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Thesis title

Understanding pathogenesis of *Neisseria gonorrhoeae* at three distinct human mucosal niches, studying bacterial/host interaction during bacterial adhesion and invasion.

Academic discipline
Biological systems/bio industries

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1. Abstract

Neisseria gonorrhoeae (Ng) is the etiological agent of gonorrhea, a sexually transmitted infection (STI), which is a major global public health concern. Ng primarily acts as a mucosal colonizer, attaching to various epithelial surfaces through distinct structures such as the opacity proteins (Opa), which mediate adhesion to cervical epithelial cells via carcinoembryonic antigen-related cell adhesion molecules (CEACAM) receptors. A Luminex-based assay was developed to measure the interaction of diverse gonococcal strains with recombinant CEACAM 1, 3, 5 and 6. The ability of anti- Ng GMMA investigational vaccine serum to inhibit bacterial interaction or binding to the distinct CEACAM human ligands was also monitored. Furthermore, we characterized the adhesion of different Ng strains in 2D in vitro models, using immortalized cells representative of distinct sites including urethral, cervix, and oropharynx. Moreover, these cell lines were used to set up adhesion-inhibition assays with polyclonal and monoclonal antibodies. To further investigate the Opa-CEACAMs interactions during a time-course of infection in a complex tissue environment and under more physiologically relevant conditions, we used a 3D complex model of the human ectocervix tract. The binding of Ng to the CEACAMs was shown to be Opa-specific and different anti-GMMA sera differentially inhibited the interaction of a representative panel of strains with diverse CEACAM ligands, presumably depending on the alleles of Opa expressed by the diverse strains. Bacterial adhesion inhibition experiments showed that Ng GMMA sera is able to inhibit bacterial adhesion to Ect 1 E6/E7, End 1 E6/E7 and Detroit 562 cell lines. Moreover, we successfully infected the 3D model, as shown by confocal microscopy images and supported by CFU counts of tissue homogenates. The implementation of a 3D model allowed us to track the Ng infection for up to 48 hours. We observed a dose-dependent response of gonococcal infection on the models at 1- ,24- and 48- hours post-infection (hpi). Small groups of adherent bacteria were observed at 1 hpi, while large microcolonies and aggregates of Ng were evident at 24 hpi. Invasion of the epithelial cells was shown at 24 hours and progressed to 48-hours both intracellularly and extracellularly through junctions. Preliminary data suggest that bacterial adhesion inhibition could be achieved with mouse antisera- Ng GMMA on a 3D model infection. This study highlights the critical role of Ng-CEACAMs interaction in the female urogenital tract, demonstrating how 3D models allow for a more accurate characterization of Ng infection, providing a more detailed and complete Ng infection profile in association with CEACAM molecules, uncovering novel bacterial CEACAM interaction not identified in 2D cell line infections.

2. Introduction

2.1. *Neisseria gonorrhoeae*

Neisseria gonorrhoeae (Ng) is a gram-negative diplococcus, also known as the gonococcus responsible for the Sexually Transmitted Infection (STI) and it is the etiological agent of gonorrhea, that remnants a major global public health concern (Unemo et al. 2019). Ng has been evolving alongside humans for hundreds of years.

In 2020 the world health organization (WHO) has estimated 82.4 million new infections with *N. gonorrhoeae* among adults aged 15 to 49 years (Rowley et al. 2019).

This obligate human host-adapted pathogen was described for the first time in 1879, by the German microbiologist Albert Neisser in Gram-stained microscopy of urethral discharge (Quillin and Seifert 2018; Unemo et al. 2019). The Neisseria genus currently consists of at least twenty-three species, about half are human-restricted species, some are animal-restricted, and some can be isolated from mucosal surfaces in both humans and animals. The two species of paramount clinical relevance and most extensively researched are *N. gonorrhoeae*, and *Neisseria meningitidis* (the meningococcus), which is capable of triggering cerebrospinal meningitis and septicemia. *N. gonorrhoeae* also has a connection to a number of other commensal *Neisseria* species, particularly those colonizing the pharynx, as *Neisseria lactamica*, *Neisseria polysaccharea*, *Neisseria cinerea*, *Neisseria subflava*, *Neisseria flavescens*, *Neisseria perflava*, *Neisseria mucosa*, *Neisseria elongata*, and *Neisseria sicca* (Rotman and Seifert 2014). Primarily, *N. gonorrhoeae* infect the genital

mucosa, however, it also has the capability to colonize the ocular, nasopharyngeal, and anal mucosa (J. S. Lee et al. 2002; Lin, Adamson, and Klausner 2021; Noble, Cooper, and Miller 1979).

N. gonorrhoeae has developed antimicrobial resistance (AMR) via several mechanisms (Unemo and Shafer 2014). The US Centers for Disease Control and Prevention (CDC) and the WHO have recognized antimicrobial-resistant *N. gonorrhoeae* as a critical menace to public health (Lin, Adamson, and Klausner 2021). *N. gonorrhoeae* has consequently been ranked as a high-priority pathogen on the WHO "*Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery and Development of New Antibiotics"* (Lin, Adamson, and Klausner 2021).

The rampant spreading of gonorrhea, along with constrained therapeutic resources in both low-income countries and less wealthy sectors in developed nations, constitutes a considerable hazard. The prospect of gonorrhea becoming resistant to treatment could trigger a sharp rise in infection incidences and related health issues. This emphasizes the immediate requirement for potent preventive actions and widely available treatment solutions to counteract this persistent and prevalent sexually transmitted infection (Annabel Baddely; Rachel; Baggaley; Maeve Brito De Mello; Meg Doherty; Boniface; Dongmo Nguimfack; Philippa Easterbrook; Nathan Ford; Cheryl Case Johnson; Olufunmilayo Lesi; Niklas Luhmann; Ismail Maatouk; Antons Mozalevskis; Busisiwe Msimanga; Morkor Newm 2024; Hasdiana 2018).

2.2. Epidemiology of sexually transmitted infection

The Seventy-fifth World Health Assembly appreciated the Global health sector strategies, on, respectively, HIV, viral hepatitis, and sexually transmitted infections, including *N. gonorrhoeae*, for the period 2022–2030 (Seventy-Fifth World Health Assembly. 2022). The proposed strategies aim to direct the healthcare industry to eradicate AIDS, viral hepatitis B and C, and STIs by 2030. This is part of the broader initiative to fulfill the 2030 Agenda for Sustainable Development. (Annabel Baddely; Rachel; Baggaley; Maeve Brito De Mello; Meg Doherty; Boniface; Dongmo Nguimfack; Philippa Easterbrook; Nathan Ford; Cheryl Case Johnson; Olufunmilayo Lesi; Niklas Luhmann; Ismail Maatouk; Antons Mozalevskis; Busisiwe Msimanga; Morkor Newm 2024). Each day more than 1 million adults aged 15–49 years are estimated to acquire one of four curable STIs: syphilis (caused by *Treponema pallidum*), gonorrhoea (caused by *Neisseria gonorrhoeae*), chlamydia (caused by *Chlamydia trachomatis*), and trichomoniasis (caused by *Trichomonas vaginalis*). In 2016, the WHO estimated that there were 86.9 (95% uncertainty interval 58.6–123.4) million incident global cases of gonorrhoea (global prevalence 0.9%), it shows in Figure **1A.** In 2020, the WHO approximated that there were 82.4 (with an interval 47.7 –130.4) million global cases of gonorrhoea. The **Figure 1B**, the graph shows projections from WHO about the anticipated decline in STIs from 2020 to 2030. According to these projections, total STI cases are expected to decrease significantly, from 374 million in 2020 to 150 million by 2030. Notably, the decline is most substantial for syphilis and gonorrhea, while chlamydia, although showing a reduction, is expected to remain the most prevalent. This expected decline is associated with several global initiatives by the WHO. These include: the screening programs and early intervention strategies, HPV vaccination coverage, improved access to healthcare services, and the implementation of more effective treatment protocols (Beno,

Silen, and Yanti 2022). The distribution and prevalence of gonorrhea vary greatly due to factors such as demographics, socioeconomics, sexual orientation, cultural aspects, prevention measures, screening and diagnostics available (Unemo et al. 2019; Uprety and Cárdenas 2017).

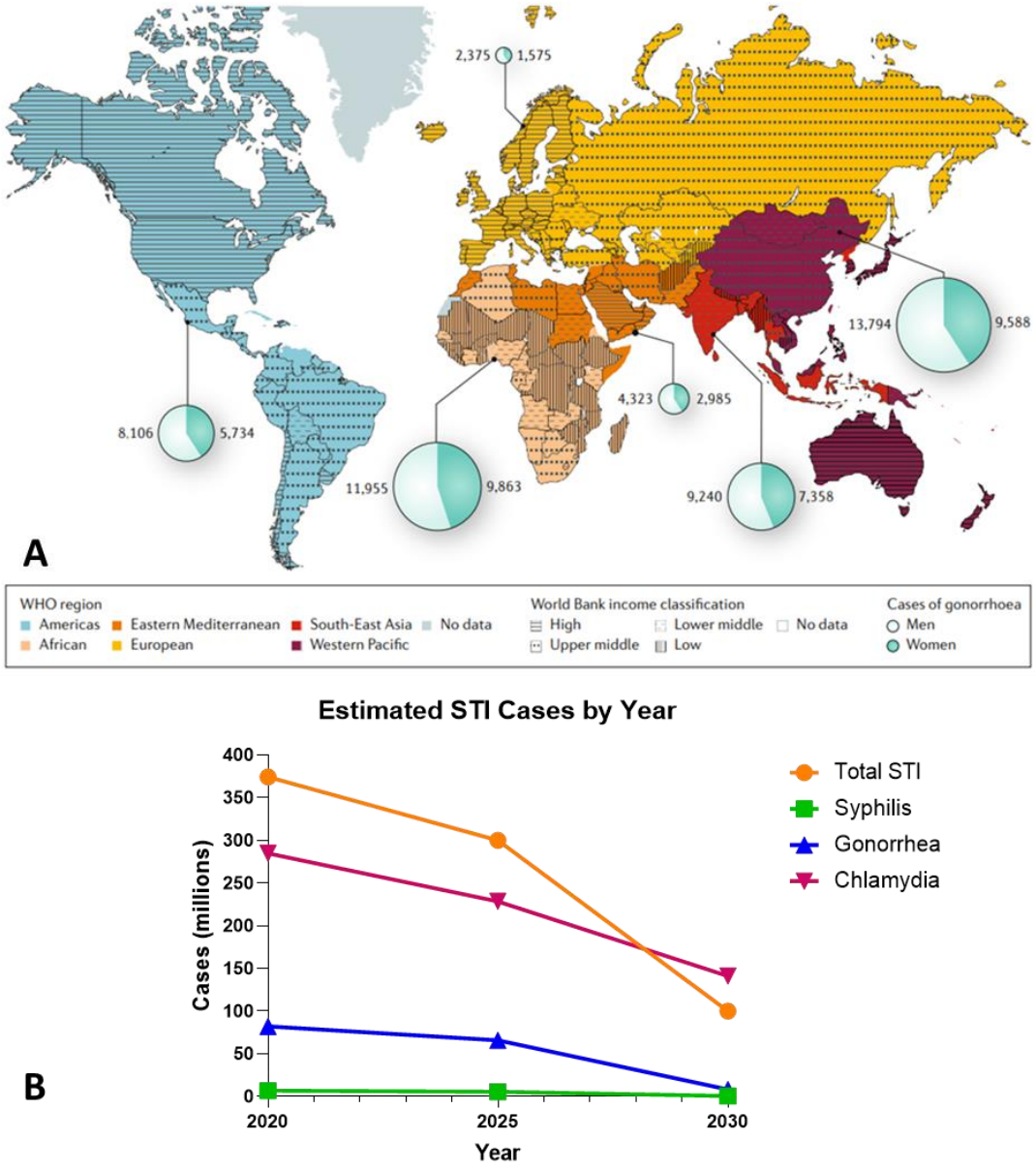

Figure 1. **Estimated new global cases of gonorrhoea in 2016**. **A)** Approximate numbers (in millions) of incident cases of gonorrhoea in (15–49 years of age) by WHO region (Rowley et al. 2019). These statistics equate to 20 new cases of gonococcal infections for every 1,000 women and 26 for every 1,000 men worldwide. The African region, as per WHO, recorded the highest incidence with 41 cases per 1,000 women and 50 per 1,000 men. This was followed by the Americas region under WHO, with 23 cases per 1,000 women and 32 per

1,000 men. The European region, according to WHO, had the lowest incidence with 7 cases per 1,000 women and 11 per 1,000 men. The World Bank Income Classification is also shown (Rowley et al. 2019; Unemo et al. 2019). **B)** Incidence of STIs from 2020 to 2030, indicating a significant decline in syphilis, gonorrhea, and chlamydia cases. The graph is based on data from a WHO document. (Beno, Silen, and Yanti 2022).

In the late 1980s, countries, particularly industrialized ones, saw a decline in gonorrhea rates due to prevention and care strategies based on established determinants of STIs. However, this decline was temporary, and rates have been increasing since the late 1990s. *Factors* contributing to high STI rates include ethnicity, economic disparities, sexual preferences and access to assistance, as well as the characteristics of the pathogen itself (Aral, Fenton, and Holmes 2007; Fenton and Lowndes 2004; Ito, Yamamoto, and Morita 2021; Mohammed et al. 2018). The **Figure 2** provides disaggregated information on new cases of STIs by sex.

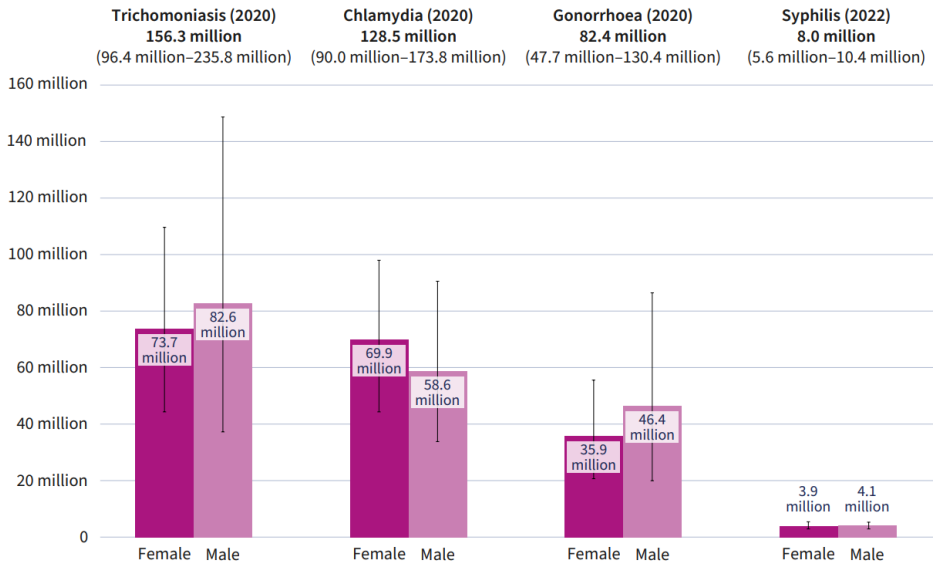

Figure 2. **Distribution of new cases of four curable STIs among adults (aged 15–49 years), by sex, global, 2020 and 2022**. Sources: Global progress report on HIV, viral hepatitis, and sexually transmitted infections, 2021 (32). Global HIV, Hepatitis and STIs Programs (HHS), WHO, 2024.

The escalation in gonorrhea case rates over the past 5-10 years can be attributed to a multitude of factors, as evidenced in both well-developed and emerging health systems.

Specifically, in regions such as the United States (US) and the European Union/European Economic Area (EU/EEA), there is a discernible correlation between gonococcal infection rates and determinants such as socioeconomic status and ethnic background. This correlation underscores the multifaceted nature of gonorrhea's epidemiology and the necessity for comprehensive, context-specific public health strategies (Unemo et al. 2019). In the United States (US) in 2017 there was a significant disparity in the incidence of reported gonorrhea cases, with the rate being approximately eightfold higher in Black populations compared to their White counterparts. Elevated rates were also documented among American Indians, Native Hawaiians, Alaskan Natives and Hispanic descent. Furthermore, a concerning trend was observed over a four-year period from 2013 to 2017, during which the total number of gonorrhea cases in the United States escalated by 67% (Unemo et al. 2019; Uprety and Cárdenas 2017). Furthermore, the data from 1989 to 2017 showed that the percentage of gonococcal isolates recovered from men who have sex with men (MSM) in the US increased from 3.9% to 38.5% (Ito, Yamamoto, and Morita 2021). Within the EU/EEA, there has been a substantial surge in reported gonorrhea cases, with an increase exceeding 200% since 2008. The demographic most affected by STIs is young adults, specifically those aged 15-24 years. Furthermore, a significant proportion of these cases, approximately 25-30%, has been attributed to men who have sex with men (MSM) in recent years (Fenton and Lowndes 2004; Mohammed et al. 2018). The current data indicates a concerning trend: the global community is not on course to achieve the set targets for reducing sexually transmitted infections (STIs). Contrary to the goal of decreasing rates, we are witnessing an escalation in new infections. This trend is particularly pronounced in high-income countries that have robust surveillance systems in place, where the notification rates for STIs, including gonorrhea, are on an upward trajectory. This highlights the urgent need for reassessment and reinforcement of our current strategies to combat STIs (**Fig. 3**),(Annabel Baddely; Rachel;

Baggaley; Maeve Brito De Mello; Meg Doherty; Boniface; Dongmo Nguimfack; Philippa Easterbrook; Nathan Ford; Cheryl Case Johnson; Olufunmilayo Lesi; Niklas Luhmann; Ismail Maatouk; Antons Mozalevskis; Busisiwe Msimanga; Morkor Newm 2024).

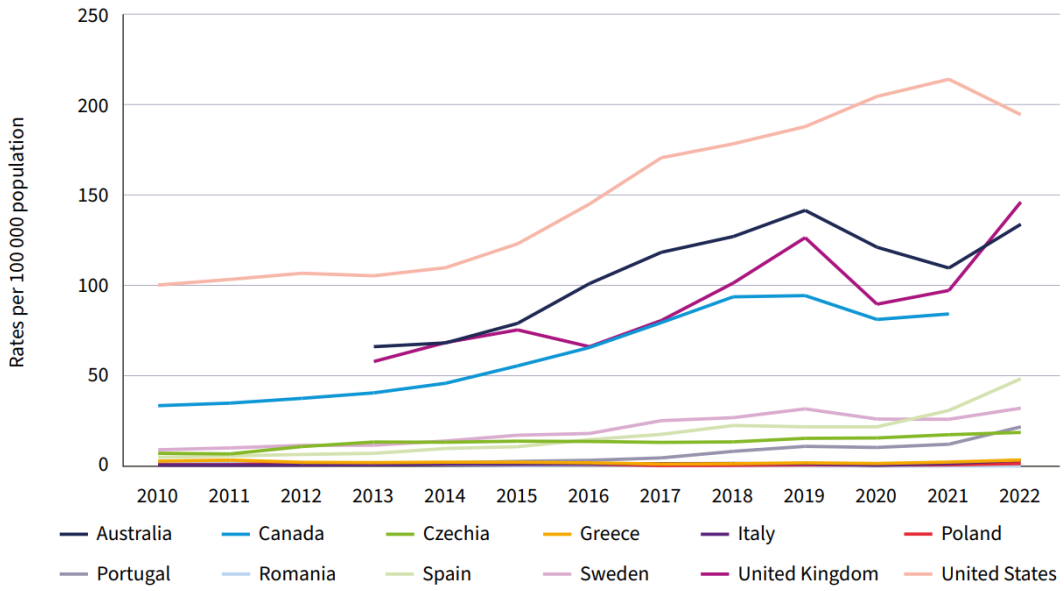

Figure 3. Gonorrhoea case notification rates per 100 000 population in selected high-income countries, 2010–2022. The provided sources include annual surveillance reports and online databases from various regions, detailing the prevalence and rates of sexually transmitted infections (STIs). These sources are: 1. Australia: The Kirby Institute's annual surveillance report on HIV, viral hepatitis, and STIs in 2023. 2. Europe: The Surveillance Atlas of Infectious Diseases online database by the European Centre for Disease Prevention and Control in 2024. 3. Canada: The Public Health Agency of Canada's online database on notifiable diseases in 2024. 4. United Kingdom: The UK Health Security Agency's data on new STI diagnoses in England from 2013 to 2022. 5. United States: The Centers for Disease Control and Prevention's report on STIs from 1941 to 2022.These sources provide comprehensive data on the incidence and trends of STIs in these regions.

The WHO is actively partnering with various entities to accelerate the creation of innovative tools against sexually transmitted infections (STIs). This collaboration has led to the development of numerous new products for STI prevention, diagnosis, and treatment, many of which have recently completed or are nearing the end of their clinical trials. These include outer membrane vesicle-based meningococcal B vaccines against gonorrhoea. Whole Genome Sequencing (WGS) is transforming our understanding of gonorrhea epidemiology. This technology enables the tracking of how antimicrobial resistance spreads over time and

geography, as well as the identification of N. gonorrhoeae strains that remain susceptible to treatment. It is particularly valuable for studying various populations and subpopulations, including those at higher risk. (Unemo et al. 2019).

2.2.1. Antimicrobial Resistance (AMR)

Antimicrobial resistance is a global public health concern. National surveillance of gonococcal antimicrobial resistance has expanded, with 74 countries reporting data in 2021 to the WHO Gonococcal Antimicrobial Surveillance Program on the proportion of gonococcal isolates with decreased susceptibility or resistance to different drugs **Fig.4.**

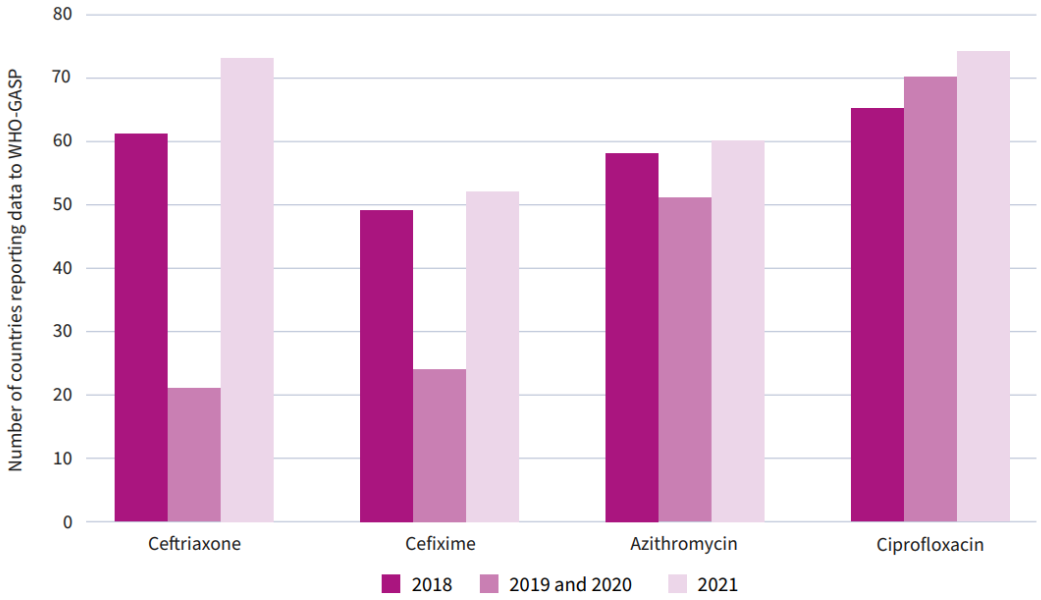

Figure 4. Gonococcal Antimicrobial Surveillance Program. Number of WHO Member States reporting data on gonococcal antimicrobial susceptibility or resistance to the WHO Gonococcal Antimicrobial Surveillance Programme (WHO-GASP) for four drugs used to treat gonorrhoea, 2018–2021.

WHO has enhanced gonorrhoea antimicrobial resistance surveillance in 14 sentinel countries across all six WHO regions, along with an additional 73 countries capable of tracking the emergence and spread of resistance to the last-line treatment for gonorrhoea. Nine countries reported decreased susceptibility to ceftriaxone, the last-line treatment for gonorrhoea, at

levels of 5% and above. The monitoring of patterns of resistance in gonorrhoea – through regular collection and analysis of data on the susceptibility of gonorrhoea to various antibiotics over time, identifying shifts in resistance patterns and sequencing the genomes of gonorrhoea – supports the change in global and national guidelines. This can mitigate further antimicrobial resistance development and spread. It will also inform the strategic positioning of new gonorrhoea treatment and point-of-care tests for STI antibiotic stewardship. (Annabel Baddely; Rachel; Baggaley; Maeve Brito De Mello; Meg Doherty; Boniface; Dongmo Nguimfack; Philippa Easterbrook; Nathan Ford; Cheryl Case Johnson; Olufunmilayo Lesi; Niklas Luhmann; Ismail Maatouk; Antons Mozalevskis; Busisiwe Msimanga; Morkor Newm 2024).

Various authoritative reports from organizations such as the WHO and the CDC clearly classify gonococcus as an urgent pathogen. This classification indicates that current available therapies are becoming increasingly ineffective due to the emergence of antibiotic resistance. Consequently, the urgency to develop a specific vaccine for gonococcus is more pressing than ever. However, as previously mentioned, the WHO currently recommends the meningococcal vaccine (4CMenB, Bexsero) as a preventive measure. This approach underscores the importance of integrated prevention strategies to combat the spread of antibiotic-resistant sexually transmitted infections. The principal molecular strategies employed by bacteria to advance in antimicrobial resistance mechanism is through protective changes to antibiotic targets, reduced antibiotic intake, increased antibiotic expulsion via efflux pumps, and the production of antibiotic-inactivating enzymes. The action of almost all major classes of antibiotics is inhibited through *N. gonorrhoeae* in multiple mechanisms, as depicted in **Figure 5**.

