terminal domain of Neisseria heparin binding antigen (NHBA), one of the main antigens of a novel vaccine against Neisseria meningitidis.* J Biol Chem. **286**(48): p. 41767-75.
50. Turner, D.P., K.G. Wooldridge, and D.A. Ala'Aldeen, *Autotransported serine protease A of Neisseria meningitidis: an immunogenic, surface-*

exposed outer membrane, and secreted protein. Infect Immun, 2002. **70**(8): p. 4447-61.
51. van Ulsen, P., et al., *A Neisserial autotransporter NalP modulating the processing of other autotransporters.* Mol Microbiol, 2003. **50**(3): p. 1017-30.
52. Hendrixson, D.R., et al., *Human milk lactoferrin is a serine protease that cleaves Haemophilus surface proteins at arginine-rich sites.* Mol Microbiol, 2003. **47**(3): p. 607-17.
53. Qiu, J., et al., *Human milk lactoferrin inactivates two putative colonization factors expressed by Haemophilus influenzae.* Proc Natl Acad Sci U S A, 1998. **95**(21): p. 12641-6.
54. Flower, D.R., A.C. North, and C.E. Sansom, *The lipocalin protein family: structural and sequence overview.* Biochim Biophys Acta, 2000. **1482**(1-2): p. 9-24.
55. Wallez, Y., et al., *Src kinase phosphorylates vascular endothelial-cadherin in response to vascular endothelial growth factor: identification of tyrosine 685 as the unique target site.* Oncogene, 2007. **26**(7): p. 1067-77.
56. Lampugnani, M.G., et al., *A novel endothelial-specific membrane protein is a marker of cell-cell contacts.* J Cell Biol, 1992. **118**(6): p. 1511-22.
57. Carman, C.V., et al., *Transcellular diapedesis is initiated by invasive podosomes.* Immunity, 2007. **26**(6): p. 784-97.
58. Engelhardt, B. and H. Wolburg, *Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house?* Eur J Immunol, 2004. **34**(11): p. 2955-63.
59. Feng, D., et al., *Pathways of macromolecular extravasation across microvascular endothelium in response to VPF/VEGF and other vasoactive mediators.* Microcirculation, 1999. **6**(1): p. 23-44.
60. Millan, J., et al., *Lymphocyte transcellular migration occurs through recruitment of endothelial ICAM-1 to caveola- and F-actin-rich domains.* Nat Cell Biol, 2006. **8**(2): p. 113-23.
61. Nieminen, M., et al., *Vimentin function in lymphocyte adhesion and transcellular migration.* Nat Cell Biol, 2006. **8**(2): p. 156-62.
62. Dejana, E., *Endothelial cell-cell junctions: happy together.* Nat Rev Mol Cell Biol, 2004. **5**(4): p. 261-70.
63. Muller, W.A., *Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response.* Trends Immunol, 2003. **24**(6): p. 327-34.
64. Vestweber, D., *VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation.* Arterioscler Thromb Vasc Biol, 2008. **28**(2): p. 223-32.
65. Andriopoulou, P., et al., *Histamine induces tyrosine phosphorylation of endothelial cell-to-cell adherens junctions.* Arterioscler Thromb Vasc Biol, 1999. **19**(10): p. 2286-97.
66. Weis, S.M. and D.A. Cheresh, *Pathophysiological consequences of VEGF-induced vascular permeability.* Nature, 2005. **437**(7058): p. 497-504.
67. Hammerling, B., et al., *The complexus adhaerens of mammalian lymphatic endothelia revisited: a junction even more complex than hitherto thought.* Cell Tissue Res, 2006. **324**(1): p. 55-67.

68. Calkins, C.C., et al., *The Armadillo family protein p0071 is a VE-cadherin- and desmoplakin-binding protein.* J Biol Chem, 2003. **278**(3): p. 1774-83.
69. Venkiteswaran, K., et al., *Regulation of endothelial barrier function and growth by VE-cadherin, plakoglobin, and beta-catenin.* Am J Physiol Cell Physiol, 2002. **283**(3): p. C811-21.
70. Kowalczyk, A.P., et al., *VE-cadherin and desmoplakin are assembled into dermal microvascular endothelial intercellular junctions: a pivotal role for plakoglobin in the recruitment of desmoplakin to intercellular junctions.* J Cell Sci, 1998. **111 (Pt 20)**: p. 3045-57.
71. Schmelz, M. and W.W. Franke, *Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells: the syndesmos connecting retothelial cells of lymph nodes.* Eur J Cell Biol, 1993. **61**(2): p. 274-89.
72. Schmelz, M., et al., *Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells: II. Different types of lymphatic vessels.* Differentiation, 1994. **57**(2): p. 97-117.
73. Bazzoni, G. and E. Dejana, *Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis.* Physiol Rev, 2004. **84**(3): p. 869-901.
74. Ivanov, D., et al., *Expression of cell adhesion molecule T-cadherin in the human vasculature.* Histochem Cell Biol, 2001. **115**(3): p. 231-42.
75. Dudek, S.M. and J.G. Garcia, *Cytoskeletal regulation of pulmonary vascular permeability.* J Appl Physiol, 2001. **91**(4): p. 1487-500.
76. Esser, S., et al., *Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells.* J Cell Sci, 1998. **111 (Pt 13)**: p. 1853-65.
77. Mehta, D. and A.B. Malik, *Signaling mechanisms regulating endothelial permeability.* Physiol Rev, 2006. **86**(1): p. 279-367.
78. Schnittler, H.J., *Structural and functional aspects of intercellular junctions in vascular endothelium.* Basic Res Cardiol, 1998. **93 Suppl 3**: p. 30-9.
79. van Hinsbergh, V.W. and G.P. van Nieuw Amerongen, *Endothelial hyperpermeability in vascular leakage.* Vascul Pharmacol, 2002. **39**(4-5): p. 171-2.
80. Shasby, D.M., et al., *Histamine stimulates phosphorylation of adherens junction proteins and alters their link to vimentin.* Am J Physiol Lung Cell Mol Physiol, 2002. **282**(6): p. L1330-8.
81. Baumeister, U., et al., *Association of Csk to VE-cadherin and inhibition of cell proliferation.* Embo J, 2005. **24**(9): p. 1686-95.
82. Potter, M.D., S. Barbero, and D.A. Cheresh, *Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state.* J Biol Chem, 2005. **280**(36): p. 31906-12.
83. Weis, S., et al., *Src blockade stabilizes a Flk/cadherin complex, reducing edema and tissue injury following myocardial infarction.* J Clin Invest, 2004. **113**(6): p. 885-94.
84. Lampugnani, M.G., et al., *Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, beta-catenin, and the phosphatase DEP-1/CD148.* J Cell Biol, 2003. **161**(4): p. 793-804.

