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Investigating pyroptotic pathways in a mouse model of meningococcal meningitis: insights into the role of HrpA/HrpB Two-Partner Secretion System

Tutor

Prof. Paola Salvatore

Candidate

Dr. Giuseppe Mantova

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LIST OF ABBREVIATIONS

β-2 AR: β-2 adrenergic receptor
ABC: ATP-binding cassette
APC: antigen-presenting cell
ASC: apoptosis-associated speck-like protein
BBB: blood brain barrier
CAPS: cryopyrin-associated periodic syndrome
CEACAM: carcinoembryonic antigen cell adhesion molecule
CdiA: Transporter of Pathogenicity Island A
CdiA-CT: Transporter of Pathogenicity Island A carboxy-terminal region
CSF: cerebrospinal fluid
CNS: central nervous system
DAMP: damage-associated molecular pattern
DYNLT1: Tctextype 1
GASDMD: gasdermin-D
GASDME: gasdermin-E
GC: gonococcal
HBMEC: human brain microvascular endothelial cells
i.cist.: intracisteral injection
i.n.: intranasal injection
i.p.: intraperitoneal injection
IL-1β: interleukin-1β
IL-18: interleukin-18
IM: inner membrane
ISS: Istituto Superiore di Sanità
LAMP: lysosome-associated membrane protein
LBP: LPS-binding protein
LOS: lipooligosaccharide
LPS: lipopolysaccharide
LRR: leucine-rich repeat
MD2: myeloid differentiation protein 2
MDM: macrophage-derived monocytes
MLST: multi-locus sequence typing
NACHT: nucleotide-binding and oligomerization domain
NI: no-infected
NLR: nucleotide-binding domain leucine-rich repeat-containing receptors
NLRP3: NOD-like receptor family pyrin domain-containing 3
OM: outer membrane
OMV: outer membrane vesicles
PBS: phosphate buffered saline
PAMP: pathogen-associated molecular pattern

PPM: protease peptone medium
PRR: pattern recognition receptors
PYD: pyrin domain
PVDF: polyvinylidene difluoride
SD: standard deviation
SDS-PAGE: sodium dodecyl sulphate - polyacrylamide gel electrophoresis
ST: sequence type
T1SS: Type I secretion system
Tat: twin-arginine translocation
US: United States
Tfp: type IV pili
TLR2: Toll-like receptor 2
TLR4: Toll-like receptor 4
TPS: two-partner secretion
TS: tryptic soy
VDAC: Voltage-Dependent Anion Channel

ABSTRACT

Neisseria meningitidis emerges as a prominent causative agent of septicemia and cerebral inflammation worldwide among humans. Although it establishes in the upper respiratory tract of asymptomatic and healthy carriers, it exhibits the remarkable capacity to evade the host's immune defenses and traverse from the bloodstream to the brain, provoking unregulated localized inflammation. The shift between transitory colonization to a devastating illness appears to be the result of a complex interplay of various factors from both the host organism and the bacterial side. Among these factors, the HrpA/HrpB two partner secretion (TPS) system has emerged as a key player in establish meningococcal meningitis. Recent *in vitro* study demonstrates the involvement of this secretion system in the formation of biofilm, the escaping from internalization vacuoles within host cells and the attachment to the dynein motor, ultimately culminating in the initiation of pyroptosis process**.** Pyroptosis, a form of pro-inflammatory cell death, has been posited as a one of principal effectors of inflammation associated with invasive meningococcal infection. Based on *in vitro* evidence about the role of HrpA/HrpB TPS, it was assessed its involvement in establishing meningococcal meningitis and activating pyroptotic pathway in a suitable animal model. It was employed a model of meningococcal meningitis based on intracisternal infection in adult BALB/c mice, using the reference Serogroup C meningococcal strain 93/4286 (ET-37) and an isogenic mutant, 93/4286Ω*hrpB*, deficient in the synthesis of the HrpB transporter. The virulence of the *hrpB*-defective strain was assessed in the mouse model of meningococcal meningitis by analyzing the survival of the animals at different time points. Preliminary data showed high survival rates in animals infected with the *hrpB*-defective mutant compared to those infected with the wild type. Comparative analysis revealed a stark difference in the mutant strain's ability to replicate within the mouse brain compared to the wild type strain. Histological analysis further reinforced the attenuated virulence trait of 93/4286Ω*hrpB* mutant strain, with the presence of inflammatory infiltrates only in the brains of mice infected with the wild type strain. Furthermore, the study shed light in the role of the pyroptotic pathway in the damage induced by *N. meningitidis* in mouse brain. Western blot analysis demonstrated that *N. meningitidis* activate pyroptotic pathway in the mouse brain: the activation of caspase-11, caspase-1 and gasdermin-D (GASDMD) were markedly reduced in the brain of mice infected with *hrpb* mutant strain. These findings offer valuable insights about the role of pyroptosis process in the context of invasive meningococcal disease and suggest a promising avenue for potential adjunctive therapies based on pyroptosis inhibitors.

1. BACKGROUND

1.1 *Neisseria meningitidis:* biology and epidemiology.

Neisseria meningitidis (meningococcus) is a gram-negative diplococcus with commensal properties, belonging to the bacterial family *Neisseriaceae*, residing the upper respiratory tract of humans (Figure 1). This microorganism shares close genetic ties with *Neisseria gonorrhoeae* (gonococci), the causative agent of the sexually transmitted infection gonorrhea. Within the *Neisseria* genus, *N. meningitidis* and *N. gonorrhoeae* stand as the sole pathogenic members, specifically for humans (Lui G et al., 2015). Meningococcus exhibits growth on various culture media, including blood agar, tryptic soy (TS) blood agar, Thayer-Martin agar, and gonococcal (GC) agar, under conditions of 35-37°C temperature and 5-10% (v/v) carbon dioxide (Rouphael NG and Stephen DS 2012).

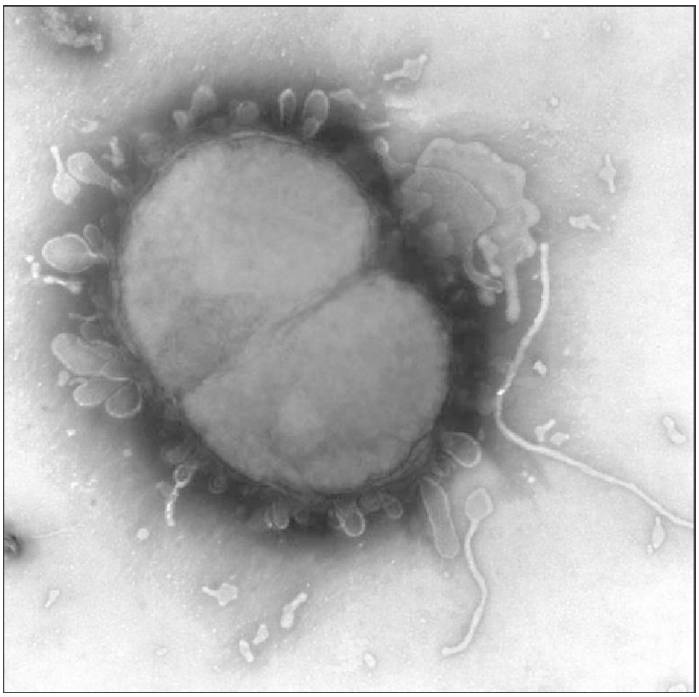

Figure 1. *Neisseria meningitidis*. Electron microscope image of *Neisseria meningitidis* showing the classical diplococcal arrangement (Gorringe A, 2009).

The transmission of *N. meningitidis* occurs via aerosols and can persist asymptomatically in up to 30% of the population for extended periods exceeding 2 years. However, under certain condition, meningococcus can acquire invasive

characteristics, penetrating the epithelial barriers of the nasopharynx, entering the bloodstream and leading to septicemia. From this, it can traverse the blood-brain barrier (BBB), gaining access to the central nervous system (CNS) and locating itself within the subarachnoid space of the meninges, resulting in meningitis (Caugant DA and Maiden MC, 2009).

Meningococcal meningitis and sepsis are debilitating diseases that continue to affect individuals, with incidence rates varying between 0.5 and 1000 cases per 100,000 individuals depending on the epidemiological region. The most vulnerable groups include infants under 1 year of age, teenagers, and young adults and individuals with complement system deficiencies and those with asplenia (Winstanley FP et al, 1985; Rosenstein NE et al., 2001). The onset of the disease is swift, with case fatality rates ranging from 10% to 20%, even with available antibiotic treatment. Among survivors, 10-20% experience long-term sequelae, including hearing loss, limb amputation, skin scarring, and neurodevelopmental deficits. Up to 36% of survivors develop impairments in physical, cognitive, or psychological functioning (Viner RM et. al., 2012; Vyse A et al., 2013).

