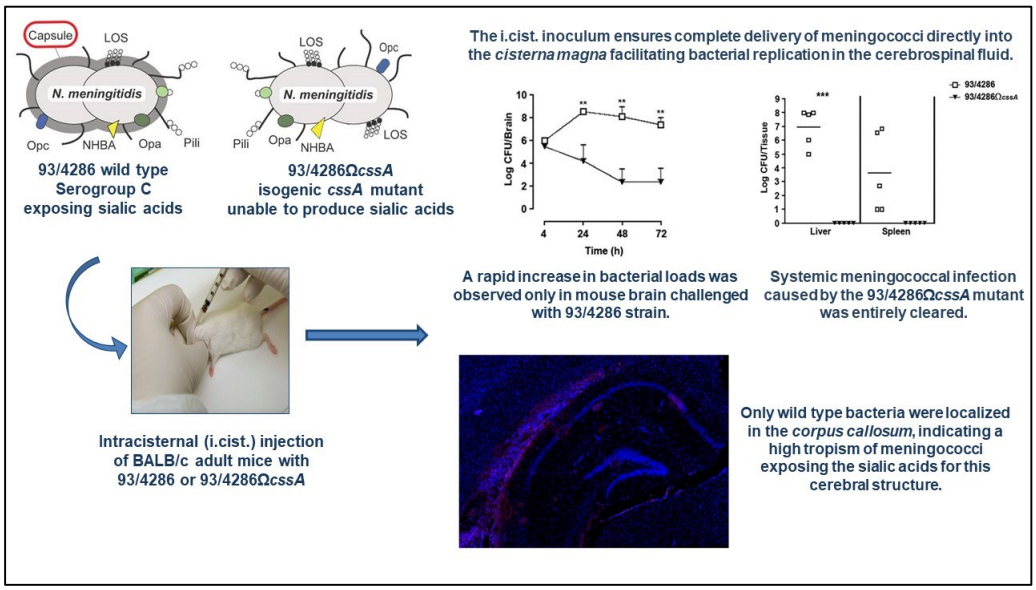
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXII CYCLE

Elena Scaglione

Role of meningococcal surface-exposed sialic acids in a murine infection model



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Role of meningococcal surface-exposed sialic acids in a murine infection model

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LIST OF ABBREVATIONS

µ: growth rate
App: Adhesion and penetration protein
BBB: blood-brain barrier
CEACAM: carcinoembryonic antigen-related cell-adhesion molecule
CFU: colony forming units
CMP-Neu5Ac: cytidine-5'-monophosphate-N-acetyl-neuraminic acid
CNS: central nervous system
***cps*:** capsular polysaccharide locus
CSF: cerebrospinal fluid
DMEM: Dulbecco's modified Eagle medium
fHbp: factor H binding protein
GC: gonococcus medium
i.cist.: intra-cisternal
i.n.: intranasally
i.p.: intraperitoneally
IMD: invasive meningococcal disease
LD_{50}: 50% lethal dose
LOS: lipo-oligosaccharide
LPS: lipopolysaccharide
MM: meningococcal meningitis
MspA: meningococcal serine protease A
NadA: *Neisseria* adhesion A
NeuNAc/Neu5Ac: N-acetyl-neuraminic acid
NHBA: *Neisserial* heparin binding antigen
NhhA: *Neisseria* hia/hsf homologue
OD_{600}: optical density at 600 nm
RT-PCR: reverse transcriptase PCR
SD: standard deviations
GBS: *Streptococcus agalatiae*
Tfp: type IV pili
UDP: uridine 5'-diphosphate
WHO: World Health Organization

ABSTRACT

Neisseria meningitidis is a leading cause of sepsis and meningitis worldwide in humans. It colonizes the upper respiratory tract of healthy and asymptomatic carriers, but it is able to elude host immune defenses and spread from the bloodstream to the brain causing uncontrolled local inflammation. Both host and bacterial factors seem to be involved in this switch from harmless transitory colonization to devasting disease. Among its virulence factors, surface-exposed sialic acids occupy a prominent position. In serogroup C *N. meningitidis*, the *cssA* gene encodes for an UDP-N-acetylglucosamine 2-epimerase that catalyzes the conversion of UDP-N-acetyl-α-D-glucosamine into N-acetyl-D-mannosamine and UDP in the first step of sialic acid biosynthesis. This enzyme is required for the biosynthesis of the (α2→9) linked polysialic acid capsule and for lipooligosaccharide sialylation. In this study, was used a reference serogroup C meningococcal strain 93/4286 and an isogenic *cssA* knockout mutant 93/4286Ω*cssA* in order to investigate the pathogenetic role of surface-exposed sialic acids in a meningitis model based on intracisternal inoculation of BALB/c mice. The primary results obtained from the 93/4286Ω*cssA* *in vitro* characterization, confirmed that the inactivation of *cssA* gene did not alter the bacterial fitness of this mutant and therefore its invasive abilities in a mouse host. Furthermore, the results obtained from the *in vivo* experiments, confirmed the key role of surface-exposed sialic acids in meningococcal pathogenesis. The LD_{50} of the wild type strain 93/4286 was about four orders of magnitude lower than that of the *cssA* mutant. Compared to the wild type strain, the ability of mutant to replicate in brain and spread systemically was severely impaired. Evaluation of brain damage evidenced a significant reduction in cerebral haemorrhages in mice infected with the mutant in comparison with the levels in those challenged with the wild type strain. Histological analysis showed that the experimental murine model has reproduced the typical characteristics of bacterial meningitis, particularly in animals infected with the wild type strain. Moreover, the 80% of the mice infected with the reference strain had a massive bacterial localization accompanied by a significant inflammatory infiltrate in the *corpus callosum*, indicating a high tropism of the meningococci exposing the sialic acids towards this brain structure and its specific involvement in meningococcal meningoencephalitis. This study proposes a new role of microbial surface-exposed sialic acids in the interplay between *N. meningitidis* and the host in the pathogenesis of meningococcal disease. Meningococcal meningitis still represents an important challenge for human health worldwide. Therefore, this model could be functional for the study of invasive meningococcal disease since *N. meningitidis* is a strictly human pathogen characterized by a complex infectious cycle and it is considered the paradigm of genetic variation.

1. BACKGROUND

1.1 General characteristics of *Neisseria meningitidis* and epidemiology of meningococcal disease

Neisseria meningitidis (meningococcus) is a Gram-negative diplococcus that can cause septicaemia and meningitis in susceptible individuals (Hollingshead and Tang 2019) (Figure 1). Together with *N. gonorrhoeae*, the causative agent of the sexually transmitted infection gonorrhoea, represent the only two species highly pathogenic for humans belonging to *Neisseria* genus. Many species of *Neisseria* inhabit the nasopharynx as commensal, including *N. meningitidis* (Dwilow and Fanella 2015).

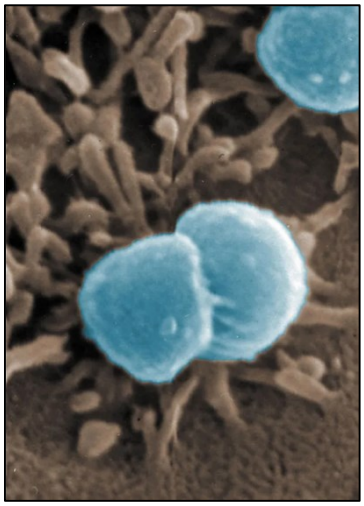

Figure 1: ***Neisseria meningitidis.*** The figure shows the typical diplococci arrangement of *N. meningitidis* (Feavers 2000).

Indeed, meningococcus can be isolated in the oropharyngeal cavity of healthy individuals and therefore assume the characteristics of commensal bacteria in the human respiratory tract, frequently causing localized asymptomatic nasopharyngeal infections. However, under certain conditions it can take on invasive capabilities, penetrate the pharyngeal mucosa and entering into the bloodstream causing septicaemia and, in more severe conditions, it reaches the central nervous system (CNS), cross the blood-brain barrier (BBB), and it can settle in subarachnoid area of the meninges causing local inflammation (Caugant and Maiden 2009). The *Neisseria spp.* optimally multiply in damp and oxygen-poor environments containing 5-10% CO_2 at 35-37°C and are able to grow anaerobically in presence of NO_2. According to different ability to use simple sugars as sources of energy, we can differentiate the *Neisseria* species: meningococci ferment both glucose and maltose, while the gonococci ferment only glucose. These microorganisms have specific nutritional requirements and

grow on different enriched culture media like chocolate-agar and gonococcal (GC) agar. Outside the host, pathogenic *Neisseria* are sensitive to adverse environmental conditions for growth and can undergo autolysis and die in a few days even at room temperature.

A distinctive feature of *N. meningitidis* that differentiates it from *Neisseria* non-pathogenic commensal species and from *N. gonorrhoeae* is the presence of a polysaccharide capsule that completely covers the cell. In particular, *N. meningitidis* strains can be classified into 13 serogroups, based on structural differences in their capsular polysaccharide (Mubaiwa et al. 2017). Six serogroups cause the majority of invasive meningococcal disease (IMD) worldwide: A, B, C, W135, X and Y (Rouphael and Stephens 2012). The capsules of serogroups B, C, W, and Y are composed of polysialic acid (B and C), or sialic acid linked to glucose or galactose (Swartley et al. 1997), while the capsules of serogroups A and X are composed of *N*-acetyl mannosamine-1-phosphate (Liu et al. 1971) and *N*-acetylglucosamine-1-phosphate (Bundle et al. 1973), respectively.

Further classification of *N. meningitidis* is based on differences in their lipopolysaccharide (LPS) and the outer membrane proteins PorB and PorA or using the multi-locus sequence typing (MLTS) that classifies strains into STs (sequence types) based on the difference among seven housekeeping genes (Hollingshead and Tang 2019). Recently, MLST has been replaced by core genome MLST. This approach groups isolates into lineages based on a core set of genes, which are found in almost all isolates. Core genome MLST provides higher resolution than MLST, and has demonstrated that disease isolates are a diverse recombining population from which hyper-invasive lineages have independently emerged on several occasions (Bratcher et al. 2014; Maiden and Harrison 2016; Hollingshead and Tang 2019).

Meningococcal disease occurs worldwide but with significant variation in rates of disease based on circulating clonal complexes, serogroup, geographic location, population susceptibility and age (Dretler et al. 2018). *N. meningitidis* is found as a colonizer, without clinical manifestations, in the nasopharyngeal tract of about 18% of the normal population representing an asymptomatic carrier for this pathogen allowing its transmission to other individuals. The percentage of carriers is very high in adolescents (≤27%), while it is much lower in adults (≤8%) and children (<5%) (Borrow et al. 2016). The incidence of IMD is highest in three specific age groups: children under 5 years old, adolescents and young adults aged 16 through 21 years, and adults aged 65 and older (Cohn et al. 2013; MacNeil et al. 2017). As for the geographical distribution, worthy of note is the so-called “Meningitidis Belt”, a region of sub-Saharan Africa that includes 18 countries from Senegal to Ethiopia, where epidemic waves of meningococcal meningitis develop periodically every 8-12 years. Traditionally, these epidemics were caused by serogroup A strains, which accounted for over 90% of cases (Trotter et al. 2017); while serogroups W-135, C and X are responsible for the development of localized epidemic outbreaks (Agier et al. 2017). Serogroup B meningococcus is the cause of endemic disease in much of the developed world, including North America,

Canada, Western Europe, Australasia and South America. In recent years there has been a dramatic increase in the number of cases of IMD caused by serogroup W in the UK (United Kingdom), associated with a high case fatality rate (Nadel and Ninis 2018). Serogroup Y meningococcus is becoming an increasingly important cause of meningococcal disease in the USA and is more recently being increasingly reported from the UK (Health Protection Report 2017).

According to recent World Health Organization (WHO) data, meningococcal serogroup C is still one of the most widespread serogroups in the world (www.who.int/emergencies/diseases/meningitis/serogroup-distribution 2018.pdf) (Figure 2). Recently, a new meningococcal meningitis clone of serogroup C has been expanding in sub-Saharan Africa, associated with a huge risk of a major epidemic in the next 2 years (WHO 2018, [https://www.who.int/emergencies/diseases/meningitis/meningitis-cepidemic-risk/en/]).

In addition, since January 2015, in Tuscany, Italy, there has been an unexpected increase in cases of IMD (a total of 43 cases, of which 10 were fatal) due to infection with serogroup C *N. meningitidis*. Thirty-five out of the samples analyzed in this study were confirmed as clonal complex (CC) 11 (genotype: C:P1.5-1,10-8:F3-6:ST-11) (Stefanelli et al. 2016).

Current mortality remains 10-15% in developed countries, and ranges higher (20%) in the developing world (Rosenstein et al. 2001; Rouphael and Stephens 2012; Borrow et al. 2016; MacNeil et al. 2017; Trotter et al. 2017). Morbidity related to sequelae of meningococcal disease remains high, with up to 20% of survivors experiencing long-term disabilities including developmental delays, deafness, and loss of limbs (Stephens et al. 2007; Lingani et al. 2015; MacNeil et al. 2017).

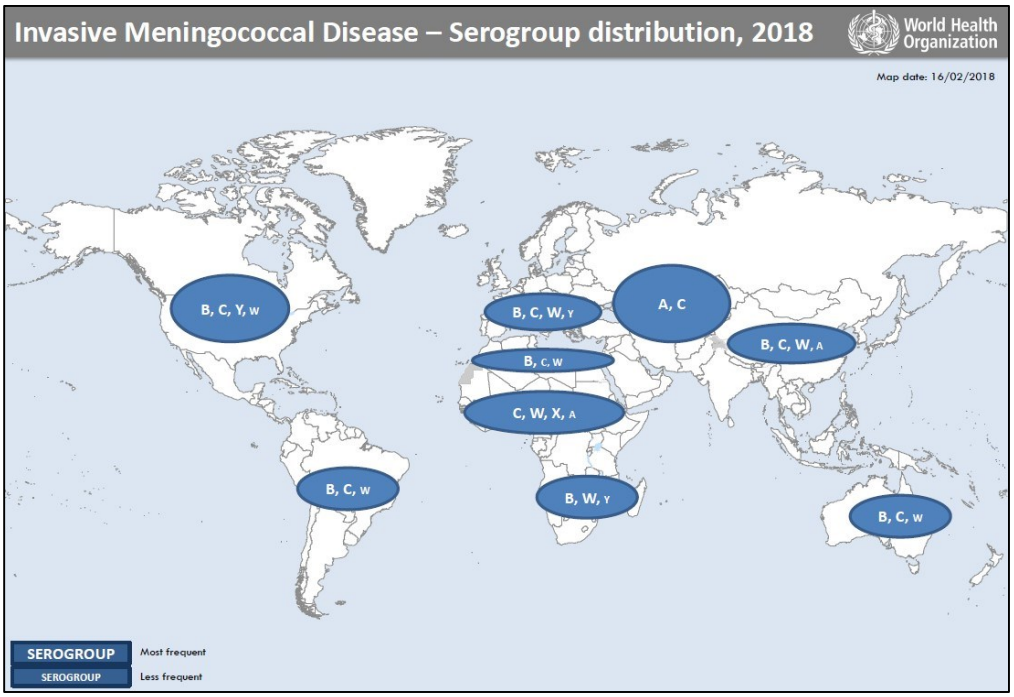

Figure 2: Global distribution of meningococcal serogroups
The figure shows the worldwide distribution of the main meningococcal serogroups. In capital character are shown the most frequent serogroups in the respective geographical areas. The data emphasize the new geographic distributions of meningococcal serogroup C one of the most widespread serogroups in the world (Adapted from WHO 2018).

1.2 Clinical features of meningococcal disease and its treatment and prevention

Humans are the only natural reservoir for the meningococcus which is found primarily as a commensal inhabitant in the nasopharynx, and may be found in over 25% of individuals during adolescence. Prompt recognition of meningococcal infection and early aggressive treatment are essential in order to reduce mortality, which occurs in up to 10% of those with IMD (Nadel and Ninis 2018). Transmission occurs by semi-direct inter-human aerosol contamination through inhalation of droplets and nasal infectious secretions; the infection rarely occurs indirectly, because the meningococcus shows a low resistance to environmental physical agents, sunlight, drying and common disinfectants. It is unclear why the meningococcus invades the bloodstream after nasopharyngeal colonization to cause invasive disease. It is likely that bacterial virulence factors, environmental conditions and innate host susceptibility all play important roles (Nadel and Ninis 2018). In the early stages of disease, the clinical picture is non-specific and may be confused with trivial viral illnesses. The most common clinical syndrome is acute bacterial meningitis with fever, headache, neck stiffness, and photophobia (Rosenstein et al. 2001; Stephens et al. 2007; Rouphael et al. 2009). The disease appears after a short incubation, during which the germ from the mucosa rhino-pharyngeal reaches the bloodstream resulting in bacteraemia, sometimes with fulminant sepsis, and localizes at aracnoidal level and *pia mater* of CNS. IMD in infants, children and young adults usually presents as two main clinical syndromes, shock and meningitis, which may exist on their own or co-exist. The meningococcal septic shock is characterized by a fulminant host inflammatory response to bacterial invasion. The disease may rapidly progress to cardiovascular failure and multi-organ failure. Shock is present in ∼20% of patients with IMD and is associated with high mortality and morbidity (Nadel and Ninis 2018). Meningitis is the most common clinical manifestation of IMD. It is an acute sieropurulenta inflammation of the brain and spinal meninges characterized by the formation of a mucopurulent exudate: the fluid is turbid, cloudy or purulent.

Because of easy transmission, fast onset and the severity of associated diseases, diagnosis and therapeutic intervention must be timely. The traditional method of confirming IMD is by direct detection of either gram-negative diplococci on gram stain or isolation of *N. meningitidis* from sterile body fluids, like cerebrospinal fluid (CSF) or blood (Ragunathan et al. 2000; Stephens and Apicella 2014). To overcome the limitations of traditional culture techniques, several additional methods have been used (Dwilow and Fanella 2015). The diagnosis can be done by direct identification of antigen by Latex Agglutination Test from sterile fluids but limitations of this test include poor overall sensitivity and specificity, poor sensitivity for certain serogroups, and inability to identify certain serogroups prohibiting its usefulness as a routine test (Rebelo et al. 2006). The limits of traditional culture techniques of Latex Agglutination Test have been overcome thanks to the advent of new molecular

technologies based on nucleic acid amplification tests (NAATs) like Real-Time PCR able to detect meningococcal DNA in body fluids with a higher sensitivity, specificity, negative predictive value, and positive predictive value. It is useful in situations where antibiotics have been given prior to testing and it can also determine subtypes that were previously untypeable (Bryant et al. 2004).