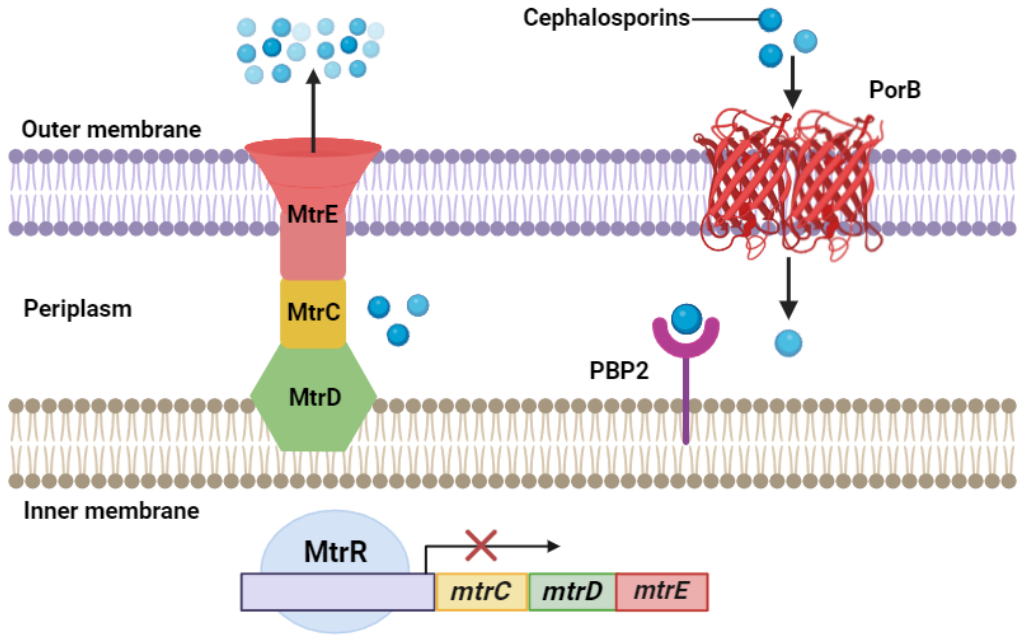

Figure 5. *N. gonorrhoeae* in Antibiotic resistance. The causative agent of gonorrhea has evolved significant antibiotic resistance via several mechanisms. These include mutations in the penA gene, encoding the penicillin-binding protein 2 (PBP2) targeted by cephalosporins, and the operation of the MtrC–MtrD–MtrE efflux pump and its repressor MtrR. Additionally, the PorB porin protein, encoded by PorB, contributes to resistance but requires a concurrent mtrR mutation. These intricate resistance mechanisms highlight the need for continued research and novel therapeutic approaches. The illustration is inspired by (Quillin and Seifert 2018) and created with BioRender.com.

Considerable investigation has been conducted into the mechanisms of β-lactam resistance in *N. gonorrhoeae*. The periplasmic transpeptidase, penicillin-binding protein 2 (PBP2), encoded by the penA gene, is the primary target of cephalosporins; a majority of resistant strains exhibit mosaic mutations in penA (Quillin and Seifert 2018; Zhao et al. 2009). The MtrC–MtrD–MtrE pump and its repressor, MtrR, play a role in *N. gonorrhoeae* resistance via antimicrobial efflux (Quillin and Seifert 2018; Zhao et al. 2009). Variations of the key porin protein, encoded by PorB, contribute to β-lactam resistance, but concurrent mutation in mtrR is necessary for resistance (Hagman et al. 1995; Quillin and Seifert 2018). It is essential to note that strains of *N. gonorrhoeae* suspected of multi-resistance undergo genotyping procedures. This is achieved through techniques such as multilocus sequence

typing (MLST) and multi-antigen sequence typing (NG-MAST). These methodologies facilitate the genetic characterization of the strains, thereby enhancing our understanding of their transmission dynamics and patterns of antibiotic resistance. This will not only enable the development of new therapies but also enhance our understanding of how resistance persists within a population and spreads among different strains (Quillin and Seifert 2018).

2.3. *N. gonorrhoeae* host adaptation and pathogenesis

As *Neisseria gonorrhoeae* colonizes genital, rectal, oral and ocular mucosa, it expresses a repertoire of factors that enable replication and survival in these environmental niches and modulate and evade the host immune system (Quillin and Seifert 2018).

Infections caused by *N. gonorrhoeae* can manifest as either symptomatic or asymptomatic in both genders, with a higher prevalence of symptomless infections observed in females (Unemo et al. 2019). However, it's worth noting that asymptomatic infection rates in males can reach up to 40% (Martín-Sánchez et al. 2020). The substantial occurrence of symptomless infections due to gonococcus often results in individuals being unaware of their infection status, thereby escalating the likelihood of transmission and spread. Furthermore, the presence of a gonococcal infection can enhance the susceptibility of an individual to contract other sexually transmitted diseases, including HIV (Little 2006; Masi and Eisenstein 1981; Quillin and Seifert 2018; Sandstrom 1987). The outcome of asymptomatic infections remains largely unknown due to the lack of treatment-seeking behavior in these individuals. However, there are indications that such infections typically resolve on their own within a few months, although in rare cases, the infection can persist for several years.

Gaining a deeper understanding of the strategies employed by *N. gonorrhoeae* to interact with and evade the host's immune response is crucial. This knowledge will aid in improving infection prevention measures, developing more accurate diagnostics, enhancing surveillance, and facilitating the creation of vaccines and novel therapeutic approaches (Quillin and Seifert 2018).

2.3.1. Symptomatic and asymptomatic infections

Differences in the developmental and embryological origins of cells lining the urogenital tracts of men and women have endowed these microenvironments with different surface molecules that act as receptors and co-receptors for *N. gonorrhoeae* and lead to differences in the mechanisms by which Ng survives in the male and female urogenital niches (Jennifer L. Edwards and Apicella 2004). Gonorrhea can affect symptoms in the genitals, anus, or throat, appearing 1-14 days after exposure. In men there is a purulent exudate from the penis and resultant painful urination. Women often have no symptoms, but if present, they may include pain during urination, irregular bleeding, or vaginal discharge. All genders can experience anal infections, leading to discharge, bleeding, itchiness, and discomfort. Throat infections are often symptomless, but can cause redness, pain, and a sore throat. Infants born to infected mothers may develop eye infections, preventable with appropriate medication (Johnson 2008).

Symptomatic infection is characterized by a robust inflammatory response and a purulent discharge composed almost entirely of gonococci and recruited polymorphonuclear leukocytes (PMNs; neutrophils), (Criss and Seifert 2012; Rotman and Seifert 2014; Shafer

and Rest 1989). Gonococci can ascend to the upper genital tract, leading to serious diseases, such as epididymitis in men and cervicitis, endometriosis, and pelvic inflammatory disease (PID) in women (a major cause of infertility).

Gonococcal PID is complication of cervical infection, leading to significant issues such as chronic pain, ectopic pregnancy, and infertility in women. PID refers to inflammation of the female upper genital tract (UGT), including conditions like salpingitis, endometritis, and others (Quiroz 1999).

In rare cases, gonococci can disseminate to the blood stream from the initial site of infection, causing disseminated gonococcal infection (DGI) and the associated complications of arthritis and endocarditis (Braxton et al. 2017; Hasdiana 2018).

Such *N. gonorrhoeae* infections most frequently result in urethritis in men and cervicitis in women, but urethritis in women is also observed (Schmale, Martin, and Domescik 1969).

2.3.2. Transmission

The process of transmission is often the least explored aspect of infections, and this holds true for Ng infections as well. For a pathogen to be successful, it must have the ability to transmit effectively to new hosts. Ng is an obligate human colonizer, it cannot survive outside its host (Gottlieb et al. 2020; Quillin and Seifert 2018; Unemo et al. 2019; Whelan et al. 2021). The spread of this pathogen from one host to another is heavily reliant on sexual networks, which facilitate the movement of the pathogen from the central, high-risk population, where most infections occur, to the peripheral, medium-risk group. This group then transmits Ng back to the central group and to their respective partners. High-risk

populations typically comprise individuals who engage in unprotected sex with multiple partners. Often, individuals are not aware that they are part of a larger sexual network (H. A. Harvey et al. 2000; Papp et al. 2014). Ng has the ability to attach to sperm and can be easily transmitted from men to their partners through ejaculates, which usually contain a high concentration of bacteria (Cohen et al. 1994; James-Holmquest et al. 1974). However, the mechanism through which efficient transmission from women to their partners is achieved is less clear. It is believed that for Ng to successfully bind to and enter the urethral epithelial cells in men, its surface must be devoid of sialic acid. This suggests that bacterial sialidases, secreted by the cervicovaginal microbiota in women, must first remove sialic acid from *N. gonorrhoeae* lipooligosaccharide (LOS) to enable efficient transmission from women to men (Ketterer et al. 2016).

2.3.3. Establishment of infection: Adherence, colonization, and invasion: the gonococcal antigens

Upon transmission, N. gonorrhoeae initiates interaction with the mucosal epithelium, where it replicates and prepares for spread to additional hosts. *N. gonorrhoeae* is predominantly a colonizer of the mucosa, adhering to various epithelial surfaces: Urethral , Cervical , Endometrial , Fallopian tube , Rectal , Pharyngeal and Conjunctival epithelial cells (Little 2006; Quillin and Seifert 2018). The initial event that sets the stage for infection and the first step in pathogenesis is the adherence of bacteria to the mucosal epithelium. This adherence is facilitated by specific bacterial surface structures (**Fig.6**), such as type IV pili, opacity (Opa) proteins, lipooligosaccharide (LOS), and the principal outer membrane protein porin (PorB).

In the early stages of infection, the attachment and subsequent colonization of Ng largely rely on the formation of type IV pili microcolonies on the surface of the epithelial cell (Craig, Pique, and Tainer 2004). Type IV pili are outer membrane structures that play an essential role in facilitating early cellular adherence, twitching motility, and in evading the immune system through changes in antigenic properties and phase variation (Edwards, and Apicella, 2004; Higashi et al. 2007).

The process of adhesion to the epithelial surface, mediated by the pilus, allows the gonococci to approach the cell surface closely. The interplay between Opa proteins and carcinoembryonic antigen-related cell adhesion molecule (CEACAM) receptors, along with other molecules such as heparin sulfate, plays a significant role in ensuring adherence (Jerse et al. 1994). The interaction involving Opa and CEACAM could be one of the primary interactions facilitating adherence (Simms and Jerse 2006; Qigui Yu et al. 2013). Opa proteins, which are outer membrane proteins, are responsible for facilitating adherence after the initial contact made by type IV pili. They also assist in avoiding the immune system by causing multigene phase variation, leading to changes in antigenic properties (Stern et al. 1986). Both type IV pili and Opa proteins are expressed during the infection in all genders and are deemed crucial for the colonization of the mucosal epithelium of the genital tract and other infection sites.

Key surface elements, such as porin and LOS, significantly influence colonization. Porin, a channel for nutrients, is one of the most copious proteins found in the outer membrane of gonococci. It has the ability to bind complement factors like C4b-binding protein (C4BP) and factor H, and it can inhibit the oxidative burst of neutrophils and prevent neutrophil apoptosis (R. A. Jones, Jerse, and Tang 2024). LOS is the predominant component of the outer membrane of Ng, it plays a

fundamental role in the bacterium's ability to adhere to and invade host cells. Structurally, LOS comprises lipid A, a core oligosaccharide composed of heptose sugars and other sugars, which contribute to the overall structure and function (e.g., HepI and HepII), and variable glycan chains, including lacto-N-neotetraose (LNnT) and globotriose, which mimic host glycans, allowing the bacterium to evade immune detection. Sialylation of LOS, achieved by scavenging CMP-N-acetylneuraminic acid from the host, inhibits complement activation and fosters serum resistance. (Ketterer et al. 2016). In addition to colonizing the mucosal epithelium, Ng can penetrate epithelial cells, particularly targeting non-ciliated cervical and urethral epithelial cells, especially when LOS is desialylated (Edwards, and Apicella, 2004). The interaction between LOS and asialoglycoprotein receptors can facilitates invasion in the male urethra, while complement receptor 3 (CR3) is involved in invasion within the lower cervical genital tract (Quillin and Seifert 2018).

To thrive in the dynamic environments of the host's genital tract, rectum, and oropharynx, Neisseria gonorrhoeae must rapidly adapt to varying conditions (Quillin and Seifert 2018). These mechanisms enable the bacterium to respond effectively to environmental variables, including metal availability, reactive oxygen species, and antimicrobial peptides. Transcriptional regulators, such as MtrR, MtrA, and MpeR, play pivotal roles in controlling the expression of the MtrC–MtrD–MtrE efflux pump, which is essential for the export of antimicrobial peptides (**Fig. 6**), (Zalucki, Dhulipala, and Shafer 2012). Additionally, N. gonorrhoeae can switch between aerobic and anaerobic respiration via a truncated denitrification pathway, regulated by copper-dependent enzymes and oxygen-sensing proteins. Iron homeostasis is maintained by the ferric uptake regulator (LpbA–LpbB, HpuA–HpuB, and TbpA–TbpB), underscoring the complexity of its adaptive regulatory networks (Mercante et al. 2012).

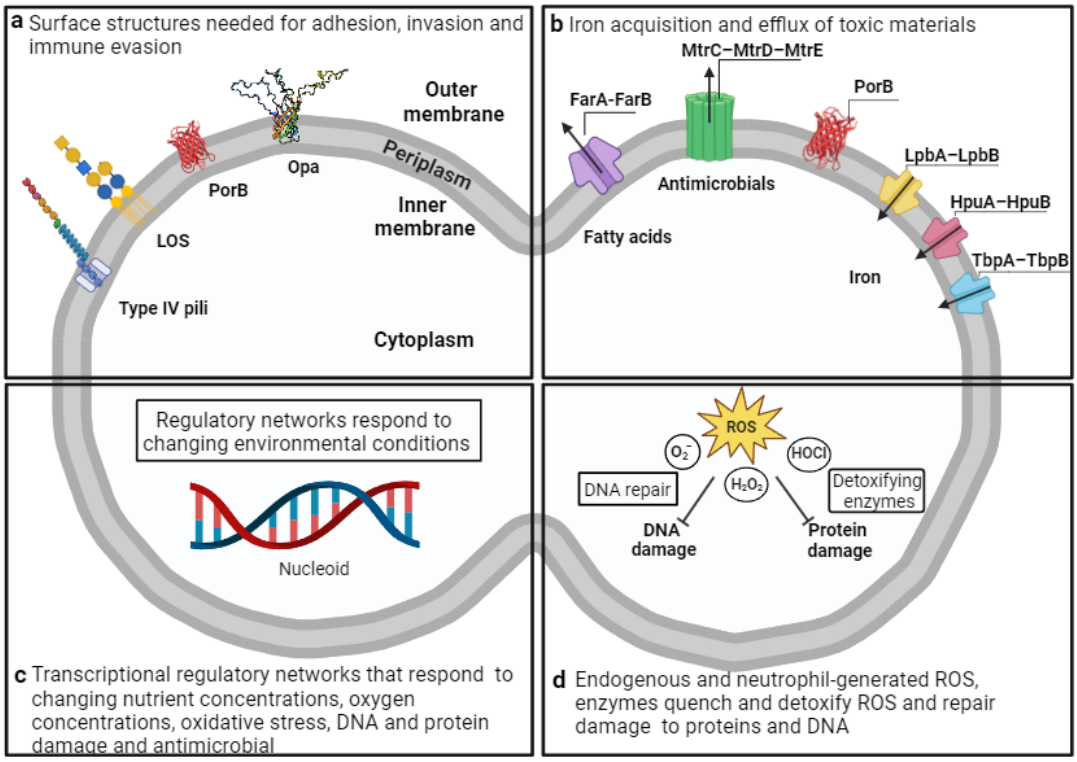

Figure 5 ***Neisseria gonorrhoeae*** **pathogenesis factors**. As host-restricted pathogen, Ng has a limited set of pathogenic and colonization factors compared to other Gram-negative bacteria. It uses surface structures like type IV pili, LOS, porin, and Opa proteins to adhere to, occasionally invade host cells, and evade the immune system. Efflux pumps protect Ng from antimicrobials and fatty acid stress, while membrane transporters help it acquire nutrients. The FarA–FarB and MtrC–MtrD–MtrE pumps manage fatty acid transport and antimicrobial peptides, respectively. The LpbA–LpbB, HpuA–HpuB, and TbpA–TbpB complexes are involved in iron transport and balance. Transcriptional regulators induce programs to adapt to changing environmental conditions during infection, with co-regulated regulons responding to iron levels, oxidative conditions, and oxygen concentration. Protective enzymes like catalase and MsrA–MsrB neutralize reactive oxygen species, including those produced by neutrophils. The illustration is inspired by (Quillin and Seifert 2018) and created with BioRender.com.

2.4. *N. gonorrhoeae* interactions with epithelial cells

2.4.1. The male urethral tract

In men, gonococcal infection frequently manifests as acute urethritis, a condition that arises from the inflammatory reaction to the invading gonococci (Ramsey et al. 1995). A primary symptom often used to diagnose gonorrhoea in men is typically the presence of a discharge

resembling pus, which is linked to the invasion of neutrophils and the sloughing off urethral epithelial cells (Sintsova et al. 2015). Research involving human subjects has established an incubation period of up to 40 hours from the point of infection to the emergence of clinical symptoms, during which gonococci are not cultivable from the male urethra (Schneider et al. 1995). The hypothesis is that gonococci infiltrate the safeguarded system of epithelial cells early in the disease process, which facilitates both their survival and replication (Edwards, and Apicella, 2004). As known, pili are responsible for the initial cell-to-cell contact crucial for colonization. Despite the known necessity of pili expression for invasion, the receptor interacting with pili during male infection has not been identified *In vivo*.

In vitro, recognized receptors for pili include complement receptors such as CD46 and CR3, along with other integrins that contain the I-domain, like the α1β1 and α2β1 integrins. The presence of CR3 is not observed inside the male urogenital epithelium, suggesting that CD46 and other I-domain-containing integrins could be potential interesting to be studied(Gill et al. 2003; Gill and Atkinson 2004). Accordingly, *in vitro* interactions between gonococcal pili and male urethral epithelial cells have shown a predilection for binding through the I-domain-containing integrins, α1β1 and α2β1, across CD46. This preference plays a crucial role in initiating the adherence and colonization of gonococci (Jennifer L. Edwards and Apicella 2005). The invasion of male urethral epithelial cells by gonococci is made possible through the interactions between the asialoglycoprotein receptor (ASGP-R) and LOS located on the gonococcal cell membrane. This interaction leads to the creation of an actin pedestal beneath the bacterium (Apicella et al. 1996; Hillery A. Harvey et al. 1997). Once the pedestal is formed, the bacterium is endocytosed via the ASGP-R in a process that is both actin-(Giardina et al. 1998) and clathrin-dependent (Hillery A. Harvey et al. 1997). This process

subsequently triggers rearrangements in the cytoskeleton, further facilitating the invasion and transcytosis of gonococci.

It's noteworthy that the interaction between Opa proteins and heparan sulfate proteoglycan (HSPG) could play a substantial role in the male urogenital tract. This supposition is backed by data from the Human Protein Atlas, which shows the expression of the HSPG2 receptor in this area. This potential interaction warrants further exploration, as it could offer new insights into the pathogenesis of *Neisseria gonorrhoeae*. (Tissue expression of HSPG2 - Summary - The Human Protein Atlas)

2.4.2. The female reproductive tract

In most cases, Ng initiates infections of the mucosal surfaces in the urogenital tract, with a great affinity for the columnar and transitional epithelial cells. Nonetheless, Ng is also able to adhere to the stratified squamous epithelium found in the ectocervix (J. L. Edwards et al. 2000; Qian Yu et al. 2019).

Gonococcal interactions with epithelial cells in the female reproductive tract (FRT) exhibit complex mechanisms that differ between the lower genital tract (LGT) and the upper genital tract (UGT) (Alzamil, Nikolakopoulou, and Turco 2021; Chumduri and Turco 2021; Epshita A. Islam et al. 2018). The female reproductive tract is composed of a diverse array of epithelial cell types. This includes non-polarized squamous epithelial cells in the vagina and ectocervix, collectively referred to as the LGT. These areas are structured with multiple layers of flattened cells, providing a form of mechanical defense. The endocervix, uterus, and fallopian tubes, collectively known as the UGT, are characterized by a single layer polarized columnar cells.

These cells are responsible for the production of mucus and facilitate the transportation of sperm and ova (Prendiville and Sankaranarayanan 2017). The cellular composition of the female genital tract is complex, with a variety of receptors that are instrumental for the development of *N. gonorrhoeae* infections. This includes cellular junctions like tight junctions (TJ), adherens junctions (AJ), and desmosomes (D), all of which are vital for preserving the integrity of the epithelial tissue (Blaskewicz, Pudney, and Anderson 2011). Cellular junctions in the female genital tract play a crucial role in maintaining epithelial integrity and regulating the passage of molecules and cells through the intercellular space (**Fig. 7**).

Tight Junctions (TJ)

- Structure and Function: Composed of proteins such as claudins, occludins, and ZO-1, forming a nearly impermeable barrier between epithelial cells. Maintain the cell polarity and regulate the passage of solutes between cells.
    - **Claudins and Occludins**: These proteins are critical for TJ function, creating a selective barrier that controls the movement of molecules and ions through the paracellular space.
    - **ZO-1** (**Zonula Occludens-1**): Binds to claudins and occludins, contributing to the stability and organization of TJs.

Adherens Junctions (AJ)

- Structure and function: Composed primarily of cadherins such as E-cadherin, which are linked to the actin cytoskeleton via anchoring proteins like catenins. Mediate cell-cell adhesion and maintain tissue cohesion and stability.

- **E-Cadherin**: Crucial for AJs, this protein plays a key role in epithelial tissue integrity and cell signaling.

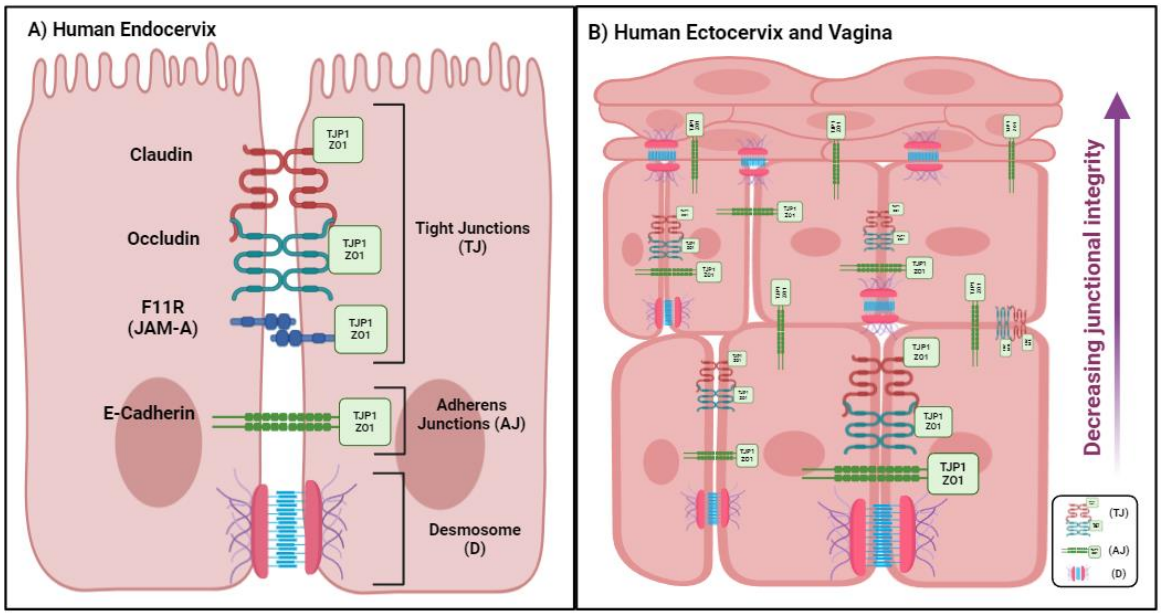

Figure 6. Localization of Selected Interepithelial Adhesion Molecules in the Female Lower Genital Tract. **A)** The endocervical epithelium is characterized by the presence of tripartite junctions. Tight junctions (TJ), found near the apical region, play a crucial role in sealing the epithelial layer and maintaining cellular polarity. Located just below these tight junctions, adherens junctions (AJ) are essential for cell-cell adhesion, featuring E-cadherin as a principal component, which is linked to the actin cytoskeleton through proteins such as vinculin, and alpha and beta catenins. Desmosomes (D), situated at the most basal level, provide the tissue with mechanical strength and resilience. The extracellular elements of desmosomes, desmoglein and desmocollin, interact with intermediate filaments of adjacent cells via a complex intracellular scaffolding system. **B)** In the stratified squamous epithelium of these areas, most TJ are present in the parabasal layer, immediately above the basal cells that contact the basement membrane. AJ is particularly numerous in this region. As epithelial cells move towards the apical surface, the integrity of these junctions diminishes. Eventually, these cells become cornified, lose all intercellular connections, and are shed into the lumen. The illustration is inspired by (Blaskewicz, Pudney, and Anderson 2011) and created with BioRender.com.

The Squamous Epithelium, which constitutes the Ectocervix and Vagina, is characterized by:

- **CEACAM 5** (Carcinoembryonic antigen-related cell adhesion molecule): This receptor mediates the adhesion of gonococci via interaction with Opa proteins (Epshita A. Islam et al. 2018) .

- **HSPG** (Heparan Sulfate Proteoglycans): These receptors interact with Opa proteins, further facilitating bacterial adhesion (Qian Yu et al. 2019).
- **Complement Receptor** (**CR3**): There is an integrin heterodimer organized of an alpha (αM or CD11b) and a beta (β2 or CD18) subunit. The primary ligand for CR3 is the complement (C0) inactivation product, iC3b. Can be bound by pili to facilitate bacterial invasion into host cells, allowing *N. gonorrhoeae* to evade the immune response and establish within tissues (Jennifer L. Edwards et al. 2001).

The Columnar Epithelium, which constitutes the Endocervix and Uterus, is characterized by:

- **CEACAM1** (Carcinoembryonic antigen-related cell adhesion molecule): This receptor mediates the adhesion and penetration of gonococci via interaction with Opa proteins (Epshita A. Islam et al. 2018).
- **HSPG** (Heparan Sulfate Proteoglycans): These receptors interact with Opa proteins, further facilitating bacterial adhesion.
- **Integrins** (**α5β1**, **αVβ3**): These integrins are transmembrane receptors, which interact with gonococcal pili, promoting bacterial adhesion to the epithelial surface. This interaction also activates intracellular signaling pathways that facilitate bacterial invasion
- **Iron-Binding Protein Receptors** (**Transferrin**, **Lactoferrin**): These are essential for gonococcal iron acquisition, which is crucial for Ng growth.

2.4.3. Hormonal influence on immune response and pathogen interactions in FRT

A crucial aspect to consider in the understanding and characterization of sexually transmitted diseases is how the female genital tract undergoes continuous changes, driven by hormones, throughout the distinct phases of the menstrual cycle. The menstrual cycle involves the regulation by pituitary and ovarian hormones, which lead to morphological changes in both the ovarian surface epithelium (OSE) and the endometrium. The ovaries are located near the fimbriae of the fallopian tubes and are held in place by the ovarian ligaments attached to the uterus. They perform two key roles: producing and releasing mature oocytes into the fallopian tubes and functioning as endocrine glands by secreting estrogen (E2) and progesterone (P4). These hormones regulate the cyclical changes in the uterine lining (endometrium), playing a crucial role in fertility and overall health of the female reproductive tract. Histologically, the ovaries consist of an outer cortex and an inner medulla. The cortex houses follicles, which contain oocytes and supporting follicular cells, while blood vessels and lymphatics are present in the medulla. Ovarian function is regulated by the hypothalamic-pituitary-ovarian (HPO) axis. At the start of each menstrual cycle, follicle-stimulating hormone (FSH) from the pituitary gland promotes follicle maturation. These follicles produce E2 and inhibin, which in turn inhibit further FSH production through a negative feedback loop. Rising E2 levels then trigger the release of luteinizing hormone (LH), leading to ovulation, where the oocyte is released from the ovarian surface epithelium (OSE) (Prendiville and Sankaranarayanan 2017; Schomberg 1978).