Meningococci are characterized through serological typing, with serogrouping as conventional methodology (Frasch CE et al., 1985; Slaterus KW, 1961). Further subcategorization into serosubtype, serotype, and immunotype is contingent upon discrepancies in their polysaccharide capsule, lipopolysaccharide (LPS), and outer membrane proteins PorB and PorA. Conspicuously, one of the distinctive attributes that distinguishes *N. meningitidis* from commensal *Neisseria* strains and *N. gonorrhoeae* is the presence of an all-encompassing capsular polysaccharide enveloping the bacterial cell's surface. At least 13 distinct meningococcal serogroups have been defined (Branham SE, 1953) based on the chemical composition of their polysaccharide capsules, of which only six serogroups (A, B, C, W-135, X, and Y) are pathogenic. Serogroups B, C, W, and Y capsules consist of polysialic acid or sialic acid linked to glucose or galactose (Swartley JS et al., 1997), while serogroups A and X capsules are composed of *N*-acetyl mannosamine-1-phosphate and *N*-acetylglucosamine-1-phosphate, respectively (Liu TY et al., 1971). One of the most widely employed approaches for typing *N. meningitidis* is multi-locus sequence typing (MLST) (Maiden MC et al, 1998; Maiden MC and Harrison OB, 2016). This methodology categorizes meningococci into various sequence types (STs) based on polymorphisms found in seven housekeeping genes. Currently, MLST has been replaced by core genome MLST. Core genome MLST has demonstrated that disease isolates are a diverse recombining population from which hypersensitivity linages have independently emerged on several occasions (Bratcher et al. 2014; Hollingshead S and Tang CM 2019).

Meningococcal infection occurs as sporadic, hypersporadic, and epidemic disease. Approximately 1.2 million instances of meningococcal infection arise annually, leading to approximately 135,000 fatalities globally. The prevalence and characteristics of the disease exhibit considerable variability across different

periods, geographical regions, age groups, and bacterial serogroups. (Harrison LH et al., 2009) (Figure 2).

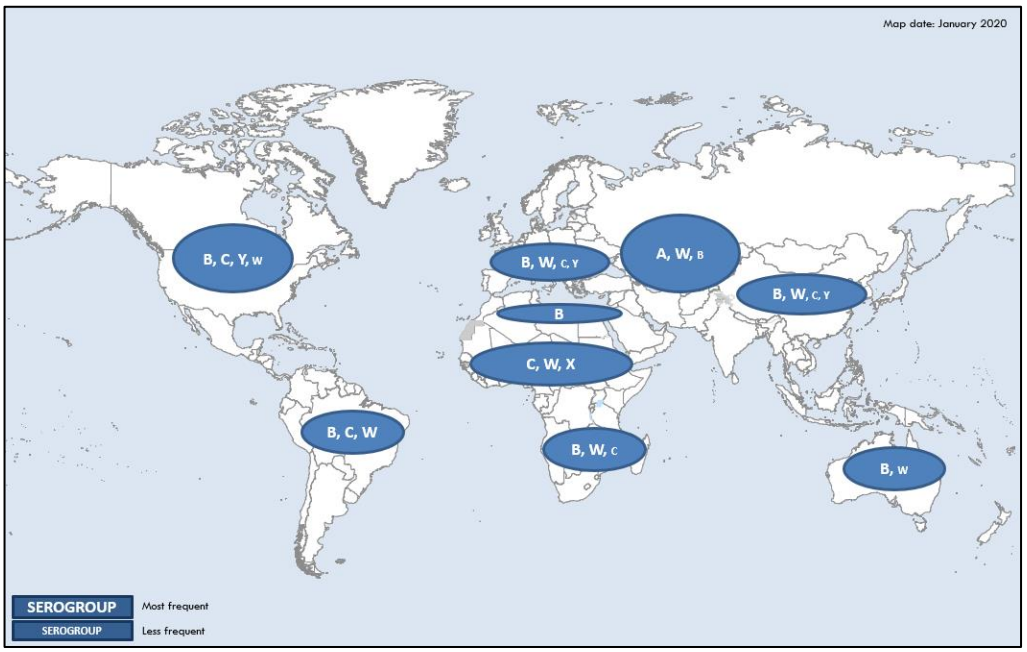

Figure 2. Map of the distribution of *N. meningitidis* serogroups. The data representation displays the worldwide distribution of the primary meningococcal serogroups. Evident in uppercase characters are the dominant serogroups specific to their respective geographic regions. The data accentuates the shifting geographical prevalence of meningococcal serogroup C, which ranks among the most widespread serogroups globally (World Health Organization, 2020).

In particular, serogroup A was responsible for the most extensive meningococcal epidemic within the sub-Saharan African region, commonly referred to as the "meningitis belt" (Hart CA et al., 1997). Serogroup B typically exhibits a lower disease incidence compared to serogroups A and C; however, prolonged outbreaks of serogroup B disease lead to substantial morbidity and mortality. Currently, serogroup B stands as the primary causative agent of endemic disease in developed nations, contributing to 30-40% of cases in the United States (US) and up to 80% in Europe (Wyle FA et al., 1972). Serogroup C is responsible for a portion of endemic cases and localized epidemic outbreaks in developed countries, constituting 30% of disease cases in the US and Europe (Jackson LA et al., 1995). Serogroup Y has emerged in the United States, accounting for over a quarter of meningococcal disease cases in the past decade. Additionally, over the last 20 years, serogroup W-135 has emerged, triggering an epidemic in Saudi Arabia similar to the serogroup A outbreak (Cohn AC et al., 2010).
In 2022, as reported by the Istituto Superiore di Sanità (ISS), 57 cases of invasive meningococcal disease were reported, with an incidence of 0.1 cases per 100,000

inhabitants, indicating an increase compared to 2021, where only 25 cases were recorded (incidence of 0.04 cases per 100,000 inhabitants). Furthermore, in the 2022, the incidence of invasive meningococcal disease was higher in children under 1 year, followed by the age groups of adolescents/young adults aged 15-24 years and 10-14 years. It is noteworthy that the 1–4-year age group, which in 2020 and previous years was the second most affected, showed a progressive decline in incidence rates in 2021 and 2022. This phenomenon can be assessed in consideration of the implementation of the National Vaccination Prevention Plan 2017-2019, valid until 2022, which includes completing the vaccination against meningococcus B at the 13th month of life, against meningococcus C between the 13th and 15th month of life, and against meningococcus serogroups A, C, W, and Y between the 12th and 18th year of age. Among the various capsular serogroups, serogroup B was the most frequently identified in the entire population over the past three years. Considering the cases from the three-year period 2020-2022, serogroup B was predominant in all age groups. In the age group with the highest incidence (<1 year), the proportion of serogroup B compared to other serogroups increased, reaching 100% of cases in 2021 and 2022. In the young adult age group of 15-24 years, the proportion of serogroup B cases remained high in the last three years, accounting for 82% of cases in 2022. In the age group of 10-14 years, serogroup B determined 100% of cases in 2022. (https://www.epicentro.iss.it/meningite/epidemiologia-italia).

1.2 *N. meningitidis*: virulence factors and infectious cycle.

The virulence of *N. meningitidis* is influenced by a multitude of factors that enhance adhesion and colonization of the epithelial mucosa, survival within the bloodstream and damaging host cells through the release of exotoxins.
N. meningitidis natural environment is the human nasopharynx from where bacteria are transmitted person to person by aerosol droplets or direct contact with contaminated fluids. In the nasopharynx, *N. meningitidis* grows on the top of mucus-producing epithelial cells surrounded by a complex microbiota (Coureuil M et al., 2019). The interaction of *N. meningitidis* with human endothelial cells leads to the formation of typical microcolonies that extend overtime to ultimately fill in the microvessels. In susceptible individuals, meningococci can survive in the extra cellular fluids including blood and cerebrospinal fluid (CSF), becoming invasive and causing septicemia and/or meningitis (Stephens DS, 2009) (Figure 3).