The primary goal of treating infected individuals is early antibiotic treatment to immediately stop proliferation of *N. meningitidis* (Rosenstein et al. 2001). Before passive immune or antibiotic treatment was available, the mortality of systemic meningococcal disease was 70-90% (Flexner 1913). Once *N. meningitidis* has been isolated from a sterile fluid, appropriate treatment regimens include intravenous penicillin G, ceftriaxone, or cefotaxime. The duration of therapy that is generally recommended is 7 days, although shorter courses of therapy have been found to be effective and are in routine use in some areas (Briggs et al. 2004; Nathan et al. 2005). All meningococci in CSF are killed within 3-4 hours after intravenous treatment with an adequate dose of antibiotic (Kanegaye et al. 2001), and concentrations of endotoxin, cytokines and chemokines in plasma fall by 50% within 2 hours (van Deuren et al. 1995; Øvstebø et al. 2004; Møller et al. 2005). In order to control the development of an endemic and outbreak-associated cases, chemoprophylaxis with third-generation cephalosporins, ciprofloxacin, and rifampicin is recommended for close contacts. To eradicate nasopharyngeal carriage of meningococcus, current recommendations for chemoprophylaxis include household contacts, child care contacts or others who have close or intimate contact with respiratory secretions within the 7 days preceding clinical illness of the index patient, although length of communicability is not well established (Pickering et al. 2012). While antibiotic therapy has decreased mortality, the continued 10–20% mortality and the long-term morbidity remain significant issues. Thus, the ideal strategy to manage *N. meningitidis* is through immunization for disease prevention (Dretler et al. 2018). Attempts to create an effective vaccine against *N. meningitidis* date back to the late 1960s with the introduction of polysaccharide vaccines targeting serogroups A and C by Gotschlich and colleagues (1969), which elicited bactericidal antibodies in human volunteers, but produced short lived, T-cell independent responses (Goldschneider et al. 1969; Gotschlich et al. 1969) (Figure 3). Late in the 20th century meningococcal vaccine development efforts became focused on improving the T-cell mediated immune response through conjugation of the polysaccharide to a carrier protein like tetanus or diphtheria toxoid: coupling the capsular polysaccharide to a protein carrier, changes the human immune response from T-cell independent to T-cell dependent engaging helper T cells in maximizing a predominant high affinity IgG immune response (Dretler et al. 2018; Hollingshead and Tang 2019). Meningococcal serogroup C conjugate vaccines were first introduced in 1999 in the UK (Figure 3). This vaccine is strongly immunogenic, giving relatively long-lasting immunity and immunological memory, and it also has a significant impact on decreasing nasopharyngeal carriage in vaccines and therefore confers herd immunity or community

protection (Nadel and Ninis 2018). Between 2010 and 2017 a partnership between the WHO and Program for Appropriate Technology in Health led to the development of MenAfriVac, a serogroup A conjugate vaccine for use in the Meningitis Belt for a mass vaccination campaigns (Hollingshead and Tang 2019). Following immunization, cases of serogroup A meningococcal disease plummeted, driven by the direct effects of vaccination and by herd protection (Trotter et al. 2017; LaForce et al. 2018). To date effective quadrivalent conjugate polysaccharide vaccines have become available against the meningococcal serogroups A, C, Y, and W (Nadel and Ninis 2018). Unlike the other disease-causing serogroups, the polysaccharide capsule of serogroup B is poorly immunogenic. The polysaccharide capsule of serogroup B is composed of polysialic acid (α2–8 N-acetylneuraminic acid) which has structural similarity to human neural cell glycopeptide. Therefore, there is a degree of immune tolerance to this polysaccharide that hindered progress on developing an effective protein/polysaccharide conjugate vaccine for serogroup B (Nadel 2012). Some regional successes have occurred using outer-membrane vesicle (OMV) based vaccines for local outbreak control in Cuba, Norway and New Zealand (Oster et al. 2005) (Figure 3). The dominant antigen in OMVs is the outer membrane porin PorA, which is antigenically variable. Thus the major drawback of OMV vaccines is that they are largely PorA variant specific and provide limited cross-protection. This design approach was not useful for the development of a vaccine able to prevent the disease in other countries (Dwilow and Fanella 2015). To overcome these limitations, the "reverse vaccinology" which identified serogroup B vaccine candidates from whole genome sequences, was used (Pizza et al. 2000) (Figure 3). Thanks to the "reverse vaccinology", genes that encoded potential targets on the bacterial cell surface and would thus be possible targets for an immune response were found in the *N. meningitidis* serogroup B genome. The first multicomponent serogroup B vaccine, 4CMenB (Bexsero® Novartis Vaccines), includes four components: factor H binding protein (fHbp), *Neisserial* adhesion A (NadA), *Neisserial* heparin binding antigen (NHBA), and OMV containing PorA (Pizza et al. 2000). Another vaccine effective against serogroup B, manufactured by Pfizer (Trumemba©), contains antigenic components from subfamilies A and B of meningococcal fHbp. This vaccine has been widely used in North America in outbreaks of serogroup B disease and has been shown to be safe and effective (Duffy et al. 2017). Although new vaccines hold great promise, meningococcal disease remains incompletely controlled by immunization, largely as a consequence of the diversity of *N. meningitidis* populations. An ideal vaccine for prevention of meningococcal disease would be effective and affordable against the all invasive meningococcal serogroups, would elicit long-lasting immunity in all age groups, and provide significant herd immunity (Chang et al. 2012). The evaluation of new meningococcal vaccines for the global elimination of meningococcal disease presents a number of challenges. Future directions and challenges for meningococcal vaccine research include combination vaccines containing ACYW(X) and B, improving on the serotype coverage of the serogroup B vaccines, tackling issues of waning effectiveness

for a longer lasting immune responses and introducing meningococcal vaccines globally to maximize effectiveness (Dretler et al. 2018).

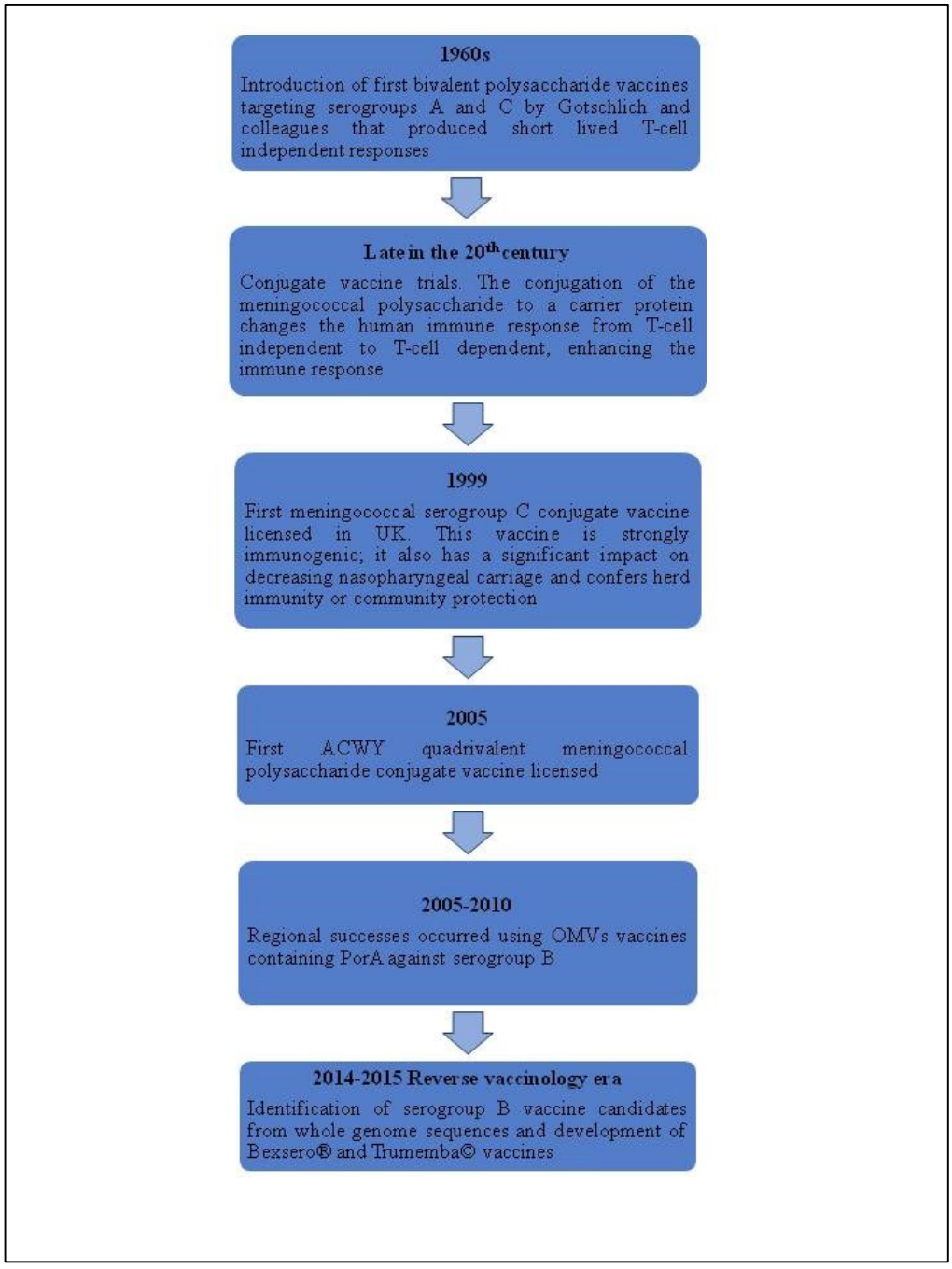

Figure 3: Outline of meningococcal vaccine developments
The figure summarizes the key historical points in the development of human meningococcal vaccines.

1.3 Infection cycle and pathogenic factors of *N. meningitidis*

The infectious cycle of *N. meningitidis* involves the colonisation of different environments. The first is the human nasopharynx from where bacteria are transmitted from person to person by aerosol droplets and grow on the top of mucus-producing epithelial cells surrounded by a complex microbiota (Coureuil et al. 2019) (Figure 4). Once *N. meningitidis* penetrates the mucus barrier of the upper respiratory tract and attaches to human epithelial cells, a series of interactions take place that result in effacement of the epithelial surface and microcolony formation (Stephens 2009). After initial adherence is achieved, bacteria must overcome physical clearance (e.g. cilia, mucus) (Livorsi et al. 2011). Under certain circumstances, meningococci have the ability to survive in the extra cellular fluids including blood and CSF, become invasive and cause septicaemia and/or meningitis (Simonis and Schubert-Unkmeir 2016). In the blood-stream, the organism can rapidly produce and release endotoxin, which stimulates cytokine production and the alternative complement pathway (Kirsch et al. 1996). In about half of bacteremic persons, *N. meningitidis* crosses the BBB into the CSF, and meningitis follows. In order to cause invasive disease, meningococci use virulence factors to colonize, spread and penetrate mucosal/epithelial surfaces and grow in the human host, avoid immunologic and other host defences, and ultimately damage the host by cellular activation, inactivation or direct toxin-mediated mechanisms (Livorsi et al. 2011).

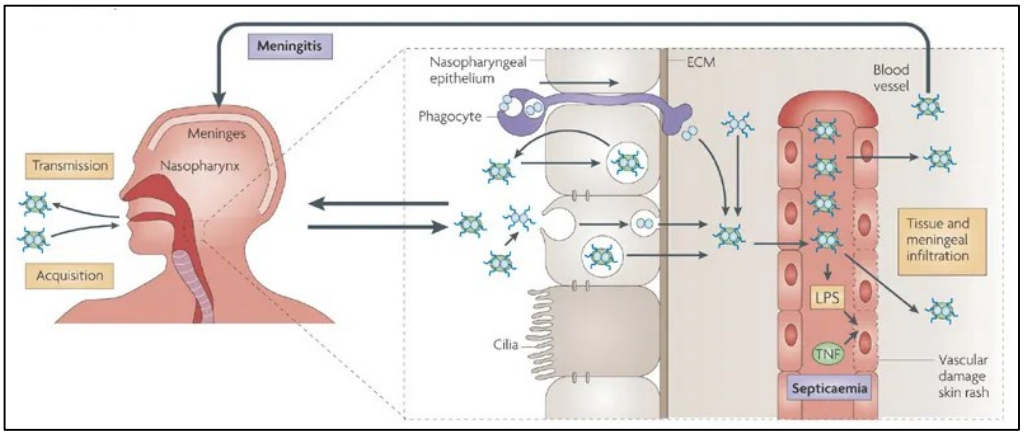

Figure 4: The infection cycle of meningococcus
The figure shows the infectious cycle of *N. meningitidis*. This microorganism, may be acquired through the inhalation of respiratory droplets and it establishes close interactions between its ligands and human receptors expressed on the epithelial cell surface of the upper respiratory tract. Besides transcytosis, *N. meningitidis* can cross the epithelium either directly following damage to the monolayer integrity or through phagocytes in a 'Trojan horse' manner. Once inside the bloodstream, *N. meningitidis* may survive, multiply rapidly and disseminate throughout the body and the brain, where after crossing the BBB it can reach the meninges and can cause meningitis (Adapted from Virji 2009).

The pathogenic *Neisseria* expresses a variety of surface structures that interact with host cells. These include the major outer membrane proteins (e.g. pili, the opacity proteins Opc and Opa and the major porins PorA and PorB) as well as other minor outer membrane structures and adhesins (Merz and So 2000; Virji 2009). The human nasopharynx is lined with columnar epithelium that forms a cellular barrier and the majority of the cells in the epithelial layer are ciliated, although there are areas of non-ciliated cells, to which *N. meningitidis* preferentially adheres (Stephens et al. 1983; Virji et al. 1994). Initial adhesion is mediated by Type IV pili (Tfp) and is followed up by a more intimate adhesion facilitated by proteins such as Opa and Opc (Virji et al. 1994). Pili are filamentous, polymeric pericellular glycoproteins, up to 6 µm in length. While other adhesins are initially concealed by the capsule, pili are able to overhang this structure and mediate primary contact and attachment to epithelial cells (Simonis and Schubert-Unkmeir 2016). Tfp are involved in bacterial movement, also known as twitching motility and in transformation competence. The *pilus* fibre is composed of a single structural component, the major pilin, PilE (encoded by *pilE* gene) and of three minor pilins that are responsible for Tfp-associated phenotypes (Coureuil et al. 2019). Indeed, in addition to PilE, several other pilin proteins, including PilD, PilG and PilF, and the minor pilins PilX, PilV and ComP, contribute to the structure and function of Tfp (Nassif et al. 1994; Tønjum et al. 1995; Hélaine et al. 2005; Carbonnelle

et al. 2006; Cehovin et al. 2013). A single meningococcus may express 100-1000 pili composed of a repeating protein pilin from the outer membrane. Recombination events in meningococcal gene *pilE*, can lead to genetic loci rearrangements and expression of antigenic variation in pilin, which aids in avoidance of host immune responses (Livorsi et al. 2011). Tfp are also essential for the natural competence of *N. meningitidis* as they enable cells to uptake exogenous DNA by recognition of DNA uptake sequences which are scattered throughout the genome. Initial attachment of *N. meningitidis* to endothelial cells requires the interaction of Tfp with human cell receptors. Several receptors were proposed as being able to interact with Tfp: the complement regulatory protein CD46, the laminin receptor, or the platelet activating factor receptor and CD147 that has been recently identified as an important adhesion receptor on both brain and peripheral endothelial cells (Coureuil et al. 2019). Intimate association is mediated by opacity proteins. *N. meningitidis* is able to express two classes of opacity proteins, Opa and Opc a family of transmembrane molecules that form β-barrel structures in the outer membrane, with the Opa proteins having four and the Opc proteins five surface loops (Stern et al. 1986; Virji et al. 1996; Prince et al. 2002). Opa proteins bind to the non-glycosylated face of the N-terminal Ig domain of carcinoembryonic antigen-related cell adhesion molecule (CEACAM) receptors (Sadarangani et al. 2011) and its expression is subject to antigenic and phase variation. Opc is particularly implicated in host-cell invasion of endothelial cells and, unlike the Opa proteins, is antigenically stable because its expression is controlled at the transcriptional level by the length of a polycytidine stretch within the *opcA*-promoter region (Sarkari et al. 1994). In addition to the major adhesion, several antigens have been described as having a role in adhesion. In particular, autotransporters are a class of proteins secreted by meningococcus composed of an N-terminal signal sequence, a central functional passenger domain(s), and a C-terminal translocator domain. *N. meningitidis* has different autotransporters like NadA and *Neisseria* hia/hsf homologue (NhhA), Adhesion and penetration protein (App) and Meningococcal serine protease A (MspA) and autotransporters AutA and AutB (Hollingshead and Tang 2019) (Figure 5). NadA and NhhA are trimeric autotransporter which bind to extracellular matrix proteins, heparan sulfate and laminin and facilitates attachment to host epithelial cells (Sjölinder et al. 2008). App and MspA are widely expressed in virulent *N. meningitidis* strains, are processed and released during bacterial-host cell adhesion. Upon release, these domains bind to different receptors, and can mediate endocytosis; once inside the cell, App and MspA can traffic to the nucleus and can induce cell death via a caspase-dependent pathway (Khairalla et al. 2015). AutA and AutB are structurally related. AutA is found in pathogenic and commensal *Neisseria spp*., while AutB is only found in the meningococcus and gonococcus. They are subject to phase variation and are both secreted on the cell surface, where AutA induces auto-aggregation while AutB promotes biofilm formation and impedes the transit of the meningococcus through epithelial cells (Arenas et al. 2015; Arenas et al. 2016).

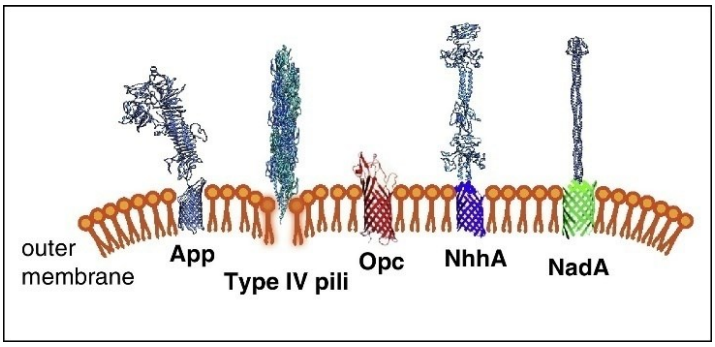

Figure 5: Graphic illustration of meningococcal outer membrane proteins involved in colonization
App (adhesion and penetration protein); Type IV pili (Tfp); Opc (opacity protein C); NhhA (Neisseria hia/hsf homologue) and NadA (Neisserial adhesin A) (Pizza and Rappuoli 2015).

N. meningitidis expresses two distinct porins, PorA and PorB, through which small hydrophilic nutrients diffuse into the bacterium via cation or anion selection (Rouphael and Stephens 2012). Porins are also involved in host cell interactions and as targets for bactericidal antibodies: PorB is the major porin that inserts in membranes, induces Ca^{2+} influx and activates Toll-like receptor 2 (TLR2) and cell apoptosis; PorA is a major component of vesicle-based vaccines and a target for bactericidal antibodies (Rouphael and Stephens 2012). The interaction of bacterial adhesins/invasine with its epithelial cell receptors establishes a "cross talk" between meningococcus and the target cell following which the bacteria are incorporated into vesicles and translocated to the membrane baseline before being exocytosis submucosal space by transcytosis (Nassif 1999). The intracellular survival, transcytosis process through the basolateral tissues and dissemination into the bloodstream are less well studied (Stephens 2009). In the intracellular environment meningococci reside within a membranous vacuole. Meningococcal intracellular survival is determined by IgA1 protease, which degrades lysosome-associated membrane proteins (LAMPs), thus preventing phagosomal maturation and upregulation of capsule expression. *N. meningitidis* is also able to acquire iron through specialized transport systems, like the haemoglobin binding receptor (HmbR), transferring binding protein (TbpAB) and lactoferrin binding protein (LbpAB) (Perkins-Balding et al. 2004). Thanks to these mechanisms, meningococcal intracellular survival is guaranteed. Once in the blood, the meningococcus benefits from the same virulence factors that ensure its survival in mucus to survive and proliferate in the blood: the polysaccharidic capsule and the lipo-oligosaccharide (LOS) (Coureuil et al. 2019). Meningococcal LOS is a major component of the outer membrane and plays an important role in activation of the innate immune system and inflammatory signalling underlying the pathogenesis of fulminant sepsis and meningitis (Brandtzaeg 2006; Plant et al. 2006). Neisserial LOS consists of lipid A that anchors the LOS into the outer

membrane, an inner core composed of two 3-deoxy-D-manno-2-octulosonic acid and two heptose residues, and an outer core of oligosaccharide chains (α, β, γ) attached to the heptose residues (Jennings et al. 1999). Lipid A is the meningococcal endotoxin: it induces the release of cytokines within the vasculature, including Interleukin 6 (IL-6), Tumor necrosis factor α (TNF-α), reactive oxygen species, nitric oxide and chemokines. This strong pro-inflammatory response can result in endothelial damage, tissue necrosis and multiple organ failure (Livorsi et al. 2011). In the meningococcal LOS, sialic acid can be a terminal component of the oligosaccharides chain, with a structural pattern identical to the human I and i antigens, example of host molecular mimicry and immune escape mechanisms (Mandrell et al. 1988). Phase and antigenic variations lead to different oligosaccharide chain and inner core composition, dramatically altering the antigenic properties of LOS (Jennings et al. 1999). The differences in phenotypes and LOS expression form the basis of the classification into different immunotypes (Ll-12) (Jennings et al. 1999).