The menstrual cycle (**Fig. 8**) consists of three phases: menstrual, proliferative, and secretory. During the proliferative phase, FSH stimulates the growth of ovarian follicles, which in turn

increase the production of estrogen E2. Elevated estrogen levels promote the proliferation of the functional layer of the endometrium. Around mid-cycle, the surge in E2 causes a spike in luteinizing hormone LH, leading to ovulation, where the oocyte is released. Following ovulation, the secretory phase begins, characterized by the dominance of P4, and induces the differentiation, or decidualization, of the endometrium, preparing it for potential implantation. If implantation does not occur, progesterone levels decline, triggering menstruation. This process involves the shedding of the functional layer of the endometrium, while the basal layer remains intact to facilitate the regeneration of the endometrium in the next cycle. (Auersperg et al. 2001; MICHA 2017; Ng et al. 2014).

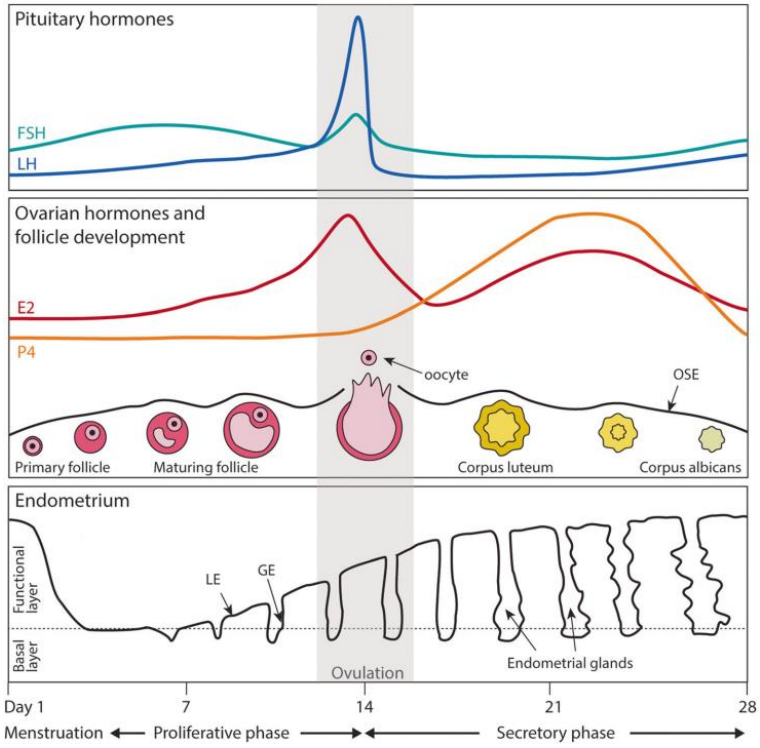

Figure 7. **The menstrual cycle comprises three phases: proliferative, secretory, and menstrual.** During the proliferative phase, FSH stimulates the growth of ovarian follicles, resulting in increased estrogen (E2) levels that promote endometrial proliferation. Mid-cycle, a peak in E2 triggers an LH surge, leading to ovulation. The secretory phase follows, dominated by progesterone (P4), which prepares the endometrium for implantation. If implantation does not occur, P4 levels drop, causing the functional layer to shed during menstruation, while the basal layer remains intact (Chumduri and Turco 2021).

Estrogen and progesterone (**Fig. 8**), the primary female sex hormones, have significant effects on the immune cells in the female reproductive tract, which can influence susceptibility to infections such as gonorrhea. E2 is able to modulate the function and trafficking of various immune cells, including T cells, B cells, neutrophils, macrophages, and dendritic cells. In the context of the lower female reproductive tract, estrogen appears to inhibit neutrophil trafficking in the vagina. It also influences the production and composition of gel-forming mucins, which can impact vulnerability to specific pathogens during pregnancy and at different stages of the menstrual cycle. Although the P4 promotes neutrophil trafficking in the vagina, which can enhance clearance of certain pathogens. It also limits the activation of dendritic cells and promotes the differentiation of regulatory T cells. Infections caused by microbes pose a significant risk to women's reproductive health. Despite the fact that sex hormones regulate reproductive cycles and pregnancy (**Fig. 9**), their influence on host-pathogen interactions and immune function in the female reproductive tract is not well-studied (Collins, McCutcheon, and Petroff 2022). Moreover, the fluctuating endocrine environment throughout pregnancy could affect the susceptibility of women to ascending infections at different times. Bacterial vaginosis, characterized by the dominance of anaerobic bacteria, leads to chronic community dysbiosis and increases the risk of coinfection with sexually transmitted pathogens (Breshears et al. 2015).

Understanding how hormonal changes affect the balance between *Lactobacillus* and the vaginal mucosa is key to understanding vulnerabilities to pathogenic microorganisms that compete with *Lactobacillus i*n the lower reproductive tract (Chee, Chew, and Than 2020). *Lactobacillus* spp. utilize several strategies to inhibit pathogenic competition. They convert glycogen, deposited by vaginal epithelial cells, into lactic acid, thereby maintaining a low physiological pH that is unfavorable for pathogens such as *G. vaginalis* and *N. gonorrhoeae*.

The isomer of lactic acid is also likely significant, with species producing D-lactic acid, but not L-lactic acid, offering protection against *C. trachomatis* infection (Jordan and Geisler 2017). Given that most intrauterine microbial infections originate in the lower reproductive tract, it is crucial for future research to determine how various hormonal conditions affect the susceptibility of the lower reproductive tract to infection. This will help to understand the temporal aspects of infection susceptibilities throughout pregnancy.

- **Immune Modulation During High Progesterone Levels**

During the luteal phase (**Fig. 8**), elevated progesterone triggers several immunological modifications to optimize the female reproductive tract for potential embryo implantation. One key adaptation is an increase in luminal immunoglobulins (**Fig. 9**), which enhance pathogen defense while maintaining immune tolerance. Progesterone also promotes neutrophil recruitment via chemokines CXCL1 and CXCL2 (Collins, McCutcheon, and Petroff 2022; Schaefer et al. 2014), providing controlled immune protection. T cell differentiation shifts towards a Th2-dominant response, reducing inflammation by increasing anti-inflammatory cytokines like IL-4 and IL-10 (L. Xu et al. 2013). Additionally, macrophages adopt an M2 anti-inflammatory phenotype (Collins, McCutcheon, and Petroff 2022), facilitating tissue repair and supporting embryo implantation by limiting pro-inflammatory mediators such as nitric oxide (NO) and IL-12 (**Fig. 9**).

- **Immune Modulation During High Estrogen Levels**

In the estrogen-dominated follicular phase (**Fig. 8**), the immune landscape of the female reproductive tract becomes more robust, optimizing its defense against potential infections. Estrogen enhances mucosal barrier function by stimulating the production of MUC5B (**Fig. 9**), a mucin that reduces cervical mucus viscosity, facilitating sperm passage while trapping

pathogens. It also upregulates antimicrobial peptides, such as SLPI, HNP1, HNP2, lysozyme, and lactoferrin, which offer potent innate immune protection (Moriyama et al. 1999; Valenti et al. 2018; Yarbrough, Winkle, and Herbst-Kralovetz 2015). However, some peptides like HBD-2 and elafin decrease, reflecting a nuanced immune modulation. Dendritic cells become more efficient at antigen presentation during this phase, upregulating MHC class II molecules and costimulatory markers to boost T cell responses (Collins, McCutcheon, and Petroff 2022). This leads to heightened IL-6 and IL-12 production, enhancing immune protection. Meanwhile, macrophages remain in an M2-polarized state, contributing to tissue repair and anti-inflammatory activities while also playing a key role in iron metabolism, crucial for controlling bacterial growth. The immune response is further modulated by estrogen's suppression of Th17 cell activity, which reduces pro-inflammatory responses. Instead, T cells shift towards a Th2 phenotype, producing cytokines such as IL-4 and IL-10, favoring a less inflammatory, more tolerogenic environment (Krausgruber et al. 2011; Liu et al. 2012). The impact of estrogen on Treg cells, which are critical for maintaining immune tolerance, remains an area of ongoing investigation.

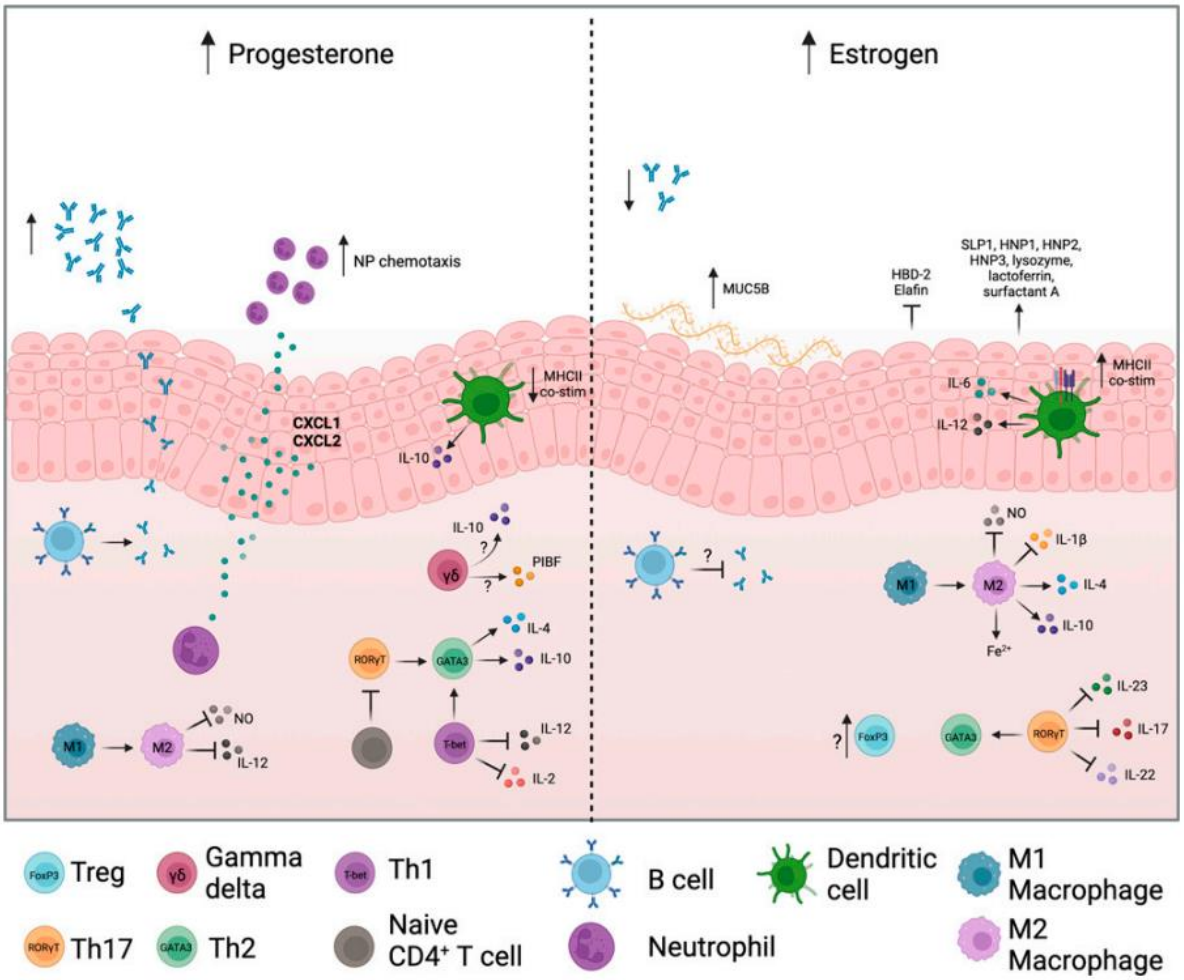

Figure 9. Modulation of the Immune Microenvironment in the Lower Female Reproductive Tract by Progesterone and Estrogen. Left Panel: For the duration of cycles of elevated progesterone, luminal immunoglobulin concentrations increase, and vaginal epithelial cells produce CXCL1 and CXCL2, promoting neutrophil chemotaxis. Naive T cells exhibit reduced differentiation into Th17 cells and favor a Th2 phenotype, with elevated IL-4 and IL-10 production. The impact of progesterone on γδ T cell IL-10 and PIBF production in the lower female reproductive tract remains to be determined. Macrophages adopt an M2 phenotype, characterized by reduced NO and IL-12 production. Dendritic cells downregulate MHC class II and costimulatory molecules while increasing IL-10 secretion. **Right Panel**: During periods of high estrogen, there is enhanced production of MUC5B, reducing mucus viscosity. Antimicrobial peptides such as SLP1, HNP1, HNP2, lysozyme, lactoferrin, and surfactant A increase, while HBD-2 and elafin decrease. Dendritic cells increase MHC class II and costimulatory molecule expression while increasing IL-6 and IL-12 production. Lower levels of immunoglobulins are observed in the lumen. Macrophages shift towards an M2 phenotype, secreting increased IL-4 and IL-10 and depositing extracellular iron. Th17 cells decrease effector cytokine production and skew towards a Th2 phenotype. The effect of estrogen on Treg abundance in the lower female reproductive tract remains uncertain (Collins, McCutcheon, and Petroff 2022).

Moreover, undetected *Neisseria gonorrhoeae* infections in women possibly will ascend into the UGT to cause an inflammatory response that establishes as PID, the consequences are influenced by the severity and duration of inflammation, and whether it is confined to the endometrial, fallopian tube, ovarian, and/or other tissues (S. X. Xu and Gray-Owen 2021).

Bacterial recovery is most common in the follicular/ proliferative phases (McCormack and Reynolds 1982; Sweet et al. 1986), which coincide with estrogen-controlled changes in mucus viscosity and uterine contractions that move fluid upward (Collins, McCutcheon, and Petroff 2022; Mitchell and Prabhu 2013). These normal physiological changes, in additional to sexual intercourse and retrograde menstruation, can provide opportunities for N. gonorrhoeae and other microbes to ascend, with the outcome dependent on the load and virulence of the microbe as well as the resulting immune response (Darville 2021). **Figure 10** shows the canalicular flow of infection after gonococcal colonization of the endocervix.

Interestingly, *N. gonorrhoeae* infection of mice expressing CEACAM 1 during estrus resulted in greater bacterial association and penetration of endometrial tissue than in wild-type mice, although tissue invasion did not affect bacterial clearance nor was there increased tissue damage (Epshita A. Islam et al. 2018).

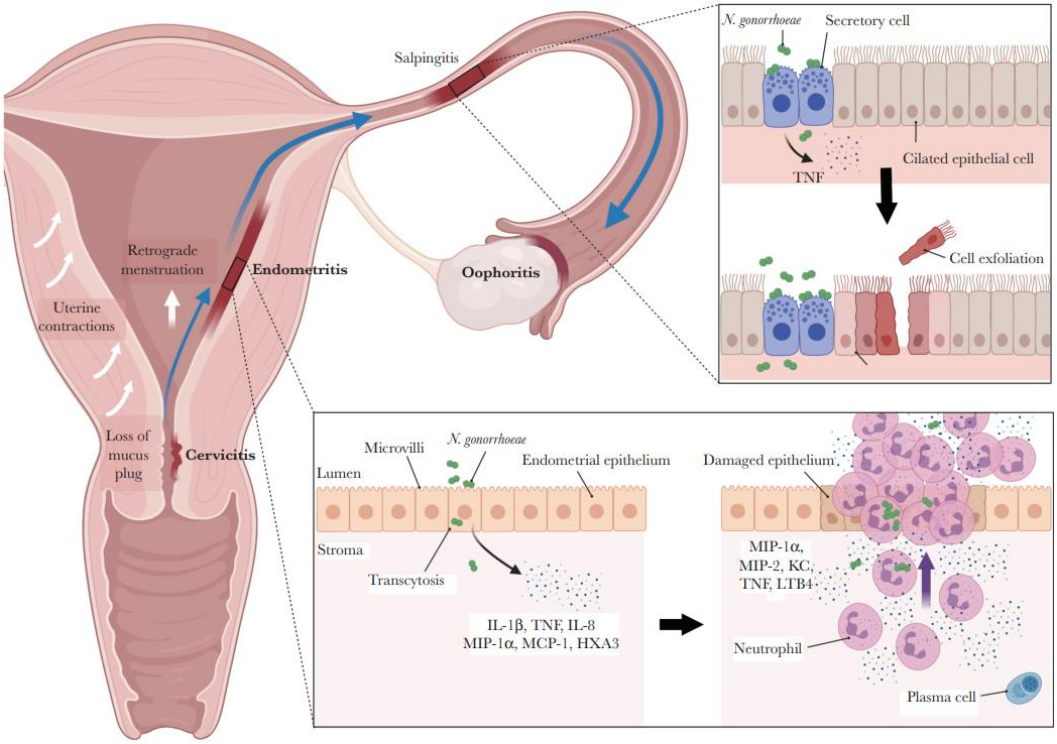

Figure 10. The progression of infection following gonococcal colonization of the endocervix. Once Neisseria gonorrhoeae (Ng) establishes infection, several factors, including hormone-controlled mucus plug loss, uterine contractions, sexual intercourse, and retrograde menstruation, can drive bacterial ascent into the upper genital tract (UGT). This ascent can result in endometritis, salpingitis, or oophoritis. In the endometrium, Ng interaction with epithelial cells leads to bacterial transcytosis and the release of proinflammatory cytokines (IL-1β and TNF) and chemokines (IL-8 and hepoxilin A3), which attract neutrophils. The subsequent production of additional chemotactic factors (LTB4, KC, and MIP-2) by neutrophils creates a feedback loop, amplifying the inflammatory response and causing tissue damage. In the fallopian tubes, Ng binds to nonciliated secretory cells, releasing peptidoglycan and lipo-oligosaccharide fragments that trigger a strong inflammatory response. TNF production results in the death and sloughing of ciliated cells, leading to irreversible scarring and potential fertility impairment. The illustration was created by (S. X. Xu and Gray-Owen 2021) using BioRender.com.

2.5. CEACAM Receptors

Cell-to-cell adhesion plays a crucial role in directing cells to their appropriate position during embryonic development and in facilitating the assembly of individual cells into functional tissues and organs. The immunoglobulin superfamily of cell adhesion molecules (IgCAMs) represents a substantial group of cell surface glycoproteins, with deep evolutionary origins

in the animal kingdom, that are specialized in cell-to-cell adhesion (N. Beauchemin et al. 1999).A significant member of the IgCAM superfamily is the carcinoembryonic antigen (CEA). This antigen participates in both homotypic and heterotypic interactions with other closely associated IgCAMs. Notably, it serves as a clinically pertinent diagnostic marker in monitoring colon tumors.

CEA has been classified within the CEA-related cell adhesion molecule (CEACAM) family, a subset of IgCAMs that, to our knowledge, is exclusive to mammals (Nicole Beauchemin and Arabzadeh 2013). A comprehensive overview of the domain structure of human CEACAMs, the count of recognized splice variants, and the presence of orthologues in other mammalian species is depicted in Figure 11.

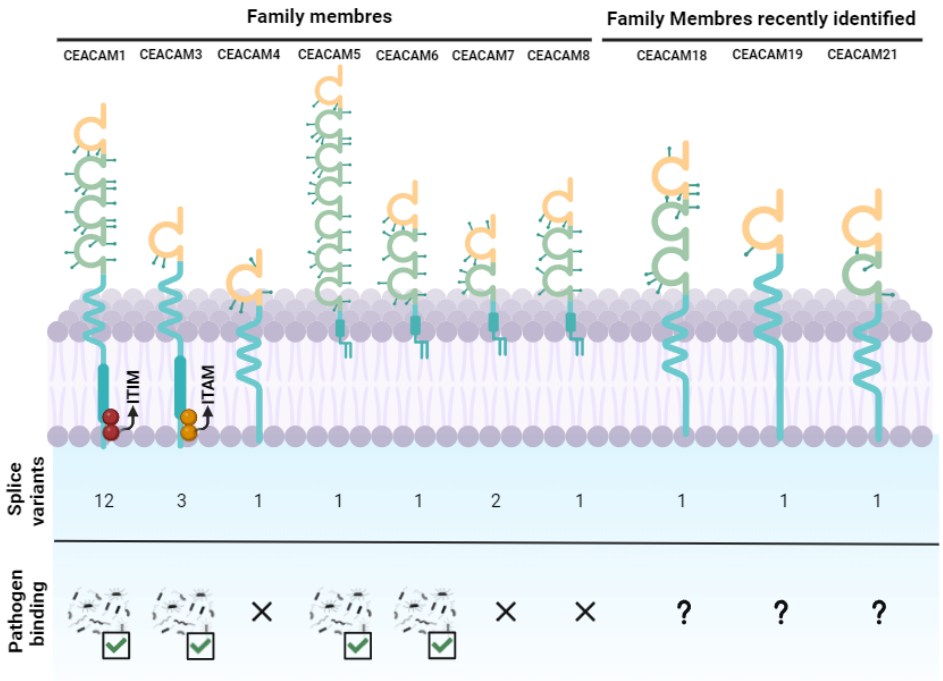

Figure 11. Summary of the H-CEACAM family This includes a schematic illustration of the primary isoforms of the various human CEACAM family members. Additional details about alternative splice variants can be accessed at the provided website: http://cea.klinikum.uni-muenchen.de/. The illustration is inspired by (Kuespert, Pils, and Hauck 2006) and created with BioRender.com.

The members of CEACAM family have undergone several name changes over time. For instance, the protein initially known as biliary glycoprotein (Bgp), which was subsequently classified as the CD66a antigen, is being nowadays mentioned to as CEACAM1. (Hammarström 1999). Although CEACAMs have been the subject of research for many years due to their association with tumor-related characteristics, recent studies suggest that members of this family play a crucial role in regulating various physiological processes (Hauck et al. 2006). Altogether CEACAMs feature an N-terminal V set fold from the immunoglobulin (Ig) superfamily, up to three type 2 immunoglobulins, a transmembrane domain, then a cytoplasmic domain. These components facilitate adhesion through both homophilic (CEACAM1, CEACAM5, and CEACAM6) and/or heterophilic (CEACAM1–CEACAM5, CEACAM5–CEACAM6, and CEACAM6–CEACAM8) interactions (Fig. 12) (Gray-Owen and Blumberg 2006; Thomas et al. 2023). Individually CEACAM exhibits a unique pattern of expression (Prall et al. 1996; Schölzel et al. 2000) . The roles of CEACAM1, CEACAM5, and CEACAM6 are crucial and vary depending on the cell type, particularly in relation to certain facets of cancer biology and immunology (Nicole Beauchemin and Arabzadeh 2013; Dankner et al. 2017).

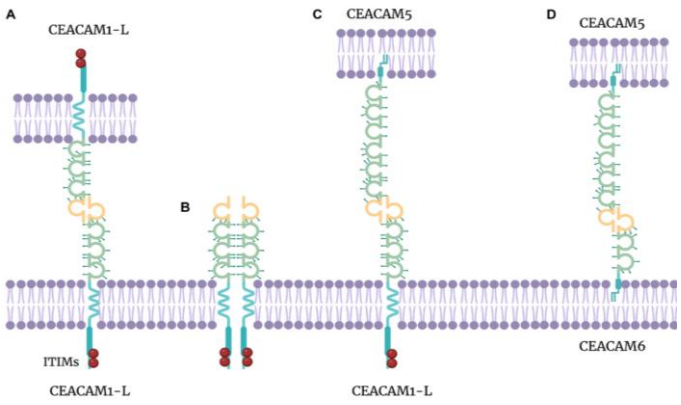

Figure 12 Interactions involving CEACAM. (A) Trans-homophilic binding of CEACAM (CEACAM1-L – CEACAM1-L). (B) Cis-homodimer formation of CEACAM (CEACAM1-L – CEACAM1-L). (C) Trans-heterophilic binding of CEACAM (CEACAM5 – CEACAM1). (D) Heterophilic binding of CEACAM (CEACAM5 – CEACAM1) The illustration is inspired by (Thomas et al. 2023) created with BioRender.com.

- **CEACAM 1**

CEACAM 1, previously known as Bgp or CD66a, exhibits the broadest tissue allocation among all identified family members. This receptor is not exclusively present on epithelial cells but is also found on a variety of leukocytes. Moreover, its expression can be stimulated in other cell types, including endothelial cells and T cells.

CEACAM 1 exists in several splice variants, with most isoforms featuring a transmembrane domain and either a long (L) or short (S) cytoplasmic domain. The 'L' isoforms contain 70 amino acids (71 in humans) within the cytoplasm, including multiple serine, threonine, and tyrosine residues that can serve as targets for phosphorylation and can be involved in signal transduction and protein-protein interactions (Kuespert, Pils, and Hauck 2006). Conversely, the 'S' isoforms encode only 10 cytoplasmic residues and do not contain any phosphorylation sites (Singer, Scheffrahn, and Öbrink 2000). Equally isoforms are simultaneously expressed in most tissues that express CEACAM 1, and the proportion between the two isoforms influences the result of the signaling process (Crossin and Krushel 2000). Notably, two tyrosine residues located within the long cytoplasmic domain form a functional immunoreceptor tyrosine-based inhibitory motif (ITIM), (Chen et al. 2001). This motif seems to underpin the inhibitory activity of CEACAM 1 in various scenarios.

Several past studies have suggested a role for CEACAM 1 in angiogenesis. For instance, soluble CEACAM 1 has been shown to promote angiogenesis by encouraging the proliferation, chemotaxis, and formation of capillary-like tubes in human microvascular endothelial cells in vitro. Given these findings, it's not unexpected that CEACAM 1 seems to play a part in tumor angiogenesis, as it is expressed on small endothelia associated with tumors but not on large and dormant blood vessels (Kilic et al. 2005; Oh et al. 2004).

Additionally, it has been observed that CEACAM 1 can depress the proinflammatory response in infected epithelial cells (Slevogt et al. 2008) and restrain leukocyte activities that are essential for initiating an adaptive immune response (H. S. W. Lee, Ostrowski, and Gray-owen 2008; Qigui Yu et al. 2013).

Beyond its involvement in these areas, CEACAM 1 also interacts with pathogens, notably *Neisseria gonorrhoeae*. The Opa proteins bind specifically to the GFCC'C'' face of CEACAM 1, particularly at residues located in the C, C', and F strands as well as the CC' loop. These areas are critical for the binding interaction, which also overlaps with regions responsible for homophilic or heterophilic interactions of CEACAM 1.