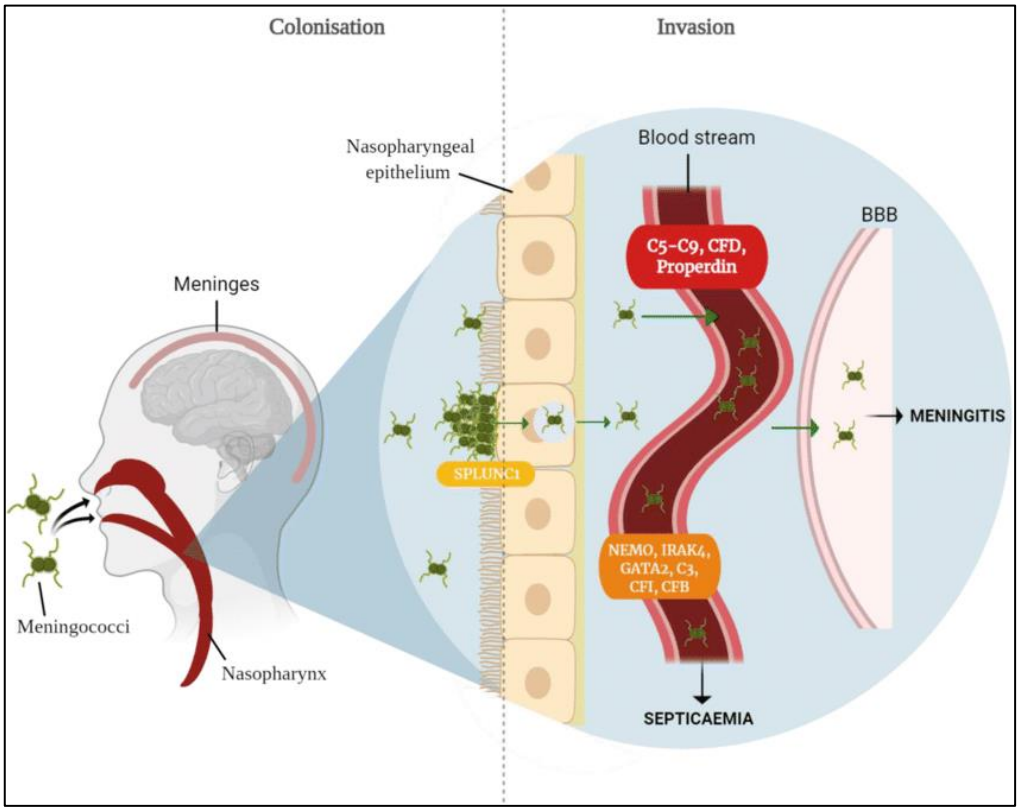

Figure 3. The infection process of *N. meningitidis*. The depiction outlines the infectious cycle of *N. meningitidis*. This microorganism can be acquired through the inhalation of infected respiratory droplets and establishes intimate interactions between its ligands and human receptors expressed on the epithelial cell surface of the upper respiratory tract. In addition to transcytosis, *N. meningitidis* has the capacity to traverse the epithelium, either directly following damage to the monolayer integrity or by exploiting phagocytes in a 'Trojan horse' fashion. Once it gains access to the bloodstream, *N. meningitidis* can survive, proliferate rapidly, and disseminate throughout the body and the brain. Subsequently, it can traverse the BBB, reaching the meninges, and potentially causing meningitis (Adapted from Virji M., 2009**)**.

The initial phase of meningococcal colonization involves adherence to nasopharyngeal epithelial cells, a process mediated by type IV pili (Tfp). These structures play pivotal roles in various stages of the infectious process, including adhesion to endothelial cells, the formation of bacterial aggregates, motility and natural transformation (Goldschneider I et al., 1969). Tfp are helical polymer structures formed primarily by the major subunit PilE, which is generated through the cleavage of prepilin by the prepilin peptidase PilD (Freitang NE et al., 1995). In addition to the major subunit, Tfp are composed of various minor subunits that play a crucial role in adhering to epithelial mucosa. PilQ functions

as a molecular gateway by forming pores in the outer bacterial membrane, allowing the pili to extend beyond the bacterial cell (Pelicic V et al., 2008). The biosynthesis of these pili involves the collaborative efforts of PilF, PilM, PilN, PilO, and PilP, while their maturation is facilitated by PilF, PilH, PilI, PilJ, and PilW (Takahashi H et al., 2012; Carbonnelle E et al., 2009; Cehovin A et al., 2011). 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 (Bernard SC et al., 2014). Recent studies have unveiled the intriguing ability of PilE and PilV subunits to activate the β-2 adrenergic receptor (β-2 AR), consequently promoting the translocation of *N. meningitidis* across the cerebral endothelium (Coureuil M et al., 2010; Lecuyer H et al., 2012). Indeed, Tfp are also crucial for the natural competence of meningococci, as they facilitate the uptake of exogenous DNA that can serve various functions, including act as a template for DNA repair, a nutrient source, and a mechanism for genetic variation (Berry JL et al., 2015; Proft T et al., 2009). This process fosters horizontal gene transfer and enables the generation of an extensive genetic diversity.

Intimate adhesion is mediated by opacity proteins. *N. meningitidis* strains commonly express two types of opacity proteins located on the outer membrane: OpA and OpC (Pizza M and Rappuoli R, 2015). This family of transmembrane molecules forms β-barrel structures in the outer membrane. OpA proteins are characterized by having four transmembrane loops, while the OpC proteins possess only five loops. (Malorny B et al., 1998). OpC is exclusively expressed by meningococci and is encoded by a single gene, whereas OpA proteins are expressed in both meningococci and gonococci and are encoded by multiple genes (Rouphael NG and Stephens DS, 2015). Both types of proteins can recognize and bind carcinoembryonic antigen cell adhesion molecule (CEACAMs). During the inflammatory process, high levels of CEACAMs are expressed, facilitating interactions with opacity proteins and promoting attachment and invasion by *N. meningitidis* (Virji M et al., 2000; Sadarangani M et al., 2011).

Although Pili, OpA, and OpC represent the most important and extensively studied meningococcal adhesins, other surface antigens also play a crucial role in the infectious process. NhhA (*Neisseri*a hia/hsf homologue) is a trimeric autotransporter that binds extracellular matrix proteins, heparan sulfate, and laminin, facilitating attachment to host epithelial cells (Scarselli M et al., 2006; Sjolinder H et al., 2008). The autotransporters App and MspA are both immunogenic and are also involved in host-cell adhesion (Turner DP et al., 2006; Hadi HA et al., 2001). Passenger domain of these proteins can be cleaved by the protease NaIP or autocatalysis. These domains bind to different receptors and can mediate endocytosis and traffic to the nucleus, where they interact with histone H3, leading to host cell death through a caspase-dependent pathway (Serruto D et al., 2003). AutA and AutB are structurally related, and both are secreted on the cell surface. AutA induces auto aggregation, while AutB promotes biofilm formation and impedes the transit of meningococci through

epithelial cells (Arenas J et al., 2015; van Ulsen P et al., 2006). Msf and NadA are trimeric autotransporters that induce bactericidal antibodies against meningococcus. Msf interacts with extracellular matrix components such as heparan sulfate, laminin, and fibronectin, while NadA binds to receptor proteins differentially expressed in various epithelial cell lines (Griffiths NJ et al., 2011; Hill DJ et al., 2015).

Neisseria meningitidis expresses two distinct porins, PorA and PorB, which are transmembrane proteins characterized by a trimeric β-barrel structure. These porins facilitate the passage of small hydrophobic nutrients with cationic or anionic selectivity (Rouphael NG and Stephens DS, 2012). Notably, *Neisseria* porins have been demonstrated to function as adjuvants in the immune response by activating B cells and other antigen-presenting cells (APCs). Specifically, PorB, in addition to inducing calcium ion influx, triggers toll-like receptor 2 (TLR2) activation and induces apoptosis in the host cell (Massari P et al., 2003). While, PorA represents a predominant component of vesicle-based vaccines and serves as the primary target for bactericidal antibodies (Vermont CL et al., 2003) (Figure 4).

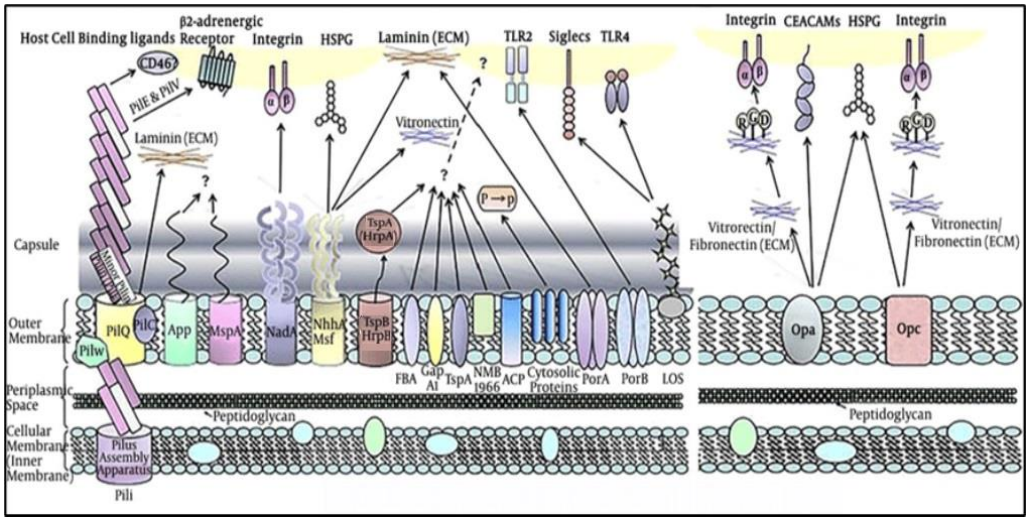

Figure 4. *N. meningitidis* surface antigens and their interaction with host epithelial cells. The *N. meningitidis* surface antigens and their engagement with host epithelial cells are depicted (Adapted from Mehrabi AT et al., 2020).