1.4 Role and biosynthesis of the capsular polysaccharide

There is a convincing evidence of the capsule requirement for *N. meningitidis* virulence. Almost all clinical isolates, recovered from blood or CSF from infected patients are encapsulated (van Deuren et al. 2000). Furthermore, the presence of capsular polysaccharide is associated with virulence and serum resistance to both phagocytosis and complement-mediated killing via alternative pathway (AP) activation, resulting in enhanced survival in the bloodstream and CNS. Several mechanisms are activated by the different meningococcal serogroups in order to inhibit the AP activation. Serogroup B and C capsules reduce AP activation by improving the binding of a complement inhibitory protein (factor H) to C3b, thus limiting the deposition of C3 on the bacterial membrane (Tzeng et al. 2016). The presence of capsular polysaccharides in serogroups W and Y, although paradoxically improve the activation of the AP by acting as a target for the deposition of C3, makes these serogroups resistant to AP (Ram et al. 2011). Finally, the capsular polysaccharide of serogroup A also increases resistance to serum bactericidal activity, but does not influence AP-mediated C3b deposition (Tzeng et al. 2016). Moreover, the capsular polysaccharides of the main serogroups (A, B, C, W, Y), can inhibit the classical complement pathway, interfering with the recruitment of C1q by antibody with consequent reduction of C4b deposition (Agarwal et al. 2014).
There was also demonstrated that the meningococcal capsule is important for bacterial survival within human cells. Encapsulated serogroup B meningococci have been reported to escape the phagosome of human epithelial cells. During the advanced phases of the infectious process, these multiply in the cytosol and diffuse intracellularly and towards the surrounding cells through a direct interaction with the microtubule cytoskeleton (Talà et al. 2008). In *in vitro*

condition, has been reported that the serogroup B capsule directly mediates the interactions between bacteria and microtubules, with the inhibition of tubulin polymerization (Talà et al. 2014). The meningococcal capsule also protects the bacteria against cationic antimicrobial peptides (CAMP), including human cathelicidin LL-37, preventing that the peptides to reach the bacterial membrane (Jones et al. 2009). But on the other hand, expression of the capsule hinders colonization and invasion of the nasopharyngeal barrier by masking adhesins/invasins during the early stages of infection. Indeed, on the mucosal surfaces, the meningococcal capsules can provide anti-adherent properties by covering the adherence ligands, causing the loss of meningococci on the mucosal surfaces and facilitating the transmission of encapsulated meningococci (Stephens et al. 1993; Virji et al. 1993).

The capsular polysaccharides have different composition among the six meningococcal serogroups responsible for the diseases worldwide: serogroup A *N. meningitidis*, expresses a homopolymeric (α1→6) N-acetylmannosamine 1-phosphate capsular polysaccharide; in serogroups B and C capsular polysaccharides are both homopolymers of sialic acid (N-acetylneuraminic acid) but with different linkages: (α2→8)-sialic acid for serogroup B and (α2→9)-, and partially O-acetylated sialic acid for serogroup C; the serogroup Y capsular polymer is composed of alternating D-glucose and partially O-acetylated sialic acid, while the serogroup W capsular polysaccharide is an alternating D-galactose and partially O-acetylated sialic acid; the serogroup X capsular polysaccharide is a homopolymer of (α1→4) N-acetylglucosamine 1-phosphate (Tzeng et al. 2016) (Figure 6).

Serogroup	Structural Repeating Unit	Acetylation
A	ManNAc-(1-P → 6)- 3 α | OAc	(+)
B	α NeuNAc-(2 → 8)-	(-)
C	NeuNAc-(2 → 9)- | | α 7-OAc 8-OAc	(+)
Y	6-Glc-(1 → 4)- NeuNAc-(2→ 6)- α | α OAc	(+)
W	6-Gal-(1 → 4)- NeuNAc-(2→ 6)- α | α OAc	(+)
X	α GlcNAc-(1-P → 4)-	(-)

Figure 6: Biochemical composition of the capsular polysaccharide in pathogenic *N. meningitidis*

The figure shows the capsular polysaccharide structures of serogroups A, B, C, Y, W and X composed by different structural repeating unit: ManNAc, N-acetyl-D-mannosamine; NeuNAc, N-acetyl-neuraminic acid (sialic acid); GalNAc, N-acetyl-galactosamine; GlcNAc, N-acetyl-glucosamine (Modified by Tzeng et al. 2016).

Recently, a new unique nomenclature has been proposed to define the genetic organization of the capsular polysaccharide locus (*cps*) in all encapsulated meningococcal serogroups established by genome sequence analysis (Harrison et al. 2013). The *cps* locus is composed by genes involved in polysaccharide biosynthesis and cell surface translocation, divided into 6 regions: A–D, D′, and E (Figure 7). Genes in region A encode enzymes for biosynthesis of the capsular polysaccharide, while genes in regions B and C are implicated in the translocation of the high molecular weight polysaccharides to the cell surface (Frosch et al. 1989). In particular, in all serogroups *cps* loci are arranged in this order D-A-C-E-D′-B, revealing a conserved genetic synthesis mechanism and a replication of the genomic rearrangements (Figure 7). While the genes in regions B–D, D′ and E are conserved, genes in region A are diverse among the different serogroups due to the distinct biochemical composition of the capsular polysaccharide (Harrison et al. 2013). Furthermore, the GC content found in regions A and C is much lower than the overall GC content of meningococcal genome, indicating that these regions are the result of horizontal recombination events.

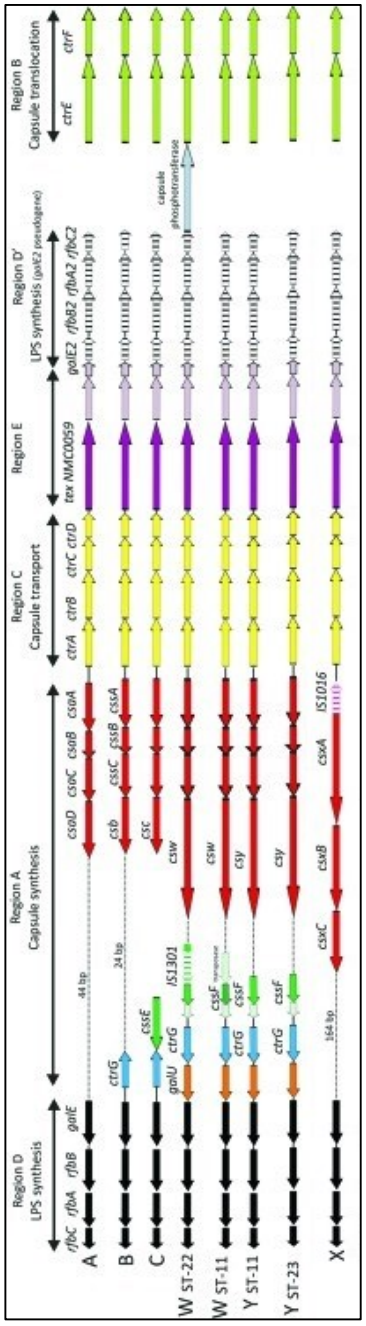

Figure 7: Genetic organization of the *cps* locus among *N. meningitidis* serogroups A, B, C, W, Y and X

The figure shows the genetic organization of *cps* locus of *N. meningitidis*. Letters on left represent serogroups while arrows indicate gene orientation. The region A encodes enzymes for biosynthesis of the capsular polysaccharide; regions B and C encode enzymes involved in the translocation and transport of the capsular polysaccharides to the cell surface (Adapted from Harrison et al. 2013).

In according to Harrison and co-authors (2013), the new *cps* locus nomenclature provides that: the capsule biosynthesis genes within region A are termed *cs* (for capsule synthesis) and the sialic acid capsule biosynthesis genes are termed *css* (*cs* for capsule synthesis and *s* for sialic acid capsule) followed by a letter representing the serogroup and by a capital letter defining each gene; the polysialyltransferase genes belonging to serogroups B and C are designated *csb* and *csc*, respectively; in serogroups W and Y there is a sialyltransferase with a glycosyltransferase function distinct from the serogroups B and C genes, so for serogroups W and Y we have *csw* and *csy* genes, respectively; the *O*-acetyltransferases is termed *cssE* for serogroup C and *cssF* for serogroups W and Y; the nomenclature for *ctrG*, involved in surface translocation of sialic capsules, has been maintained; finally, genes within region C that encode the capsule transport genes are called *ctrA–D* and genes within region B, involved in capsule translocation, are designated *ctrE* and *ctrF*.

The region A of *cps* locus in serogroup A is 4,365 bp long and contains 4 genes, *csaA–D*: *csaA*, encodes the UDP-N-acetyl-d-glucosamine 2-epimerase; *csaB*, is the polymerase linking ManNAc-phosphate monomers together with *csaC*, encoding an O-acetyltransferase, which transfers acetyl groups to ManNAc; *csaD*, is involved in either capsule transport or in cross-linking of the capsule to the meningococcal cell surface (Swartley et al. 1998; Gudlavalleti et al. 2004). Serogroups B, C, W and Y contain sialic acid in their capsular polysaccharides. The region A is 5,313 bp long in serogroup B, 6,690 bp long in serogroup C, and about 7,581 bp in serogroups W and Y (Harrison et al. 2013). All four serogroups contain the conserved *cssA–C* genes for cytidine-5′-monophosphate-N-acetylneuraminic acid synthesis followed by *csb*, *csc*, *csw*, and *csy* genes that are the capsule polymerases and determine the functional and nucleotide specificity for these serogroups. Serogroups C, W, and Y contain O-acetyltransferase genes designated *cssE* and *cssF*. Serogroups B, C, W and Y contain also the *ctrG* gene which synthesizes an essential protein to allow the correct expression of sialic acid polysaccharides (Hobb et al. 2010). Finally, serogroups W and Y contain an additional gene in region A, *galU*. This gene encodes for an UTP-glucose-1-phosphate uridylyltransferase and its sequence has also been found in *Streptococcus pneumoniae* isolates, confirming a role in the virulence of different bacterial species (Bonofiglio et al. 2005). In the serogroup X, the region A is 4,467 bp long and is composed by only 3 genes, *csxA–C*, where *csxA* encodes a putative capsule phosphotransferase protein (Harrison et al. 2013).

The biosynthesis of capsular polymers occurs in the cytoplasm at the cytoplasmic membrane, thanks to the four polycistronic genes, *ctrA-D* of region C, that encode proteins involved in the transport of capsular polysaccharide across the inner and outer membranes (Tzeng et al. 2016). In particular, the genes *ctrE* and *ctrF* of region B, are required for surface expression of a properly anchored capsule polymer. The transport of capsule polysaccharide is mediated by an ABC (ATP binding cassette) transport protein characterized by the hydrophobic outer and inner membrane proteins

CtrA and CtrB, respectively; the integral inner membrane–associated protein, CtrC; and the ATP binding protein, CtrD (Harrison et al. 2013).
Considering the different environments that meningococci can encounter in the human host during the infectious process and the different effects due to the presence or absence of the capsular polysaccharide on the host-pathogen interactions, it is not surprising that the meningococci have developed multiple strategies to alter and regulate the expression of the capsule (Tzeng et al. 2016). The main mechanisms, that may not be universal for all meningococci, are represented by: i. transformation and capsule switching; ii. on-off/off-on phase variation of capsule biosynthesis controlled by slipped strand mispairing (SSM); iii. on-off/off-on switch via insertion/excision of IS elements in the biosynthesis genes; iv. transcriptional regulation by control of the intergenic promoter region (Tzeng et al. 2016). Through the transformation and capsule switching mechanism, *N. meningitidis* is able to influence the expression of meningococcal capsule. Due to genetic identity of parts of the capsule loci, events of capsule switching might occur as result of horizontal exchange, by transformation and recombination in serogroup specific capsule biosynthesis genes. As a consequence, the bacterium changes its capsular phenotype (Swartley et al. 1997; Tsang et al. 2005; Simoes et al. 2009). So, the ability to change capsules presumably offers advantages for meningococci due to the escape from the naturally acquired and vaccine-induced anti-capsular immunity (Tzeng et al. 2016). On/off expression of capsule is controlled by a number of genetic events including SSM. For example, SSM of a poly-cytosine tract presents within the *cssD* gene, causes a frame shift mutation with premature termination of its translation and consequently off expression of capsule in serogroup B (Hammerschmidt et al. 1996). Another mechanism of regulation of capsule expression is mediated by the insertion of an *IS1301* element into *cssA* gene that causes a reversible disruption of this gene in *N. meningitidis* serogroup B and C (Hammerschmidt et al. 1996). The transcriptional regulation by control of the intergenic promoter region of capsule biosynthesis genes, was proposed by Morelle and co-authors (2003). It was found that CrgA protein, a LysR family transcriptional regulator, is induced upon meningococcal contact with epithelial cells with consequent down-regulation of capsule synthesis in *N. meningitidis* serogroups A (Morelle et al. 2003). Another transcriptional regulation mechanism is mediated also by two-component systems (TCSs). Indeed, to successfully colonize and invade hosts, many bacterial pathogens use TCSs to detect microenvironment and coordinate gene expression in response to signals emanating from the host (Tzeng et al. 2016). Meningococci encode relatively few (4 pairs) TCSs and capsule expression is affected through one of these, the MisR/MisS system. It was demonstrated that the capsule production is increased in *misR* and *misS* mutants, thus implying a negative regulatory role mediated by the MisRS TCS (Tzeng et al. 2008). The response regulator, MisR, was shown to bind directly to the divergent promoter region (Bartley et al. 2013), but the regulatory sequence has not been defined. Thus, this environmental sensing two-component system plays a role in regulating meningococcal capsule

expression, however, the environmental signal detected by the MisRS system remain to be defined (Tzeng et al. 2016).
In conclusion, the ability to alter or regulate capsule expression provides significant biologic selective advantages to the meningococcus. Loss or down-regulation of capsule expression is important during the early phase of infection, because its absence enhances the cell attachment and entry, the microcolony formation and facilities the carriage state at human mucosal surfaces. On the contrary, the presence of capsule is essential during the latest phases of infection because it may alter recognition by capsule-specific bactericidal antibodies resulting in immune escape (Tzeng et al. 2016). Full knowledge of the molecular mechanisms applied by meningococcus to regulate the expression of the capsule is therefore necessary in order to implement early diagnosis and the production of new vaccines against *N. meningitidis*.

1.5 Role of sialic acids in bacterial infections

As mentioned before, the serogroup C capsular polysaccharide is a homopolymer of N-acetylneuraminic acid with (α2→9)- linkage and partially O-acetylated. The biosynthetic pathway of polysialic acid capsule has been well characterized in *N. meningitidis* serogroup B (Frosch et al. 1989). In *N. meningitidis* serogroup C, the biosynthetic pathway of polysialic acid capsule is identical to serogroup B except for the last enzymatic step catalyzed by a different polysialytransferase responsible for the specific linkage of serogroup (Claus et al. 1997). The pathway is composed by different enzymatic reactions. It starts with the conversion of UDP-N-acetyl-D-glucosamine into N-acetyl-D-mannosamine and UDP (uridine 5′-diphosphate) catalyzed by UDP-N-acetylglucosamine 2-epimerase (encoded by *cssA* gene). N-acetyl-D-mannosamine is subsequently used for the synthesis of N-Acetylneuraminc Acid (Neu5Ac) by its condensation with phosphoenolpyruvate. Before polymerization of the Neu5Ac molecules occurs, the monomer is converted to an activated state, the cytidine-5'-monophosphate- N-Acetylneuraminc Acid (CMP-Neu5Ac), the substrate for the α2→9-polysialyltransferase, encoded by *csc* gene Serogoup C (Claus et al. 1997; Murkin et al. 2004).
Sialic acids are a family of nine-carbon carboxylated sugars, which include more than 50 different members classified based on various substituents on carbons 4, 5, 7, 8, and 9. The substituent on carbon 5 defines the four most common types of sialic acids: neuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), and 2-keto-3-deoxy-nonulosonic acid (Kdn) (Figure 8). They can be found as terminal sugars of glycoconjugates such as glycoproteins and glycolipids on cell surfaces of vertebrates and higher invertebrates (Chen and Varki 2010; Varki et al. 2011). By modulating contact-dependent mechanisms, sialic acids and their metabolism play key roles in many physiological and pathological processes, including nervous system embryogenesis, regulation of immune system, cancer metastasis, and bacterial and viral infection (Angata and Varki 2002; Petridis et al. 2004; Chen and

Varki 2010; Chen et al. 2011; Li and Chen 2012; Chang and Nizet 2014). In addition, the highest concentration of sialic acid in humans is found in the brain and is widely used throughout the CNS. In particular, it is known that within the brain and in the CNS, the polysialic acid chains are associated with the neural cells adhesion molecules of neurons, glia cells and ganglions (Rutishauser 2008; Wang 2012). Finally, an important reservoir of sialic acids is found in intestinal, lung and vaginal mucin glycans playing a role in mucin mediated bacterial aggregation and hydroxyl radical scavenging (Ho et al. 1995; Slomiany et al. 1996; Ogasawara et al. 2007).

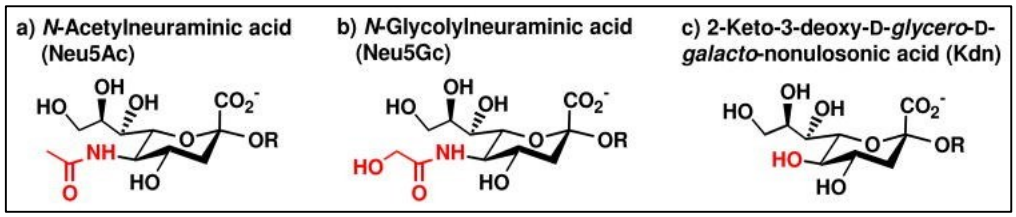

Figure 8: Naturally existing sialic acids and their chemical composition
The figure shows the chemical composition of the common types of sialic acids: N-Acetylneuraminic acid, Neu5Ac; N-Glycolylneuraminic acid, Neu5Gc; and 2-Keto-3-deoxy-D-glycero-D-galacto-nonulosonic acid, Kdn (Adapted from Chen and Varki 2010).