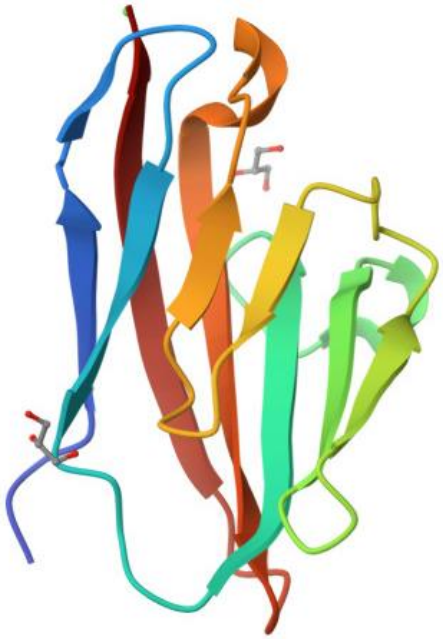

Figure 13 Crystal structure of the N terminal domain of human CEACAM 1. Binding target of the opacity proteins during invasion of Neisseria meningitidis and N. gonorrhoeae**.** PDB DOI: https://doi.org/10.2210/pdb2GK2/pdb

- **CEACAM 3**

Beyond CEACAM 1, CEACAM 5, and CEACAM 6, which are expressed on epithelial cells, quite a few bacterial adhesins also bind to CEACAM3. This family member is exclusively expressed on human granulocytes (Chen and Gotschlich 1996; Unit, Cancer, and Zimmermann 1993). CEACAM 3 possesses a single extracellular IgV-like domain but does not participate in homo- or heterophilic interactions with other family members (Kuespert, Pils, and Hauck 2006). The most unique characteristic of CEACAM 3 is found in the 71-amino-acid cytoplasmic domain, which includes an amino acid sequence that resembles an immunoreceptor tyrosine-based activation motif (ITAM), (Gray-Owen and Blumberg 2006; Sadarangani, Pollard, and Gray-Owen 2011).

Numerous studies have shown that CEACAM 3 undergoes phosphorylation by protein tyrosine kinases from the Src family, specifically Hck and Fgr, which are activated during bacterial infection of human phagocytes (Hauck et al. 1998). Bacteria bound to CEACAM 3 are effectively engulfed through a process dependent on the actin cytoskeleton by cell lines transfected with CEACAM 3 as well as human phagocytes, and this occurs independently of opsonin (McCaw et al. 2003).

The swift internalization facilitated by CEACAM 3 necessitates a functional ITAM and the activation of the small GTPase Rac, a crucial modulator of actin cytoskeleton dynamics and the oxidative burst (McCaw et al. 2003). The uptake and killing of bacteria that bind to CEACAM by primary human granulocytes can be hindered by antibodies that block CEACAM 3 or by introducing the phagocytes to dominant-negative variants of Rac. However, dominant-negative forms of the closely related GTPase Cdc42 do not have the same effect (McCaw et al. 2003).

These findings suggest that CEACAM 3, which lacks an identified endogenous ligand and has no homologue in other mammalian species, is a unique member of the CEACAM family. It appears to have evolved to enable the innate immune system to control human-specific bacterial pathogens.

Remarkably, CEACAM 3, which is exclusively expressed by human neutrophils, appears to function as a decoy receptor that activates the neutrophil upon binding with N. gonorrhoeae. The engagement of CEACAM 3 results in highly efficient opsonin-independent phagocytosis of the attached bacteria, triggers a potent bactericidal response, and stimulates an increase in the production of proinflammatory cytokines by neutrophils (Sintsova et al. 2014). In conclusion, the Opa-expressing *N. gonorrhoeae* binding to CEACAM 3 on neutrophils seems to be efficiently engulfed and killed (Criss and Seifert 2012), provoking an inflammatory response that draws more neutrophils to the site of infection.

- **CEACAM 5**

CEACAM5, also known as CEA, consists of one N-terminal variable domain and six C2-like Ig domains (Thompson et al. 1987). Its glycosylphosphatidylinositol (GPI) linker facilitates membrane anchoring (**Fig. 11**), (Hefta et al. 1988).

This type of semi-penetrating membrane anchorage is also observed in several other human CEACAM family members (but not in rodents). This could be one of the factors contributing to the detectability of soluble CEA in serum in tumorigenic environment (Wautier and Wautier 2022). The expression of this family member is typically limited to epithelial cells, with CEA being most prevalent on the apical surface of the gastrointestinal epithelium.

However, it is also found on other mucosal epithelia such as the nasopharynx, lung, and urogenital tract, as well as in sweat glands (Quillin and Seifert 2018),(Thompson et al. 1987),(Hefta et al. 1988).

CEACAM 5 typically serves as a molecule facilitating adhesion, but it also plays roles in modulating differentiation (Benchimol et al. 1989), influencing immune responses (Blat et al. 2014). CEACAM 5 is an FDA-approved diagnostic tumor marker for colon cancer and holds potential as a prognostic marker (Thirunavukarasu et al. 2011).

The association between high levels of IL-6, a key pro-inflammatory cytokine, and the increase in CEACAM5 is known in the context of colorectal cancer cells (Holmer et al. 2015). Analogously, gonococcus is known to trigger a significant inflammatory response, with IL-6 playing a central role in this process (Darville 2021). The increase of CEACAM5 could be regulated by elevated levels of IL-6, potentially induced by gonococcal infection.

- **CEACAM 6**

CEACAM 6 has been identified as playing a role in immune-related diseases. It is highly expressed in conditions such as hyperplastic polyps and colon adenomas (Schölzel et al. 2000), as well as in various types of cancer including breast, pancreatic, mucinous ovarian, gastric, and lung adenocarcinoma. CEACAM 6 is also found in high levels in several organs and tissues, including the colon, liver, stomach, gallbladder, skin, and tongue (Prall et al. 1996). Furthermore, CEACAM 6 is expressed in the squamous epithelia, esophagus, and cervix (Schölzel et al. 2000).

CEACAM 6 is a glycosylphosphatidylinositol (GPI)-anchored protein characterized by an N-terminal variable domain followed by two C2-like immunoglobulin domains (**Fig.11**), (Hefta et al. 1988). It plays a significant role in cell signaling by activating the Src-FAK pathway in a dose-dependent manner, leading to the phosphorylation of key components such as MEK1/2 and ERK, (Thomas et al. 2023). In contrast, CEACAM 5 does not influence this signaling pathway, underscoring that CEACAM 6 specifically forms trans-dimers to initiate Src-FAK signaling. This distinct functionality highlights the unique contributions of CEACAM 6 in cellular signaling processes (Hefta et al. 1988).

2.5.1. CEACAMs and immune-cell function

- **Neutrophils**

Opa-expressing *Neisseria* predominantly engage with neutrophils, thus earning Opa the name “leucocyte association proteins” (Swanson et al. 1975). Opa proteins facilitate the binding to neutrophils, promoting non-opsonic phagocytosis and triggering the bactericidal oxidative burst (Criss and Seifert 2012; Swanson et al. 1975). Neutrophils express CEACAM 1, CEACAM 3, and CEACAM 6 , with CEACAM 3 being responsible for the bactericidal response via ITAM-dependent signaling (Gray-Owen, Lorenzen, et al. 1997). In fact, the neutrophils utilize CEACAM 3 as a decoy receptor to trap Opa-expressing bacteria aimed at colonizing other tissues (Schmitter et al. 2004). Different Opa variants either encourage adhesion or enhance phagocytosis. In meningococci, the non-opsonic interaction is more prevalent in strains found in the nasopharynx, while those isolated from the blood can adhere to endothelial cells but resist phagocytosis (Sadarangani, Pollard, and

Gray-Owen 2011). In detail, the binding of Opa to CEACAM 3 triggers phosphorylation by Src-family kinases, which downregulates SHP-1 phosphatase, recruiting Syk and initiating downstream signals involving phospholipase C, Rac GTPase, and phosphatidylinositol 3-kinase (Hauck et al. 1998; Sarantis and Gray-Owen 2007). This leads to efficient bacterial uptake via actin-dependent mechanisms. Additionally, CEACAM 3 can activate Rac through direct interaction with Vav, enhancing rapid phagocytosis (Schmitter et al. 2007). While the contribution of Syk-dependent and independent pathways to bacterial uptake remains unclear, it is evident that CEACAM 3-dependent bactericidal responses rely on Syk (**Fig. 14**), (Sarantis and Gray-Owen 2007). Several CEACAM receptors on neutrophils work together to enhance adhesion and uptake; however, CEACAM 1 and CEACAM 6 do not promote bacterial killing following internalization (Gray-Owen, Lorenzen, et al. 1997).

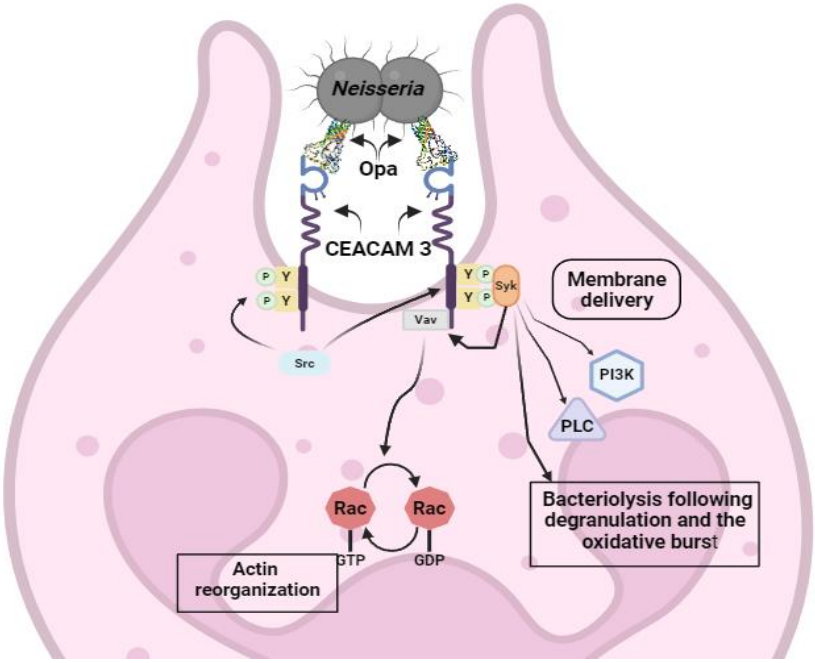

Figure 14 Internalization of Neisseria by neutrophils is mediated by Opa proteins binding to CEACAM 3. When *Neisseria* adheres to neutrophils, Opa variants that bind CEACAM 3 recruit these receptors to the adhesion site. Opa binding induces tyrosine phosphorylation in the CEACAM 3 ITAM motif through Src-family kinases like Hck and Fgr. This activation triggers Syk kinase recruitment, initiating a signaling cascade involving phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), and the small GTPase Rac, leading to actin reorganization and phagocytosis. Additionally, CEACAM 3's ITAM can activate Rac through direct interaction with the guanine nucleotide exchange factor Vav. The illustration is inspired by (Sadarangani, Pollard, and Gray-Owen 2011) created with BioRender.com.

- **T lymphocytes**

The reaction of T lymphocytes, especially CD4+ T lymphocytes, plays a crucial role during an infection with pathogenic *Neisseria* (**Fig. 15**). These cells are instrumental in guiding the scale and quality of the humoral immune response (Hedges et al. 1999). T lymphocytes also play a significant role in the creation of immunological memory and potentially in cell-mediated immunity. This is particularly pertinent in the context of vaccine development.

The Role of T Lymphocytes in Ng Infections and Opa-CEACAM1 Interactions

The interaction between *Neisseria gonorrhoeae* and T lymphocytes, particularly CD4+ T cells, is crucial for the bacteria's ability to evade the immune system and establish persistent infections. Gonococcal Opa proteins, particularly those that bind to CEACAM 1, are central to this process, suppressing T-cell activation and proliferation (Boulton and Gray-Owen 2002). The phosphorylation of Src kinase following Opa-CEACAM 1 engagement leads to a cascade of immune inhibition. Recruitment of SHP-1 and SHP-2 phosphatases deactivates the signaling required for full T-cell activation (**Fig. 15**). This inhibition correlates with a targeted halt in cell division, avoiding cytotoxicity but preventing immune activation (Science 2004). This suppression prevents T cells from responding effectively to activation signals such as IL-2 or CD3 engagement, creating a highly controlled evasion strategy that enables *N. gonorrhoeae* to persist within the host for extended periods (Boulton and Gray-Owen 2002). In addition to impairing CD4+ T-cell responses, recent studies have shown that Opa-CEACAM 1 interactions influence the function of regulatory T cells (Tregs), which may further contribute to immune suppression in gonococcal infections. By modulating the activity of Tregs, gonococci could dampen both the inflammatory and adaptive immune responses, allowing the bacteria to evade clearance (H. S. W. Lee, Ostrowski, and Gray-

owen 2008). In this study (Boulton and Gray-Owen 2002), When gonococci express Opa50, which binds to heparan sulfate proteoglycans (HSPGs), rather than CEACAM 1, CD4+ T cells show increased activation, indicated by upregulation of CD69; This suggests that the specific Opa variant plays a key role in determining the immune response, with CEACAM 1-binding Opa proteins being responsible for immune suppression**.** Recent studies have further clarified that different Opa variants interact with other CEACAM family members, broadening the immunosuppressive effects beyond T lymphocytes, as CEACAM 3 and CEACAM 5 are also involved in limiting neutrophil and monocyte responses (Sadarangani, Pollard, and Gray-Owen 2011).

Comparative insights from meningococcal infections

While *Neisseria meningitidis* shares many structural and functional similarities with *N. gonorrhoeae*, its interaction with the immune system, particularly through Opa proteins, exhibits notable differences. Opa proteins from *N. meningitidis* are known to provoke strong T-cell proliferative responses, particularly in the context of outer membrane vesicles (OMVs) derived from meningococci (Wiertz et al. 1996). These OMVs, containing different Opa variants, have shown robust immunogenicity, which has been leveraged in vaccine development (Bjune et al. 1991; P. Oster et al. 2007; Philipp Oster et al. 2005). However, like gonococci, certain Opa-expressing meningococcal strains also suppress T-cell activation via CEACAM1 binding (H. S. W. Lee et al. 2007). This suppression appears to be variant-specific, as CEACAM 1-binding Opa proteins inhibit T-cell responses, while non-CEACAM 1-binding Opa variants do not. Thus, while *N. meningitidis* can evoke strong immune responses in some cases, CEACAM 1-binding Opa variants create a similar immune evasion strategy to that of *N. gonorrhoeae*, contributing to immune suppression and bacterial survival in the host (H. S. W. Lee et al. 2007).

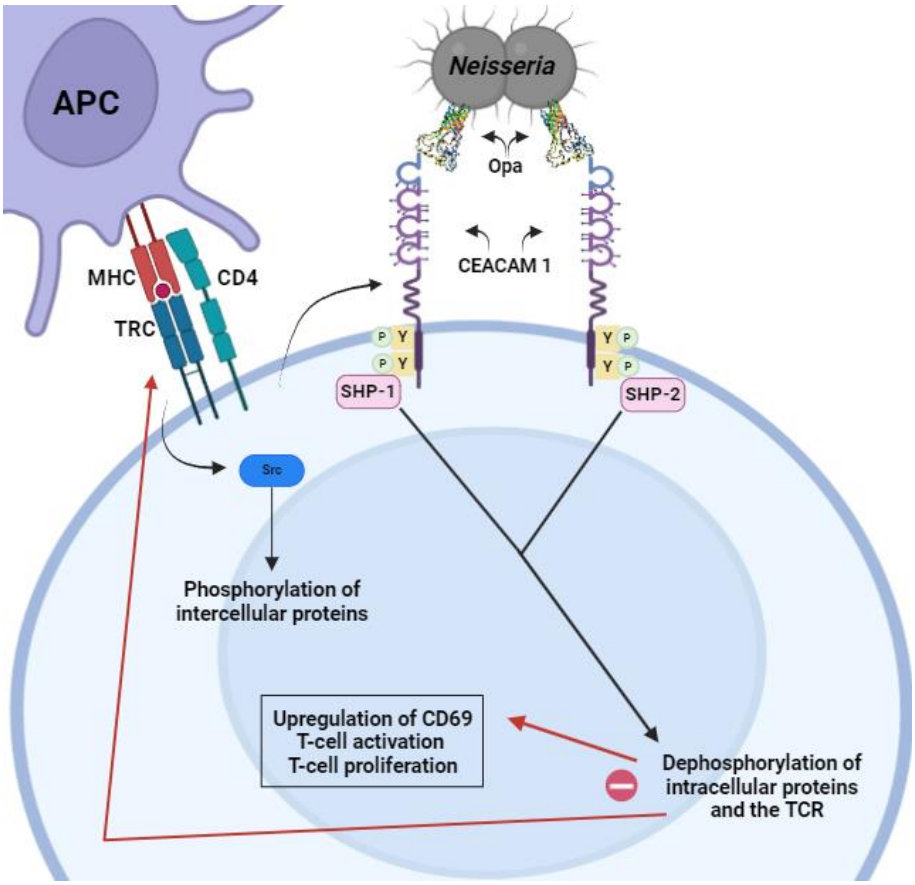

Figure 15 A potential mechanism for Opa-mediated suppression of CD4+ T cells involves binding to CEACAM 1. Normally, when the T-cell receptor (TCR) recognizes an antigen presented by antigen-presenting cells (APCs) through MHC class II, it triggers Src kinase activity. This results in tyrosine phosphorylation of CEACAM 1 and the activation of downstream pathways that promote T-cell activation. However, when Opa binds to CEACAM 1, it recruits tyrosine phosphatases SHP-1 and SHP-2 to the ITIM. These enzymes dephosphorylate CEACAM 1, the TCR, and other intracellular proteins, effectively blocking T-cell activation. The illustration is inspired by (Sadarangani, Pollard, and Gray-Owen 2011) created with BioRender.com.

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- **B lymphocytes**

Recurrent gonorrhea infections are frequent, and one contributing factor may be the gonococcus's ability to suppress the immune response, particularly antibody production (Hedges et al. 1999). Studies have shown that Opa proteins inhibit antibody production in human B lymphocytes expressing CEACAM 1 during infection (Pantelic et al. 2005). This interaction between *N. gonorrhoeae* and CEACAM 1 in peripheral B lymphocytes leads to

cell death, a result also observed in the DT40 B cell line. Interestingly, this CEACAM 1-mediated cell death pathway bypasses SHP-1 and SHP-2 inhibitory signals, instead relying on Bruton's tyrosine kinase. Consequently, Opa–CEACAM 1 interactions may reduce immunoglobulin expression by inducing B cell death. However, CEACAM 1 is not universally linked to apoptosis in all contexts (Qigui Yu et al. 2006), raising the possibility that Opa-driven B cell association could trigger activation-induced cell death rather than being directly caused by CEACAM 1 signaling (Sadarangani, Pollard, and Gray-Owen 2011).

2.6. Neisserial Opa Protein

Proteins known as Opacity-associated (Opa) adhesins, found in the outer membrane of *Neisseria*, play a fundamental role in enabling bacterial interaction with diverse host cell types. These include epithelial cells of mucosal surfaces and a range of immune cells. This suggests that they have a significant influence on the immune response. The receptor affinity of Opa proteins can be commonly classified into two main groups: those that attach to the CEACAM family members (Chen and Gotschlich 1996; Gray-Owen and Blumberg 2006; Epshita A. Islam et al. 2018; Qigui Yu et al. 2013) and those that attach to HSPGs (Bos et al. 2002; Chen et al. 1995). The attachment of Opa proteins to CEACAMs mediates the interaction with human mucosal, endothelial, and leucocytic cells, which are typically encountered during a *Neisserial* infection. (Gray-Owen and Blumberg 2006; Quillin and Seifert 2018; Unemo et al. 2019).

2.6.1. Expression of Opa genes

Gonococci and meningococci are known to commonly encode for 11 or 4 Opa proteins respectively, with specific genetic loci isolated across the genome (Bhat et al. 1992). While Opa genes are incessantly transcribed, the expression of Opa proteins experiences phase variation at the translational stage. This is attributed to the existence of the pentameric coding repeat (CR) sequence CTCTT found within the sequence that encodes the amino-terminal leader peptide (Stern et al. 1986). Alterations in the count of repeats occur due to slipped-strand mispairing throughout the process of DNA replication, leading to consistent frameshifting. The rate of phase variation in gonococci is estimated to be around 10^{-3} for each cell per generation, and this takes place irrespective of homologous recombination (Mayer 1982). Moreover, the strength of the promoter has an impact on the phase variation

of gonococcal Opa genes, as well as on the regulation of Opa expression levels (Belland et al. 1997; Sadarangani, Pollard, and Gray-Owen 2011).

Furthermore, *N. gonorrhoeae* possesses a phase-variable methyltransferase, known as ModA13, which alternates between active and inactive states of gene expression. This regulates the alterations in polynucleotide repeats that transpire during the replication process (Gawthorne et al. 2012; Srikhanta et al. 2009). This occurrence, known as the ModA13 phase-variation, plays a significant role in manipulating the expression of virulence factor genes and the formation of biofilms (Quillin and Seifert 2018).

Consequently, a specific bacterial population can comprise bacteria that express no Opa proteins, a single Opa protein, or several Opa proteins, each at different expression levels. This is in stark contrast to Opc, another primary adhesin, which is absent in the gonococcus and is encoded by a solitary gene in the meningococcus. The fluctuating expression of Opc is regulated at the transcriptional stage, influenced by changes in the length of a polycytidine tract located in the promoter region. The capacity of bacteria to survive without Opa proteins is likely a crucial strategy for evading the immune system. This is because Opa proteins are recognized for their ability to stimulate antibody production following a meningococcal infection and post-immunization with vaccines that include outer membrane complexes or vesicles (Bjune et al. 1991; Mandrell and Zollinger 1989; Philipp Oster et al. 2005).

2.6.2. Structure and function of Opa proteins

Opa proteins are estimated to encompass eight antiparallel beta strands, which create a barrel-like structure in the bacterial outer membrane. This structure is connected by four extracellular loops (De Jonge et al. 2002). The first three loops (Loop 1, 2 and 3) contain regions that are variable (Malorny et al. 1998), and the diversity in these sequences bestows specific affinities for host receptors. Predominantly, sequence variation is noticed within the second and third loops (**Fig. 16**), which are often referred to as hypervariable (HV1 and HV2, respectively). The first loop exhibits some sequence resemblances among Opa proteins; hence it is referred to as semi-variable (Fox et al. 2014; Stern et al. 1986).

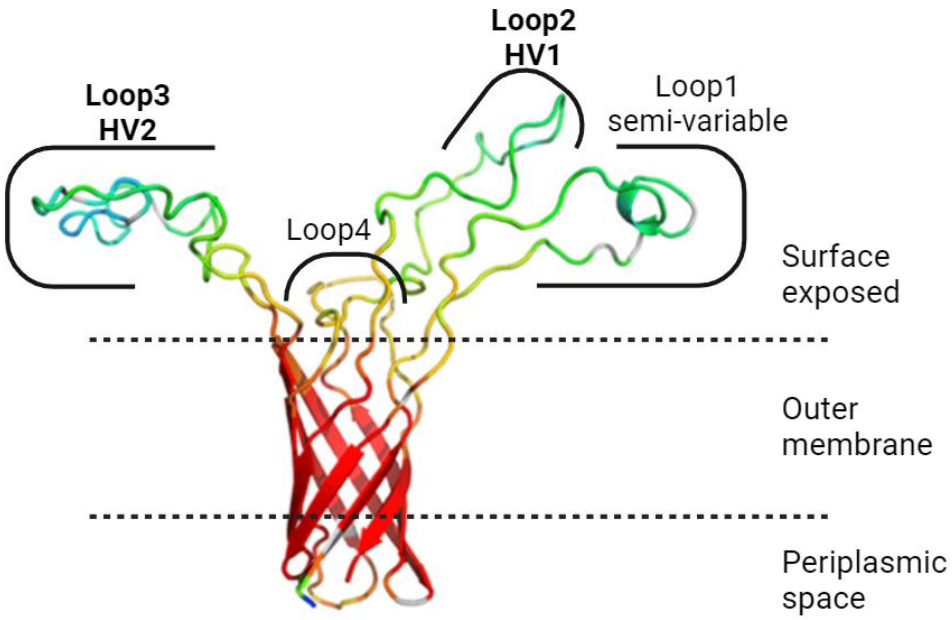

Figure 16 MS11 Opa60 suggests an eight-stranded beta-barrel structure, interconnected by four presumptive surface-exposed loop regions and three periplasmic turns. Variable sequences are found in three out of the four loop regions: a semi-variable region is situated in the first loop, while highly variable regions (HV1 and HV2) are present in the second and third loops. The illustration is created with BioRender.com.

One of the challenges in measuring Opa diversity has been the absence of a uniform system for naming alleles (Martin J. Callaghan, Jolley, and Maiden 2006). The Opa sequence database, hosted at the University of Oxford, was partially established to bring uniformity to Opa categorization. Since of 28 September 2010, the database contains 338 unique Opa alleles (Neisseria.org - Serving the Neisseria Research Community). This encompasses 26 distinct semi-variable regions, 96 HV1 variations, and 127 HV2 sequences. If every potential combination of these variable regions were to exist, it would result in over 300,000 Opa alleles. This implies a limitation in allowable combinations, which could be attributed to the stability of the resultant protein structures or a survival benefit associated with specific variants. (Sadarangani, Pollard, and Gray-Owen 2011). Interestingly, the diversity of Opa contained in the meningococci seems to exhibit a high degree of structure at the population level. This is characterized by the grouping of a restricted number of allelic variants within clonal complexes, as determined by multilocus sequence typing (Martin J. Callaghan et al. 2008; Martin J. Callaghan, Jolley, and Maiden 2006; Sadarangani, Pollard, and Gray-Owen 2011). This observation has not been made in research concerning gonococcal Opa genes. This could potentially be due to the augmented count of Opa loci in each chromosome, which notably enhances the possibility for intragenomic recombination (Sadarangani, Pollard, and Gray-Owen 2011).

2.6.3. The role of other Neisserial adhesins during infection

Although pili, Opa proteins, and Opc proteins continue to be the most extensively researched adhesins in *Neisseria*, other significant adhesins such as NadA, NhhA, App, MspA, and HrpA also exist (Capecchi et al. 2005; Peak et al. 2000; Serruto et al. 2003). Up until now, these have only been identified in *N. meningitidis*, except for App, which is also found in *N.*

gonorrhoeae. Pili plays a crucial role in facilitating the primary connection between the organism and epithelial cells. Opc assists in the adhesion of bacteria to epithelial and endothelial cells by interacting with HSPGs and the extracellular matrix (ECM) proteins, namely vitronectin and fibronectin. Certain research indicates that Opc plays a significant role in meningococcal meningitis, contributing to the penetration of the endothelial barrier between the blood and the brain (Sadarangani, Pollard, and Gray-Owen 2011).