The interplay between meningococcus and the target cell begins when bacterial adhesins or invasins interact with epithelial cell receptors. This interaction initiates a communication pathway that leads to the bacterial incorporation into vesicles, followed by translocation to the baseline of the membrane (Stephens DS, 2009). Subsequently, the bacteria penetrate the submucosal space through transcytosis. Meningococcal survival within host cells depends on IgA1 protease, which is responsible for the degradation of lysosome-associated membrane proteins (LAMPs), thus preventing phagosomal maturation and upregulation of capsule expression (Virdarsson G et al., 2005). *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) (Hill DJ et al., 2010).
These mechanisms facilitate the survival of *N. meningitidis*, allowing it to subsequently continue its infectious process within the circulatory system. In this environment, the capsule is expressed, allowing the microorganism to evade the host's immune system defenses. *N. meningitidis* can exhibit both encapsulated and non-encapsulated forms, but strains causing invasive diseases, as isolated from sterile sites such as blood and CSF, are almost invariably encapsulated (Rouphael NG and Stephens DS, 2012). The capsule plays a crucial role in the microorganism's survival within the bloodstream, providing resistance against antibody and complement-mediated killing, while also inhibiting phagocytosis by immune system cells (Uria MJ et al., 2008). Another crucial virulence factor for the survival of meningococcus is lipooligosaccharide (LOS). Meningococcal LOS is a major component of the outer membrane. It 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 MP et al., 1999). Neisserial LOS plays an important role in activation of the innate immune system and inflammatory signalling underlying the pathogenesis of fulminant sepsis and meningitis (Kahler CM et al., 1998; Plant L et al., 2006). In particular, cells of the innate immune system express pattern recognition receptors (PRRs) that specifically recognize microbial structures (Franchi L et al., 2009; Cassel SL et al., 2008). PRRs include Toll-like receptors (TLRs), located on the cell surface, and nucleotide-binding domain leucine-rich repeat-containing receptors (NLRs), which detect pathogens in the cytosol of eukaryotic cells (Idosa BA et al., 2019). Among these, the NLR family pyrin domain-containing 3 (NLRP3) can be activated by a wide range of microbial stimuli, including LOS, mediating the activation of the pyroptotic process (Davis BK et al., 2011; Yang CS et al., 2012) with the formation of a cytoplasmic multiprotein complex called the inflammasome. This facilitates the activation of caspase-1, resulting in the maturation of pro-forms of interleukin-1β (IL-1β) and IL-18 into biologically active cytokines (Cassel SL et al., 2008; Mariathasan S et al., 2006).

1.3 Bacterial Secretome: the two-partner secretion system HrpA/HrpB in *N. meningitidis.*

Bacteria have the ability to secrete proteins into the extracellular space, often with the function of acquiring nutrients. In pathogenic strains, secreted proteins, play a crucial role in various phases of the infectious cycle, particularly during the colonization of host organism tissues and the modulation of the immune response (Lee VT and Schneewind O et al., 2001). All proteins secreted by a bacterial strain constitute its secretome. Over the years, the secretome of *N. meningitidis* has received less attention despite its obvious involvement in all infectious stages of the bacterium (Van Ulsen P and Tommassen J et al., 2006). To date, the availability of sequenced genomes from various meningococcus strains. Serogroup A strain Z2491 (Parkhill J et al., 2000), serogroup B strain MC58 (Tettelin H et al., 2000), and serogroup C strain FAM18 (http://www.sanger.ac.uk/Projects/N_meningitidis) has provided the opportunity to study and identify secreted proteins and their associated transport channels.
In Gram-negative bacteria, various mechanisms exist for protein secretion. These pathways have been categorized into six distinct classes: type I-IV secretion, autotransporters, and TPSs. Protein secretion can occur in either a single step or a two-step process. In the two-step pathway, proteins are transported through the inner membrane (IM) and the outer membrane (OM) using two different mechanisms. Transport through the IM is mediated by the Sec machinery (de Keyzer J et al., 2003) or twin-arginine translocation (Tat) mechanism (Berks BC et al., 2003). Sec protein is conserved in *Nesseriaceae* family and comprises several components. The chaperone SecB prevents protein aggregation and guides them to the IM by facilitating their interaction with the heterotrimeric translocation complex, SecYEG. Energy for translocation is provided both by ATP hydrolysis through the motor protein SecA and the proton gradient induced by the SecDF-YajC complex (Tommassen J and Arenas J, 2017) (Figure 5).

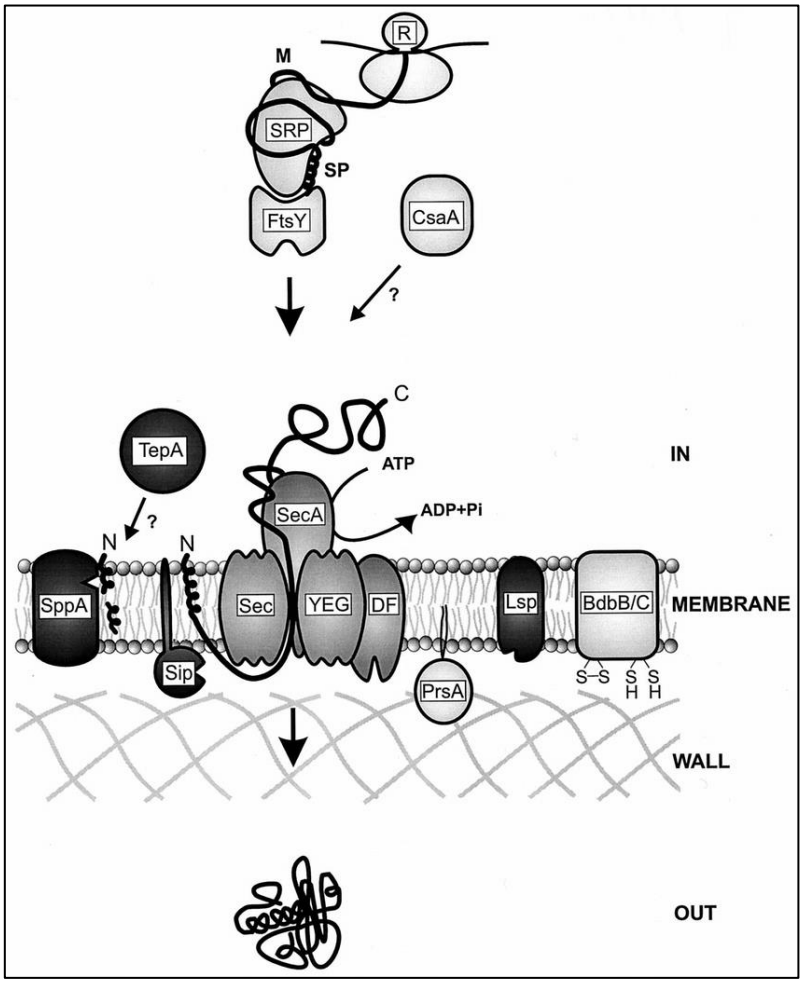

Figure 5. Main components of the Sec-dependent protein secretion machinery. Secretory proteins are ribosomally synthesized as precursor proteins with an N-terminal signal peptide. Cytoplasmic chaperones, such as SRP/FtsY and CsaA, maintain the precursors in a translocation-competent state and facilitate their targeting to the translocase in the membrane, comprising SecA, SecY, SecE, SecG, and SecDF. During or shortly after translocation, the preprotein is cleaved by one of the type I signal peptidases (Sip) and cleaved by the lipoprotein-specific signal peptidase (Lsp). SppA and TepA may participate in the degradation of cleaved signal peptides, while the folding of various secreted proteins relies on the activities of PrsA and BdbB/C that contribute to the quality control of secretory proteins. Upon passage through the cell wall, the mature protein is released into the environment. (Adapted from Tjalmsa H et al., 2000)

Tat mechanism, along with its components TatA, TatB, and TatC, is conserved in *N. meningitidis*. This transport system recognizes specific proteins that possess the consensus motif 'twin-arginine' ([S/T]R-R-x-F-L-K) in their N-terminal region and facilitates their translocation (Palmer T and Berks BC, 2012). The transport across the IM into the periplasmic space can be mediated by both the type II secretion system, autotransporters, and TPSs. In *N. meningitidis*, no type II secretion system has been identified; therefore, proteins are secreted only through two of these mechanisms (Jacob-Dubuisson F et al., 2004) (Figure 6).

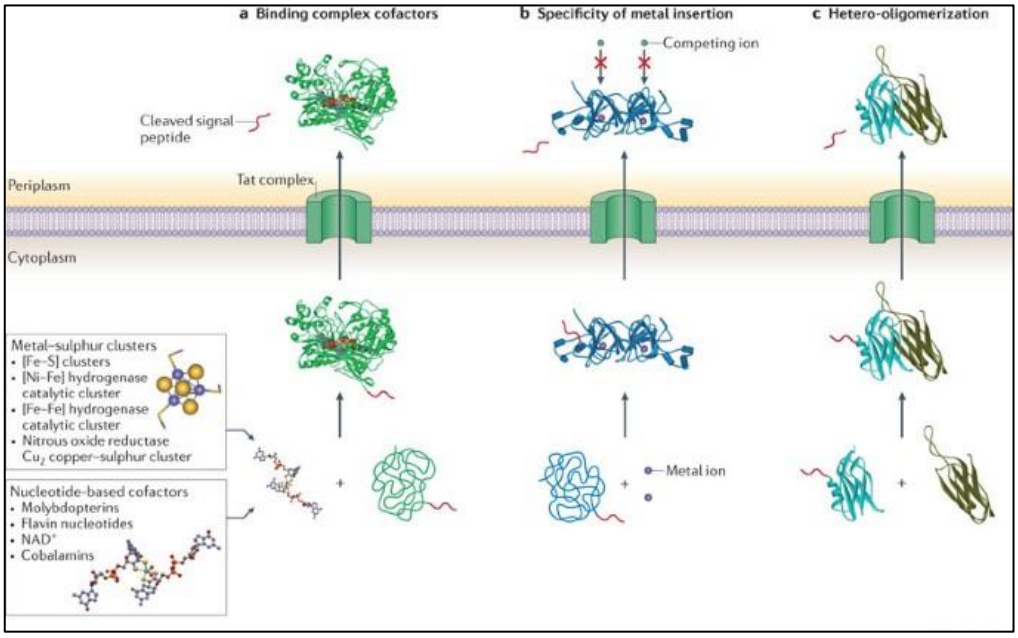

Figure 6. Tat machinery export pathway. Tat targeting allows proteins to acquire their cofactor before transport across the cytoplasmic membrane **(a)**, Tat system allows a protein to obtain metal ions under controlled conditions in the cytoplasm **(b)**, Tat pathway allows the formation of hetero-oligomeric complexes in the cytoplasm and then the transport by a signal peptide **(c)** (Adapted from Palmer T and Berks BC, 2012).