The presence of Neu5Ac has been reported as terminal and internal residues on the surface of different pathogenic bacteria, other than *N. meningitidis* like, *Haemophilus influenzae*, *Streptococcus agalactiae* (GBS) and *Campylobacter jejuni* (McGuire and Binkley 1964; Mandrell and Apicella 1993; Vimr and Lichtensteiger 2002). The incorporation of sialic acid in the surface structures of the bacterial cell (whether they are capsules containing sialic acid or sialylated LPS) allows to hypothesize that bacterial pathogens can mask themselves as host cells and therefore circumvent and or counteract the host's immune responses through a strategy of "molecular mimicry" (Harvey et al. 2001). Bacteria have developed two main pathways to obtain sialic acid: *de novo* biosynthesis, used by a *N. meningitidis* and *C. jejuni*, or acquisition from the environment (Vimr and Lichtensteiger 2002; Vimr et al. 2004). Regarding the ability of bacteria to obtain sialic acids from the environment, many pathogens secrete a sialidase that releases sialic acid from a diverse source of host sialo-glycoconjugates (Corfield 1992); however, other sialic acid-utilizing bacteria, for example *H. influenzae*, lack genes for a sialidase but rely on sialic acid derived from the host (Bouchet et al. 2003). Presumably free sialic acid is made available to such pathogens by other, sialidase-expressing bacteria living in the same niche (Shakhnovich et al. 2002), or by host sialidases that are activated in the course of inflammation (Sohanpal et al. 2004; Sohanpal et al. 2007). This latter process is part of the normal recycling of sialic acid and it has recently been suggested that host cells could use free sialic acid to help

them cope with oxidative stress (Iijima et al. 2007). There is also evidence that the sialylated bacterial surfaces of *C. jejuni*, *N. meningitidis* and *S. agalactiae* (in particular the LPS for the former two species and capsule for the latter) can interact with the Sia-binding immunoglobulin (Ig)-like lectins (Siglec) family, which are normally expressed on the surface of human immune system cell types (Jones et al. 2003; Avril et al. 2006; Carlin et al. 2007). This led to the hypothesis of direct cellular interactions between these cell types and bacterial cells, which may represent yet another way in which the bacterial cell-surface sialylation could modulate the host's immune response (Jones et al. 2003; Avril et al. 2006; Carlin et al. 2007).

There are different cases of how pathogenic bacteria can use sialic acids in their lifestyles in order to colonize, persist and cause disease in mammalian host (Severi et al. 2007). Among them, GBS is a major cause of neonatal pneumonia, septicemia and meningitis and GBS colonization during pregnancy increases the incidence of preterm rupture of membranes and premature birth (Ferrieri et al. 1977; Galask et al. 1984; Thigpen et al. 2011). GBS expresses a α2-3-linked sialylated capsule which is an important virulence factor that contributes to the evasion of host immune defense mechanisms (Rubens et al. 1987) and promotes survival of GBS *in vivo* (Shigeoka et al. 1983; Wessels et al. 1989). In addition to interfering with host complement functions by blocking C3b deposition and limiting C5a production (Marques et al. 1992; Takahashi et al. 1999), GBS can involve multiple Siglec inhibitors through its sialylated capsule, in order to inhibit the host immune response and provide a survival advantage to the pathogen (Carlin et al. 2007). Furthermore, *C. jejuni* strains with sialylated LOS, a principal cause of human foodborne enteritis, are a likely immunological trigger for some cases of the neurological disease Guillain–Barre', occurring subsequent to *C. jejuni* enteritis (Chang and Nizet 2014). This disease is an acute peripheral neuropathy caused by autoantibodies elicited to recognize *C. jejuni* LOS but that aberrantly target peripheral nerve gangliosides that share identical oligosaccharide structures (Hughes and Cornblath 2005; Yuki 2005). Finally, the Non typeable (non-encapsulated) *H. influenzae* (NTHi), a normally human nasopharyngeal commensal organism, expresses LOS structures which are sialylated and formes structures which mimick human glycosphingolipid antigens (Mandrell et al. 1992). Even if NTHi has lost the ability to synthesize Neu5Ac, it developed a number of mechanisms to acquire, control, and incorporate this important sugar into its membrane structures (Apicella 2012). The incorporation of Neu5Ac into LOS structures protects NTHi against host adaptive and innate immune response as well as serving as a mechanism for sustaining itself within biofilms. Recent evidence suggests that this also may be the source of the evolution of human antibodies to non-human sialic acid structures which can lead to inflammation in the host (Apicella 2012).

1.6 Animal model for the study of meningococcal meningitis

N. meningitidis is specialized exclusively in human colonization and infection. It has a narrow host range and, therefore, has limited *in vivo* pathogenesis studies due to the lack of suitable animal models that reproduce the human meningococcal disease. As a result, it had led to fundamental gaps in the comprehension concerning the pathogenesis of septicemia and meningitis caused by meningococcus. In the last decades, the development of many *in vitro* systems allowed the identification of several meningococcal virulence factors (Pagliarulo et al. 2004; Talà et al. 2014; Colicchio et al. 2015). Although these valuable studies provided important insights to understand the role of these factors for a successful meningococcal infection, these models did not allow assessment of the consequences of bacterial interactions with the humoral and cellular immune system and even less with the whole tissue. *In vivo* animal models of infection are of great relevance as well for the evaluation of protection degree conferred by vaccine formulations. As a human-tropic pathogen, meningococcus possess appropriate determinants necessary for successful infection such as surface structures (i.e., type IV pili and opacity proteins) and iron uptake systems for human receptors and transport proteins (i.e., transferrin and lactoferrin) (Schryvers and Stojiljkovic 1999; Virji et al. 1996; Plant and Jonsson 2003) to properly adhere, survive and invade the human host. Finally, the genetic variation abilities of the pathogen to evade and/or block the human immune response further contribute to the high species tropism (Tinsley and Heckels 1986; de Vries et al. 1996). Therefore, the absence of specific host factors, involved in the interaction, can block steps of the pathogen's life cycle, establishing significant difficulties in the development of small animal models summarizing the meningococcal life cycle.

Over the past decades, several approaches have been developed to improve our understanding of the meningococcal infectious cycle. Infections of two animal model, mouse and rat, either intraperitoneally (i.p.) or intranasally (i.n.), were developed to reproduce meningococcal disease (Salit and Tomalty 1984; Salit and Tomalty 1986; Mackinnon et al. 1992; Mackinnon et al. 1993; Oftung et al. 1999; Yi et al. 2003; Newcombe et al. 2004; Gorringe et al. 2005). The laboratory mouse is probably one of the more versatile animals for inducing experimental meningococcal infection. However, the i.p. way of infection leads to the development of severe sepsis although it does not mimic the natural route of infection, whereas the i.n. route of infection was useful to evaluate meningococcal pathogenesis, even though it may induce lung infection prior to sepsis (Salit and Tomalty 1984; Salit and Tomalty 1986; Mackinnon et al. 1992; Mackinnon et al. 1993; Oftung et al. 1999; Yi et al. 2003; Newcombe et al. 2004; Gorringe et al. 2005). The i.p. mouse model was instrumental to assess the protection from the meningococcal challenge (Oftung et al. 1999; Newcombe et al. 2004; Gorringe et al. 2005). The mouse model of meningococcal colonization based on the i.n. route of infection has been developed with infant mice, as they are more susceptible to meningococci, to

reproduce an invasive infection mimicking the course of the meningococcal disease in humans (Salit and Tomalty 1984; Salit and Tomalty 1986; Mackinnon et al. 1992; Mackinnon et al. 1993; Yi et al. 2003). Moreover, to promote meningococcal replication in the murine host, a growing number of technical strategies were also applied including the administration of the iron to the animals to improve the infection, the use of high bacterial *inoculum*, mouse-passaged bacterial strain as well as the employment of infant or immunocompromised animal hosts (Holbein et al. 1979; Salit and Tomalty 1984; Saukkonen 1988; Mackinnon et al. 1992; Gorringe et al. 2005). Expression of specific human factors like CD46 (Johansson et al. 2003) or transferrin (Zarantonelli et al. 2007) has increased the susceptibility of mice to this human tropic bacterium; the employment of the human skin xenograft model of infection has also been useful to evaluate the adhesion ability of meningococci to human endothelium (Join-Lambert et al. 2013; Melican et al. 2013). Collectively, the recent development of humanized transgenic mice has improved the understanding of the meningococcal pathogenesis and its host interactions.

Subsequently, it was developed a new murine model of meningococcal meningitis based on the inoculation of bacteria into the *cisterna magna* of adult mice with mouse-passaged bacteria (Colicchio et al. 2009). Clinical parameters and the survival rate of infected mice demonstrated the establishment of meningitis with characteristics comparable to those seen in the human host, as well as, the microbiological and histological analyses of the brain. Bacteria were also recovered from blood, liver and spleen of infected mice, and bacterial loads from peripheral organs correlated with the infectious dose. In particular, this model was employed to evaluate the virulence of a *N. meningitidis* isogenic mutant strain defective in the L-glutamate transporter GltT (Monaco et al. 2006; Colicchio et al. 2009). The intra-cisternal (i.cist.) route of infection ensures complete delivery of the meningococci directly into the *cisterna magna*, which in turn facilitates bacterial replication in the CSF and induces meningitis with features that mimic those present in humans (Colicchio et al. 2009; Ricci et al., 2014). This route of inoculation is physiologically more accessible (Sturges 2009) and less traumatic than the intracranial subarachnoidal route, already used for the development of meningitis due to *Streptococcus spp.* (Chiavolini et al. 2004; Zhang et al. 2018). Although it does not represent the natural way of infection of meningococcus, the injection of bacteria in this area was instrumental for the induction of meningococcal meningitis, as shown by mouse survival, bacterial loads, clinical parameters, and by histological analysis (Colicchio et al. 2009; Ricci et al. 2014).

2. AIM OF THE STUDY

N. meningitidis is a successful pathogen: it can exploit multiple environments within the human host, acquiring nutrients at each site for its persistence, propagation, and dissemination. In particular, *N. meningitidis* has acquired to colonize humans a range of virulence factors to enable survival within its chosen niche. However, much of our knowledge about cellular and molecular biology of this human pathogen and its virulence determinants, including capsular polysaccharide, lipooligosaccharide, and a number of surface-adhesive and secreted proteins, comes from cell and organ culture systems or animal models that, however, fail to reproduce the complexity of the infectious cycle in the human host (Hill et al. 2010). Indeed, several experimental animal model have been evaluated over the last decades; up to now, however, an animal model able to reproduce the pathological events characteristic of meningococcal disease, is the meningococcal meningitis (MM) mouse model based on i.cist. infection of bacteria (Colicchio et al. 2009). The aim of the present study was to evaluate the role of surface-exposed sialic acids in the establishment of meningitis and meningoencephalitis in BALB/c adult mice when the bacteria are directly injected i.cist. using the murine MM model. This method has been particularly useful for the characterization of meningococcal infection in murine host, as well as for the assessment of the virulence. In order to explore the role of meningococcal surface-exposed sialic acids during infection, we have used the reference serogroup C meningococcal strain 93/4286 and an isogenic *cssA* knockout mutant 93/4286Ω*cssA*, defective in UDP-*N*-acetylglucosamine 2-epimerase that catalyzes the first step of sialic acid biosynthesis, i.e., the conversion of UDP-*N*-acetyl-α-D-glucosamine into *N*-acetyl-D-mannosamine and UDP (Murkin et al. 2004). Before starting *in vivo* experiments, to exclude any differences during bacterial replication induced by this gene knockout, the growth rate of reference strain and *cssA*-defective mutant was evaluated in *in vitro* conditions, and an *in vitro* competition assay was also performed to rule out any alteration in 93/4286Ω*cssA* bacterial fitness. Therefore, to identify any changes in the expression of principal virulence factors, a semiquantitative analysis of the expression of various surface adhesins was performed in both 93/4286Ω*cssA* mutant and the reference strain. In *in vivo* experiments, the 50% lethal dose (LD_{50}) of these strains were determined as well as their abilities to replicate in the mice brain and in the peripheral organs. To investigate the infectious dynamics and histopathological correlates of the disease in the MM mouse model, histological evaluation, cerebral bleeding analysis, and localization of bacteria in brain structures were carried out.

3. MATERIALS AND METHODS

3.1 Bacterial strains and growth conditions

The meningococcal strains used in this study are the serogroup C strain 93/4286 and the sialic acid-deficient isogenic mutant 93/4286Ω*cssA*. The 93/4286 strain belonging to the ET-37 hypervirulent lineage (CC ST-11) was kindly provided by Novartis Vaccine and Diagnostics, Siena, Italy. Meningococci were cultured on GC agar/broth (Oxoid S.p.A., Milan, Italy) supplemented with 1% (vol/vol) Polyvitox (Oxoid, containing vitamin B_{12} 0.1 mg, L-glutamine 100 mg, adenine 10 mg, guanine 0.3 mg, p-aminobenzoic acid 0.13 mg, L-cystine 11 mg, NAD (Coenzyme 1) 2.5 mg, cocarboxylase 1 mg, ferric nitrate 0.2 mg, thiamine 0.03 mg, cysteine hydrochloride 259 mg, glucose 1 g in 10 mL of distilled water) at 37°C with 5% CO_2. When needed, erythromycin (Sigma-Aldrich/Merck KGaA, Darmstadt, Germany) was added to a final concentration of 7 μg ml^{-1}. Meningococci were also cultured in Dulbecco's modified Eagle medium (DMEM) (Microgem, Naples, Italy) with 10% fetal bovine serum, heat inactivated (Microgem), and 2mM L-glutamine (Microgem).

To evaluate the fitness of each strain, at every stage of growth, serial dilutions were plated on GC agar in the presence or absence of erythromycin and incubated at 37°C with 5% CO_2 for 24 h. After growth, viable cell counts were determined by the colony forming units (CFU) method. The growth rate μ (h^{-1}) of the *cssA* mutant and the wild type strain was calculated as described by Hall and coworkers (2014) by formula:

$$\mu = ((\log_{10} N - \log_{10} N_0)\ 2.303)/(t - t_0)$$

and the number of generations (*G*) and time (*t*) per generation were calculated as described by Billington and coworkers (1999). All experiments were performed in triplicate with three independent cultures; the results obtained were analyzed and graphically reported by using GraphPad Prism (version 4) software, and statistical significance was examined by the Student's *t* test.

Pairwise competition experiments were used to estimate the *in vitro* fitness of the *cssA*-defective mutant relative to that of the wild type strain (Colicchio et al. 2015). The 93/4286 wild type strain and the *cssA*-defective mutant were grown, respectively, on GC-agar and GC-agar supplemented with erythromycin (7 μg ml^{-1}), overnight at 37°C with 5% CO_2. Day after, equal numbers of CFU of the wild type strain and *cssA*-defective mutant were resuspended in GC-broth and mixed together (1:1). The bacteria were allowed to grow together competitively in antibiotic-free GC broth at 37°C until the optical density at 600 nm (OD_{600}) of 1.0 is reached. The initial and final number of parental and mutated bacterial cells was determined by standard plate counting on antibiotic-free GC agar followed by pick-and-patch testing, in which a portion of each colony was streaked onto GC agar medium with or without erythromycin (7 μg ml^{-1}). Competition assays were carried out across a small number of generations starting with an initial inoculum of about 10^6

bacteria to reach a final number of about 10^9 bacteria. Indeed, with fastidious bacteria such as pathogenic *Neisseria spp.*, growth from a lower initial inoculum may be difficult (generally due to susceptibility to pH and divalent cation concentrations and a tendency toward autolysis) and/or affected by considerable variability that may mask eventual differences between competing strains (Annear and Wild 1982).
The experiments were performed four times with independent cultures, and the statistical significance of the results was examined by the Student's *t* test. The number of generations (*G*) of the isogenic mutant and parental strain that occurred in the mixed broth was calculated as described by Billington et al. (1999):

$$G = (\log B - \log A)/\log 2$$

where *A* is the number of CFU per milliliter at time zero and *B* is the number of CFU per milliliter at an OD_{600} of 1.0. The difference in fitness ($D_{0\ -\ 1.0\ OD}$) between two competing strains was calculated using the function described by Sander et al. (2002):

$$D_{0\text{-}1.0OD} = \ln[(R_t/S_t : R_{t\text{-}1}/S_{t\text{-}1})^{1/g}]$$

where R_t is the number of the antibiotic resistant cells at 1.0 OD_{600} and R_{t-1} is the number of resistant cells at time 0, respectively. While S_t is the number of the antibiotic susceptible cells at 1.0 OD_{600} and S_{t-1} is the number of susceptible cells at time 0, respectively.
Hence, $D_{0–1.0OD}$ can be interpreted as the natural logarithm of the quotient of the growth rates of the competing strains. $D_{0–1.0OD}$ is equal to 0 if there is no difference in fitness between the competing strains; $D_{0–1.0OD}$ is negative if erythromycin resistance reduces bacterial fitness, and $D_{0–1.0OD}$ is positive if resistance increases bacterial fitness relative to that of the erythromycin-susceptible competitor strain.
The relative fitness of *cssA*-defective mutant respect to wild type strain was calculated using the formula:

$$g_R/g_S$$

The cost per generation (cpg) was calculated by formula (Sander et al. 2002):

$$cpg = 1 - e^{D0\ -\ 1.0\ OD}$$

Inocula for mouse challenge were prepared by cultivating bacteria in GC broth until mid-logarithmic phase. Viable cell counts were determined, and bacteria were frozen at -80°C with 10% glycerol until use. *Escherichia coli* strain DH5α (F-, φ80dlacZΔM15, Δ (lacZYA-argF) U169, deor, recA1, endA1, hsdR17 (rk-, mk +), Phoa, supE44, λ-, thi-1, gyrA96, relA1) (Invitrogen) was used in cloning procedures. This strain was grown in Luria-Bertani (LB) (Oxoid) medium. To allow plasmid selection, LB medium was supplemented with ampicillin (50 μg ml^{-1}) (Sigma-Aldrich/Merck KGaA).

3.2 DNA procedures, plasmids, and transformation of *N. meningitidis*

High-molecular-weight genomic DNA from *N. meningitidis* strains was prepared as previously reported (Bucci et al. 1999). Bacterial strains were

grown in GC broth at 37°C with shaking until the OD_{600} was 1.0. After growth, the cells were harvested by centrifugation at 3500 rpm for 20 minutes and the pellets were lysed with lysozyme 1 mg/ml, SDS 1% and proteinase K 20 µg/ml. The extraction of DNA and purification from proteins and lipids was achieved by the phenol/chloroform treatment. The precipitation of chromosomal DNA was carried out with 3M sodium acetate (pH 6.6) and 2.5 volumes of ethanol 100%. The extracted DNA was then resuspended in 10mM Tris-HCl (pH 7.5), 1mM EDTA (pH 8) and RNAase A 50µg/ml. DNA fragments were isolated by using agarose slab gels and recovered by electroelution as described previously (Sambrook and Russel 2001). Oligonucleotide synthesis and DNA sequencing were performed by Ceinge-Advanced Biotechnologies, Naples, Italy. DNA sequence analysis was carried out by using the GeneJockey Sequence Processor software (Biosoft) and the multiple-sequence alignment tool Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

To construct the pDEΔ*cssA* vector, a genomic fragment of *cssA* (also known as *siaA*, *synA*, *or neuA* before proposal of a unified nomenclature for capsule loci [Harrison et al. 2013]) (644 bp) was amplified from genomic DNA of 93/4286 strain using the primers:

CssAXbaF: (5'-ATTGAACCTCTAGAGGTCATGATTCACGGCGACCG-3')
CssAXbaR: (5'-TGGCGTTCTAGAACATCAATTGAAGGGACACCG-3').