- **Opa proteins as a serogroup B meningococcal vaccine candidate**

The extensive variety of Opa proteins has resulted in them being overlooked as a potential candidate for a meningococcal vaccine, although anti-Opa antibodies have been detected following invasive disease (Martin J. Callaghan, Jolley, and Maiden 2006). However, the revelation that only a restricted set of Opa variants have been linked with hyperinvasive serogroup B meningococci worldwide over the past six decades has rekindled interest in their potential as a vaccine target (M. J. Callaghan et al. 2011; Martin J. Callaghan, Jolley, and Maiden 2006). The process of vaccinating mice with either recombinant or liposomal Opa proteins resulted in the production of a significant amount of antibodies specifically targeting meningococci, thereby validating the concept of this method (Arenas et al. 2010; M. J. Callaghan et al. 2011). An analysis of the highly variable regions from a set of 227 samples of invasive meningococcal disease in the UK from 1996 to 2001 (M. J. Callaghan et al. 2008) suggests that a vaccine incorporating the six most prevalent Opa variants could theoretically provide coverage for 90% of cases.

2.7. Molecular interactions between Opa and CEACAM

Binding of CEACAM by Opa proteins requires a conformational interaction between both hypervariable regions of Opa, whereas the semi-variable region appears to be dispensable (**Fig. 16**) (Bos et al. 2002). The removal of the conserved fourth loop led to a drastic drop in Opa expression, leaving its specific effect on CEACAM binding untested. Antibodies against the HV2 region of a gonococcal Opa protein hindered neutrophil interactions (Elkins and Rest 1990). Chimeric Opa proteins, with hypervariable regions from different CEACAM-binding Opa proteins, mostly lost their receptor-binding activity, suggesting a necessary interaction between HV1 and HV2 for CEACAM binding (Bos et al. 2002; Sadarangani, Pollard, and Gray-Owen 2011). This raises the question of how CEACAM binding sites are conserved despite the high sequence variation in Opa proteins' hypervariable regions (Bos et al. 2002; Sadarangani, Pollard, and Gray-Owen 2011).

The *Neisseria meningitidis* strain H44/76 exhibits four separate Opa proteins. OpaA and OpaJ are specifically inclined towards CEACAM1, while OpaB and OpaD exhibit binding properties with both CEACAM 1 and CEACEAM 5 (De Jonge et al. 2003). A critical sequence motif for CEACAM 1 binding was discerned via alanine scanning mutagenesis within the conserved residues of the hypervariable regions across all Opa proteins. Hybrid Opa variants, integrating various HV1 and HV2 combinations, displayed diminished CEACAM 1 and CEACAM 5 binding (De Jonge et al. 2003).. This strongly infers a conserved CEACAM binding site, enabled by the interplay between HV1 and HV2 regions. A relatively conserved HV2 binding motif, consisting of Gly172, Ile174, and Gln176, was also identified (De Jonge et al. 2003). Interestingly, similar motifs are present in nearly all CEACAM-binding Opa variants. This suggests that despite the high degree of Opa sequence

variation, the receptor-binding contacts are conserved, or these residues are integral to the functional conformation of all Opa variants.

Isolates of meningococci and gonococci that express Opa proteins bind to the N-domain of CEACAM, a region that is largely preserved among CEACAM family members. This attachment takes place within the initial 108 amino acids (Gray-Owen, Dehio, et al. 1997; Popp et al. 1999; Virji et al. 1996). The recognition process is facilitated by the CEACAM protein structure itself, not by glycosylated components. Therefore, variations in glycosylation are unlikely to affect the responses mediated by Opa-CEACAM (Bos et al. 1998; Watt et al. 1996).

Through the use of chimeric constructs from various N-domains, specific binding regions have been identified for certain groups of Opa variants. These regions likely dictate the differential recognition of CEACAM receptors by Opa proteins. The binding interface of the CEACAM1 N-domain consists of non-glycosylated regions, primarily composed of the beta strands C″, C′, C, F, and G. Similar structural motifs are found in other receptors within the CEACAM family that interact with Opa proteins. This region represents a significant sequence divergence between human and animal CEACAMs (Voges et al. 2010), potentially explaining why *Neisseria* species are strictly human pathogens. This also underscores the challenges in studying these interactions using animal models and highlights the growing need for more physiologically relevant systems, such as advanced *in-vitro* models. Opa proteins bind to exposed residues on the CC'FG face of CEACAMs, with Tyr34 and Ile91 playing critical roles in facilitating this interaction (Virji et al. 1999). Research has demonstrated that both HV1 and HV2 regions of Opa60 are essential for binding to CEACAM 1, and the absence of these regions prevents interaction (Fedarovich et al. 2006; Linda Columbus 2018) (**Fig. 17 A**).

Recent studies have further elucidated that Opa52 binds specifically to CEACAM 6 by engaging residues 27-29 and a crucial residue at position 32 (**Fig. 17 B**), these regions significantly contribute to the binding specificity. The structural elements βC and the C-C′ loop of CEACAM6, along with other regions such as residues 85-108, are critical in forming the appropriate spatial conformation required for Opa binding (E. Y. Jones et al. 1992; Popp et al. 1999)

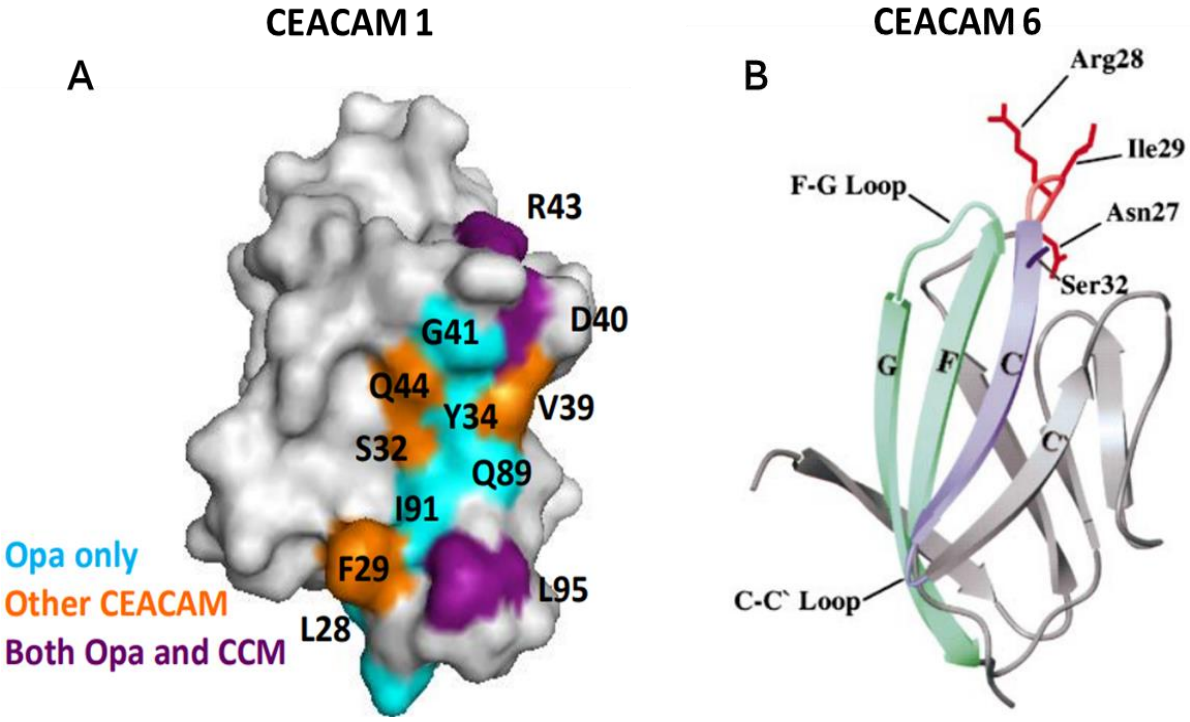

Figure 17 Model of the binding site of Opa on CEACAMs. A) The N-terminal domain of CEACAM1(PDB: 2GK2, (Fedarovich et al. 2006)) interacts with two hypervariable (HV1-HV2) loops located on the extracellular surface of Opa. Key residues of CEACAM1 involved in this interaction are shown in blue. The binding interface of the N-terminal domain of CEACAM1 also includes residues that mediate interactions with other CEACAM family members, depicted in orange. Notably, certain residues serve dual roles by interacting with both Opa proteins and other CEACAM receptors (purple),(Linda Columbus 2018). **B)** The model of the binding site of Opa52 on CEACAM6 (E. Y. Jones et al. 1992) highlights the spatial organization of residues predicted to interact with Opa52 in the folded protein structure. The binding of Opa52 involves residues 27–29 (red), the βC strand, and the C-C′ loop (blue), with residue 32 identified as critical for the interaction.

Despite the diversity in amino acid sequences, the vast majority of currently characterized Opa proteins from both meningococcal and gonococcal sources can be classified into two

primary categories. This classification is based on their binding specificity to human surface receptors: HSPG and CEACAMs.

2.7.1. Differential expression of CEACAMs in the reproductive tract of women

A recent study (Epshita A. Islam et al. 2018) was the first to investigate the individual roles of different CEACAM receptors in the tissue targeting and immune responses of *N. gonorrhoeae* within the female genital tract, using an *in-vivo* model. A key finding is the presence of CEACAMs on the epithelial cells of non-cancerous human genital tissues, which demonstrates that these receptors are available for interaction during natural gonococcal infection (Epshita A. Islam et al. 2018). The different Opa proteins bind selectively to CEACAM receptors in the female genital tract, influencing the infection's location. Opa proteins that bind to CEACAM 5 may promote infections localized in the lower genital tract (squamous epithelium). While Opa that interact with CEACAM 1 localized in the upper genital tract (columnar epithelium), it can contribute to infections ascending into the uterus. This receptor-specific interaction likely plays a key role in how gonococcal infections spread within the reproductive system (**Fig. 18**). Previous research using wild-type mice had shown that CEACAM 5 expression enhances gonococcal adherence to vaginal tissues and reduces cell detachment following infection (Fowler et al. 2010). This study (Epshita A. Islam et al. 2018) expands on that by showing that CEACAM 5 binding supports long-term colonization of the lower genital tract, with some mice remaining infected for over two weeks (Jerse 1999). Opa variants that bind to epithelial CEACAMs, such as CEACAM 1 and CEACAM

5, are selected during gonococcal infections in the genital tract, as evidenced by the higher prevalence of these variants in clinical isolates from female patients (Sintsova et al. 2015). However, Opa variants that bind to neutrophil-restricted CEACAM 3 are less commonly found in clinical isolates, suggesting a selective pressure against them (Sintsova et al. 2015).

Furthermore, this study highlights the role of Opa-CEACAM interactions in the upper genital tract. During the estrus cycle in wild-type mice, gonococcal infections typically result in mild inflammation due to the bacteria's inability to strongly associate with uterine tissues (E. A. Islam et al. 2016). However, in transgenic mice expressing human CEACAM 1, *N. gonorrhoeae* exhibits increased attachment, tissue invasion, and more pronounced inflammation in both the uterus and lower genital tract. These inflammatory responses are linked to increased levels of pro-inflammatory cytokines, driven by CEACAM-mediated bacterial attachment and penetration, as well as the recruitment of immune cells like neutrophils and macrophages (E. A. Islam et al. 2016).

The differential expression of CEACAM receptors across various tissues and stages of the female reproductive cycle plays a crucial role in determining the extent of bacterial colonization, tissue penetration, and inflammation (E. A. Islam et al. 2016).

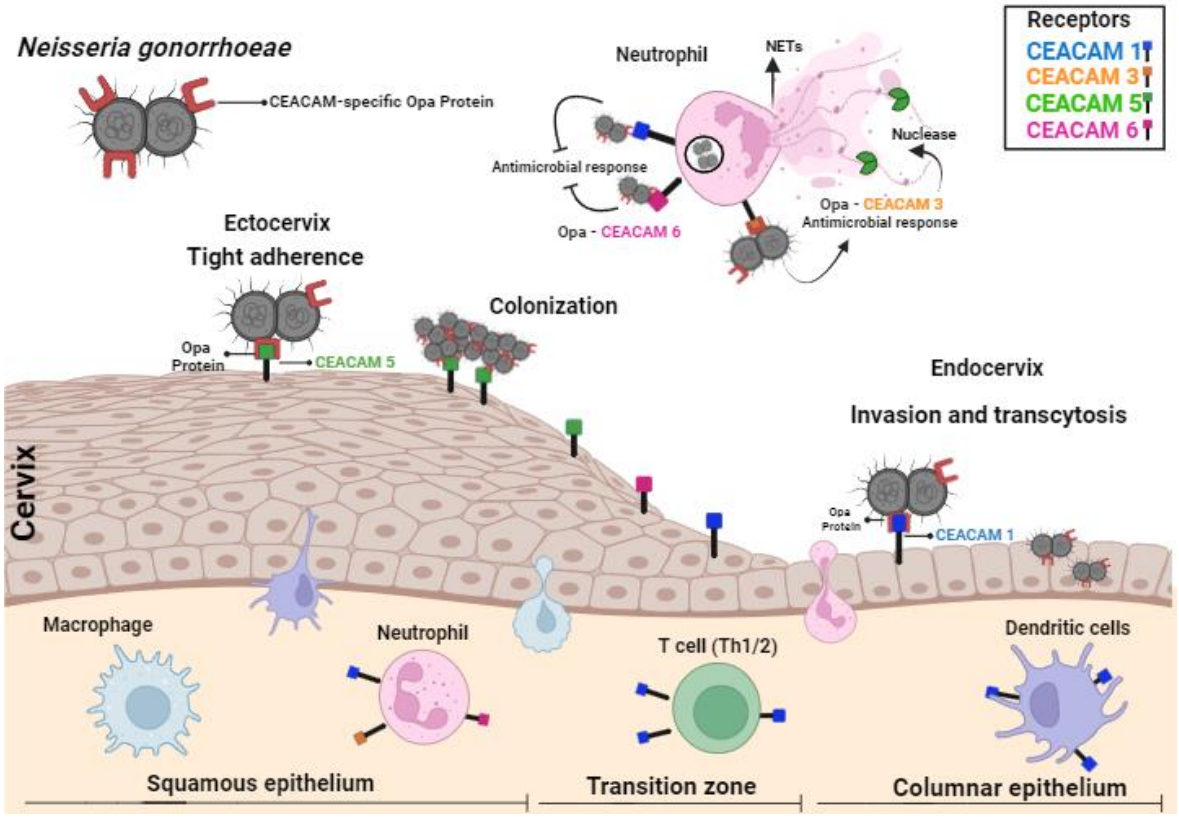

Figure 18 The expression patterns of CEACAMs in the female urogenital tract. The expression of **CEACAM 5** in the lower genital tract (**squamous epithelium**) and of **CEACAM 1** in the upper genital tract (**columnar epithelium**) suggests that different Opa protein variants, which bind specifically to these receptors, could facilitate both localized cervical infections and ascending uterine infections. The illustration is created with BioRender.com.

3. Aim of the study

The aim of this project is to deepen the understanding of the complex dynamics of host-pathogen interactions, specifically focusing on the less explored aspects of gonococcal pathogenesis. A key objective is to refine our characterization of the binding process between human CEACAM receptors and the bacterium's specific Opa proteins, and to better investigate the process of colonization. Our aim is also to develop cell models that accurately mimic the primary natural infection sites to study how *gonococcus* interacts with three distinct human mucosal niches (cervix, urethra, and oropharynx).

We have characterized a collection of 12 *N. gonorrhoeae* strains, focusing on the diversity of Opa expression profiles and their binding patterns to CEACAMs. Additionally, we have implemented a high-throughput screening method, using Luminex technology, to investigate the interaction between Opa-expressing Ng and CEACAMs. A further objective of this study is to assess the effectiveness of mouse antisera, raised against GMMA of Ng FA1090 strain in inhibiting bacterial interaction/binding to distinct human CEACAM ligands.

Lastly, we have employed 2D and 3D cell models resembling the primary natural infection sites to corroborate the *in vitro* data.

4. Material and Methods

4.1. Bacterial strains and growth conduction

A panel of representative Opa-expressing Ng strains was selected for this study: FA1090, FA1090 *ΔOpa*, SK92-679, WHO-M, WHO-N, WHO-F, WHO-G, BG27, BG8, GC14, F62 and MS11 (**Table 1**). Bacteria were grown on gonococcus medium (GC) agar plates (Difco), or liquid GC broth supplemented with 1% isovitalex (BBL) at 37°C in 5% CO2.

Strain	Reference	Year of Isolation	Country
FA1090	ATCC	1983	USA
FA1090Δ*Opa*	Jane Cannon	N/A	USA
SK92-679	ATCC	1992	USA
WHO-M	NCTC 13481	1992	USA
WHO-N	NCTC 13482	2001	Australia
WHO-F	NCTC 13477	1991	Canada
WHO-G	NCTC 13478	1997	Thailand
BG27	Clinical Isolate*	2013	UK
BG8	Clinical Isolate*	2013	UK
GC14	GSK	N/A	N/A
F62	ATCC	1960	USA
MS11	ATCC	1960	USA

Table 1. *N. gonorrhoeae* strains references. * Provided by Dr. Derryl Hill, University of Bristol, United Kingdom.

4.2. Cell lines

Ectocervical and Endocervical cell lines (Ect1/E6E7 ATCC CRL-2614 and End1/E6E7 ATCC CRL-2615) were maintained in keratinocyte serum-free medium (KSFM, catalog number 17005042) supplemented with 20% of fetal bovine serum (FBS), 0.1 μg/mL

epidermal growth factor, 0.4 mM CaCl2 and 1% antibiotics at 37°C in 5% CO2. The pharyngeal Detroit 562 cell line (ATCC CCL-138) were maintained in Eagle's Minimum Essential Medium (Catalog number 11095080) supplemented FBS to a final concentration of 10% and 1% of antibiotics at 37°C in 5% CO2. The ureteral SV-HUC 1 cell line (ATCC CRL-9520) were maintained in F12K Nutrient Mixture medium (Catalog number 21127022) supplemented with FBS to a final concentration of 10% and 1% of antibiotics at 37°C in 5% CO2, (**Table 2**).

Cell line	Reference	Year of Isolation	Country
Ect 1 E6/E7	ATCC	1996	USA
End 1 E6/E7	ATCC	1996	USA
SV-HUC-1	ATCC	1990	USA
Detroit 562	ATCC	1968	USA

Table 2. Cell lines references from ATCC: The Global Bioresource Center | ATCC.

4.3. GMMA productions

We investigated a GC-derived OMV, using on the generalized modules for membrane antigens (GMMA) technology. GMMA are membrane vesicles from Gram-negative bacteria that are genetically modified to induce hyper-blebbing and/or to show reduced reactogenicity. To generate small scale GMMA preparations of Ng GMMA from FA1090 *ΔlpxL1Δrmp* strain, cell-free supernatants were recovered by centrifugation at 8,000 × g for 15 min at 4 °C from overnight cultures of the gonococcal strain grown in liquid media at 37 °C and filtered through 0.2-µm Sartobran P H9 filter to ensure bacteria removal. After filtration, $MgCl_2.6H_2O$ was added to a final molarity of 1 mM and 50 U/L of benzonase was added for DNA removal and incubated at 4–8 °C with stirring overnight. A tangential flow

filtration step using 300K Sartocon slice cassettes was used for retention of the GMMA and buffer exchange in PBS, followed by an ultracentrifugation step (30,000 × g for 15 h) to pellet the GMMA which were subsequently resuspended in PBS and sterile filtered using a 0.2 μm filter, and the product was stored at –20 °C. Mice were immunized intraperitoneally on Days 1, 29 and 57 with 10 μg/mouse of either of the three different batches of Ng GMMA adsorbed to alum (Ng FA1090, Ng SK92-679 and Ng BG27), or alum alone.

4.4. Test coupling

The CEACAM soluble recombinant His-tagged proteins, listed in **Table 3**, were thawed, and resuspended in 1mL of DPBS. Recombinant proteins were covalently conjugated to the free carboxyl groups on Magplex Beads (Luminex Corporation, Austin, TX) using an N-hydroxysulfosuccinimide (sulfo-NHS)-enhanced carbodiimide-mediated conjugation chemistry. 5 falcon tubes were prepared with PBS BSA or Storage Buffer, MES, PBS TWEEN 0.05%, Milli-Q water, and Sodium phosphate NaH2PO4. 2 ml tubes were marked with proteins to be coupled to beads. The desired sample volume was diluted in 220 μL of MES and transferred into an Eppendorf tube. The EpMotion 5075 system was turned on, and the program was configured with the necessary parameters. Eppendorf tubes containing beads and proteins were placed in the magnetic rack, while tubes containing SNHS and EDC were positioned in rack 2. All tips were aligned, and the program was started to proceed with coupling, which lasted 3-4 hours. After completion, the samples were stored at 4°C.

Receptor	Reference	Description	Catalog
anti-human CEACAM-1	Invitrogen	Monoclonal mouse antibody	MA5-23985
anti-human CEACAM-3	Sino Biological	Policlonal rabbit antibody	11933-T24
anti-human CEACAM-5	Sino Biological	Policlonal rabbit antibody	11077-RP01
anti-human CEACAM-6	Sino Biological	Policlonal rabbit antibody	10823-T24

Table 3. Recombinant Human CEACAMs (His Tag), HPLC-verified, Sino Biological | Global Leader in Recombinant Technology for Life Science.

The coupling assay for CEACAM receptors was tested in DPBS using the monoclonal antibodies (mAbs) specified in **Table 4**, starting with a 1:100 dilution in step 3. The results were evaluated by measuring the range of Mean Fluorescence Intensity (MFI) emitted to assess the efficiency of receptor-bead attachment.

Receptor	Reference	Description	Catalog
anti-human CEACAM-1	GenoVac	Monoclonal mouse antibody	GM-0501
anti-human CEACAM-3	Sino Biological	Monoclonal mouse antibody	11933-MM06
anti-human CEACAM-5	GenoVac	Monoclonal mouse antibody	GM-0503
anti-human CEACAM-6	GenoVac	Monoclonal mouse antibody	GM-0509

Table 4. Anti-human CEACAMs antibodies tested in Luminex.

4.5. Functional Luminex assay

Ng strains binding to CEACAMs

Luminex experiments were conducted with a panel of 11 strains (FA1090, FA1090 *ΔOpa*, SK92-679, WHO-M, WHO-N, WHO-F, WHO-G, BG27, BG8, GC14. F62 and MS11). Recombinant CEACAM 1, 3, 5 and 6 proteins (10ug), were covalently bound to the Luminex

beads and Gonococcus was marked with Alexa Fluor 532 as detection of the binding. FA1090 *ΔOpa* was used as negative control in the assay. The plate was incubated for 30 minutes under agitation. After incubation, the wells were aspirated and washed three times before being fixed with 2% PFA for 15 minutes. The analysis was carried out using the Luminex FLEXMAP 3DTM reader, with the standard PMT voltage settings applied. Data acquisition was performed in real time using Bio-Plex Manager™ Software. The interaction between the recombinant CEACAM receptors and the panel of gonococcal strains was quantified as mean fluorescence intensity (MFI).

Ng strains binding inhibition to CEACAMs

Ng strains (Table 1) were labeled by adding Alexa Fluor 532 carboxylic acid, succinimidyl ester (final dilution 1:200). The bacteria were incubated for 15 minutes at 37°C, then centrifuged at 8000 RPM for 5 minutes and washed once with 1ml of DPBS to remove excess dye. The bacterial pellet was resuspended in the final volume (1mL) with 2%BSA/DPBS, and the strains were stored at -20°C. The bacteria were consistently resuspended in a 1:1 ratio, either with DPBS or test sera at the appropriate dilutions. An initial 1:50 dilution was prepared in the plate. Sera samples were serially diluted in DPBS in a 96-well plate, aiming for a final volume of 50 µL per well. Subsequently, 50 µL of the labeled bacteria suspension was dispensed into each well containing the serially diluted sera using a multichannel pipette. The plate was incubated for 15 minutes at room temperature. Each CEACAM was mixed with the bacteria and sera mixture in a filtrate plate, followed by additional incubation for 30 minutes at room temperature with shaking. The wells were then aspirated and washed three times before being fixed with 2% PFA for 15 minutes. Plate analysis was conducted using the Luminex FLEXMAP 3DTM reader, following the standard PMT voltage setting.

Data collection was performed in real time utilizing the Bio-Plex Manager™ Software, and results were expressed as mean fluorescence intensity (MFI).

Sequencing of Opa genes

N. gonorrhoeae genomic DNA from the 12 strains listed in **Table 1** was extracted from cell suspensions of overnight culture plates (GC+1% isovitalex) using the MagPurix Bacterial DNA Extraction Kit (ZP02006, Resnova) following manufacturer's instructions with slight modifications. Briefly, 220 µL of BL2B buffer were added to each bacterial pellet and the mixtures were incubated at 70°C for 10 minutes at 300rpm. Once cooled down in ice for 3 minutes, the samples were supplemented with 20 µL of DNase-free RNase A (PureLink™ RNase A (20 mg/mL) 12091021, Invitrogen) and were incubated at 37°C, 20 min at 300 rpm. Finally, 200 µL of lysate were transferred to each sample tube and genomic DNA was extracted by the Resnova Instrument (Zinexts). Genomic DNA was sequenced using the Illumina MiSeq platform and read sequences were assembled. For each strain, the resulting consensus of the assembly was used to extract each of the 11 *opa* genes and to define the ON/OFF status of the Opa proteins.

4.6. Confocal microscopy

Ect/Endo -cervical, Urethral and Oropharyngeal cells were seeded into PhenoPlate™ 96-well microplates (Revvity, 6055302). Cells were seeded at different density to reach confluence at the same time: Ect 1 E6/E7 4×10^5 cells/well, End 1 E6/E7 4×10^5 cells/well,

SV-HUC-1 3×10^5 cells/well, and Detroit $4{,}5 \times 10^5$ cells/well. On the day preceding the experiment, cells were incubated in an antibiotic-free medium. Afterwards, all cell lines were infected with fluorescent Ng strains (Oregon green, 1:200 in PBS, 15 minutes at 37 °C). After an hour of infection, samples were fixed using 4 % (v/v) formaldehyde for 20 min, blocked with DPBS containing 2% BSA for 60 min and incubated with anti-CEACAM 1 and 5 antibodies (1:100) for 3 h at room temperature (GenoVac, GM-0501 and GM-0503). Wells were washed with DPBS and incubated with goat anti-mouse Alexa Fluor 568 conjugated antibodies (ThermoFisher, A-11004). After washing in DPBS to remove the excess of dye, the samples were permeabilize using Triton 100 0,25% in PBS-BSA 1% for 30 minutes and stained with CellMask™ Plasma Membrane Stains (ThermoFisher, C10046) and DAPI (Invitrogen, D1306) for 15 min at room temperature. Samples infected were examined utilizing Opera Phenix. Images were captured using a 40 and 63 X water immersion lens, operating in confocal mode to perform a z-stack image acquisition.