Specifically, only the Type I secretion systems (T1SS) systems pathway, autotransporters, and TPSs are active for protein secretion in meningococcal strains. The T1SS systems are composed of three distinct proteins: an ATP-binding cassette (ABC) protein in the IM, a TolC-type transporter protein, and a fusion protein linking protein components on the IM with those on the OM. (Delepelaire P, 2004). This system completely surrounds the cell envelope and it is involved in all single-step secretion processes, enabling direct secretion from the cytoplasm to the cellular medium. Autotransporters are modulatory proteins consisting of a signal sequence in the N-terminal region, a translocation domain

located in the C-terminal sequence, separated by a secreted passenger domain. The signal sequence allows autotransporters to interact with the Sec mechanism for transport through the IM.

The TPS system is a two-step mechanism that facilitates the secretion of very large beta-helix proteins (containing more than 1000 amino acid residues) (Jacob-Dubuisson F et al., 2001; Jacob-Dubuisson F et al., 2004). The complex consists of a secreted protein, called TpsA, and a dedicated transporter, known as TpsB, which facilitates its release through the OM (Tommassen J and Arenas J, 2017). It has been proposed that the TPS system should be associated with autotransporters because the passage domain and the translocation domain are uncoupled (Henderson IR et al., 2000). However, there are substantial differences between these two systems, as the lack of sequence similarity between the transporter TpsB and the translocation domain of autotransporters (Jacob-Dubuisson F et al., 2000). On the contrary, TpsBs show sequence similarity with Omp85, a family of proteins that are essential for outer membrane biogenesis in mitochondria and bacteria. Omp85 proteins are also involved in the insertion of protein structures into the OM (Voulhoux R et al., 2003).

The transporter TpsB is composed of a 16-stranded β-barrel, with two POTRA domains located in the periplasmic region and an α-helix that plunges into the channel within the barrel (Clantin B et al., 2007). Indeed, the secreted TpsA proteins are characterized by an N-terminal sequence of approximately 245 amino acid residues that serves as a signal sequence for transport through the IM via interaction with the Sec machinery (Chevalier N et al., 2004). Additionally in this region, is located the TPS signal that includes two motifs, NPNL and NPNGI, enabling recognition by TpsB POTRA domain (Schonherr R et al., 1993; Jacob-Dubuisson F et al., 1997; Tommassen J and Arenas J, 2017). Based on their sequence similarity, TpsA proteins are divided into two subclasses. The first includes calcium-dependent cytolysins, while the second comprises various groups of adhesins and haemoglobin-binding proteins (Jacob-Dubuisson F et al., 2001). The interaction of TpsA with TpsB induces a conformational change in the transporter, leading to the removal of the plug helix and enabling the passage of TpsA through the β-barrel structure. After transport, the TpsA proteins initially remain tethered to the cell surface, presumably via their C-terminal domains, before being released into the medium (Jacob-Dubuisson F et al., 2004). (Figure 7)

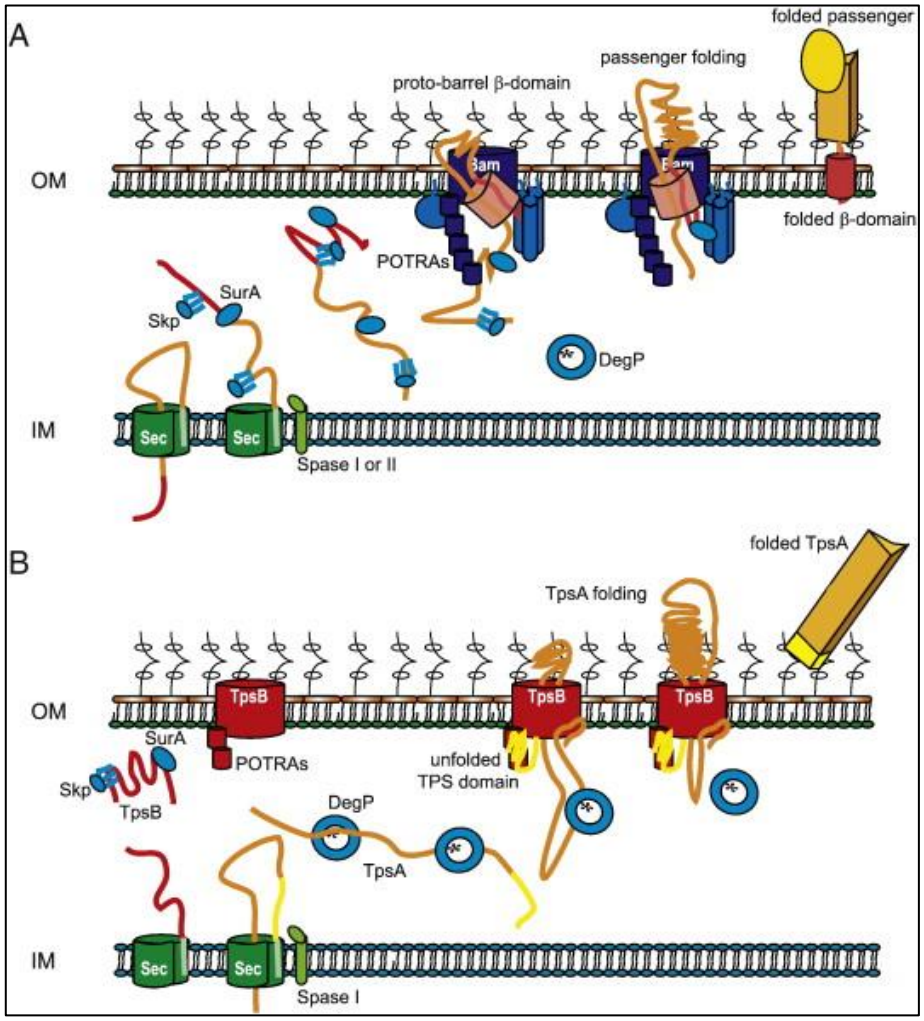

Figure 7. Schematic models of autotransporter and TPS system across the IM and OM of Gram-negative bacteria. In the first model **(A)**, autotransporter proteins are conveyed to the periplasmic space via Sec system located in the IM and to the OM through the Bam complex and periplasmic chaperones. In the second model **(B)**, the TPS system, TpsB (red) and TpsA (yellow) proteins across the IM via the Sec system to position within the OM (Adapted from Van Ulsen P et al., 2014).

The genome of *N. meningitidis* strains potentially contains up to three distinct TPS systems. Some of these systems have the ability to secrete multiple TpsA proteins (Van Ulsen P et al., 2008). For example, an examination of the genomic sequence of the reference serogroup B strain MC58 has revealed the presence of five distinct *tpsA* genes: two associated with system 1 (referred as *tpsA1a* and *tpsA1b*), two linked to system 2 (denoted as *tpsA2a* and *tpsA2b*), and one affiliated with system 3 (referred to as *tpsA3*). In contrast, the genomes belonging to serogroup C (FAM18, 053442) and serogroup A (Z2491), contains only a single *tpsA* gene aligned with system 1.
The *tpsB* and *tpsA* genes of system 1 are located within distinct genetic islands. Downstream of the *tpsA* genes, several 5'-end-truncated *tpsA*-related open reading frames (ORFs) exist, known as *tpsC* cassettes (Van Ulsen P et al., 2008). These *tpsC* cassettes share sequence homology with the central region of *tpsA* gene but possess entirely distinct 3'-terminal sequences. Research has indicated that the System 1 TPS in *N. meningitidis* functions as a toxin/antitoxin fratricide system, hindering the growth of other meningococci competing for the same niche within the human host (Arenas J et al., 2013). The TpsA1 proteins of *N. meningitidi*s mediate this growth inhibition, while the downstream ORFs provide immunity to the producing strain. Similar systems have been identified in Transporter of Pathogenicity Island A (CdiA) proteins of other Gram-negative bacteria. In these systems, contact-dependent inhibitory activity resides in the carboxy-terminal region of CdiA (CdiA-CT), downstream of the VENN peptide motif. Furthermore, the CdiI anti-toxin binds to and neutralizes its corresponding CdiA-CT, but not heterologous CdiA-CT (Willett JL et al., 2015).
In addition to their role in contact-dependent inhibition, TpsA1 proteins of *N. meningitidis*, known as HrpA, derived from "hemagglutinin/hemolysin-related proteins A," have been involved in the interaction with the host. It was observed that HrpA undergoes proteolytic processing, resulting in an approximately 180 KDa form, and is secreted through an HrpB-dependent mechanism, while a small fraction of HrpA remains associated with the bacterial cell and contributes to its interaction with epithelial cells (Schmitt C et al., 2007). It was observed that, deletion of *hrpB* gene (TpsB1) in a non-capsulated and LPS-truncated strain resulted in a significant reduction in adherence to epithelial cells while HrpA protein accumulates in the bacteria and remains stable and undegraded (Schmitt C et al., 2007). The significance of HrpA in biofilm formation on human bronchial epithelial (HBE) cells, in both encapsulated and non-encapsulated strains of *N. meningitidis*, was also demonstrated (Neil RB et al., 2009). Additionally, there is evidence suggesting that HrpA plays a crucial role in intracellular survival by aiding bacteria in escaping from the internalization vacuole into the cytoplasm. Furthermore, this protein functions as manganese-dependent cell lysins (Talà A et al., 2008). Consistent with these findings, *hrpB* and *hrpA* genes are upregulated upon contact with host cells, in the intracellular environment of host cells, and under anaerobic conditions (Neil RB e Apicella MA 2009, Talà A et al., 2008) (Figure 8).