Amplification reaction programs were as follows: 45 s of denaturation at 94°C, 45 s of annealing at 65°C, and 60 s of extension at 72°C for a total of 30 cycles. Reactions were carried out in a MyCycler thermal cycler (Bio-Rad, Laboratories S.r.l., Segrate, Milan, Italy). The amplicon was cloned into the XbaI site of *Neisseria-E. coli* shuttle plasmid pDEX (Bucci et al. 1999; Pagliarulo et al. 2004). Plasmid pDEΔ*cssA* was then used to genetically inactivate by single crossover the *cssA* gene (GenBank accession number NMC0054 or NMC_RS00310) of strain 93/4286 coding for the UDP-*N*-acetylglucosamine 2-epimerase. Transformation experiments were performed using 0.1 to 1 µg of plasmid DNA as previously described (Frosch et al. 1990). For transformation assay, $1x10^8$ bacteria were mixed with 0.1 to 1 µg of plasmid DNA in 1ml of protease peptone medium (PPM, containing proteose peptone; sodium chloride; soluble starch; potassium dihydrogen phosphate; dipotassium hydrogen phosphate; vitamin mix 1%; sodium hydrogen carbonate 5mM; magnesium chloride 10 mM), and incubated at 37°C for 4h at 200 rpm. After incubation time, transformants were selected on GC agar medium supplemented with 7 µg ml^{-1} erythromycin. Gene disruption was demonstrated by Southern blot hybridization using a 644-bp-long *cssA*-specific ^{32}P-labeled probe. Southern blot hybridizations were carried out according to standard protocols (Sambrook and Russel 2001). ^{32}P-labeling of DNA fragments was performed by random priming using the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dGTP (3,000 Ci $mmol^{-1}$) (Sambrook and Russel 2001). To detect capsular polysaccharide expression, a rabbit polyclonal antibody-coated latex suspension against groups C and W-135 *N. meningitidis*

strains was used in a latex slide agglutination test (BD Directigen Meningitis Combo Test; BD Italia, Milan, Italy).

3.3 Real-time RT-PCR experiments

Semiquantitative analysis of the *pilE*, *fHbp*, *hrpA*, *nadA*, *opa54*, and *nhbA* transcripts normalized to the level of expression of the *16S* rRNA gene was performed by real-time reverse transcriptase PCR (RT-PCR). Wild type strain 93/4286 and the *cssA*-defective mutant were grown up to the late logarithmic phase (OD_{600}=1.0) in GC broth. After incubation, the cells were harvested by centrifugation at 3,000 rpm for 10 minutes at room temperature. Each sample was suspended in 500 µl of GC broth and treated with 2 volumes of RNA Protect Bacteria reagent (Qiagen). The cells were collected by centrifugation at 8,000 rpm for 10 minutes at 4°C and pellets stored at -80°C. Total bacterial RNAs were then extracted by use of an RNeasy minikit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. DNA contamination was avoided by on-column treatment with an RNase-free DNase set (Qiagen) according to the manufacturer's instructions. This procedure was performed in triplicate for each strain. The concentration and integrity of the RNA samples were assessed by measurement of the A_{260}/A_{280} and A_{260}/A_{230} ratios and verified using a NanoDrop Lite spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Then, for each sample, total RNAs (2 µg) were reverse transcribed into cDNA by using a random hexamer (2.5 µM) with SuperScript RT (5 U/µl; Invitrogen) in the presence of 0.01 M dithiothreitol and 2 U/µl RNase inhibitor at 37°C for 50 minutes. The reactions were stopped by heat inactivation at 70°C for 15 minutes (Colicchio et al. 2015). For semiquantitative analysis, about 64 to 128 ng of each reverse transcription reaction mixture was used to run a real-time PCR on an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with KiCqStart SYBR Green quantitative PCR (qPCR) Ready Mix, Low ROX (Sigma-Aldrich), and with a specific primer pair as reported in Table 1. PCR was conducted according to the manufacturer's guidelines as follows: initial holding and activation at 95°C for 10 min, followed by 40 cycles at 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s. The post-PCR melt curve was performed between temperatures of 60°C and 95°C with 1% temperature increments. Previously, standard curves were analyzed to determine the efficiency of amplification and the $2^{-\Delta\Delta CT}$ method (where C_T is threshold cycle) was used for the analysis of the level (*n*-fold) of change. Samples were run in the real-time PCR in triplicate, and statistical significance was examined by a Mann-Whitney U test. To quantify gene expression, the data were expressed as fold change obtained using the $2^{-\Delta\Delta CT}$ method.

Table 1. Oligonucleotides used in the Real time RT PCR analyses.

ORF (NC_008767.1)*	Primer name	Sequence
NMC0210	pilE L	5'-CATCGTCGGTATCTTGGCAG-3'
	pilE R	5'-GGGAATGTGCCGTTGTCG-3'
NMC0349	fHbp L	5'-ACCGCTCGACCATAAAGACA- 3'
	fHbp R	5'-CCCGTCCACTTCGATTTGAC- 3'
NMC0444	hrpA L	5’-TGCAGGGGAAATTACAGGGT-3’
	hrpA R	5’-TGCGGTAGTATCAAGGGCAA-3’
NMC1969	nadA L	5’-GGGTCGGTTCAATGTAACGG-3’
	nadA R	5’-ATGGTAGGCTGCGGAAGAAC-3’
NMC1551	opa54 L	5’-ACAGTATCAGCAGCTTGGGT-3’
	opa54 R	5’-TTTTCAAGCGTCCCCAGTTG-3’
NMC2109	nhba L	5’-ATTTTCCCGCCACTTCTTCG-3’
	nhba R	5’-TCTGTGGACGGCATTATCGA-3’
Reference gene	16S univ-1	5'-CAGCAGCCGCGGTAATAC-3'
	16S-r	5'-CTACGCATTTCACTGCTACACG-3'

* the primers were designed by the alignment to the reference genome (*N. meningitidis* serogroup C strain FAM18, NCBI reference sequence with GenBank accession number NC_008767.1).

3.4 Mice, MM model, and experimental design

Eight-week-old female inbred BALB/c mice weighing 19 to 20 g were purchased from Charles River Italia (Lecco, Italy). The mice were fed with laboratory food pellets and tap water *ad libitum* and were housed under specific-pathogen-free conditions. All efforts were made to minimize animal suffering and reduce the number of mice in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The study was approved by the Ethical Animal Care and Use Committee (protocol number 2, 14 December 2012) and the Italian Ministry of Health (protocol number 0000094-A-03/01/2013). For brain histology and cerebral bleeding analysis, animal experiments were performed at the Azienda Ospedaliera Universitaria Senese and authorized by the Local Ethics Committee (Comitato Etico Locale, Azienda Ospedaliera Universitaria Senese, 21 May 2012) and the Italian Ministry of Health (document no. 131/2013, 30.05.2013).
Mice were infected by the i.cist. route as previously described (Koedel et al. 2001; Colicchio et al. 2009; Ricci et al. 2014). Bacteria for mouse challenge were grown in GC broth until the early exponential phase at an OD_{600} of 0.7, corresponding to $\approx 7x10^8$ CFU/ml and were frozen at -80°C with 10% glycerol until use. Before the infection, bacteria were thawed, centrifuged for 15 min at 1,500 x *g*, and suspended in GC broth containing iron dextran (5 mg/kg; Sigma-Aldrich/Merck KGaA). Approximately 2 h before infection, animals were injected i.p. with iron dextran (250 mg kg^{-1}). Animals were lightly anesthetized (50 mg/kg ketamine and 3 mg/kg xylazine or Zoletil 20 [30 mg/kg; VirbacSrl] and Xilor [8 mg/kg; Bio 98 Srl]), and bacteria (suspended in a total volume of 10 µl) were inoculated by hand puncturing the *cisterna magna* of mice using a 30-gauge needle (BD Italia, Milan, Italy). Mice were monitored for possible seizures due to inoculation. Clinical signs were monitored according to a previously described coma scale (Liechti et al. 2014): 1 = presence of coma; 2 = animal does not stand upright after being turned on the back; 3 = animal stands upright within 30 s; 4 = animal stands upright within 5 s, minimal ambulatory activities; 5 = normal state. When in animals was recorded pain, analgesia with meloxicam (5 mg/kg i.p. for duration of study) was administered. In particular, mice with a score of 2 were euthanized and recorded as dead for statistical analysis.
For brain histology and cerebral bleeding analysis, brains were removed and dissected into the two hemispheres. One hemisphere was fixed in 4% paraformaldehyde (PFA) in phosphatebuffered saline (PBS) (wt/vol) for histological analysis, while the other one was frozen in dry ice for assessment of intracerebral bleeding. Samples were not collected from animals found dead or humanely sacrificed before 48 h.

3.5 Animal survival and CFU counts

Different bacterial doses ranging from 10^4 to 10^6 CFU per mouse for the 93/4286 wild type strain and from 10^7 to 10^9 for the 93/4286Ω*cssA* isogenic mutant were used to inoculate animals (*n*=6/dose). Control mice were inoculated with GC broth. Every day throughout the whole experiment, animals were monitored for clinical symptoms (i.e., ruffled fur, hunched appearance, hypothermia, weight loss, lethargy, or moribund). Body weight and temperature were measured by using a digital balance (Acculab) and a thermometer (Greisinger Elettronic), respectively. Rodents were humanely killed before reaching the moribund state (Colicchio et al. 2009). Survival was recorded for a week. To determine the number of meningococci in the brain over time, animals were infected with $5x10^5$ CFU/mouse and sacrificed at different time points (4, 24, 48, and 72 h) (*n*=3/time point) after infection. To compare the virulence of the wild type strain versus that of the *cssA*-defective mutant, two groups of mice (*n*=5/group) were infected with $5x10^5$ CFU/mouse and sacrificed 48 h after challenge for organ collection. Brain, spleen, and liver were excised and homogenized in 1 ml of GC medium. Viable cell counts were performed by plating 10-fold dilutions onto GC agar plates with (mutant) or without (wild type) erythromycin.

3.6 Brain histology

Experiments were performed with a total number of 20 mice, of which 8 were infected with the wild type strain, 8 were challenged with the *cssA*-defective mutant, and 4 served as controls. Infection dose was $6x10^5$ CFU/mouse. Brain hemispheres from mice infected for 48 h were prepared for cryopreservation by incubation in 18% (wt/vol) sucrose in PBS at 4°C overnight. Hemispheres were mounted in 22-oxacalcitriol (OCT) compound and cut coronally using a Leica 3050S cryostat (Leica Biosystems, Wetzlar, Germany). Coronal sections (45 µm thick) were sampled at a frequency of every 15th slice. Additional 10-µm sections were obtained for immunofluorescence analysis. Histopathological evaluations were made on sections stained with cresyl violet for Nissl substance.

3.7 Immunofluorescence

Slices were incubated with a primary rabbit polyclonal antibody against whole-cell preparation of serogroup A, B, and C *N. meningitidis* (6122; ViroStat, Portland, ME, USA) at a dilution of 1:1,000. This antibody was reactive against both encapsulated and unencaspulated meningococci (Spinosa et al., 2007; Talà et al., 2014). Sections were washed three times with PBS and incubated with the secondary antibody (goat anti-rabbit Cy3; Jackson, West Grove, PA, USA) for 45 min at room temperature in the dark. Primary and secondary antibodies were diluted in Tris-buffered saline (TBS; Sigma-

Aldrich/Merck KGaA) containing 0.5% bovine serum albumin. After a washing step, slides were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) for 1 min, washed, and mounted with Mowiol (Merck) containing 2.5% diazabicyclooctane (DABCO; Sigma-Aldrich/Merck KGaA). Pictures were obtained using a Zeiss fluorescence microscope (Axio Imager M1; Zeiss, West Germany) equipped with a digital camera (AxioCamHRc). Overview pictures were created by combining photos obtained with a 10X objective and mosaic reconstruction using the AxioVision, version 4.8, software (Zeiss, Oberkochen, Germany).

3.8 Analysis of cerebral bleeding

Cerebral hemorrhages were assessed as described by Koedel and coworkers (2009). Briefly, brain hemispheres were cut in a frontal plane into 30-µm-thick sections, and serial sections were photographed with a digital camera at 0.3-mm intervals. For each animal, five comparable brain sections were analyzed. The bleeding spots were counted, and the relative areas of bleeding were measured by using the UTHSCSA Image Tool (Texas, USA). Cumulative bleeding areas were divided by the whole slice area and computed into a total bleeding area/whole slice area X1,000.

3.9 Statistical analysis

Bacterial counts in different organs and time points were represented as means ± standard deviations (SD) of CFU numbers isolated from single mice. Differences in growth rates in *in vitro* experiments and differences in bacterial loads between mice infected with the wild type strain or the mutant were examined by Student's *t* test. Mouse survival was estimated by Kaplan-Meier survival analysis, and differences were compared using a log rank test ($P<0.05$). Differences in expression levels of surface adhesins determined by reverse transcriptase real-time PCR and differences in cerebral bleeding were evaluated using a Mann-Whitney U test ($P<0.05$).

4. RESULTS

4.1 Construction of a *cssA*-defective isogenic mutant

The isogenic mutant 93/4286Ω*cssA* of the serogroup C reference strain 93/4286 was obtained by insertional inactivation of the *cssA* gene (locus tag NMC0054 or NMC_RS00310), coding for the UDP-N-acetylglucosamine 2-epimerase (EC 3.2.1.183). In the genome of serogroup C strains, *cssA* is the first gene of the region A capsule synthesis locus, which comprises the conserved *css*ABC (formally denominated *sia*ABC) genes for CMP-Nacetylneuraminic acid biosynthesis followed by the serogroup C-specific loci, *csc* (*siaD*$_C$) coding for α2→9 polysialyltransferase and *cssE* (*oat*$_C$) encoding the O-acetyltransferase (Harrison et al. 2013) (Figure 9A).
Southern blot analysis confirmed the insertion of the erythromycin resistance cassette by a single cross over event into NMC_RS00310 gene. By using a *cssA*-specific probe, two NdeI DNA fragments of the expected sizes (3,180 bp and 2,183 bp) were detected in the 93/4286Ω*cssA* strain, whereas only a single 791-bp NdeI fragment was observed in the wild type strain 93/4286 (Figure 9B). In addition, the absence of capsular polysaccharide expression in the mutant was confirmed by a latex slide agglutination test using antibodies against serogroup C meningococci capsular polysaccharide (Figure 10).

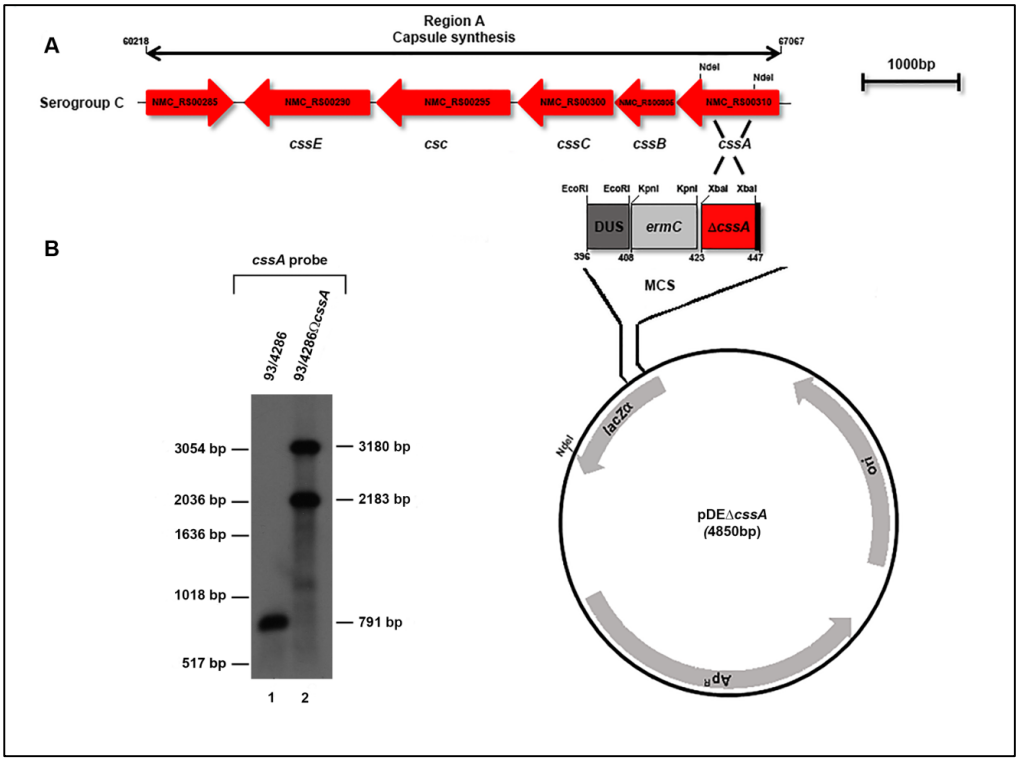

Figure 9: Knockout of the *cssA* gene in the 93/4286 strain

(A) Experimental design for *cssA* disruption by single crossover. The genetic map of the *cps* locus region A (capsule synthesis) of *N. meningitidis* serogroup C was constructed on the basis of the available nucleotide sequences of FAM18 (ET-37) in the NCBI data bank (accession number NC_008767) with arrows depicting gene orientations. The genetic determinants of plasmid pDEΔ*cssA* are indicated: i. a DNA fragment containing a DNA uptake sequence (DUS), required for efficient DNA uptake during transformation; ii. *ermC*, the erythromycin resistance gene used as a selective marker for transformation; iii. Δ*cssA*, a 644-bp XbaI DNA fragment spanning the central part of *cssA* gene; and iv. a plasmid polylinker region (indicated by a black box). The physical map of the pDEΔ*cssA* plasmid is also indicated.

(B) Southern blot analysis demonstrating inactivation of *cssA*. Chromosomal DNA was extracted from the parental strain 93/4286 (lane 1) and from an isogenic mutant, 93/4286Ω*cssA* (lane 2), obtained by transformation with pDEΔ*cssA* and selection with erythromycin. Chromosomal DNA was analyzed by Southern blotting using a *cssA*-specific probe. Bars on the right indicate *cssA*-specific fragments whose sizes were deduced on the basis of the relative migration pattern of DNA ladders (1kb DNA Ladder, Invitrogen) (bars on the left).