85. Lampugnani, M.G., et al., *Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments.* J Cell Biol, 2006. **174**(4): p. 593-604.
86. Carmeliet, P., et al., *Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis.* Cell, 1999. **98**(2): p. 147-57.
87. Gavard, J. and J.S. Gutkind, *VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin.* Nat Cell Biol, 2006. **8**(11): p. 1223-34.
88. Xiao, K., et al., *p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin.* Mol Biol Cell, 2005. **16**(11): p. 5141-51.
89. Grazia Lampugnani, M., et al., *Contact inhibition of VEGF-induced proliferation requires vascular endothelial cadherin, beta-catenin, and the phosphatase DEP-1/CD148.* J Cell Biol, 2003. **161**(4): p. 793-804.
90. Zanetti, A., et al., *Vascular endothelial growth factor induces SHC association with vascular endothelial cadherin: a potential feedback mechanism to control vascular endothelial growth factor receptor-2 signaling.* Arterioscler Thromb Vasc Biol, 2002. **22**(4): p. 617-22.
91. Belousov, V.V., et al., *Genetically encoded fluorescent indicator for intracellular hydrogen peroxide.* Nat Methods, 2006. **3**(4): p. 281-6.
92. Melican, K. and G. Dumenil, *Vascular colonization by Neisseria meningitidis.* Curr Opin Microbiol. **15**(1): p. 50-6.
93. von Heijne, G., *Mitochondrial targeting sequence may form amphiphilic helices.* The EMBO Journal 1986. **5**(6): p. 1335-1342.
94. Mitchell, D.J., et al., *Polyarginine enters cells more efficiently than other polycationic homopolymers.* J Pept Res, 2000. **56**(5): p. 318-25.
95. Borgese, N., et al., *Targeting of a tail-anchored protein to endoplasmic reticulum and mitochondrial outer membrane by independent but competing pathways.* Mol Biol Cell, 2001. **12**(8): p. 2482-96.
96. Bedwell, D.M., et al., *Sequence and structural requirements of a mitochondrial protein import signal defined by saturation cassette mutagenesis.* Mol Cell Biol, 1989. **9**(3): p. 1014-25.
97. Loschen, G., L. Flohe, and B. Chance, *Respiratory chain linked H(2)O(2) production in pigeon heart mitochondria.* FEBS Lett, 1971. **18**(2): p. 261-264.
98. Boveris, A., N. Oshino, and B. Chance, *The cellular production of hydrogen peroxide.* Biochem J, 1972. **128**(3): p. 617-30.
99. Chance, B., H. Sies, and A. Boveris, *Hydroperoxide metabolism in mammalian organs.* Physiol Rev, 1979. **59**(3): p. 527-605.
100. Li, N., et al., *Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production.* J Biol Chem, 2003. **278**(10): p. 8516-25.
101. Herrero, A. and G. Barja, *ADP-regulation of mitochondrial free radical production is different with complex I- or complex II-linked substrates: implications for the exercise paradox and brain hypermetabolism.* J Bioenerg Biomembr, 1997. **29**(3): p. 241-9.
102. Mukhopadhyay, P., et al., *Simple quantitative detection of mitochondrial superoxide production in live cells.* Biochem Biophys Res Commun, 2007. **358**(1): p. 203-8.

103. Timmerman, I., et al., *The tyrosine phosphatase SHP2 regulates recovery of endothelial adherens junctions through control of beta-catenin phosphorylation.* Mol Biol Cell. **23**(21): p. 4212-25.
104. Wright, T.J., et al., *Dynamics of vascular endothelial-cadherin and beta-catenin localization by vascular endothelial growth factor-induced angiogenesis in human umbilical vein cells.* Exp Cell Res, 2002. **280**(2): p. 159-68.
105. Vincent, P.A., et al., *VE-cadherin: adhesion at arm's length.* Am J Physiol Cell Physiol, 2004. **286**(5): p. C987-97.
106. Xiao, K., et al., *Mechanisms of VE-cadherin processing and degradation in microvascular endothelial cells.* J Biol Chem, 2003. **278**(21): p. 19199-208.
107. Schubert-Unkmeir, A., et al., *Neisseria meningitidis induces brain microvascular endothelial cell detachment from the matrix and cleavage of occludin: a role for MMP-8.* PLoS Pathog. **6**(4): p. e1000874.
108. Serruto, D., et al., *The new multicomponent vaccine against meningococcal serogroup B, 4CMenB: immunological, functional and structural characterization of the antigens.* Vaccine, 2012. **30 Suppl 2**: p. B87-97.
109. Turowski, P., et al., *Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration.* J Cell Sci, 2008. **121**(Pt 1): p. 29-37.