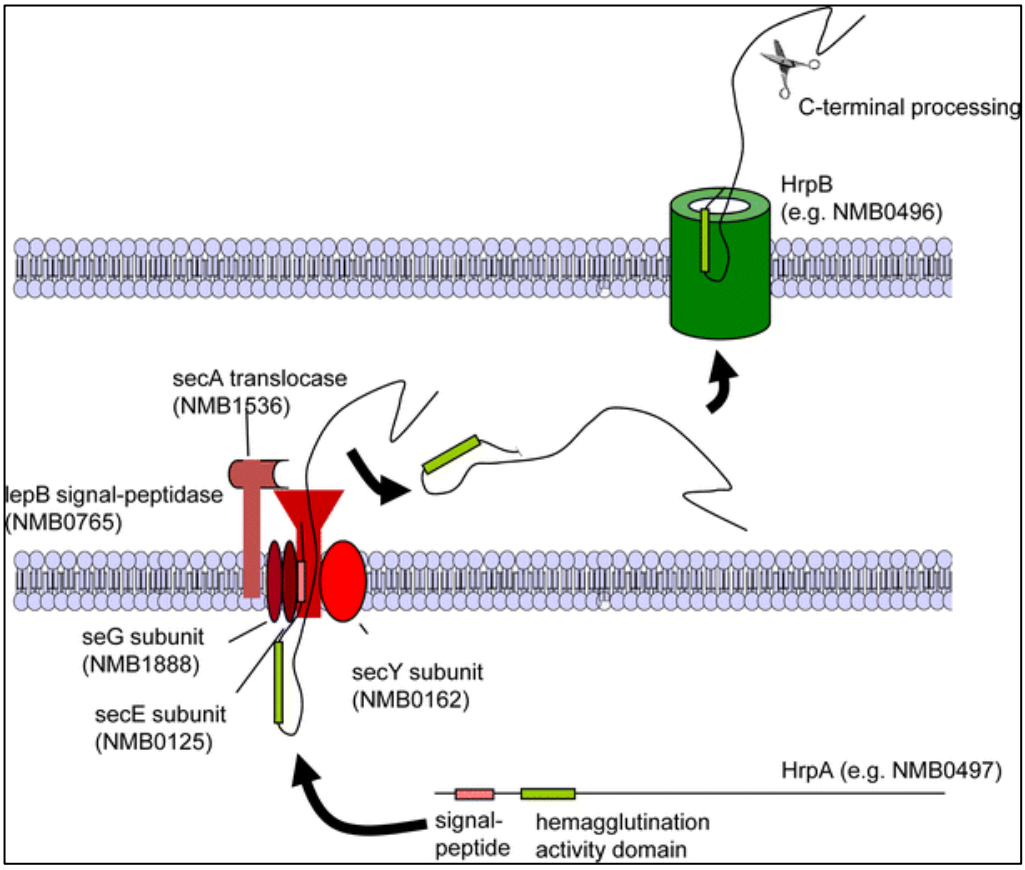

Figure 8. HrpA/HrpB pathway in *N. meningitidis*. HrpA effector proteins are translocated across the IM using the universal Sec machinery, while the passage through the OM is facilitated by the dedicated transporter HrpB (Adapted from Schielke S et al., 2010).

HrpA is a versatile protein with multiple domains and functions. A recent investigation revealed that while manganese-dependent cell lysin activity is confined to the carboxy-terminal region of HrpA, the middle region of HrpA can bind to the dynein light-chain, Tctextype 1 (DYNLT1) (Talà et al., 2022). This interaction between HrpA and DYNLT1 plays a pivotal role in meningococcal trafficking within various types of cells, including neuronal cells, and is implicated in modulating the balance between apoptosis and pyroptosis (Talà et al., 2022). Notably, *in vitro*-infected cells suggest that *N. meningitidis* tends to inhibit apoptosis while inducing pyroptosis, a form of pro-inflammatory cell death that may contribute to the inflammation characteristic of invasive meningococcal disease (Talà et al., 2022; Tunebridge AJ et al., 2006).

1.4 Pyroptosis, a defense against intracellular bacteria.

Pathogenic bacterial strains are categorized into intracellular and extracellular based on their location and interaction with host cells (Girardin SE et al., 2002). Intracellular bacterial pathogens penetrate host cells to facilitate the mechanisms of replication and the propagation of the infectious process localizing within specific niches such as plasma membrane-derived vacuoles: phagosomes or endosomes (Petit TJP and Lebreton A al., 2022). Some bacterial strains, like *Salmonella typhimurium*, are defined as vacuolar bacteria because they remain inside the vacuole, while others, like *Listeria monocytogenes*, are defined as cytosolic bacteria because once phagocytosed by the host cell, they escape from the vacuole and establish themselves within the cytoplasm (Chen YG et al., 2017). The selective pressure exerted by the virulence strategies of intracellular bacterial pathogens has led to the evolution of new host defense mechanisms, such as regulated cell death pathways, including apoptosis and lytic cell death. (Figure 9).

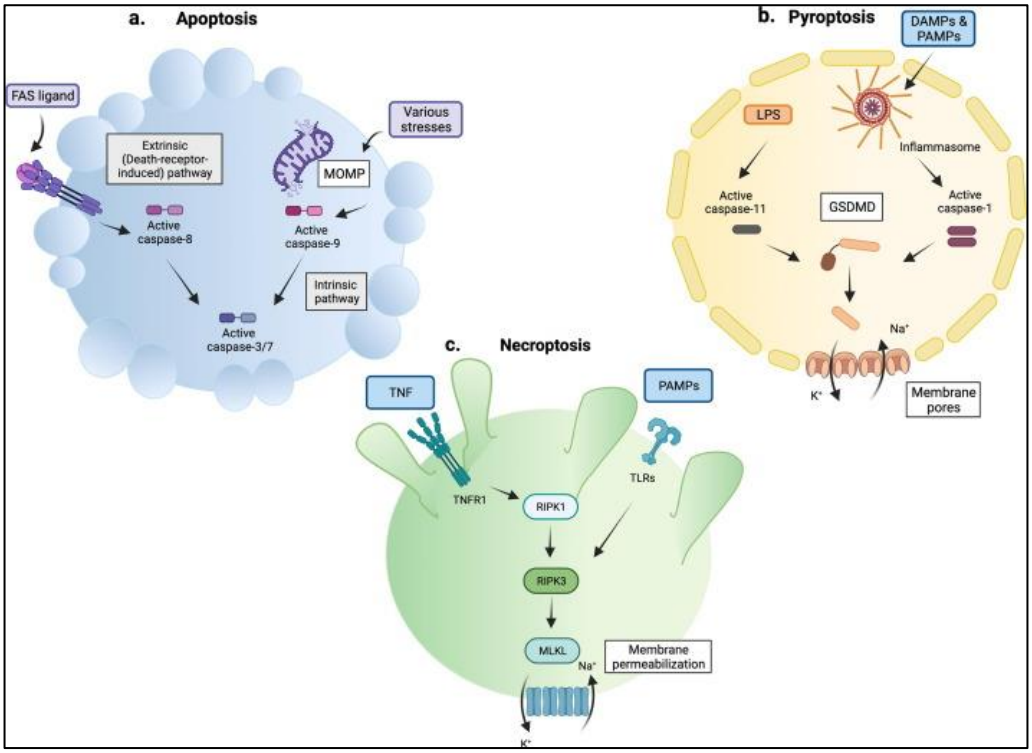

Figure 9. Schematic representation of the apoptosis, necroptosis, and pyroptosis pathways. The figure displays the various effectors that can act on the cell to induce one of the three programmed cell death pathways: apoptosis **(a)**, necroptosis **(b)**, and pyroptosis **(c)** (Adapted from Carneiro KN and Fitzgerard KA, 2022).

These defense pathways are part of innate immunity mechanisms and serve not only for destroying the replicative niche established within the host cell but also for the recruitment of immune cells to the site of the infection, allowing for the complete pathogen eradication (Aleru O and Barber MF et al., 2020). Cells undergoing on these programmed cell death mechanisms can be distinguished based on morphological and immunological hallmarks. Apoptosis is characterized by DNA fragmentation, cell blebbing, and the formation of apoptotic bodies. The apoptotic bodies are rapidly cleared by phagocytes through efferocytosis, this process does not activate the inflammatory response. In contrast, regulated lytic cell death, including pyroptosis and necroptosis, leads to the release of cellular contents that cause a pro-inflammatory response (Brokatzky D and Mostowy S, 2022). The utility of different cell death pathways can depend upon the cell type that is infected as well as the nature of the infecting pathogen. Sometimes regulated cell death pathways act redundantly, whereas in other cases one specific pathway is required to mediate pathogen's clearance.