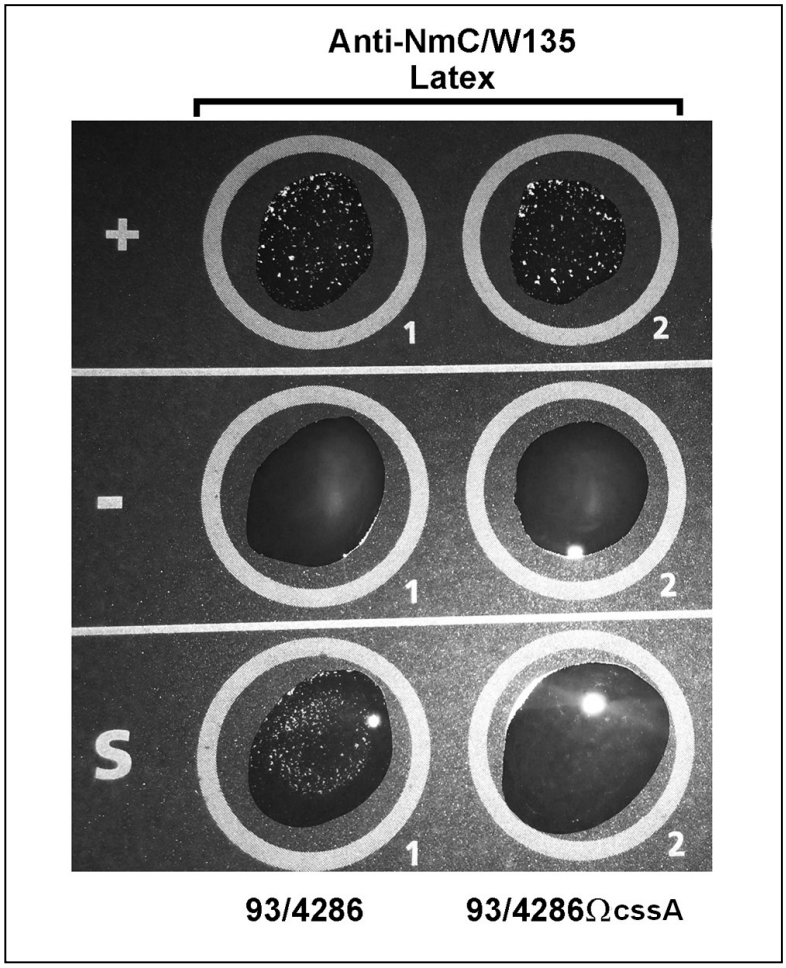

Figure 10: Latex agglutination test of 93/4286 wild type and 93/4286Ω*cssA* isogenic mutant

To detect capsular polysaccharide expression of 93/4286 wild type strain and 93/4286Ω*cssA* isogenic mutant, Anti-*N. meningitidis* serogroups C and W135 Rabbit Polyclonal Antibody-Coated Latex Suspension was used by latex slide agglutination test. The following samples were assayed: (+) Control positive (spots 1-2); (-) Control negative (spots 1-2); (S) 93/4286 strain (spot 1), 93/4286Ω*cssA* isogenic mutant (spot 2). The assay confirmed the absence of capsular polysaccharide expression in *cssA*-defective mutant.

4.2 Evaluation of 93/4286Ω*cssA* mutant fitness under *in vitro* conditions

In order to exclude any differences during bacterial replication, the μ of wild type strain 93/4286 and its derivative *cssA* mutant were preliminarily analyzed in GC broth at 37°C. Bacteria were grown up to late logarithmic phase (1.0 OD_{600nm}) at same temperature, and then bacterial viability was determined by CFU method. The *cssA* mutant exhibited growth curves comparable to those of the wild type strain, with a μ (μ=0.97±0.08) comparable to that of the reference strain (μ=0.85±0.06) without any statistically significant difference (Figure 11A and Table 2).

Determination of growth curve and growth rate are valid measures of fitness deficit associated with mutations (Colicchio et al. 2015). A fitness deficit can be also determined by using resistant and susceptible strains in competition assay, where the strains of interest are mixed together in equal proportions and left to compete head-to-head in a common environment without the selective pressure of the antibiotic (Pope et al. 2010). Thanks to *in vitro* competition assay, we are able to evaluate: the number of generations of the isogenic mutant and parental strain, the difference in fitness between two competing strains and the cost per generation. In GC medium, the *in vitro* competition index of the *cssA*-defective strain was determined (Table 3). The μ of the mutant during the logarithmic phase of growth compared to that of the wild type strain provided a relative fitness value of the mutant of 108% compared to that of the wild type strain (10.77±0.85 generations for the mutant and 9.93±0.68 generations for the wild type) (Table 3). In a competition experiment using equal numbers of CFU, the difference in fitness ($D_{0–1.0\ OD}$) was determined as 0.057±0.01, and the cost per generation was -0.058±0.01 for the mutant versus the wild type (Table 3).

Moreover, the *cssA* mutant had a growth curve similar to those of the wild type strain even in DMEM cellular culture medium (Figure 11B). In DMEM medium, the μ of the *cssA*-defective mutant (μ=0.25±0.004) was comparable to that of wild type strain (μ=0.30±0.04) (Table 2). Therefore, these data confirmed that the knockout of *cssA* locus did not seem to affect the 93/4286Ω*cssA* mutant bacterial fitness.

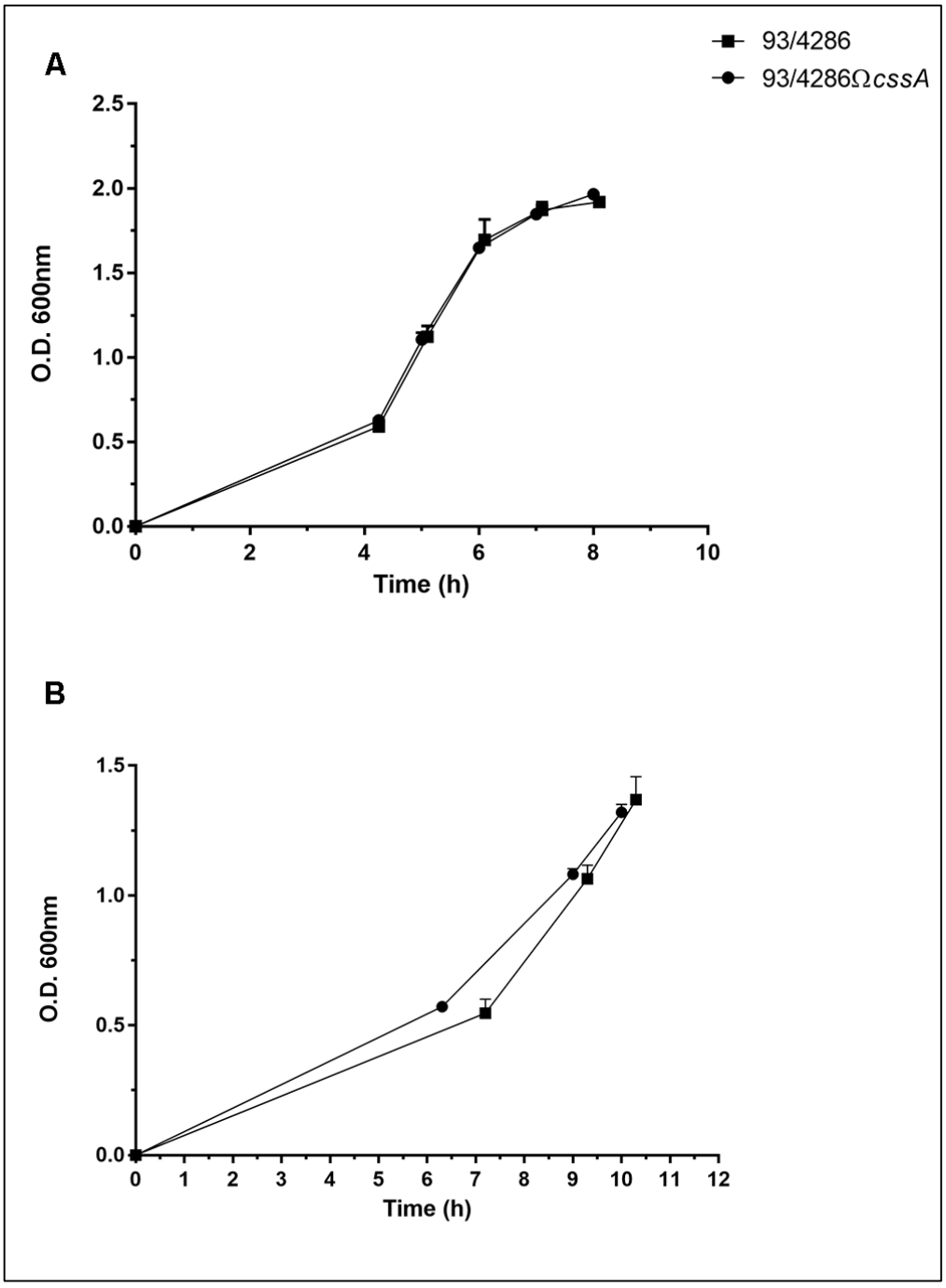

Figure 11: Growth curves of 93/4286 strain and 93/4286Ω*cssA* defective mutant in GC broth and in DMEM cellular culture medium

Growth curves of wild type strain and *cssA* isogenic mutant in GC broth **(A)** and in DMEM cellular culture medium **(B)**. The experiments were performed in triplicate with three independent cultures, and statistical significance was examined by the Student *t* test. Results are indicated as means ±SDs.

Table 2. Growth rates of 93/4286 strain and 93/4286Ω*cssA* defective mutant in GC broth and DMEM cellular culture medium

Medium	Strains	μ (h^{-1})[a]	G (0.5-1.0 OD_{600nm})[b]	*t* per generation (h)[c]
GC	93/4286	0.85 ± 0.06	0.92 ± 0.06	0.81 ± 0.06
	93/4286Ω*cssA*	0.97 ± 0.08	0.81 ± 0.06	0.71 ± 0.06
DMEM	93/4286	0.30 ± 0.04	0.96 ± 0.13	2.27 ± 0.31
	93/4286Ω*cssA*	0.25 ±0.004	0.91 ± 0.01	2.7 ± 0.05

[a] The growth rate μ (h^{-1}) was calculated as described by Hall and coworkers (2014).
[b] Number of generation (*G*) during lag phase (0.5-1.0 OD_{600nm}) as described by Billington and coworkers (1999).
[c] Time (*t*) expressed in hour (h) required to complete a single generation as described by Billington and coworkers (1999).
The values are presented as the means ± SD_s from three independent experiments.

Table 3. Fitness cost of *cssA* inactivation

Mutant strain	No. of generations[a]		Cost per generation[b]	$D_{0\text{-}1.0OD}$ [b,c]	Relative fitness (g_R/g_S)[b]
	g_S	g_R			
93/4286Ω*cssA*	9.93 ± 0.68	10.77 ± 0.85	-0.058 ± 0.01	0.057 ±0.01	1.08 ± 0.02

[a] The number of generations of the mutant strain (g_R) and of the wild type strain (g_S) was calculated as described by Billington and coworkers (1999). The values are the means ± SD_s from four independent experiments.
[b] The values are presented as the means ± SD_s from four independent experiments.
[c] The difference in fitness ($D_{0\text{-}1.0OD}$) was estimated by direct competition against an equal number of CFU of the mutant – wild type strain.

4.3 Evaluation of virulence-associated surface adhesins gene expression in 93/4286Ω*cssA* compared to reference strain

To assess whether the absence of the meningococcal capsule could inhibit the expression of the main surface adhesins of the isogenic mutant 93/4286Ω*cssA*, a Real Time RT-PCR assay was performed. Consequently, the mutant gene expression was compared to those of the reference strain.
In particular, it focused on the possible gene expression alteration of the main meningococcal virulence factors involved in the different states of the infectious process. Indeed, the *pilE* and *opa* gene expression, coding for the major outer membrane protein involved in the first intimate adhesion to host epithelial cells during the meningococcal infection, was evaluated; as well as the expression of *nadA* and *nhbA* genes encoding for proteins able to bind extracellular matrix proteins facilitating the attachment to host cells, was analyzed (Vacca et al. 2016). The gene expression of *fHbp,* coding for factor H binding protein, useful for the meningococcus to downregulate the host alternative complement pathway in order to evade host innate immunity (McNeil et al. 2013), was also assessed. Finally, the evaluation of *hrpA* expression, coding for a protein involved in adhesion and intracellular survival and vacuole escape (Schmitt et al. 2007; Talà et al. 2008; Colicchio et al. 2015), was investigated. Bacteria were grown up to the late logarithmic phase (OD_{600}=1.0) in GC broth. The *cssA* mutant exhibited a slight upregulation in the expression of pilin (*pilE*; 1.64±0.63-fold change) and nonfimbrial adhesins like opacity protein (*opa*; 1.66±0.65-fold change), *nhbA* (2.08±0.38-fold change), *nadA* (2.07±0.36-fold change), adhesin/invasin (*hrpA*; 1.98±0.38-fold change), and *fHbp* (2.08±0.23-fold change) (Figure 12). The difference in the expression levels of all analyzed genes was not, however, statistically significant. The *in vitro* characterization of the mutant strain confirmed that the *cssA* gene deletion involved in the biosynthesis of the meningococcal capsule did not introduce unexpected genetic changes that could affect the ability of 93/4286Ω*cssA* strain to persist and survive in a host.

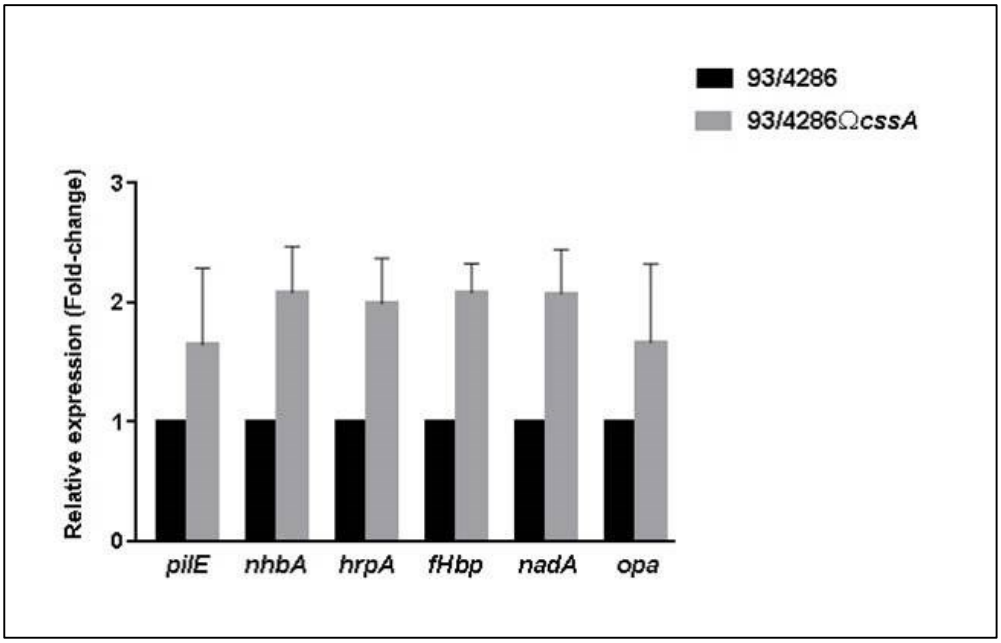

Figure 12: Characterization of the *cssA* mutant for the expression of virulence-associated surface adhesins

Expression of *pilE*, *nhbA*, *hrpA*, *fHbp*, *nadA* and *opa* genes in 93/4286 wild type and *cssA* isogenic mutant strains. The gene expression of selected ORFs were analyzed by Real time RT-PCR using appropriate specific primers. Data are expressed as relative fold change expression of mutant strain compared to the wild type strain and statistical significance was examined by Mann-Whitney U test. The expression of the constitutive *16S* gene was used to normalize. The values are presented as the means ± SD_s from three independent experiments.

4.4 Survival of mice infected with the 93/4286Ω*cssA* mutant and 93/4286 wild type strain

The virulence of the *cssA*-defective strain was assessed in the MM model by analyzing animal survival at different bacterial doses. Inbred BALB/c mice were infected directly into *cisterna magna* and before the infection animals were treated i.p with exogenous iron source, in form of iron dextran, in order to favor the bacterial multiplication in the host (Holbein et al. 1979; Salit and Tomalty 1986; Schryvers and Gonzalez 1989). The i.cist. injection was peformed at the craniocervical junction, specifically in the dorsal subarachnoid space, placing the animals in lateral recumbency and and flexing the neck moderately (90° to 100°).

In order to determine the LD_{50} of animals, three groups of mice were infected by i.cist. injection of 10^4, 10^5, and 10^6 CFU of the wild type strain 93/4286 or 10^7, 10^8, and 10^9 CFU of the mutant strain 93/4286Ω*cssA*. Preliminary data showed higher survival rates in animals infected with the *cssA*-defective mutant than in those infected with the wild type strain at the same dose. In accordance with previous results (Colicchio et al. 2009), mouse death, weight loss, and temperature drop generally occurred within the first 72 h after meningococcal inoculation.

Results with the wild type strain 93/4286 indicated that 50% and 16.6% of rodents survived meningococcal challenge with 10^4 and 10^5 CFU, respectively, while all mice died at the dose of 10^6 CFU (Figure 13A). A significant difference was observed between the three groups (log rank test, $P<0.05$). In contrast, at the lowest dose of 10^7 CFU, there was 83.3% survival in the group infected with the mutant strain 93/4286Ω*cssA*, while 50% survival was recorded in mice inoculated with 10^8 CFU (Figure 13B), indicating a 10,000-fold-increased LD_{50} of the *cssA*-defective mutant.

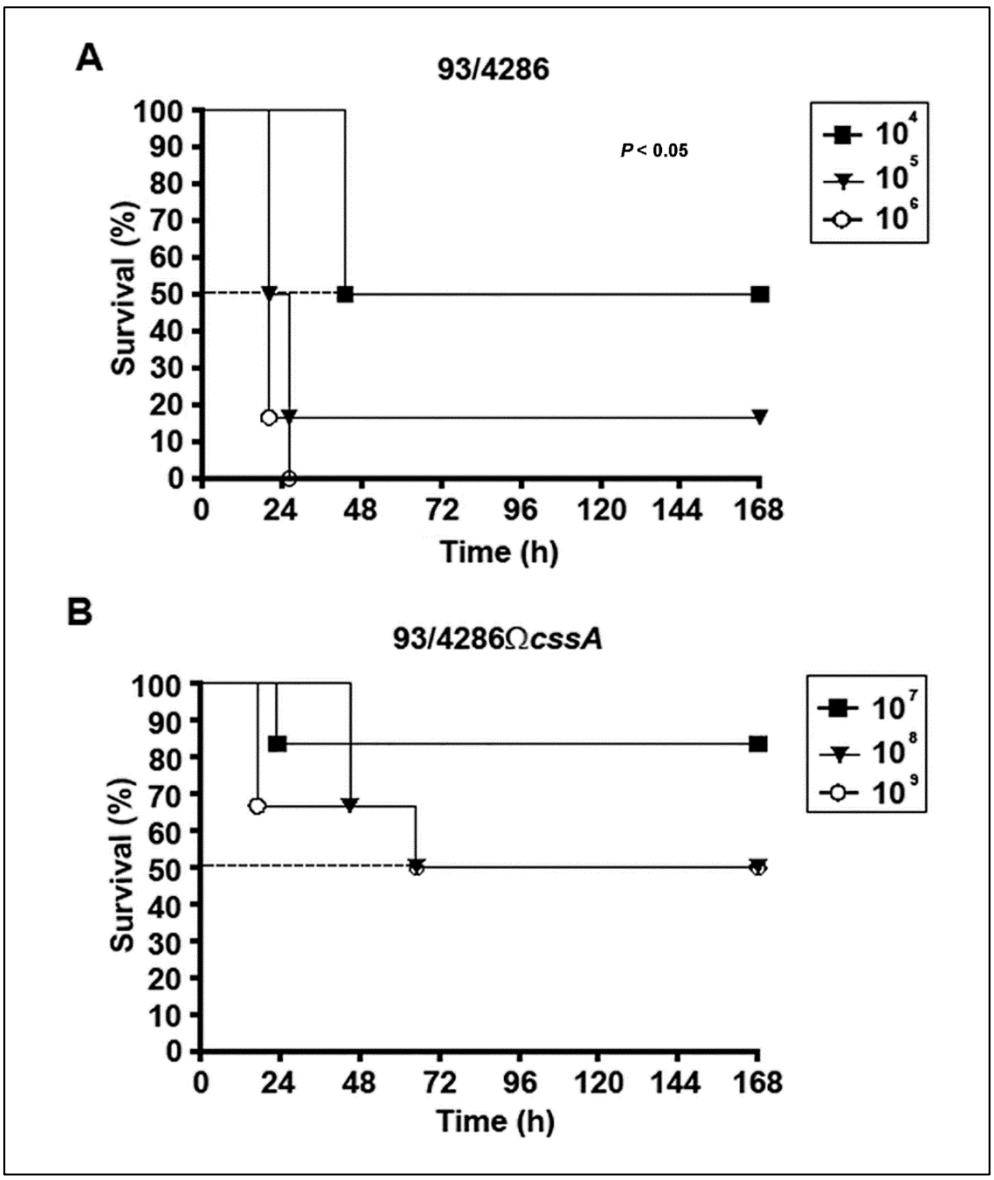

Figure 13: Survival of mice infected with a wild type or *cssA*-defective *N. meningitidis* strain

Three groups of BALB/c mice (n=6/dose) were infected i.cist. with 10^4, 10^5, and 10^6 CFU per mouse of the wild type strain 93/4286 **(A)** and with 10^7, 10^8, and 10^9 CFU per mouse of the *cssA*-defective mutant **(B)**. Mice were monitored for a week at least twice a day, and survival was recorded. Results are expressed as percent survival at different doses over time; the log rank P value was <0.05 for mice infected with the wild type strain.