4.7. Bacterial adhesion inhibition assay (BAI)

A cell-based fluorescent bacterial adhesion inhibition (BAI) assay was employed to evaluate the ability of pooled murine sera, immunized with alum and NgG, to inhibit the adhesion of various *N. gonorrhoeae* strains. NgG is a preclinical candidate generated through GMMA technology. The strains tested included homologous FA1090 and heterologous SK92-679, WHO-M, FA1090 *ΔOpa*, and BG8. The adhesion was assessed on different human cell lines: Ect1 E6/E7 and End1 E6/E7, representing ecto- and endocervical cells, Detroit 562, representing human pharyngeal cells, and the SV-HUC1 human urethral epithelial cell line.

Furthermore, we conducted tests using the monoclonal antibodies (mAbs), the anti-OpaB 1 mAb and the anti-OpaB 2 mAb, to assess their ability to inhibit the adhesion of the FA1090 strain to the four cell lines previously described. Lastly, we tested human sera from individuals vaccinated with 4CMenB vaccine (Bexsero, GlaxoSmithKline [GSK]). Cultured cells were detached from 175 cm^2-flask, after resuspension to avoid cell clumping. The cells were seeded into 96-well plates at a density of 3×10^4 cells/well. For the SV-HUC1 cells, they were cultured in F-12K Nut Mix medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotics. On the other hand, Ect 1 E6/E7 and End 1 E6/E7 cells were seeded at a density of 4×10^4 cells/well and cultured in Keratinocyte medium supplemented with 0.1 ng/mL recombinant Epidermal Growth Factor (rEGF), 20% FBS, and 0.4 mM CaCl2. For the Detroit 562 E7 cells were seeded at a density of $4{,}5 \times 10^4$ cells/well cultured in Eagle's Minimum Essential Medium (EMEM). All cells were cultured until they reached confluence. Ng strains were harvested from a fresh overnight plate culture into 10 mL of GC + 1% Isovitalex medium. Bacteria were grown at 37 °C under shaking till $OD_{600} = 0.5$, then resuspended in dPBS and labelled with Oregon Green dye for 15 min at 37 °C. Afterwards, bacteria were washed to remove excess of dye, resuspended in DPBS-2% BSA, and combined, at final $OD_{600} = 0.1$, with an equal volume of serially cell medium-diluted sera for 15 min at RT. Bacteria-sera complexes were added to cell plates and incubated for 1 h at 37 °C to allow bacterium-cell adhesion. After three washes with DPBS, samples were fixed for 20 min with 4% formaldehyde at RT and, after one washing step with DPBS, finally one volume of distilled water was added to each well. Infected samples were examined utilizing a high content screening (HCS) fluorescence microscope platform, specifically the Opera Phenix. Images were captured using a 40 X water immersion lens, operating in confocal mode to perform a z-stack image acquisition. The aggregate count of bacteria was determined using the image region identification

building block (channel 488). The overall bacterial fluorescence area was computed as a measure of adherence.

4.8. 3D Complex Model infection

The HNG-TOP-12 plates were prepared by first pre-warming 125 mL of VEC-100-MM medium at 37°C for each VEC-100 AFAB EpiVaginal kit containing 3D Human Vaginal Ectocervical MicroTissue (MatTek). Under sterile conditions, the HNG-TOP-12 lids were removed, and 5.0 mL of the pre-warmed VEC-100-MM medium was pipetted into each well of the 12-well plate before replacing the hanging-top lid. Ng strain FA1090 was harvested from fresh overnight cultures on GC plates supplemented with 1% Isovitalex. Bacteria was grown at 37 °C under shaking till OD_{600} = 0.5, then resuspended in DPBS with 2% BSA and adjusted to a final OD_{600} of 0.1. The models were infected with Ng strain FA1090 using a solution of 2% BSA in DPBS diluted in cell media (VEC-100-MM, MatTek), with an inoculum concentration of 1.16×10^8. The plates were incubated for 1 hour at 37°C with 5% CO2. Samples infected were further incubated for 24 and 48 hours at 37°C with 5% CO2 under Air-Liquid conditions, whereas those in Liquid-Liquid conditions were incubated for 24 hours. All supernatants of infection for each timepoints were collected for Luminex assay and western blot analysis. Subsequently, at each time point, samples were homogenized for CFU counts using 1% saponin in DPBS. For the bacterial adhesion inhibition (BAI) assay on the complex model, an inoculum of 1.16×10^9 bacteria was pre-incubated with anti-FA1090 GMMA sera before infection. After 1 hour of infection, the models were evaluated using the Opera Phenix high-content screening system.

Confocal microscopy

Cellular models were washed three times with DPBS and fixed with 4% paraformaldehyde (PFA). Samples were permeabilized using a solution of 1% saponin and 0.1% Triton X-100 (both from Sigma) in DPBS for 60 minutes, and then blocked with 3% bovine serum albumin (BSA) and 0.1% Triton X-100 in DPBS for 60 minutes at room temperature. Primary antibodies were incubated with the samples for 3 hours at 4°C (**Tab. 5**). The samples were then washed with the blocking solution and treated with the appropriate Alexa Fluor-conjugated secondary antibodies (Thermo Fisher) for 60 minutes (**Tab. 5**). Membranes were cut with a scalpel, mounted onto microscopy slides (Thermo Fisher), and analyzed by confocal microscopy.

Images of the membrane, acquired using the Opera Phenix High-Content Screening (HCS) System, were analyzed with Harmony High-Content Imaging (HCI) software. The analysis aimed to **1**) assess the modulation of CEACAM 1 and 5 presence across three timepoints, and **2**) evaluate *N. gonorrhoeae* bacterial infection over the same timepoints to understand infection morphology in a 3D model. The antibodies and dyes used in this study are summarized in **Table 5**.

Receptor	Reference	Description	Catalog
anti-hCEACAM1/5 (Rabbit)	Thermo Fisher	Primary antibody	GM-0501
Anti- Ng pAb (Rat)	GSK	Primary antibody	N/A
CellMask™	ThermoFisher	Plasma Membrane Stains 647	C10046
Goat anti-rabbit	ThermoFisher	conjugated -568	A-11011
Goat anti-Rat	Thermo Fisher	conjugated -488	A-11004
DAPI	Thermo Fisher	dyes 350	D1306

Table 5. Antibodies and dyes used.

5. Result

5.1. Functional Luminex assay

5.1.1. Test coupling of CEACAM receptors

To start to investigate the interaction between human CEACAM receptors and gonococcal Opa proteins, we set up a Luminex based assay to study the binding of a selected panel of Ng strains to CEACAM 1, 3, 5 and 6. (**Fig. 19**). All four CEACAM recombinant proteins, were coupled with Luminex beads at three different doses: 10, 25 and 50 µg. A test coupling to identify the best coupling dose was conducted using specific anti-CEACAM antibodies. As shown in **Figure 19**, all doses of each receptor were similarly recognized by the appropriated antibodies, leading us to use the lower dose tested. The curves obtained for CEACAM 5 receptor diverged from those of the CEACAM 3 and CEACAM 6 receptors, resembling more those of the CEACAM 1-L receptor and accounting for a less potent recognition.

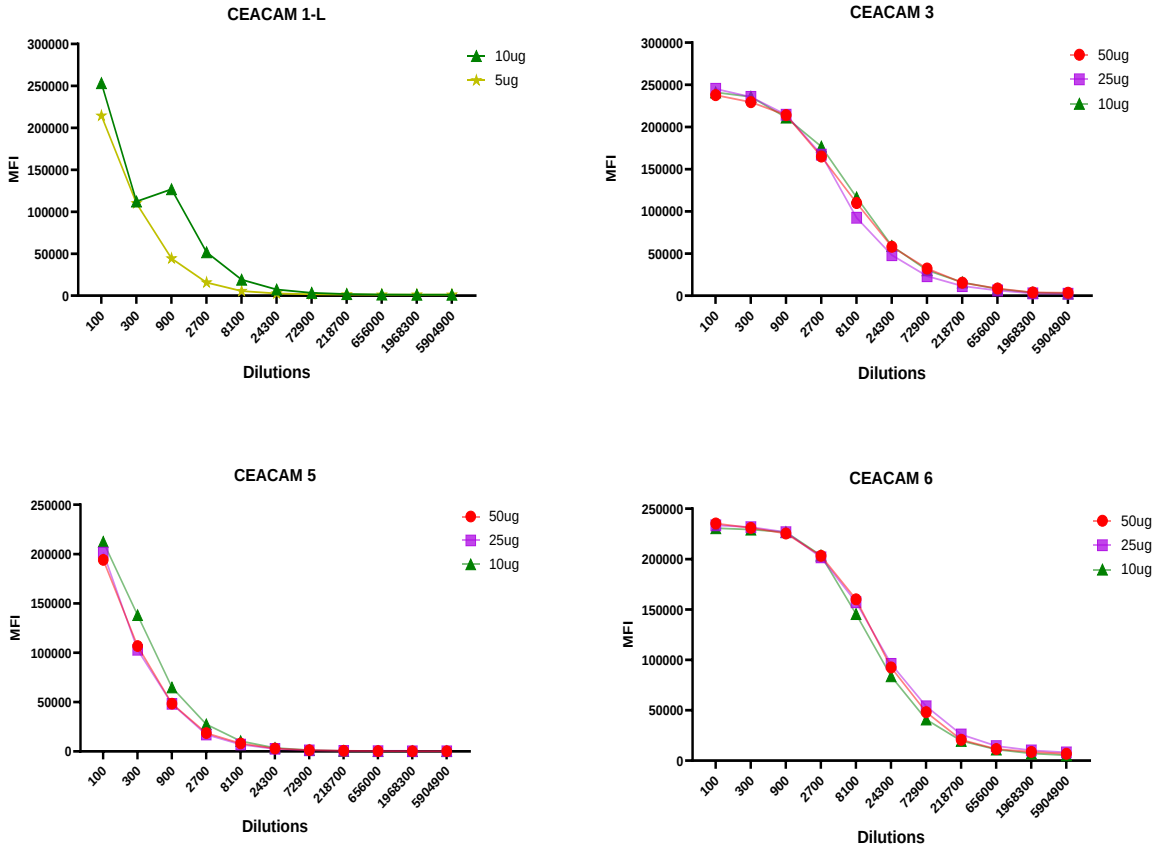

Figure 19. Luminex test coupling of CEACAM receptors. Curves represent results expressed as Mean Florescence Intensity (MFI).

5.1.2. *N. gonorrhoeae* binding to CEACAMs

We investigated the CEACAM-binding profile of a selected panel of eleven epidemiologically relevant Ng strains: FA1090, SK92-679, WHO-M, WHO-N, WHO-F, WHO-G, F62, BG27, BG8, GC14, and FA1090 Δ*Opa*, which served as a negative control.

The results from the Luminex assay showed distinct binding patterns between various *N. gonorrhoeae* strains and CEACAM receptors, which likely reflect the specific Opa proteins expressed by each strain. As shown in **Figure 20**, three different patterns of CEACAM

binders were identified. Strains WHO-G and GC14 were able to bind to CEACAM 1 and CEACAM 5. In contrast, FA1090, WHO-M, and BG8 strains bound to CEACAM 1, CEACAM 3, and CEACAM 6. Lastly, SK92-679, BG27, and WHO-F strains were shown to bind all four CEACAM receptors, demonstrating a more versatile interaction profile. Of note, strain FA1090 *ΔOpa* was not able to bind to any CEACAMs, thus confirming the involvement of Opa proteins in Ng binding to CEACAM receptors. Lastly, the Ng strains WHO-N, MS11 and F62 strains did not bind significantly any CEACAMs.

Overall, this analysis revealed that the analyzed strains exhibited different patterns of binding to the tested CEACAM receptors, probably related to different Opa expressions, likely due to phase variation.

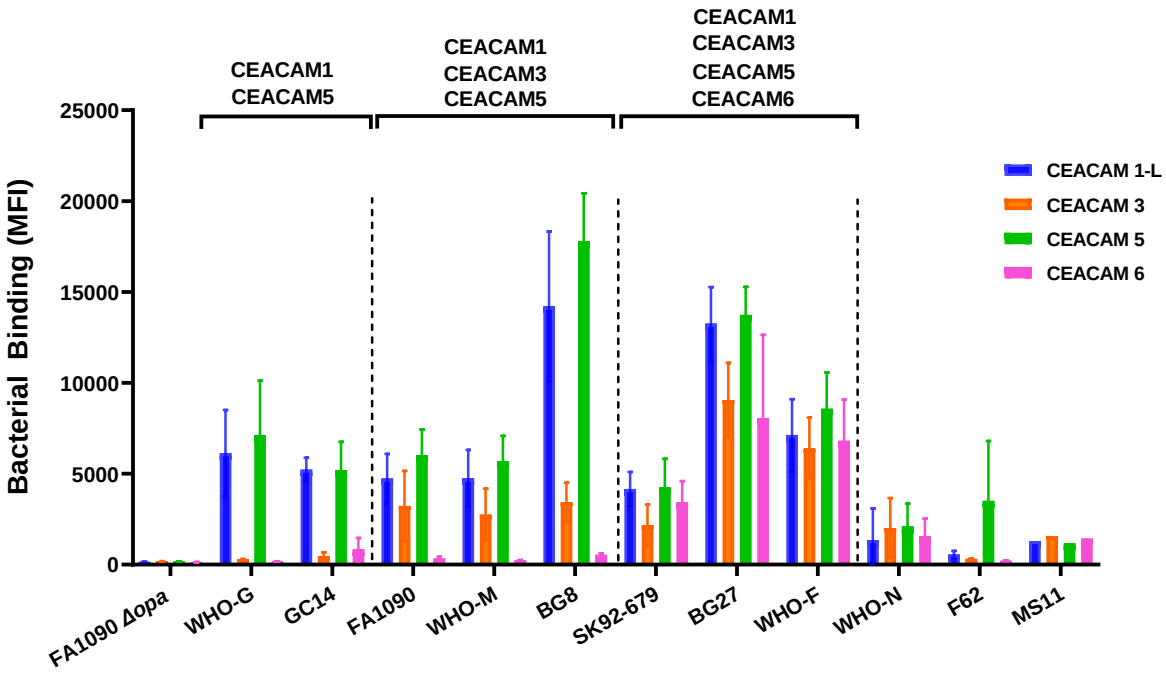

Figure 20 Bacterial adhesion to CEACAM receptors. The histograms show the mean fluorescence intensity (MFI) of the binding between recombinant CEACAM receptors and the panel of gonococcal strains under investigation. FA1090 *ΔOpa* was used as negative control in the assay. Strains are clustered according to their different binding capabilities to CEACAMs. MFI is the average of four replicates and error bars represent the standard deviation.

5.1.3. Bacterial binding inhibition to CEACAM receptors

Having elucidated the CEACAM binding patterns of the selected *N. gonorrhoeae* strains, we aimed to further explore the specific interactions between these Ng strains and CEACAM receptors. Our objective was to discern the role of anti-Opa antibodies in inhibiting the binding of various Ng strains to CEACAM receptors. This investigation is pivotal in understanding the potential of these antibodies in interfering with bacterial adhesion, a critical step in the infection process.

5.1.3.1. Anti- Ng GMMA mice sera

To determine the capacity of anti- Ng GMMA mice sera to inhibit binding to CEACAMs, a panel of *N. gonorrhoeae* strains was evaluated at Luminex, as described previously (**Figure 20**).

The heatmap in **Figure 21** showed that sera from anti- Ng GMMA immunized mice effectively inhibited the binding of both the homologous FA1090 strain (**Figure 21 A**) and heterologous strains, WHO-M, SK92-679, and BG27, to CEACAM 1 and CEACAM 5 (**Figure 21 B-C, F**). Additionally, the analysis showed significant inhibition of binding for strains FA1090, WHO-M, BG27 and BG8 to CEACAM 3 receptor (**Fig. 21 A, C, F** and **G)**, while only SK92-679 and BG27 strains were inhibited to CEACAM 6. Moreover, strains characterized in the binding profile (**Figure 20**) as non-binding to CEACAMs consistently showed this lack of interaction (**Figure 21 I**, highlighted in **gray**).

To provide further understanding, these results were cross-referenced with ON predictions from genome consensus sequences of the *Opa* genes for each strain (see **Figure 21 I**).

These results suggest a relationship between the specific Opa ON profiles of Ng strains and their binding capabilities to CEACAMs. Specifically, the Ng strains that were inhibited such as FA1090 showed Opa ON proteins A, B, C, D, F, and G; SK92-679 displayed Opa ON proteins A, B, I, and K; WHO-M showed Opa ON protein E; and BG27 showed Opa ON proteins J and K (**Fig. 21 I**).

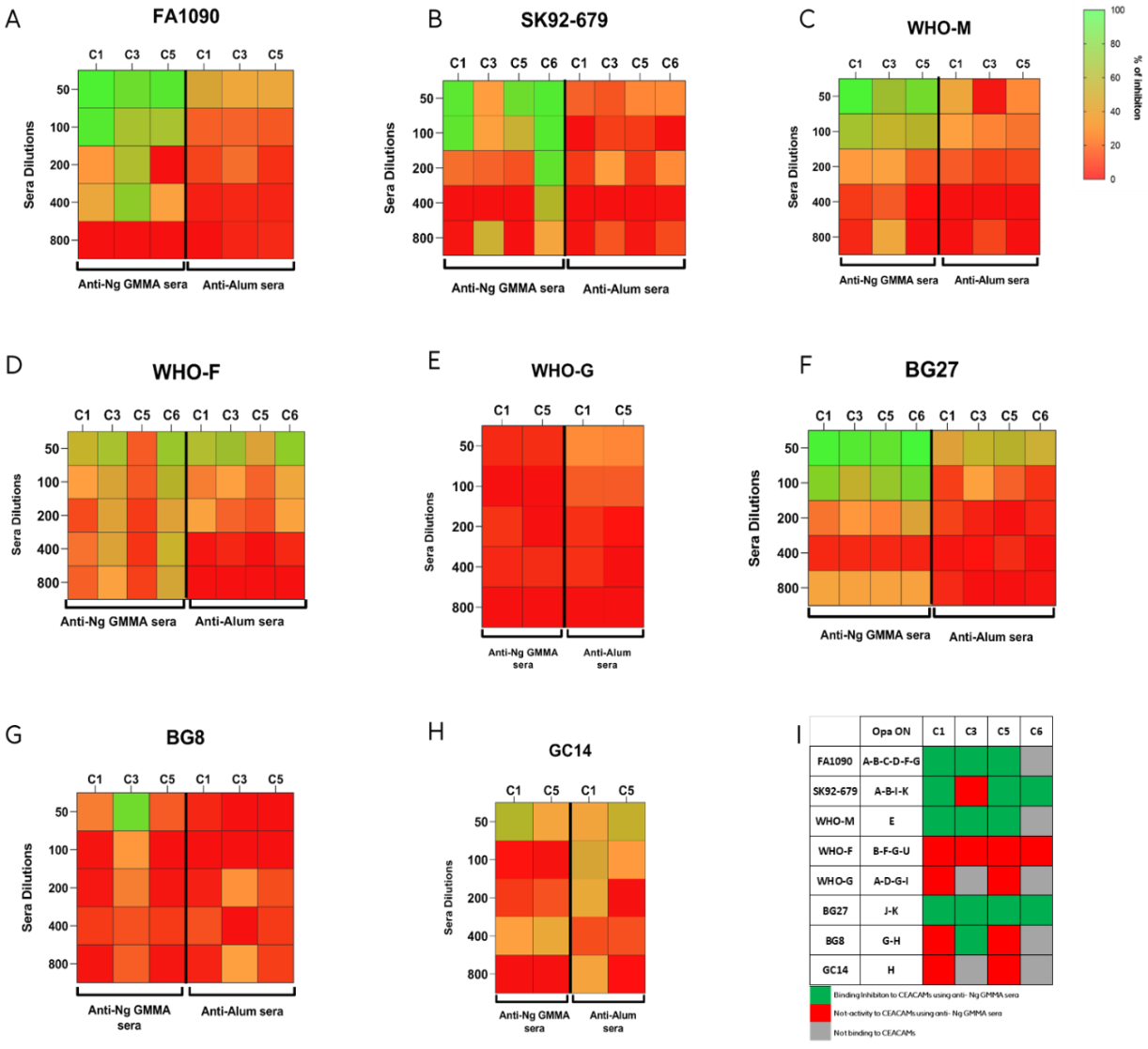

Figure 21 Bacterial binding inhibition to CEACAM receptors. The effect of anti- Ng GMMA mice sera in inhibiting the binding phenotypes of different Ng strains to CEACAMs was determined by Luminex assay compared to control. Binding inhibition is represented in green, indicating effective inhibition of receptor interaction by the sera. Red indicates strains where the sera exhibited no inhibition activity, and gray represents strains that did not bind to CEACAM receptors.

5.2. 2D Cell line models

5.2.1. Host-pathogen characterization on 2D cell line models

To deepen our understanding of the interaction between Opa proteins and CEACAMs, we investigated the presence of these receptors in two separate cell lines representative of the ecto/endo-cervical tract, using specific antibodies for CEACAM 1 and CEACAM 5. We quantified the adhesion of strains, FA1090 and BG8 to the same cell lines, Ect 1 E6/E7 and End 1 E6/E7.

As shown in **Figure 22A**, at a similar MOI the FA1090 strain adhered better to endocervical than to ectocervical cells. A similar pattern of adhesion was observed for the BG8 strain (**Fig. 22 B**).

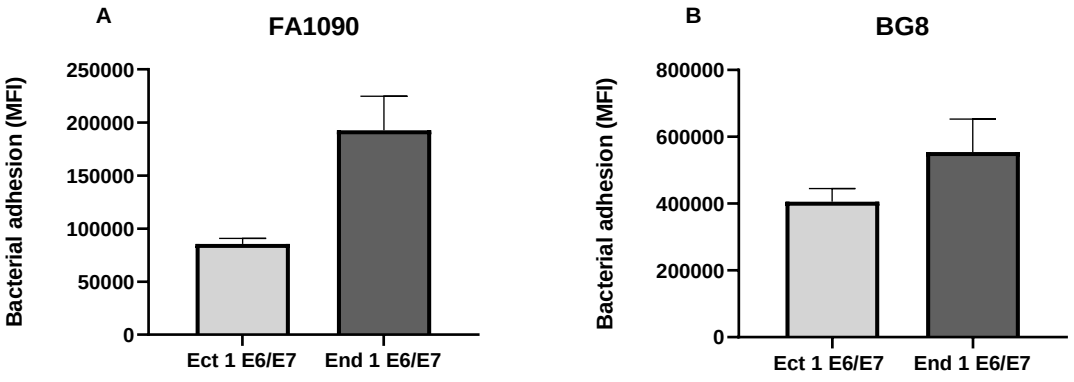

Figure 22 Bacterial Adhesion of Ng strains FA1090 and BG8 on Ect 1 E6/E7 and End 1 E6/E7 using Opera Phenix. This figure illustrates the adhesion of Ng strains to epithelial cell monolayers. **A**) the adhesion of the FA1090 strain to ecto- and endocervical cell lines. **B**) the adhesion of the BG8 strain to these same cell lines. Adherent bacteria were quantified using automated microscopy with Opera Phenix technology, with images captured at 40x magnification using a water immersion objective.

The confocal microscopy images in **Figure 23** illustrate the co-localization of two distinct Ng strains, FA1090 and BG8 with CEACAMs. Interestingly, as showed in **Figure 23 B**, in the case of Ect1 E6/E7 cell line, representative of ectocervical tract with its squamous cell morphology (in red the actin and in blue the nuclei), co-localization is observable with the CEACAM 5 receptor (in yellow), but not with CEACAM 1, when infected with the FA1090 strain (in green). The same scenario was observed also in the same cells infected with the BG8 strain, in green, (**Fig. 23 D**), where co-localization is appreciable solely, in yellow, with CEACAM 5.

In context of End 1 E6/E7 cell line (in red the actin and in blue the nuclei), representative of the endocervical tract with its columnar cell morphology, co-localization between CEACAMs and FA1090 and BG8 strains was observed (in green), as illustrated in **Figure 23 E-H**. FA1090 strain colocalized with both CEACAM 1 and CEACAM 5 receptors (in yellow). A similar trend in co-localization was observed for the strain BG8 (in green), which also binds to both CEACAM 1 and CEACAM 5 (in yellow).

Lastly, both the FA1090 and BG8 strains exhibited the highest adherence to the End1 E6/E7 cell line, as indicated by an increased percentage of bacterial adhesion, as shown in **Figure 22**. This increase of binding might be related to the presence of both CEACAM 1 and CEACAM 5 receptors.

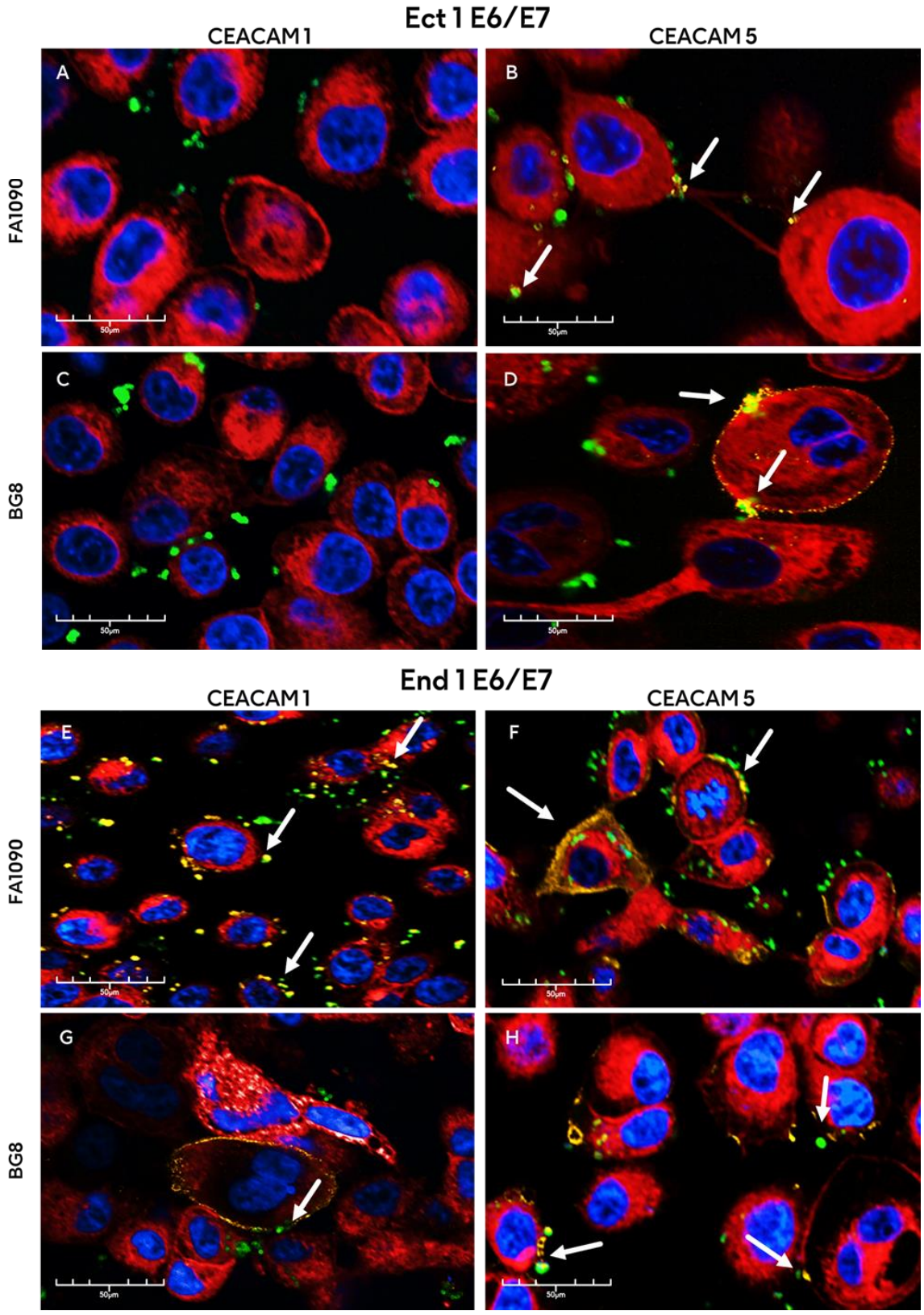

Figure 23 Characterization of adhesion and co-localization of Ng strains FA1090 and BG8 on Ect 1 E6/E7 and End 1 E6/E7 with Opera Phenix: **A-H**) DAPI (blue) nuclei; Cell mask (red) plasma membrane, CEACAM receptor antibody (orange); Ng strains Oregon green (green). The white arrows indicate the co-localization between Ng and CEACAM 1 or 5. Images taken with the 63x water microscope.