Pyroptosis (from the Greek *pyro* meaning "fire" or "fever" and *ptosis* meaning "falling") is a regulated form of necrosis and is considered part of the host organism's innate immune response (Galluzzi L et al., 2018; Robinson N et al., 2019). It is characterized by cell lysis and the release of pro-inflammatory cytokines, damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs) (Broz P et al., 2020). The release of DAMPs and PAMPs activates surrounding cells and the inflammatory response, attracting innate immune cells to the site of infection (Broz P et al., 2020). Pathogenic bacteria can trigger pyroptosis through various extracellular signals such as LPS or flagellin, as well as through intracellular signals like mitochondrial DNA and cytosolic bacterial LPS (Bauernfeind F et al., 2011; He Y et al., 2016). One hallmark of pyroptosis is the activation of the inflammasome, a multiprotein complex, which can be defined canonical or non-canonical (Brokatzky D and Mostowy S, 2022) (Figure 10).

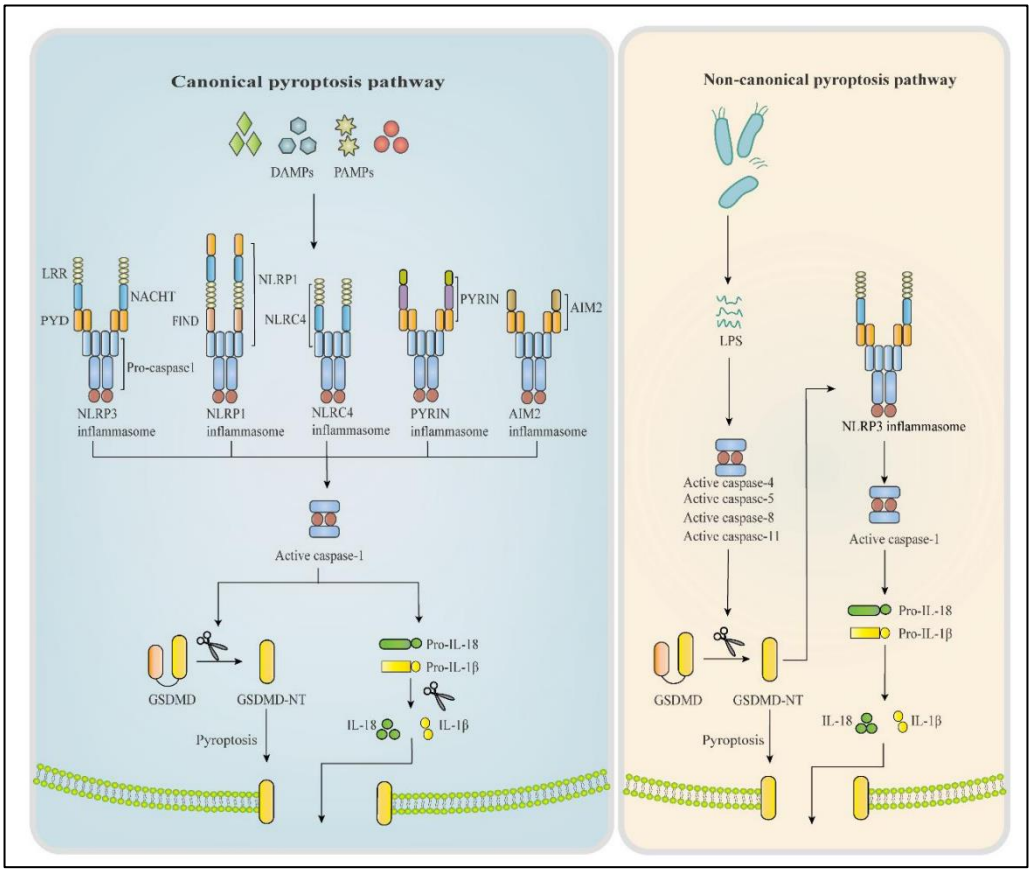

Figure 10. Mechanisms activation of the canonical and non-canonical inflammosome. (Left) Canonical inflammasome activation requires priming by a Toll-like receptor (TLR) ligand, (Right) non-canonical inflammasome activation is activated by Gram-negative bacteria. (Adapted from Lin J et al., 2020)

In the canonical inflammasome pathway, activation begins with the recognition of foreign or damaged signals such as PAMPs or DAMPs by receptors NOD-like receptor family pyrin domain-containing 3 (NLRP3). NLRP3 possesses a multi-domain structure, including a pyrin domain (PYD) at its N-terminus, a central nucleotide-binding and oligomerization domain (NACHT), and a C-terminal leucine-rich repeat (LRR) domain. NLRP3 activation is lead to the interaction between the PYD domain of NLRP3 and the PYD domain of the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) (Lu A et al., 2014). ASC functions as crucial intermediary linking NLRP3 to caspase-1. Upon binding of the PYD domains of NLRP3 and ASC, ASC assembles into a polymerized structure referred to as the ASC filament. This filament formation serves as a platform facilitating the recruitment and activation of caspase-1. Once brought into proximity with the ASC filament, caspase-1 undergoes autocleavage and attains enzymatic activity (Shi J et al., 2015; Liu X et al., 2016). Activated caspase-1, cleaves GASDMD into its active

form. Cleaved GSDMD produce two fragments: an N-terminal fragment and a C-terminal fragment. N-terminal fragment of GASDMD recognizes membrane lipids and undergoes oligomerization, leading to the formation of soluble pre-pores (Chen YG et al., 2017). This pre-pore subsequently undergoes a conformational change as it inserts into the membrane, forming large pores with an approximate diameter of 21 nm (Ruan J et al., 2018; Xia S et al., 2021). After the GASDMD pore opens, the subsequent membrane rapture is an active process mediate by NINJ1 protein (Kayagaki N et al., 2021). The process of NINJ1 activation downstream of the GSDMD pore, while not yet fully understood, is a crucial link in this pathway. Once NINJ1 is activated, it undergoes polymerization within the membrane, ultimately leading to membrane rupture. This rupture, in turn, facilitates the release of pro-inflammatory cytokines and cellular contents, thereby intensifying the inflammatory response. Consequently, interleukin-1β (IL-1β) and interleukin-18 (IL-18), both pro-inflammatory cytokines, are released into the surrounding environment, further promoting inflammation (Loomis WP et al., 2019).

Instead, the non-canonical inflammosome activation pathway begins with the detection of intracellular danger signals, typically bacterial LPS and results in activation of GASDMD by human caspase-4 or mouse caspase-11 indipendent of inflammosome formation (Brokatzky D and Mostowy S, 2022), allowing the formation of plasma membrane pores (Hagar A et al., 2013); potassium ion (K+) efflux through these GASDMD pores can lead to inflammasome formation, caspase-1 activation and IL-1β maturation (Ruhlz S and Broz P, 2015).

Recent studies have consistently indicated that pyroptosis triggered by *N. gonorrhoeae* showcases a multifaceted relationship with both the canonical and noncanonical pathways (Ritter JL and Genco CA, 2018). Evidently, noncanonical pyroptosis pathway often finds its ignition source in intracellular Gram-negative bacteria and intracellular LPS, creating a compelling nexus. This phenomenon is especially relevant when considering the intracellular dynamics of pathogenic *Neisseria* species. Intriguingly, a growing evidence suggests a direct correlation between the presence of intracellular gonococci and the manifestation of pyroptotic events within macrophage-derived monocytes (MDMs) (Ritter JL and Genco CA, 2018). Investigations into this area have revealed that treatment with cytochalasin D, an inhibitor of gonococcal internalization, exerts a diminishing effect on gonococcal-induced cell death. Furthermore, the stimulation of MDMs with gonococcal LPS serves as a potent inducer of pyroptosis, concurrently bolstering the secretion of the pro-inflammatory cytokine IL-1β (Ritter JL and Genco CA, 2018).

For *N. meningitidis*, recent studies have shown that the pyroptotic pathway can be triggered through caspase 8 activation that increase caspase-3 levels within HeLa and NSC34 cells (Talà A et al., 2022). In particular, *N. meningitidis* can expose the cytosolic lipooligosaccharide (LOS) that induce the non-canonical inflammasome pathway. GASDMD is an effector of pyroptosis and, after cleavage, its N-terminal domain constitutes a membrane pore to favor secretion of inflammatory mediators. In this study, GASDMD is inhibited by high caspase-

3 levels which are involved, instead, in the activation of gasdermin E (GASDME) (Figure 11).