4.5 The growth and the replication of 93/4286Ω*cssA* mutant is severely impaired in the mouse brain

To follow the kinetics of infection in the brain tissue of infected animals, a time course assay was performed with wild type or *cssA*-mutant strain. In order to limit a rapid fatal outcome and to permit the development of brain damage, a sub-lethal dose of live meningococci, determined by preliminary experiments for each strain, was used (Colicchio et al. 2009; Ricci et al. 2014). Therefore, animals were injected i.cist. as mentioned before with $5x10^5$ CFU of the 93/4286 or 93/4286Ω*cssA* strain and sacrificed at different time points after challenge (Figure 14). Brains were explanted, mechanically homogenized and the viable bacterial cell counts were evaluated through the CFU methods.

A rapid increase in CFU counts was observed for wild type bacteria that reached the highest numbers 24 h after inoculation (8.519±0.072 log CFU). In contrast, bacterial loads in the brain of mice challenged with the *cssA*-defective mutant progressively dropped over time, reaching 2.026±1.774 log CFU at 72 h post infection (Figure 14). Bacterial clearance from the infection site occurred in 33.3% of subjects challenged with the mutant, whereas infection was never eradicated from the brain of mice inoculated with the wild type strain.

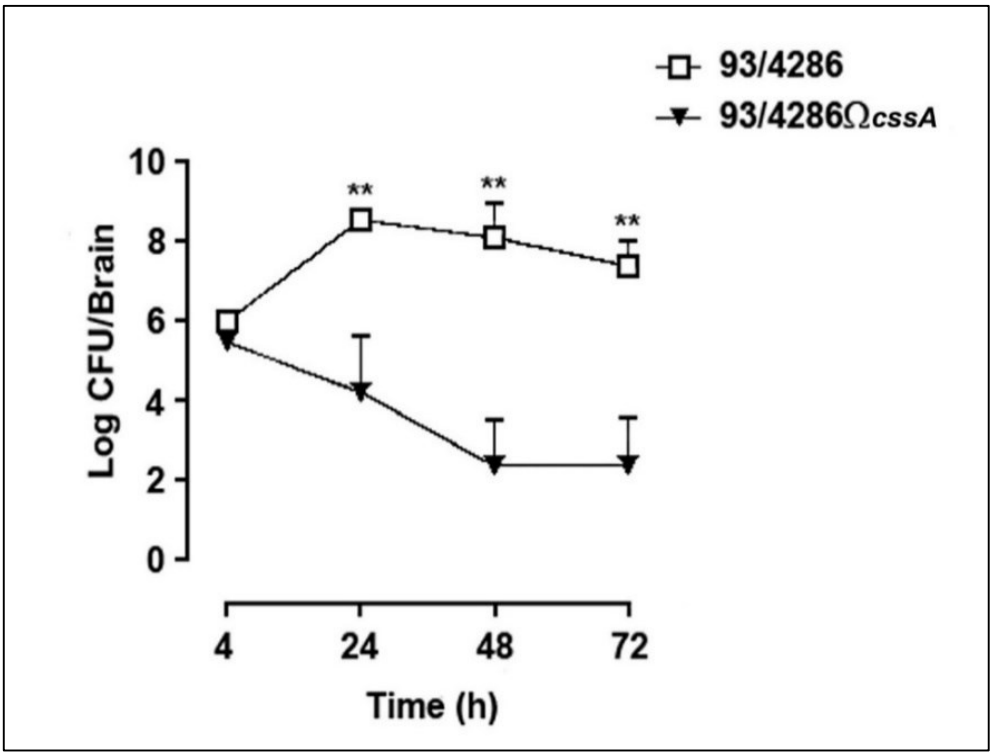

Figure 14: Bacterial loads over time in mice inoculated with the 93/4286 or 93/4286Ω*cssA* strain

Time course of bacterial loads in the brain following i.cist. infection. Two groups of BALB/c mice (*n*=20/group) were infected by the i.cist. route with $5x10^5$ CFU of either the wild type strain 93/4286 or the *cssA*-defective mutant. Animals were sacrificed at 4, 24, 48, and 72 h after infection (3 mice/time point). Brains were collected and homogenized in GC medium, and viable counts were determined. Results are expressed as means ± SD log CFU numbers per organ at different time points after inoculation. Asterisks indicate statistical significance (**, $P<0.01$).

4.6 The *cssA*-defective mutant is cleared systemically from mice with meningococcal meningitis

To evaluate clearance of bacteria from infected mice, two groups of animals were inoculated in the same manner previously described with $5x10^5$ CFU of either the 93/4286 or 93/4286Ω*cssA* strain, and bacterial viable counts in the collected and homogenized peripheral organs, spleen and liver, were determined (Figure 15).
Systemic meningococcal infection caused by the *cssA*-defective mutant was entirely cleared within 48 h from i.cist. challenge, whereas none of the animals inoculated with the wild type had eliminated bacteria from spleen and liver. In particular, it is note that during the hypoferremic phase of neisserial infection, most of hemederived iron remains combined with liver ferritin. As ferritin can be used by meningococci to obtain iron, the liver represents a target organ for bacterial replication (Larson et al. 2004). Two days after inoculation, mean CFU counts of the wild type strain in the spleen and liver were still 3.212±3.354 log CFU and 6.949±1.37 log CFU, respectively. Differences in bacterial loads in the liver between the two animal groups were statistically significant (P<0.001).

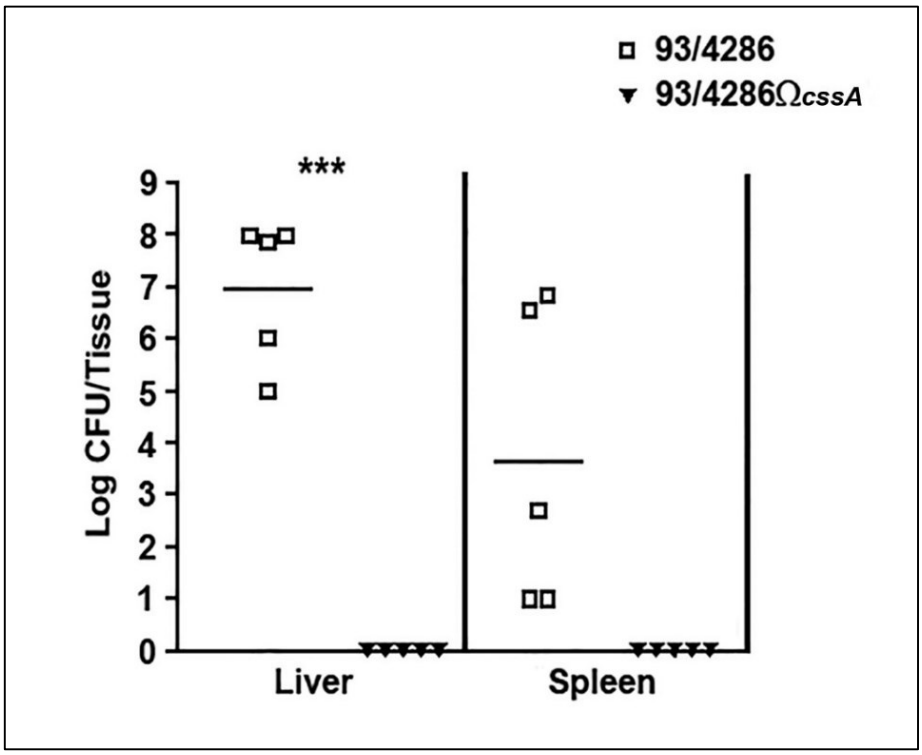

Figure 15: Bacterial loads over time in in the spleen and liver of mice inoculated with the 93/4286 or 93/4286Ω*cssA* strain
Two groups of BALB/c mice (*n*=5/group) were infected i.cist. with $5x10^5$ CFU of either the wild type strain 93/4286 or the *cssA*-defective mutant. Animals were sacrificed 48 h after infection and viable bacterial counts were determined from the target organs. Results are expressed as log CFU numbers per organ. Horizontal bars indicate mean values of bacterial titers. Each symbol represents a single animal. Asterisks indicate statistical significance (***, $P<0.001$).

4.7 Serogroup C wild type meningococci induced severe meningitis in mice

To compare the disease features induced by the wild type 93/4286 and *cssA*-defective mutant, histological analysis and bacterial immunostaining were performed on brain tissues from infected mice 48 h after infection.
BALB/c mice were challenged by the i.cist. route with the infection dose of $6x10^5$ CFU/mouse either the wild type 93/4286 or the mutant 93/4286Ω*cssA* strain. At 48 h, brains were harvested and treated for histological analysis. MM was considerably more severe in animals infected with the wild type (Figure 16A) than in those challenged with the *cssA*-defective strain (Figure 16B). Histological analysis showed the typical features of bacterial meningitis, including the presence of inflammatory cells in the subarachnoid (Figure 16C, black arrowheads) and perivascular and ventricular spaces (Figure 16D, white arrowheads). Vasculitis (Figure 16C, white arrowheads), the injury of blood vessels due to inflammation, and hemorrhages (Figure 16A, black arrows) were observed mainly in animals infected with the wild type strain. Interestingly, inflammatory infiltrates were detected in particular in the *corpus callosum* (Figure 16E), a thick band of nerve fibers that connects the left and right cerebral hemispheres. Indeed, 80% of mice infected with the wild type presented with severe inflammation in the *corpus callosum* (Figure 16E, white arrowheads). In contrast, except for one mouse, no massive evident inflammatory infiltrates, but only few immune cells, could be observed in the *corpus callosum* of animals infected with the mutant strain (Figure 16F, white arrowheads).
The presence and localization of bacteria were further investigated by immunofluorescence. Brain sections (10 μm) were treated with a rabbit meningococcal antiserum and then a goat anti-rabbit Cy3 serum. Slides were counterstained with DAPI and observed using a Zeiss fluorescence microscope. In animals infected with the wild type 93/4286 strain, immunoreactivity with a meningococcal antiserum was mostly detected in the *corpus callosum* (Figure 17A and B), in association with neutrophils in the ventricles (Figure 17C). In contrast, immunostaining of meningococci revealed no signal in the *corpus callosum* of animals infected with the *cssA*-defective mutant (Figure 17D and E). While, a weak immunoreaction was detected in association with cells only in the ventricles (Figure 17F).

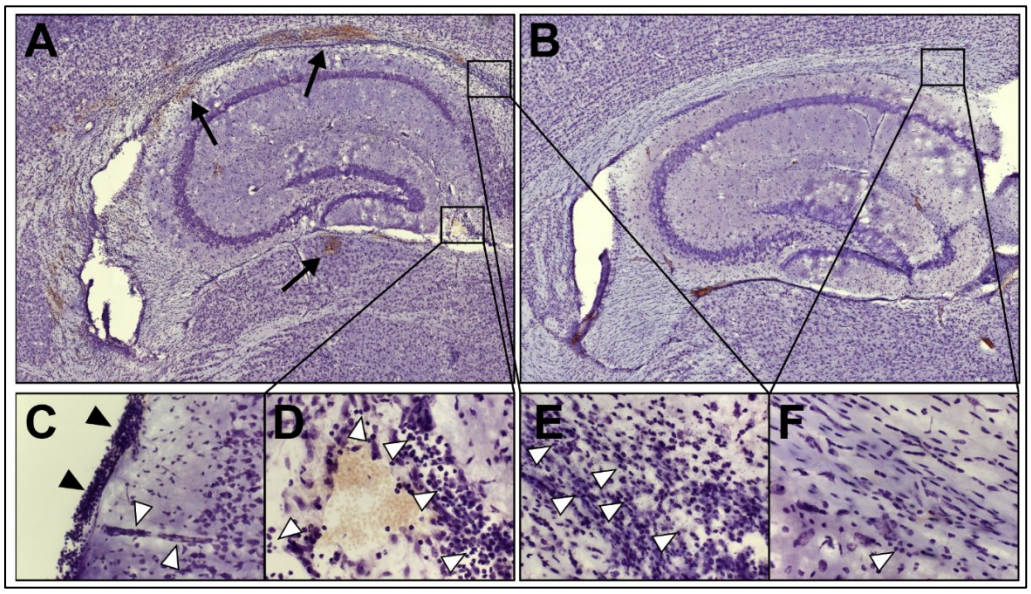

Figure 16: Cresyl violet stained sections of brains from animals infected with the wild type or *cssA*-defective *N. meningitidis* strain

Coronal sections (45 µm) were stained with cresyl violet. Images provide an overview (mosaic reconstruction from individual pictures at a magnification of X10) of the hippocampal region of a representative animal infected with 93/4286 (**A**) or 93/4286Ω*cssA* (**B**). (**C**) Overview (20X objective) of the meninges (black arrowheads) and an inflamed penetrating vessel (white arrowheads) in an animal infected with 93/4286. (**D**) Close-up view (40X objective) of the ventricular space of the animal infected with 93/4286 showing inflammatory cells (white arrowheads) and possible intraventricular hemorrhage (black star). Close-up views (40X objective) are shown of the *corpus callosum* of animals infected with 93/4286 (**E**) or 93/4286Ω*cssA* (**F**) with the presence of infiltrated inflammatory cells (white arrowheads).

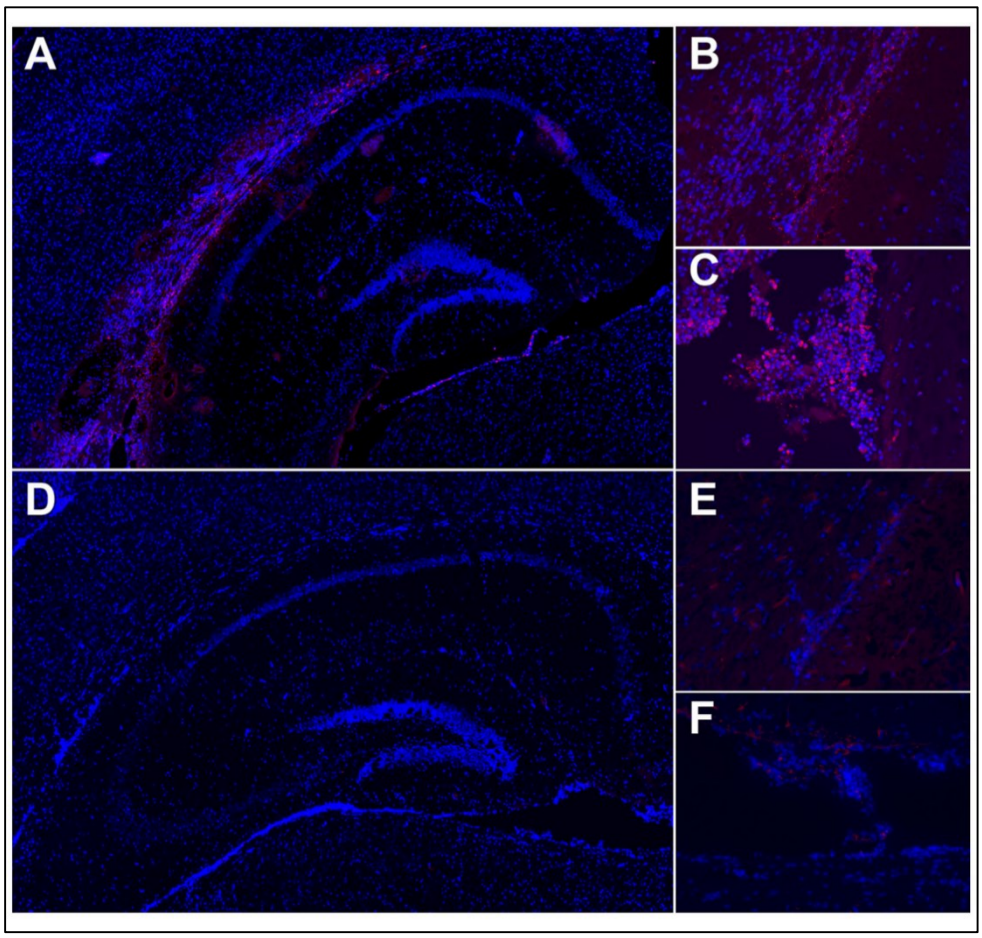

Figure 17: Immunofluorescence analysis of brain sections of mice infected with the wild type 93/4286 or mutant 93/4286Ω*cssA* strain

BALB/c mice were challenged by the i.cist. route with either the wild type 93/4286 or the mutant 93/4286Ω*cssA* strain. At 48 h, brains were harvested and treated for immunofluorescence analysis. Brain sections (10 µm) were treated with a rabbit meningococcal antiserum and then a goat anti-rabbit Cy3 serum. Slides were counterstained with DAPI and observed using a Zeiss fluorescence microscope. An overview of the hippocampal region of two representative animals infected with either the wild type 93/4286 (**A** to **C**) or the mutant 93/4286Ω*cssA* (**D** to **F**) strains is shown. In the insets (20X magnification), *corpus callosum* (**B** and **E**) and ventricles (**C** and **F**) from the brain of mice challenged with 93/4286 or 93/4286Ω*cssA* are shown. Large quantities of bacteria are detected in samples from mice infected with the wild type strain. Red, *N. meningitidis* bacteria immunostained with meningococcal antiserum; blue, DAPI.

4.8 Evaluation of intracerebral hemorrhages in mice infected with the *cssA*-defective mutant and wild type strain

Literature data showed that, cerebral bleeding and intracerebral hemorrhages were identified as a consistent readout in the brain of mice with MM (Ricci et al. 2014). To perform a quantitative analysis of brain bleeding, the number and area of cerebral bleedings were determined in mice infected by the wild type or the *cssA* defective strain. To this aim, BALB/c mice were infected with the infection dose of $6x10^5$ CFU/mouse with either the wild type strain 93/4286 (*n*=8) or the mutant strain 93/4286Ω*cssA* (*n*=8) and sacrificed at 48 h.