In our investigation of the urogenital tract, we included the male urethral SV-HUC 1 epithelial cell line in our study. This cell line was infected with the FA1090 strain, marked in green (**Fig. 24**). Notably, the results demonstrated an absence of co-localization between the *N. gonorrhoeae* strain and either CEACAM 1 or CEACAM 5 in this urethral cell line (**Fig. 24A-B).**

Finally, we investigated the cellular environment of the oropharynx by infecting the Detroit 562 tumor cell line, using FA1090 strain, indicated in green (**Figure 24C** and **D**). Notably, we observed co-localization Ng with CEACAM 5, highlighted in yellow in **section D**. Conversely, no interaction was detected with CEACAM 1, as shown in **section C**.

Overall, these results suggest that the interaction between Ng and CEACAM may depend on the specific cellular context, further highlighting the complexity of host-pathogen interactions.

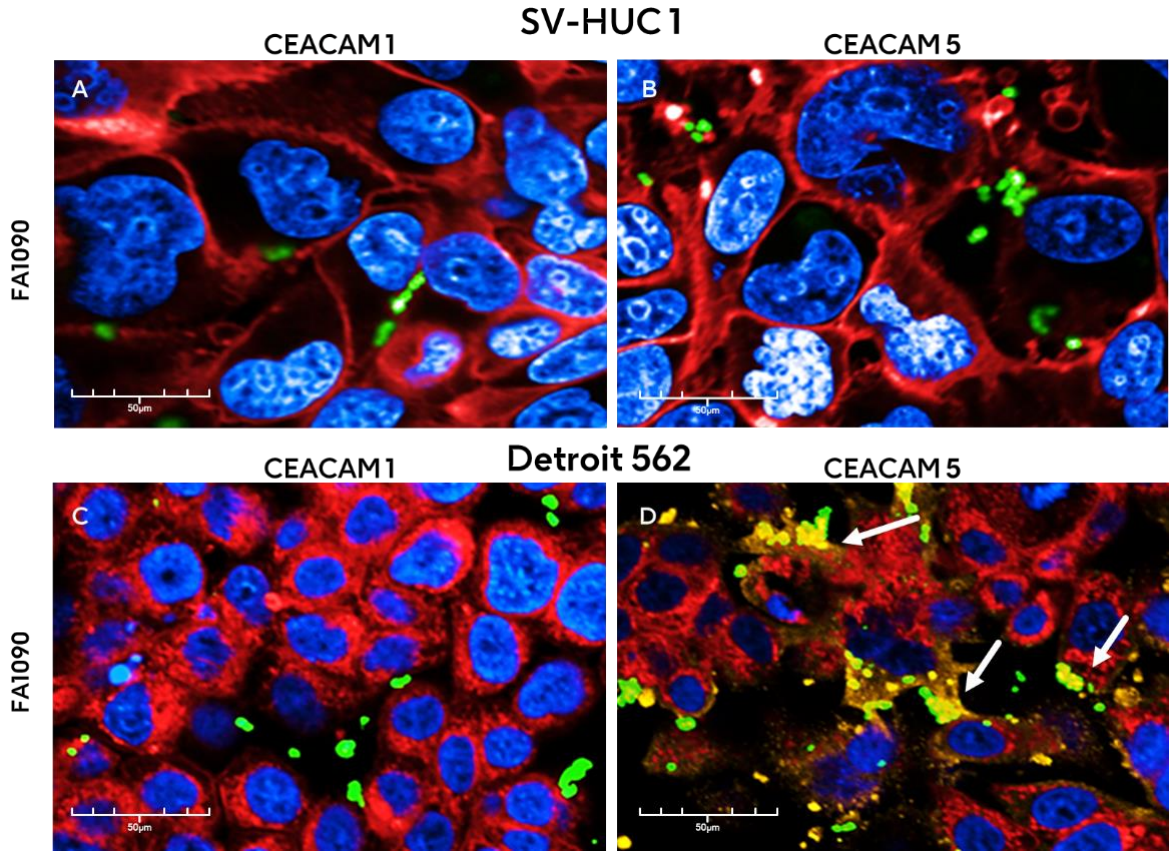

Figure 24 **Characterization of adhesion and co-localization of Ng strains FA1090 and BG8 on SV-HUC1 and Detroit 562 with Opera Phenix**: **A-D**) DAPI (blue) nuclei; Cell mask (red) plasma membrane, CEACAM receptor antibody (orange); Ng strains Oregon green (green). The white arrows indicate the co-localization between Ng and CEACAM 1 or 5. Images taken with the 40x water microscope.

To investigate the role of Opa proteins in bacterial adhesion, we have examined the interaction of a monoclonal antibody (mAb) specific to Opa B.

As shown in **Figure 25**, the Ect1 E6/E7 cell line was infected with FA1090 and SK92-679 strains, (in green). Notably, co-localization with the anti-Opa B mAb was observed exclusively with the FA1090 strain (Fig. **25A**), indicating specific antibody recognition. Instead, the anti-Opa B mAb did not exhibit interaction with SK92-679 strain (**Fig. 25B**), suggesting the absence of Opa B expression on its surface.

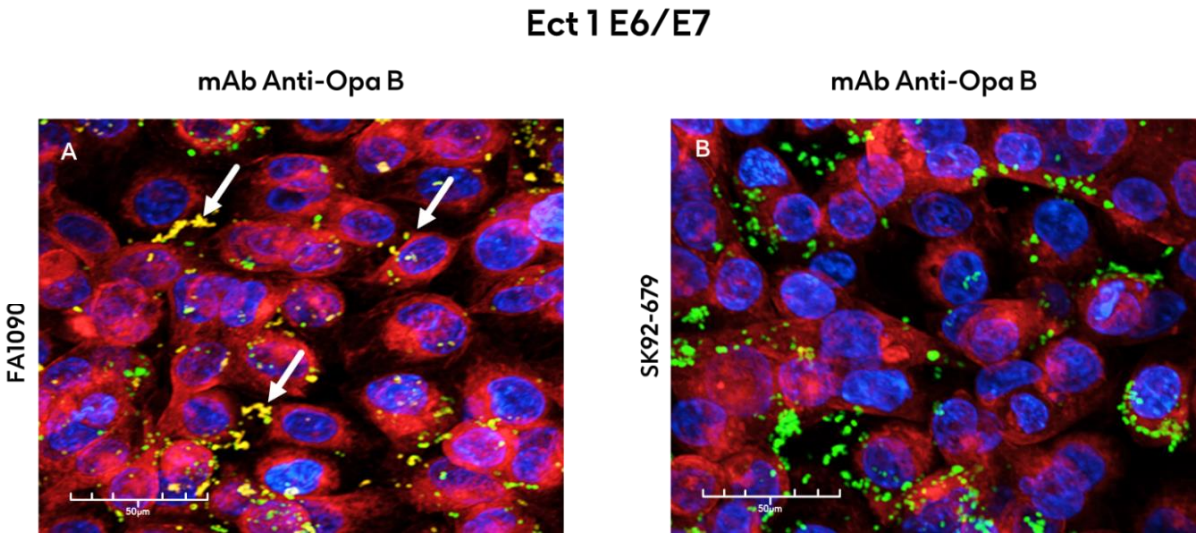

Figure 25 Adhesion and co-localization with anti-OpaB mAb and Ng strains FA1090 and SK92-679 on Ect 1 E6/E7 with Opera Phenix: A-B) DAPI (blue) nuclei; Cell mask (red) plasma membrane, Anti-OpaB antibody (orange); Ng strains Oregon green (green). The white arrows indicate the co-localization between Ng and OpaB protein. Images taken with the 63x water microscope.

5.2.2.Characterization of adhesion of *N. gonorrhoeae* strains

In our study, we evaluated the adhesion of various Ng strains to different epithelial cell lines (**Fig. 26**), with fluorescence measurements taken to quantify bacterial adhesion (MFI). Fluorescent bacterial strains labeled with Oregon Green (1:200) at an MOI of 1:25 were incubated for 1 hour with Ect1 E6/E7, End1 E6/E7, and Detroit 562 cells. The MFI values were calculated from multiple replicates, with the results presented alongside the standard deviations of the experimental measurements.

As shown in **Figure 26A**, adhesion of the Ng strains to the Ect1 E6/E7 epithelial cell line was assessed. The BG8 strain exhibited the highest level of adhesion, with an MFI > 100,000. Additionally, the SK92-679, WHO-M, and FA1090 strains presented elevated levels of adhesion. However, the FA1090 *ΔOpa* strain showed a reduced signal (MFI < 100,000), suggesting a significant role of Opa proteins in the adhesion and colonization

process on this specific cell line. These results underscore the differences in adhesion capacity among the tested strains.

We performed an analogous analysis on the endocervical cell line End1 E6/E7, as shown in **Figure 26B**. Consistent with findings from the Ect1 E6/E7 line, the BG8 strain again demonstrated the highest adhesion levels on End1 E6/E7. The SK92-679 strain showed strong adhesion, with MFI > 100,000. Both the FA1090 and FA1090 *ΔOpa* strains showed comparable adhesion, with MFI values above 100,000, despite the absence of Opa proteins in the FA1090 *ΔOpa* strain. On the contrary, the WHO-M strain exhibited lower adhesion levels, with an MFI below 100,000.

Additionally, we extended the analysis to the Detroit 562 cell line (**Fig. 26 C**), representative of the pharyngeal tract, to assess the adhesion of FA1090 and FA1090 *ΔOpa* strains. Notably, similar to observations in the ectocervical cell lines, the FA1090 strain demonstrated higher adhesion, with an MFI around 100,000, compared to the FA1090 *ΔOpa* strain.

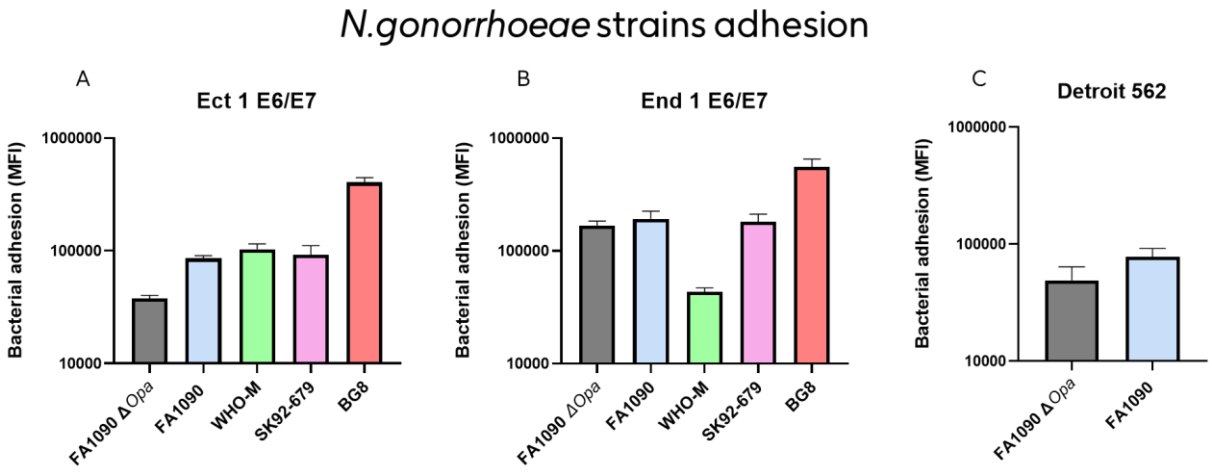

Figure 26 Bacterial Adhesion of different Ng strains on Ect 1 E6/E7, End 1 E6/E7 and Detroit 562 using Opera Phenix. The adhesion of Ng strains to epithelial cell monolayers. **A**) the adhesion of the Ng strains to ectocervical cell lines. **B**) the adhesion of the Ng strains to endocervical cell lines. **C**) Ng strains FA1090 and FA1090 *Δopa* to pharyngeal cell line. Adherent bacteria were quantified using automated microscopy with Opera Phenix technology, with images captured at 40x magnification using a water immersion objective.

5.2.3. Bacterial Adhesion Inhibition

At this stage, we evaluated the ability of the anti- Ng GMMA mice sera to induce antibodies that can inhibit adhesion to different human epithelial cell lines.

This investigation was conducted using a cell-based fluorescent bacterial adhesion inhibition (BAI) assay. We tested the homologous FA1090 strain, including its *ΔOpa* mutant, as well as the heterologous strains SK92-679, WHO-M, and BG8. The cell lines used in this study included Ect1 E6/E7, End1 E6/E7 and Detroit 562 cell line. As a negative control, we used sera obtained from mice immunized with alum.

As shown in **Figure 27 A**, the anti- Ng GMMA mice sera showed a dose-dependent ability to inhibit the adhesion of the homologous FA1090 strain to the Ect 1 E6/E7 cell line (P=0.0001). Furthermore, the adhesion of the heterologous SK92-679 strain was significantly inhibited, (P=0.0028), as shown in **Figure 27 B**. In contrast, the heterologous strain WHO-M exhibited a non-significant trend towards adhesion inhibition (**Fig. 27 C**).

Lastly, no significant inhibition was observed for the FA1090 *ΔOpa* and BG8 strains when tested with the anti- Ng GMMA mice sera (**Fig. 27 D, E**).

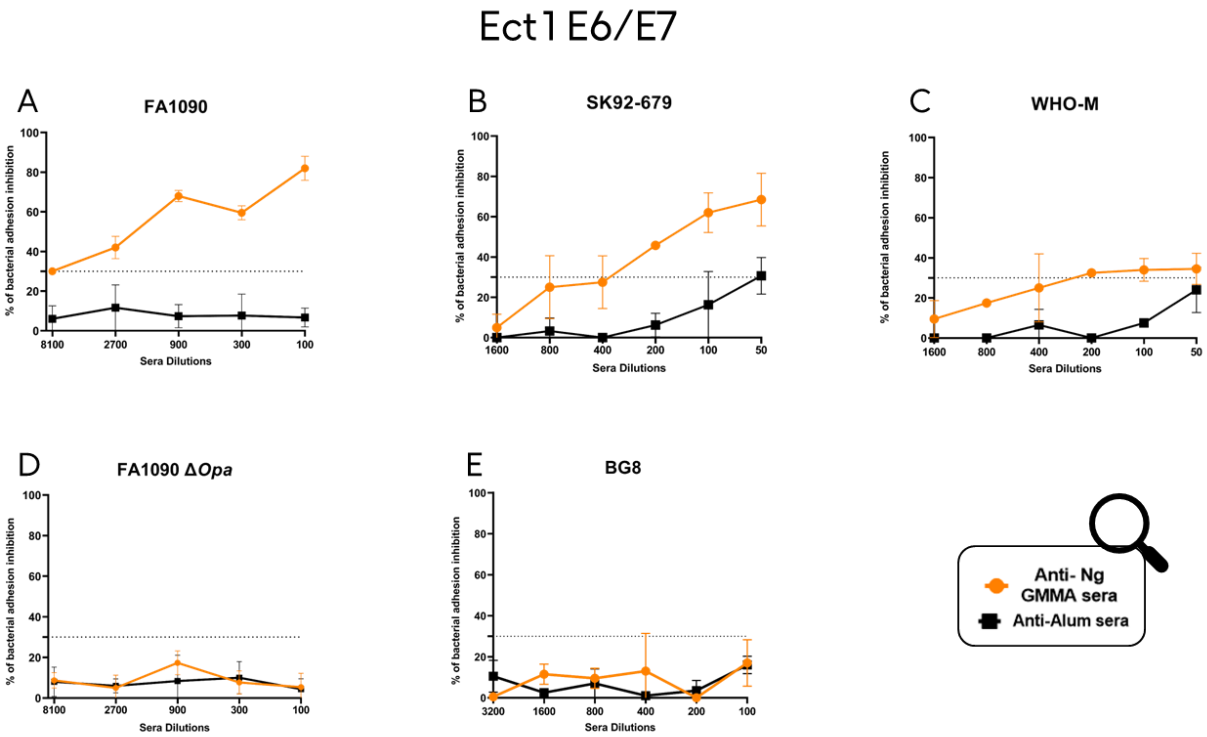

Figure 27 Bacterial Adhesion Inhibition (BAI) on Ect 1 E6/E7 using Opera Phenix. The assay consists in measuring the ability of anti- Ng GMMA mice sera to inhibit the adhesion to ectocervical epithelial cell monolayer. Sera were tested on **A**) FA1090, **B**) SK92-679, **C**) WHO-M, **D**) FA1090 *ΔOpa* and **E**) BG8. All adherent bacteria are quantified by microscopy with Opera Phenix technology automated, Images taken with the 40x water objective.

Furthermore, we tested the anti- Ng GMMA mice sera on the End 1 E6/E7 cell line. As represented in **Figure 28 A**, the mice sera demonstrated a significant ability to inhibit adhesion of the homologous FA1090 strain (P<0,0001).

Additionally, the adhesion of the SK92-679 strain was also greatly inhibited ($P < 0.0001$), as shown in **Figure 28 B**. The WHO-M strain, we observed a lower capacity for adhesion inhibition, at 40% ($P < 0.0001$), as shown in **Figure 28 C**. Even in this context, no significant inhibition was observed for the FA1090 *ΔOpa* and BG8 strains when treated with the anti-Ng GMMA mice sera (**Fig. 28 D, E**).

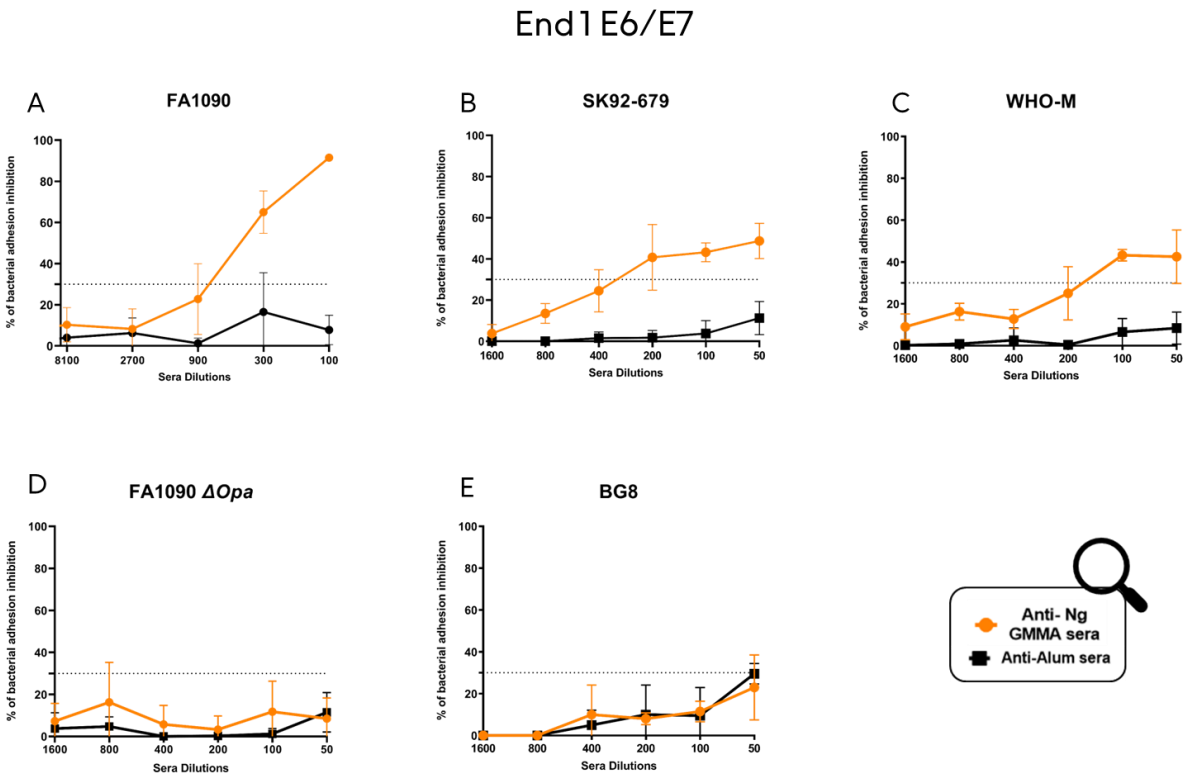

Figure 28 Bacterial Adhesion Inhibition (BAI) on End 1 E6/E7 using Opera Phenix. The assay consists in measuring the ability of Anti- Ng GMMA sera to inhibit the adhesion to endocervical epithelial cell monolayer Sera were tested on **A**) FA1090, **B**) SK92-679, **C**) WHO-M, **D**) FA1090 ΔOpa and **E**) BG8. All adherent bacteria are quantified by microscopy with Opera Phenix technology automated, Images taken with the 40x water objective.

We extended our observations to the Detroit 562 cell line, representative of the oropharyngeal tract (**Fig. 29**) and found similar results to those observed in the ecto/endo cervical female cell lines. Specifically, the anti- Ng GMMA mice sera demonstrated a significant capability to inhibit adhesion of the homologous FA1090 strain, achieving approximately more than 80% inhibition at a serum dilution of 1:100, (P=0,0093), (**Fig. 29 A**). This inhibitory capacity was not observed when the serum was tested against the FA1090 *ΔOpa* strain (**Fig. 29 B**).

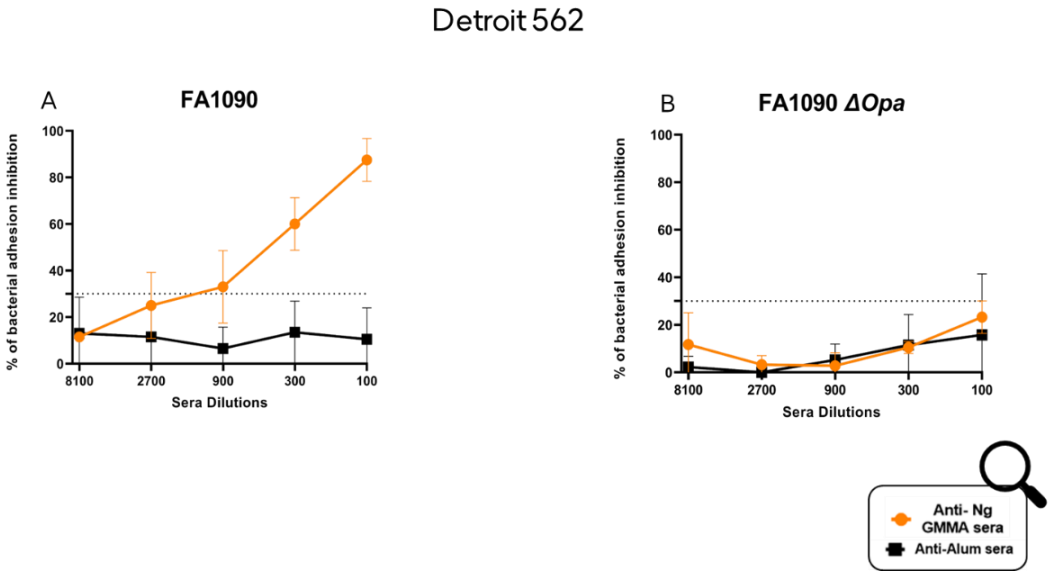

Figure 29 Bacterial Adhesion Inhibition (BAI) on Detroit 562 using Opera Phenix. The assay consists in measuring the ability of Anti- Ng GMMA mice sera to inhibit the adhesion to pharyngeal epithelial cell monolayer. Sera were tested against **A**) FA1090 and **B**) FA1090 *Δopa*. All adherent bacteria are quantified by microscopy with Opera Phenix technology automated, Images taken with the 40x water objective.

Moreover, we compared the levels of bacterial adhesion inhibition by the anti- Ng GMMA mice sera against its homologous strain, FA1090 (**Fig. 30**), across three different cell lines niches. This comparative analysis revealed a statistically significant result, with a *P* value of 0.0016, indicating a strong inhibitory effect of the mice sera across the cell lines studied.

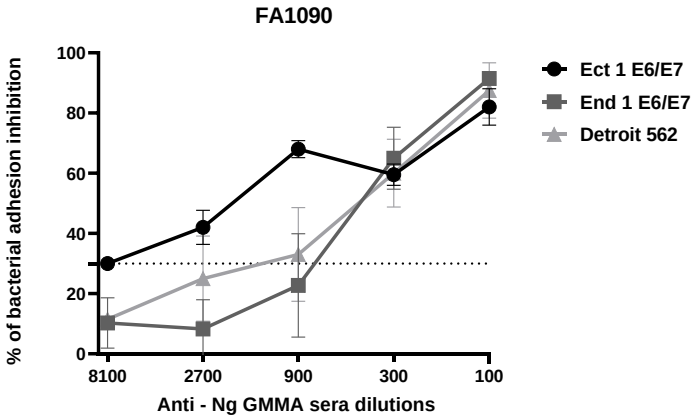

Figure 30 Bacterial Adhesion Inhibition (BAI) comparison with three different cell lines using Opera Phenix. The assay consists in measuring the ability of Anti- Ng GMMA mice sera to inhibit the adhesion to ecto/endo cervical (Ect 1 and End 1) and oropharyngeal (Detroit 562) epithelial cells monolayers. Sera were tested against FA1090 strain. All adherent bacteria are quantified by microscopy with Opera Phenix technology automated, Images taken with the 40x water objective.