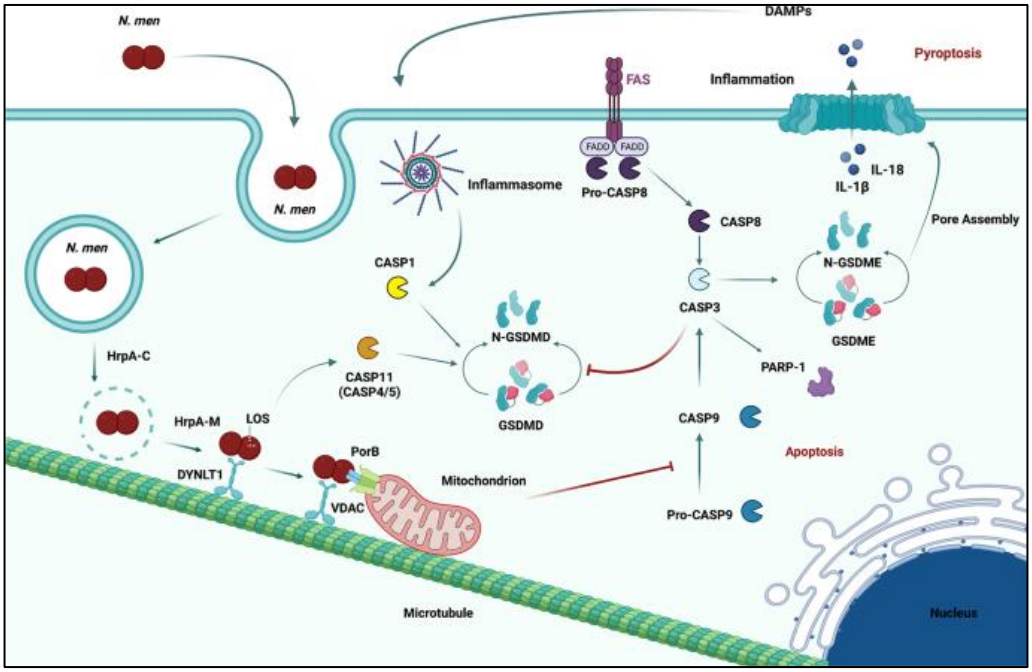

Figure 11. Mechanism of apoptosis inhibition and pyroptosis activation after *N. meningitidis* infection. *N. meningitidis* infection, the HrpA-DYNLT1 interaction relocates PorB to the mitochondria, leading to its interaction with the voltage-dependent anion-selective channel (VDAC). This interaction inhibits pro-caspase-9 cleavage and downstream pathway activation, preventing apoptosis. *N. meningitidis* can expose cytosolic LOS, triggering the non-canonical inflammasome pathway, resulting in caspase-11 cleavage in mice and caspase-4/5 in humans. (Adapted from Talà A et al., 2022)

Like the GASDMD, GASDME is activated by N-terminal cleavage determining membrane pore assembly to mediate efflux of activate interleukins and induce inflammation and pyroptosis. DAMPs derived by dead cells induce activation of inflammasome with consequent caspase-1 activation. Also, in this case the effect is aborted as a consequence of the caspase- 3 inhibition on GASDMD (Talà A et al. 2022).

1.4 Murine model of meningococcal infection.

Progress in understanding the pathogenesis of meningococcal disease has faced significant challenges due to the absence of suitable animal disease models. This condition arises from the fact that *N. meningitidis* predominantly infects humans, owing to its specific virulence factors and iron uptake systems tailored for human receptors (Plant L et al., 2004). Moreover, the phase variation mechanisms exhibited by major surface antigens of *N. meningitidis* during various infection stages have further complicated the development of relevant experimental models and effective vaccines (De Vries FP et al., 1996; Tinsley CR et al., 1986). The bulk of our knowledge regarding the cellular and molecular biology of this human pathogen and its virulence determinants, such as capsular polysaccharides, LOS, and numerous surface adhesins and secreted proteins, has been gleaned from studies conducted in cell systems, organ cultures, and animal experimental models. However, these models fail to fully replicate the complexity of the meningococcal infectious cycle as it occurs in the human host (Hill DJ et al., 2010). To date, murine models for studying meningococcal disease have relied on three primary infection routes: intraperitoneal (i.p.), intranasal (i.n.), and intracisternal (i.cist.).

In the i.p. infection model, typically involving neonatal rats, the microorganisms are injected into the abdominal region of the animal. This method is often employed after meningococci have been passage through murine hosts to enhance bacterial virulence (Saukkonen K et al., 1988). The i.p. model, although not replicating the natural infection route in humans, has proven valuable for vaccine studies (Saukkonen K et al., 1989; Newcombe J et al., 2004) by inducing septicemia and sepsis. Conversely, in the i.n. infection model is useful to analyze the pathogenesis of meningococcal disease. However, animals often develop lung infections before sepsis, which is not a typical manifestation of the disease in humans. The i.p. model has been instrumental in assessing protection against meningococcal disease (Gorringe A et al., 2005; Newcombe J et al., 2004), while the i.n. model has been crucial for understanding disease pathogenesis and remains relevant for studying the virulence of various *N. meningitidis* isolates.

Furthermore, in order to facilitate the proliferation of meningococci within the murine host, various technical strategies have been employed. These strategies encompass the administration of exogenous iron to enhance infection, the injection of mice with inocula containing a high bacterial load, the adoption of mouse-passaged bacterial strains, and the utilization of neonatal or immunocompromised animal hosts. Additionally, the introduction of specific human factors like CD46 has heightened the susceptibility of mice to this strictly human bacterium. Moreover, the incorporation of the human skin xenograft model of infection has proven valuable in assessing the adhesion capabilities of meningococci to human endothelium.

Recently, i.cist. infection model has been studied and validated **(**Colicchio R et al., 2009; Colicchio R et al., 2019; Pagliuca C et al., 2019). This model provides

the injection of mouse-passaged bacteria into the cisterna magna of immunocompetent outbred adult mice. Survival rates, clinical parameters of infected mice, and histological analysis of the brain have demonstrated the ability of the meningococcus to establish and replicate within the meninges, exhibiting infectious characteristics comparable to those seen in human host. Meningococci have also been found in peripheral organs such as the spleen and liver, indicating that this infection system can induce not only meningitis but also systemic infections in the murine host. In particular, this model was employed to evaluate the virulence of a *N. meningitidis* isogenic mutant strain defective in the L-glutamate transporter GltT (Colicchio R et al., 2009).
Furthermore, through the application of the described i.cist. murine infection model, the role of surface-exposed sialic acids in the establishment of meningitis and meningoencephalitis was evaluated (Colicchio R et al., 2019). For this purpose, the reference serogroup C meningococcal strain 93/4286 and an isogenic *cssA* knockout mutant, defective in capsule antigen production, were used. This study not only further validated the efficiency of the i.cist. method, highlighting the reduction in virulence of the isogenic mutant compared to the reference strain but, for the first time, it 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. In this cerebral region, heparan sulfate receptors are highly expressed, serving as the target for meningococcal proteins Opa, Opc, and Nhha, mediating interactions with both epithelium and endothelium. Additionally, CEACAM-1 proteins, specific for Opa adhesins/invasins, are expressed at high levels by oligodendrocytes, which are abundant in the *corpus callosum* (Colicchio R et al., 2019).

2. AIM OF THE STUDY

N. meningitidis exhibits remarkable pathogenicity, it can exploit diverse microenvironments within the human host to secure essential resources for its persistence, propagation, and dissemination. Notably, *N. meningitidis* has acquired a repertoire of virulence determinants that facilitate its establishment within the human host, ensuring prolonged survival within its unique ecological niche. Numerous studies have sought to elucidate the molecular and cellular interactions between *N. meningitidis* and its host. Various virulence-contributing factors, including capsular polysaccharides, LOS, type IV pili, IgA1 proteases, surface adhesion proteins, and iron acquisition systems, have been identified and analyzed. In the last years, the crucial role of proteins secretion systems in the progression of meningococcal pathogenicity has gained substantial importance. This observation is noteworthy, as the proteins released through these systems could potentially serve as candidates for vaccine components or as targets for therapeutic intervention, underscoring their potential usefulness in medical advancements. The HrpA/HrpB TPS system, among various protein secretion systems, plays a crucial role in the development of meningococcal meningitis. Recent *in vitro* studies of HrpA/HrpB TPS system have shown its involvement in biofilm formation (Neil BR and Apicella MA, 2010), escaping from internalization vacuoles within host cells (Talà A et al., 2008) and attaching to the dynein motor (Talà A et al., 2022). These actions ultimately lead to the initiation of the pyroptosis process. The aim of the present study was to evaluate the role of HrpA/HrpB TPS in establishing meningococcal meningitis and activating pyroptotic pathways in an experimental murine model based on i.cist. infection of female BALB/c adult mice. On this purpose, we employed the serogroup C reference strain 93/4286 and an isogenic mutant 93/4286Ω*hrpB*, which lacks the expression of HrpB transporter. This protein plays a pivotal role in the secretion of hemagglutinin/hemolysin HrpA across the OM. Before starting *in vivo* experiments, to ensure that the gene knockout did not induce any differences in bacterial replication, we assessed the growth rates of both the reference strain and the *hrpB* isogenic mutant in *in vitro* conditions. In *in vivo* experiments, we assessed the 50% lethal dose (LD_{50}) of these strains and examined their replication in the mouse brain and peripheral organs. To delve into the infectious dynamics and histopathological associations of the disease within the mouse model, we conducted a histological analysis. Moreover, to examine the potential of the 93/4286 reference strain and the 93/4286Ω*hrpB* isogenic mutant in activating the pyroptotic pathway in the murine brain, Western blot analyses were conducted focusing on the principal markers of the pyroptotic process: caspase-1, caspase-11, GASDMD, and caspase-7.