In accordance with histological data, results showed a significant reduction in macroscopical assessment of cerebral hemorrhages (Figure 18A), in the number of bleeding spots (Figure 18B; *P*=0.01), and in the hemorrhagic area (Figure 18C; *P*=0.048) in mice challenged with *cssA*-defective bacteria.

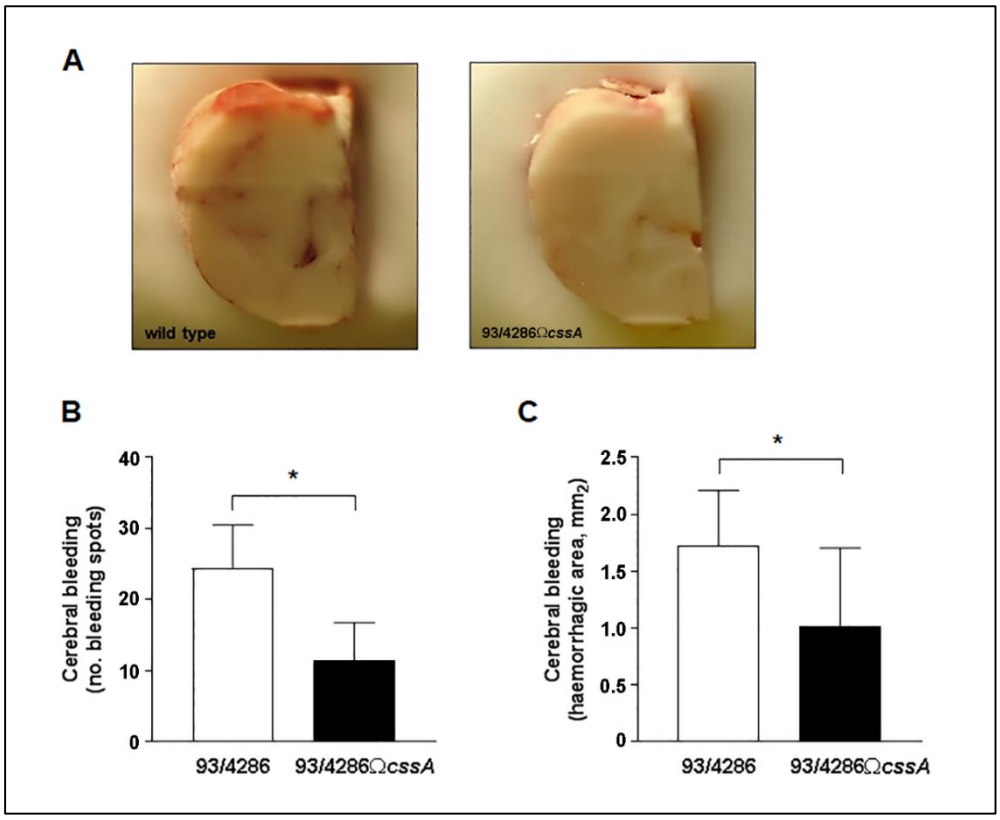

Figure 18: Cerebral bleeding in mice infected with the 93/4286 or 93/4286Ω*cssA* strain

Brains were collected and immediately frozen in dry ice. Hemispheres were cut in 30-µm cryosections and photographed to determine the number of hemorrhagic spots and the areas of bleeding. **(A)** Macroscopical assessment of cerebral hemorrhages in animals challenged with the wild type or the mutant strain. (**B** and **C**) Enumeration of bleeding spots and measurement of hemorrhagic areas were carried out on five comparable brain sections/mouse. Data are represented as means ± SD. Differences were assessed by a Mann-Whitney U test (*, $P<0.05$).

5. DISCUSSION

The crucial importance of sialic acids in the host-pathogen interplay is well exemplified in some pathogens, like *N. meningitidis*, that are able to regulate the expression of polysialic acid capsule with the 'phase variation' genetic mechanism (van der Woude and Baumler 2004). The variable expression makes it probable that any population of bacteria will express alternative glycoforms and that any expression pattern can confer a fitness advantage to the relevant bacteria in any given condition inside the host (Severi et al. 2007). Most of the attention has been focused on the role of meningococcal surface-exposed sialic acids in mediating resistance to both phagocytosis and complement-mediated killing via alternative pathway activation (Estabrook et al. 1992; Hammerschmidt et al. 1994; Jarvis 1995; Estabrook et al. 1997; Ram et al. 1999; Lewis et al. 2012; John et al. 2016), resulting in enhanced meningococcal survival in the intracellular environment (Spinosa et al. 2007; Jones et al. 2009; Talà et al. 2014), in the bloodstream, and in the CNS (Stephens 2009).

The first aim of the present study, was to validate the MM mouse model by using a reference serogroup C strain and its attenuated isogenic *cssA* knockout mutant unable to produce sialic acids. In addition, comparison of the virulence of the two strains was instrumental to further explore the pathogenesis of MM and subsequent cerebral damage by analyzing possible interactions between meningococcal surface-exposed sialic acids and brain structures.

In this study, the inbred BALB/c mouse strain was used instead of the outbred CD-1 strain that was previously employed to establish the MM murine model (Colicchio et al. 2009). Even if outbred mice have a greater genetic variability useful to identify universal effects in a more diversified cohort with results more applicable to the human population (Festing 1976; Festing 1999), this variability requires larger sample sizes to reach a sufficient statistical power and may hinder standardization procedures and targeted studies. In particular, in this study the LD_{50} of strain 93/4286 in inbred BALB/c mice was 10^4 CFU, while the LD_{50} in outbred CD-1 animals was approximately 10^7 CFU of mouse-passaged bacteria (Colicchio et al. 2009). In this regard, it is noteworthy that BALB/c mice carry the susceptibility mutation in the solute carrier family 11a member 1-encoding gene (*Slc11a1*), which truncates the encoded protein (also known as natural resistance-associated macrophage protein 1, Nramp1) and increases susceptibility to infection with many pathogens, like: *Mycobacteria spp.* and *Salmonella spp.* (Vidal et al. 1993; Vidal et al. 1995; Medina and North 1996; Medina and North 1999). It is note that the highest rate of meningococcal disease is found between young children, adolescents, and young adults (Goldschneider et al. 1969 (I); Goldschneider et al. 1969 (II); WHO and Centers for Disease Control and Prevention (U.S.) 2011); for this reason, in this MM mouse model, instead of focusing on neonatal or infant animals, 8 week-old immunocompetent adult animals were employed. To date, our results validated that the i.cist. model is functional to induce both meningitis and IMD. In particular, the i.cist. model allows to overcome the

limits of i.p. or i.n. models of infection, which are characterized by the occurrence of sepsis and bacteremia before meningitis is established (Salit and Tomalty 1984; Salit and Tomalty 1986; Mackinnon et al. 1992; Mackinnon et al. 1993; Oftung et al. 1999; Yi et al. 2003; Newcombe et al. 2004; Gorringe et al. 2005). Therefore, the i.cist. infection murine model, could be useful not only to evaluate innovative therapeutic strategy to prevent bacterial replication directly into CSF but also to analyze the efficacy of possible passive immune therapy against human pathogens. However, meningococcal infection is a multistep process, that includes nasopharynx colonization, access to bloodstream, crossing of the BBB and finally uncontrolled proliferation in the CSF. Even if the present experimental MM mouse model only reproduces some aspects of the meningococcal infection, in order to subvert in part these limitations transgenic animal model (Johansson et al. 2003; Zarantonelli et al. 2007; Join-Lambert et al. 2013; Melican et al. 2013) may be useful to mimic the human pathogenesis of meningococcal disease.

The key role of surface-exposed sialic acids in meningococcal pathogenesis was confirmed in the present experimental MM mouse model using the reference serogroup C meningococcal strain 93/4286 exposing the sialic acids on surface and an isogenic 93/4286Ω*cssA* knockout mutant.

The results obtained from the characterization of 93/4286Ω*cssA* mutant under *in vitro* conditions showed that the knockout of *cssA* locus did not seem to affect the mutant bacterial fitness, as indicated by the μ value and the relative fitness in different media comparable to those of wild type strain. In particular, the slight upregulation in the expression of virulence-associated surface adhesins found in 93/4286Ω*cssA* mutant strain, confirmed that the mutation did not generate unexpected genetic changes that could alterate the infectious ability of this bacteria in a murine model.

Therefore, the LD_{50} of the wild type strain 93/4286 was about four orders of magnitude lower than that of the 93/4286Ω*cssA* mutant (Figure 13). Compared to the wild type strain, the ability of the mutant to replicate in the brain (Figure 14) and spread systemically (Figure 15) was severely impaired. Histological analysis and bacterial immunostaining on brain tissues confirmed higher disease severity with more pronounced inflammation, vasculitis, and hemorrhages in mice infected with the wild type strain than in those challenged with the *cssA*-defective mutant (Figures 16 and 17). The histopathological finding is reminiscent of cerebral infarction that in humans represents a complication in about 25% of patients suffering from bacterial meningitis and in 9% of MM cases (Schut et al. 2012). Interestingly, 80% of mice infected with the 93/4286 wild type strain presented with severe inflammation in the *corpus callosum* (Figure 16), and most of the immuno-positive signal was localized in this brain structure by immunofluorescence with a meningococcal antiserum (Figure 17). In particular, the *corpus callosum* is the largest fibre of axons that connects the two cortical hemispheres of the mammalian brain, essential for many aspects of neural function, including integration of lateralised sensory input and regulation of higher-order cognitive, social, and emotional processing (Aboitiz and Montiel 2003; Fenlon and Richards 2015).

As expected, meningococci were also detected on the meninges, in the ventricles, and in the choroid plexus. The main function of the choroid plexus is the production of the CSF; indeed; in the choroid plexus the endothelial cells are fenestrated, a feature which is required for the production of CSF from the blood (Wolburg and Paulus 2010; Schwerk et al. 2015). In contrast, to enable shielding of the CSF from the blood, the choroid plexus epithelial cells are interconnected by dense tight junctions strands that generated a "molecular barrier" located at the epithelial cells of the choroid plexus (Schwerk et al. 2015). Massive presence of bacteria in the vessels as well as in the epithelium of the choroid plexus and ventricular system is a very common finding in histopathological examination of patients with MM (Pron et al. 1997; Guarner et al. 2004). Indeed, the choroid plexus is considered an important gateway for meningococcal traversal from the bloodstream into the CNS during meningitis in humans (Schwerk et al. 2015). It is very likely that the bacteria utilize this highly vascularized site to spread systemically from the CNS in the i.cist. mouse model of MM by using a reverse route. In contrast, the remarkable localization of meningococci in the *corpus callosum* is unexpected, suggesting a certain tropism of *N. meningitidis* for this brain structure. From a theoretical point of view, in order to accumulate within the *corpus callosum*, in the i.cist. mouse model of MM the bacteria have to leave the CSF space (since the CSF is generated within the plexus choroideus and flows toward the subarachnoid space), survive in the bloodstream, and reenter the brain. Our data seem to suggest the *corpus callosum* as a major site of bacterial reentry in the i.cist. mouse model. A reasonable hypothesis could be due to a high concentration of adhesion molecules relevant to meningococcal-host cell interactions at the level of the cerebral vessels or other structures in the *corpus callosum*.
Indeed, there is evidence in a murine model that heparan sulfate receptors (heparin sulfate proteoglycan [HSPGs]), which are targeted by meningococcal Opa, Opc, and NhhA proteins (Hill et al. 2010) and mediate the interaction with both epithelial and endothelial cells, are highly expressed in the *corpus callosum* (Kaur et al. 2009). Moreover, it is also note that both Opa and the Opc protein can bind directly or indirectly to components of the extracellular matrix (ECM) like vitronectin or fibronectin expressed on brain-vessel cells (Schubert-Unkmeir 2017). Interestingly, even if this mechanism should be further explored, various pathogens utilise ECM proteins to enhance adhesion and invasion at the brain and vascular endothelial interfaces or even as a target for degradation in order to breach cellular barriers (Schwarz-Linek et al. 2004; Hill et al. 2010; Singh et al. 2012). Further evidence to support our hypothesis could be that the carcinoembryonic CEACAM-1, which serves as a receptor for several meningococcal Opa adhesins/invasins (Hill et al. 2010), is highly expressed by oligodendrocytes, which are abundant in the *corpus callosum* (Neyazi et al. 2017). Furthermore, the CEACAM-1 pathway activates matrix metalloproteinases (MMPs) that are involved in BBB breakdown (Ludewig et al. 2013). Indeed, a recent study based on i.cist. MM mouse model demonstrated for the first time that MMP-9 is involved in the pathogenesis of MM-induced brain damage and MMP-9 inhibition significantly improves

intracranial complications (Ricci et al. 2014). In particular, MMPs are Zn^{2+}-dependent peptidases that act a major role in inflammation and tissue turnover MMP levels were found increased in the CSF of both adults and children with bacterial meningitis (Paul et al. 1998; Leppert et al. 2000; Lindberg et al. 2006). Noteworthy, oligodendrocytes specifically express the myelin-associated glycoprotein (MAG), which is a member of the Siglec family of proteins capable of binding sialic acid (Huang et al. 2012). Thus, it is possible, although speculative, that these molecular interactions could recruit the 93/4286 wild type meningococci and guide their reentry through the *corpus callosum*. In contrast, the absence of the *cssA*-defective mutant in this brain structure might be due to both/either its inability to survive in the bloodstream, as demonstrated by its complete clearance at 48 h postinfection in the peripheral organs (Figure 15), and/or to the absence of surface-exposed sialic acid.

Although *corpus callosum* involvement as a complication of MM or IMD is reported to be a rare occurrence, a case of involvement of the *corpus callosum* with cerebral ischemia and consequent callosal disconnection syndrome has been recently documented by magnetic resonance imaging and diffusion tensor tractography (Marchi et al. 2016). More recently, a case of a reversible splenial lesion of the *corpus callosum* associated with MM has also been reported (Hayashi et al. 2017). Whether the involvement of this brain structure in meningococcal meningitis/meningoencephalitis, as revealed by advanced imaging technologies, may have actually been underestimated in the past is not clear yet. In fact, the histological evidence of the localization of meningococci in the *corpus callosum* of patients who died of meningococcal disease does not yet exist. This limits our findings to the analyzed meningococcal serogroup and strain and to the i.cist. mouse model of MM used in this study.

As regards the role of sialic acids in meningococcal pathogenesis, the results of this study are consistent with the data reported in the past by Vogel and coworkers (1996) with a bacteremia model in infant rats infected with serogroup B *N. meningitidis* strain B1940 and a set of isogenic mutants defective in either capsule synthesis or LOS sialylation. Infection of infant rats with the wild type strain caused severe bacteremia, while an isogenic mutant strain defective in capsule synthesis (but expressing a sialylated LOS) caused bacteremia only when a 10^6-CFU-higher bacterial dose was used. In addition, when infant rats were infected with encapsulated meningococci that were unable to sialylate the LOS, bacteremia could never be induced, even with an infective dose as high as 10^8 CFU, suggesting that both forms of sialic acid on the bacterial cell surface are indispensable for systemic meningococcal survival in the infant rat model (Vogel et al. 1996). This study further expands these data to CNS infection dynamics, having in mind, however, all the limitations of an i.cist. mouse model that exploits a non-natural infection route. Histological analysis and bacterial immunostaining indicate surface-exposed sialic acid as a main determinant for meningococcal intracellular growth/survival as reported before (Spinosa et al. 2007; Talà et al. 2014) and also as a possible mediator in the interaction between meningococci and neuronal cells in the pathogenesis of

IMD. The possible molecular interactions between bacterial protein and brain structures previously described may explain the massive localization of wild type meningococci in the *corpus callosum* in our MM model, proposing a new role of microbial surface-exposed sialic acids in the interplay between *N. meningitidis* and the host in the pathogenesis of meningococcal disease that, however, should be further explored.

In particular, this MM mouse model is transversal and it can be applied to strains belonging to different serogroups. Indeed, in order to understand the role of exposed sialic acids in meningococcal pathogenesis, future studies will employ this MM mouse model with the other meningococcal serogroups responsible for diseases worldwide, characterized by a different chemical composition of capsular polysaccharides compared to serogroup C. These further studies, aimed also to identify the adhesion molecules involved in the interactions of the meningococcus to the host cells at the level of the *corpus callosum*, could confirm the tropism for this brain structure for the different meningococcal serogroups. In particular, it will be interesting to investigate the molecular mechanisms that regulate the *N. meningitidis* interaction capabilities with neuronal cells in *in vitro* cellular systems and how these diverge amoung the meningococcal serogroups according to the capsular polysaccharides chemical composition.

It is also note that the meningococcal cell components can trigger a massive inflammatory response inducing the release of proinflammatory molecules and increasing the permeability of the BBB attracting leukocytes to the CNS (Coutinho et al. 2013). In the future, to better understand the inflammatory response induced by meningococci, using the MM animal model described above further studies will be carried out to measure the levels of cytokines and chemokines commonly expressed during bacterial meningitis in the CSF. Since the inflammatory response was associated with the occurrence of sequelae after the disease (Azuma et al. 1997; Brandt et al. 2006), the full understanding of the inflammation profile induced by *N. meningitidis* should contribute to a more detailed clarification of meningococcal pathogenesis.

6. CONCLUSION

N. meningitidis is a strictly human pathogen due to the high specificity of both meningococcal surface structures and iron uptake systems for human receptors and transport proteins (Virji et al. 1996; Schryvers and Stojiljkovic 1999; Plant and Jonsson 2003). The lack of valuable animal models of disease due to the narrow host range, along with the meningococcal high degree of genetic (phase and antigenic) variation of surface structures, has greatly hindered progress in understanding the pathogenesis of meningococcal disease and developing effective vaccines.

In the present work, an MM mouse model based on i.cist. inoculation of bacteria was validated to induce meningococcal meningitis in adult mice. To our knowledge, no other model of meningococcal meningitis has been developed in laboratory mice infected by i.cist. route; in the past, this way has been explored to provide models of meningococcal disease in both rat (Trampuz et al. 2007) and rabbit (Tuomanen et al. 1989). Although this model does not mimic the initial phases of colonization and invasion of meningococcus, survival and clinical parameters of infected mice and microbiological and histological analyses of the brain demonstrated the establishment of meningitis with features comparable to those of the disease in humans. Meningococci grew well not only in the CSF, but they were also able to remain in the spleen and liver compartments. This MM mouse model was also useful to evaluate the key role of surface-exposed sialic acid in meningococcal pathogenesis using the reference serogroup C meningococcal strain 93/4286 and an isogenic 93/4286Ω*cssA* knockout mutant.

Compared to the reference strain, the ability of this mutant to replicate in the brain and to spread systemically has been severely compromised. Histological analysis and bacterial immunostaining on brain sections confirmed a greater aggressiveness of the disease, associated with inflammation, vasculitis and hemorrhages in animals infected with the wild type strain compared to those infected with the *cssA*-mutant strain. Interestingly, for the first time was found that 93/4286 wild type bacteria localized in the *corpus callosum*, indicating a high tropism of the meningococci exposing the sialic acids towards this cerebral structure and its specific involvement in meningococcal meningoencephalitis. In conclusion, this study confirmed that the CNS infection dynamics depend on the meningococcal exposed sialic acid that may probably be a mediator of interaction between *N. meningitidis* and neuronal cells in the IMD, a hypothesis that will be further studied.

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