5.2.3.1. Anti-Opa mAbs in BAI

Additionally, we evaluated the inhibitory effect of two distinct anti-OpaB murine monoclonal antibodies (mAbs), anti-OpaB 1 and anti-OpaB 2, on the adhesion of the FA1090 strain to the tested cell lines (**Fig. 31 A**) Ect 1 E6/E7, **B**) End 1 E6/E7, **C**) SV-HUC 1, **D**) Detroit 562). In this context, we have included the SV-HUC 1 cell line, representative of the urogenital male tract, considered the potential involvement of another Opa-dependent receptor, HSPG.

As shown in Figure **31A**, the two anti-Opa mAbs exhibit a high inhibitory power, ranging from 80% to 90%, against the Ect1 E6/E7 cells. The mAbs were tested starting from concentrations of 5 µg/mL. The anti-OpaB 1 mAb demonstrated nearly 90% inhibition at the highest tested dose, maintaining its inhibitory effect even at 0.02 µg/mL. In contrast, the anti-OpaB 2 mAb achieved approximately 80% inhibition at 5 µg/mL and 70%-80% at 1.7 µg/mL.

For the End 1 E6/E7 cell line (**Fig. 31B**), a similar inhibition trend was observed with both mAbs at the tested concentrations. However, both mAbs lost their inhibitory effects starting at a concentration of 0.1 µg/mL.

In both the Ect 1 E6/E7 and End 1 E6/E7 cell lines, the *P* value was less than 0.0001, indicating a significant inhibitory effect of the mAbs.

In the case of the SV-HUC 1 cell line (**Fig. 31 C**), both mAbs were able to inhibit adhesion by 50-70% at the initial dilution points, starting of 10 µg/mL. with a *P* value of less than 0.0001.

Lastly, in the Detroit 562 cell line (**Fig. 31D**), representative of the oropharyngeal tract, both mAbs inhibited adhesion by 50%-70% at initial dilution points, beginning at 10 µg/mL (P = 0.0148). It is important to note that the inhibitory effect of the mAbs did not exhibit a dose-dependent pattern. Furthermore, the standard deviation across different dilution points was notably high. This variability might be due to the tumoral nature of the Detroit 562 cell line, which can lead to greater response variability compared to non-tumoral cell lines.

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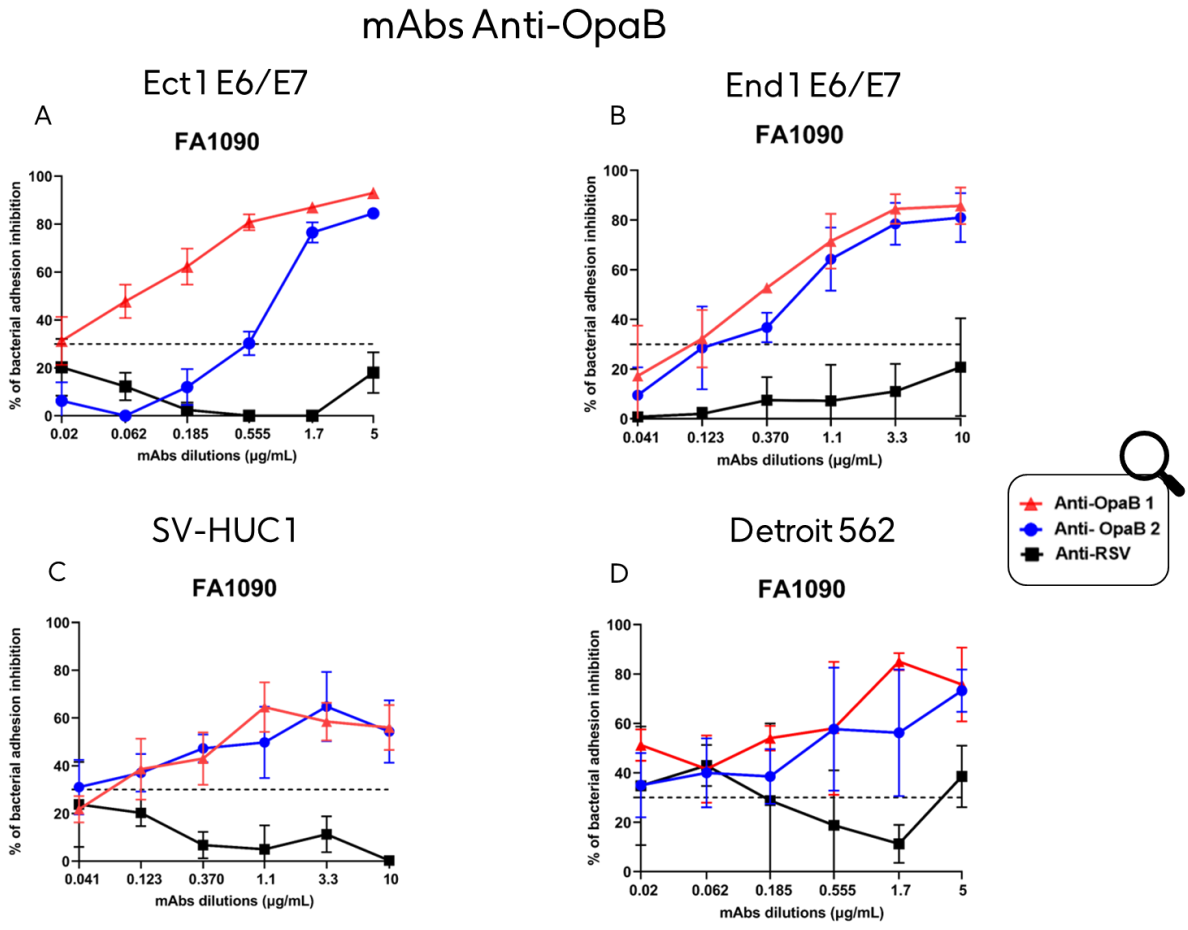

Figure 31 Bacterial Adhesion Inhibition (BAI) comparison with different cell lines using Opera Phenix. The assay consists in measuring the ability of anti- OpaB mAbs to inhibit the adhesion of four epithelial cell monolayers. **A**) Ectocervical, **B**) Endocervical, **C**) Urethral and **D**) Oropharyngeal. The two mAbs were tested on FA1090 Ng strain. All adherent bacteria are quantified by Opera Phenix technology automated, Images taken with the 40x water objective.

In addition to our investigations with mice sera against gonococcus, we explored the potential of evaluating human sera from individuals vaccinated with Bexsero using the bacterial adhesion inhibition assay. We tested two commonly used laboratory Ng strains, FA1090 and SK92-679.

As illustrated in **Figure 32A**, for the FA1090 strain, no strong inhibitory effect was observed when comparing Post2- and Pre-vaccination sera. Similarly, the inhibition of adhesion for the SK92-679 strain was not significant.

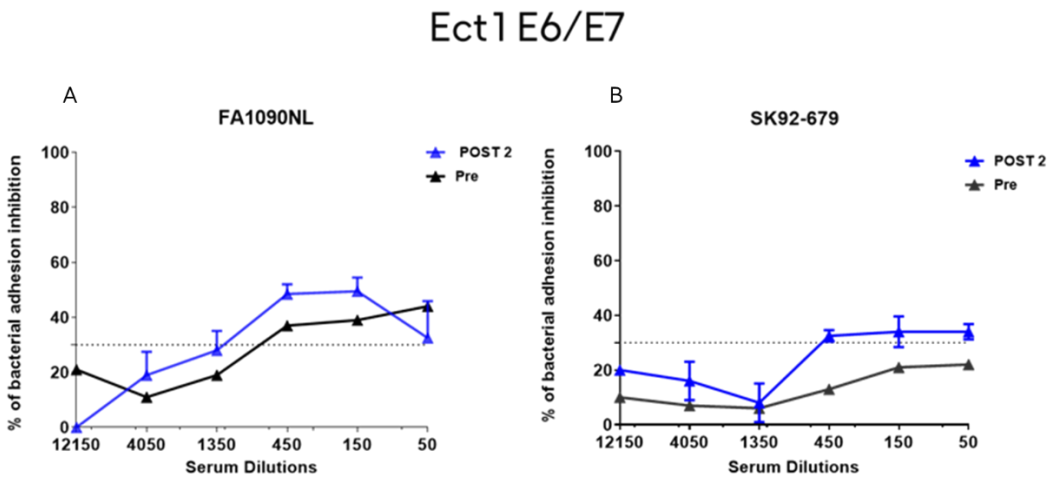

Figure 32 Bacterial Adhesion Inhibition (BAI) with Ect 1 E6/E7 cell lines using Opera Phenix. The assay consists in measuring the ability of human sera post2 to inhibit the adhesion against FA1090 and SK92-679 strains to ectocervical epithelial cell monolayers. All adherent bacteria are quantified by microscopy with Opera Phenix technology automated, Images taken with the 40x water objective.

5.3. 3D Cell Complex *In-vitro* Model (CIVM)

5.3.1. Host-pathogen characterization on 3D (CIVM)

To investigate host pathogen interaction in more physiological conditions we have used a 3D Cell Complex In-vitro Model (from MatTek). Initially, we confirmed the expression of both CEACAM 1 and CEACAM 5 (indicated in yellow) localized on the apical part of the cell membranes (in red) within this experimental model (**Fig. 33**). Notably, both receptors are organized in a pattern that reflects their native distribution in human tissue.

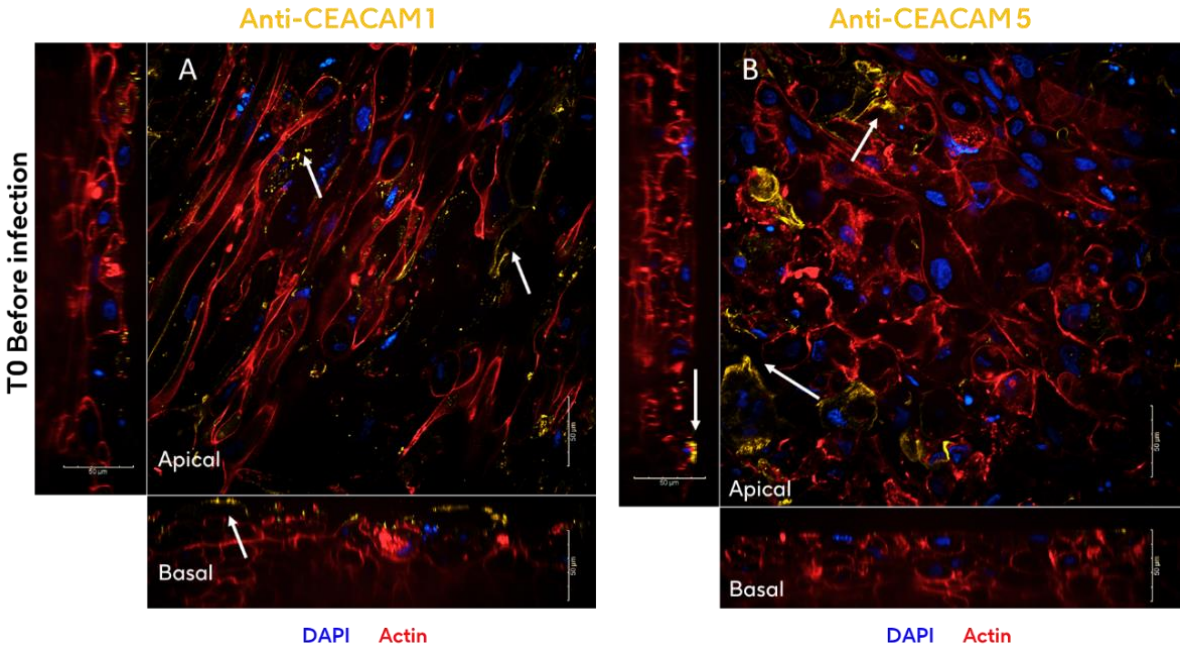

Figure 33 Characterization of 3D Human Vaginal Ectocervical MicroTissue using Opera Phenix: The localization of CEACAM 1 and 5 are indicated with the white arrows, T0 before the infection. **A**) Anti-CEACAM 1 in yellow, nuclei in blue and plasma membrane in red. **B**) Anti-CEACAM 5 in yellow, nuclei in blue and plasma membrane in red. Images taken with the 40x water microscope, with Opera phenix.

Our initial approach, we were able to effectively monitor the progression of the infection at three distinct timepoints: 1- hour post-infection (hpi), 24 hpi, and 48 hpi (**Fig. 36**).

As shown in **Figure 34 A-C**, confocal imaging highlights adhesion and extracellular invasion of Ng strain FA1090, revealing specific cell-membrane interactions and receptor clustering.

At 1 hpi, we observed gonococci adhering to the cells at the apical surface, attributed to the presence of CEACAM 1 and 5 (**Fig. 34A**). Additionally, evidence of bacteria successfully invading the tissue extracellularly is indicated by the white arrow on the left in **Figure 34A**.

It is well-documented in the literature (Song et al. 2020) that gonococcus can invade by disrupting E-cadherin cell-cell binding. Notably, even 1 hour post-infection, the complex model preserved its original thickness (50 μm) and cell morphology, mirroring the uninfected models at the T0 timepoint, as reported in **Figure 34**.

The results shown In **Figure 34 B**, observed at 24 hours post-infection (hpi), suggest early signs of colonization, The clustering of CEACAM receptors upon Opa binding was observed, which may facilitate endocytosis-like uptake, enabling extracellular invasion. This interaction appears to contribute to the bacterial persistence detected in the cellular environment.

Additionally, **Figure 34 B** shows the presence of both extracellular and probable intracellular *N. gonorrhoeae* strain FA1090 (white square, gonococcus in blue). However, the intracellular presence of gonococci need to be further investigation for definitive confirmation. Within our gonococcus-infected model, interactions between the FA1090 strain (in green) and CEACAM 1/5 (in yellow) receptors were noted. Observations of cell

morphology suggest that the apoptosis pathway may be inhibited, with only a subset of cells displaying morphological changes indicative of apoptosis, such as chromatin condensation and cell rounding as showed in **Figure 34 B**.

At the final timepoint of 48 hpi, a significant increase in the presence of gonococci (in green) was observed on the apical surface of the cellular model (**Fig. 34C**). Additionally, bacterial proliferation within the model was associated with progressive damage to the host cells, ultimately leading to cell death. This observation suggests that between 24 and 48 hpi, the infected cells likely died and detached from the cellular substrate.

Another notable observation was the persistent expression of CEACAM 1/5 receptors (in yellow) by the cells, even at 48 hours post-infection (hpi) (**Fig. 34D**). Throughout the infection, the bacteria maintained their ability to interact with these receptors via Opa-CEACAM interactions. This persistence suggests a mechanism by which the bacteria might evade host defenses, continuing to engage with these receptors even in advanced stages of infection.

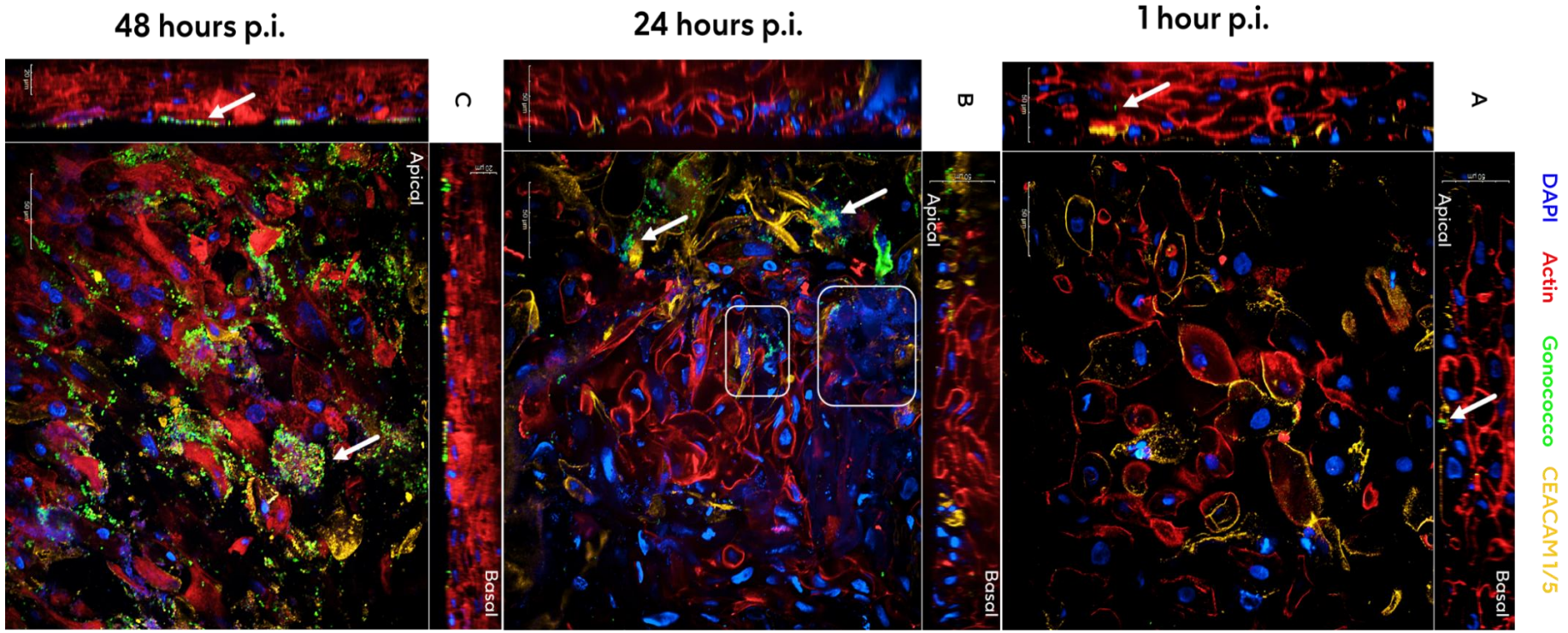

Figure 34 Characterization of infection of N. gonorrhoeae on a 3D Human Vaginal Ectocervical MicroTissue using Opera Phenix: The models were infected with FA1090 for 1h and underexamined the time courses on infection. The CEACAM 1/5 and Ng strain FA1090 were analyzed the co-localization. DAPI (blue) nuclei, Cell mask (red) plasma membrane, CEACAM 1/5 receptor antibody (yellow); Anti-Ng strain FA1090 (green). **A**) 1 h.p.i., the arrows indicate gonococci adhere the model, images taken with the 40x water microscope. **B**) 24 h.p.i., the arrows indicate gonococci co-localization with CEACA 1/5 on the apical side of the model, while the square indicate the extracellular invasion, images taken with the 40x water microscope. **C**) 48 h.p.i., the arrows indicate gonococci co-localization with CEACA 1/5 on the apical side of the model, images taken with the 40x water objective..

From the analysis of the Colony Forming Units (CFU) in the tissue homogenate, as shown in **Figure 35 A**, it was evident that the Ng strain FA1090 effectively has infected and proliferated within complex cellular models. The increase in CFU over the infection timepoints demonstrated the strain's high adaptability and potential virulence.

In panel **B**, we reported a Ng-CEACAM binding assay regarding Ng strain FA1090 and CEACAM 1 and 5. In this scenario, the supernatants of infected models, were collected at 1, 24 and 48 hpi, were tested for binding to CEACAM 1 and 5 receptors. Interestingly, the bacteria in the supernatants have bound to CEACAMs increasingly depending on infection time. Of note, CEACAM 5 appears to be more involved in the binding process compared to CEACAM 1, notably at 48 hpi (**Fig.35 C**).

In conclusion, it was remarkable that the non-adhering bacteria present in the supernatants collected at different time points post-infection were still able to bind CEACAM, suggesting that the FA1090 strain remains Opa positive.

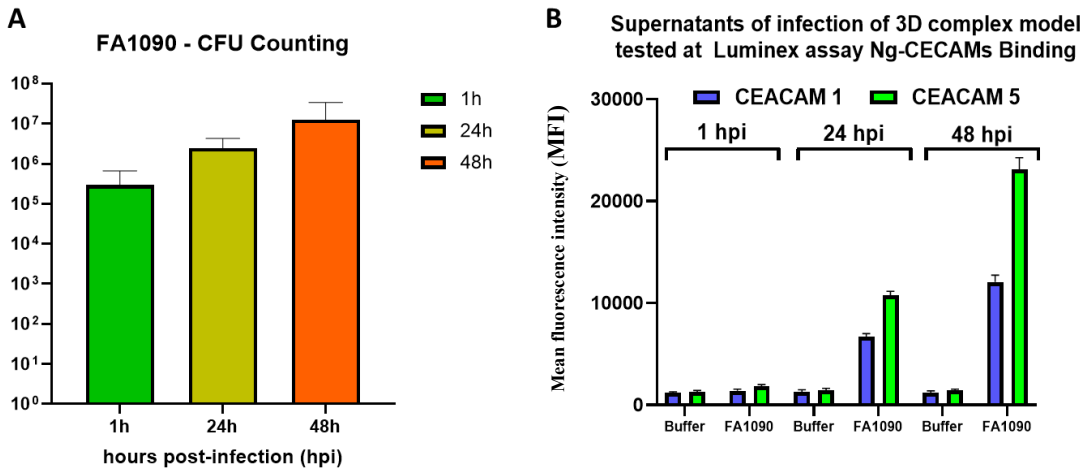

Figure 35 Ng strain effectively infects the 3D complex models: A) CFU count of homogenates of the infected models **B**) bacterial binding to CEACAM receptors: Binding of Ng strain, grown on a 3D complex model, to distinct CEACAM receptors was measured by Luminex assay as mean fluorescence intensity (MFI).

Subsequently, experiments were conducted to investigate alternative methods of infection on complex model. Interestingly, after 24 hours of infection in liquid-liquid conditions (**Fig. 36**), significant bacterial colonization was observed on the apical surface of the cells.

We identified a co-localization between the FA1090 strain (visualized in green) and CEACAM 1/5 (visualized in yellow) on the apical surface, as shown in **Figure 36**. The Ng FA1090 strain successfully interacted with the tissue, showing evidence of adherence and potential invasion.

The nuclei appeared large and regular (blue), as shown in **Figure 36**, with uniformly distributed chromatin and no signs of condensation or fragmentation. The cellular membranes remained intact (red), without any signs of blebbing, which suggests an absence of damage or altered permeability. Additionally, the overall cellular morphology was well-preserved, with no evidence of swelling or cytoplasmic vacuolization, both of which are associated with cellular stress or damage. These observations indicate that the cells did not exhibit any morphological characteristics of apoptosis or cellular stress despite exposure to the *Ng* FA1090 strain.

Furthermore, we observed aggregates of gonococci bound to CEACAMs, which were located at a distance from the cellular membranes. Interestingly, the release of CEACAM 5 into the supernatant was detected. This release was confirmed by Western blot analysis (**Fig. 36**).

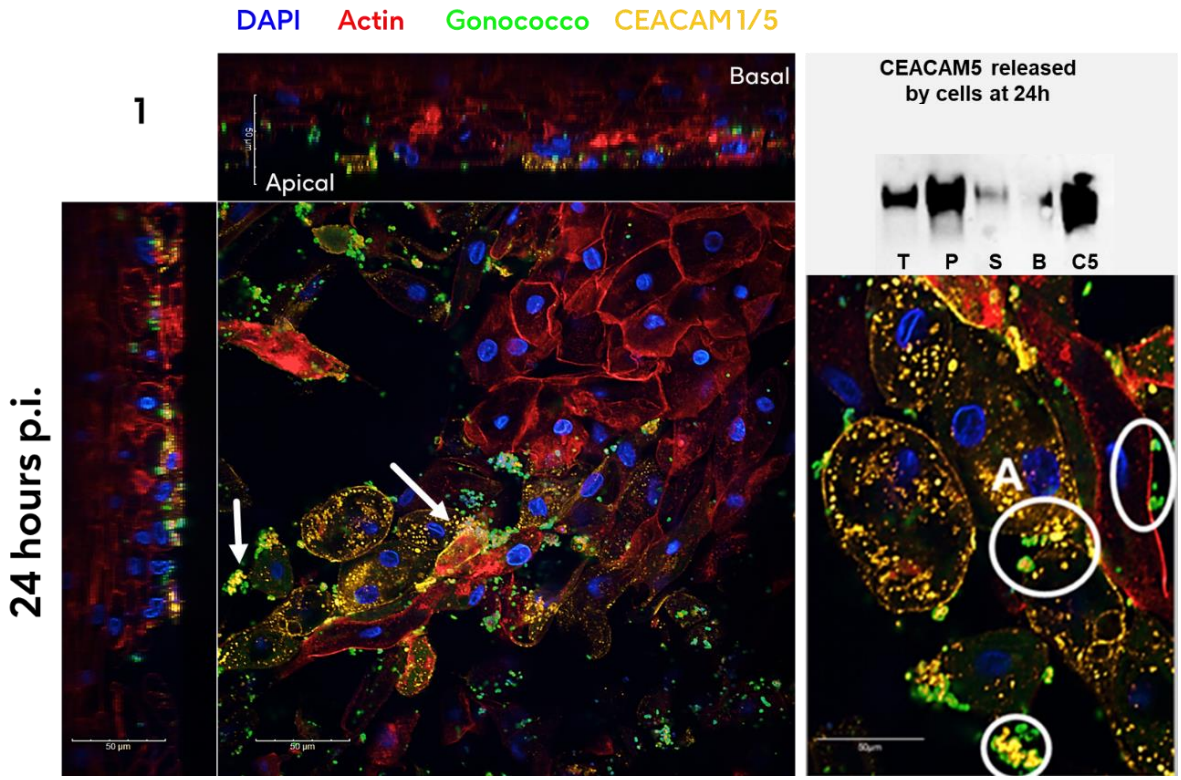

Figure 36 Characterization of 24 hours of infection of *N. gonorrhoeae* (Liquid-Liquid) on a 3D Human Vaginal Ectocervical MicroTissue using Opera Phenix: The models were infected with FA1090 for 24h in liquid-liquid condition. The CEACAM 1/5 and Ng strain FA1090 were analyzed for co-localization. DAPI (blue) nuclei, Cell mask (red) plasma membrane, CEACAM 1/5 receptor antibody (yellow); Ng strain FA1090 (green). The arrows indicate gonococci co-localization with CEACAM 1/5 on the apical side of the model, images taken with the 40x water objective. The western blot indicates the presence of CEACAM 5 in supernatant on infection; (T is the Total supernatant of infection after 24 h.p.i., P is the Pellet of T, S is the Supernatant of P, B is the supernatant buffer of model without infection).

Analysis of the tissue homogenate (**Fig. 37A**) showed that the model was infected with a bacterial load ranging between 10^5 and 10^6 CFUs, compared to the initially inoculated dose of 10^8.

Furthermore, after 24 hours of infection in liquid-liquid culture, FA1090 showed effective binding to both CEACAM1 and CEACAM5 receptors (**Fig. 37 B**), as highlighted in **Figure 35B**. These observations suggest that CEACAM5 may play a predominant role in interactions between the pathogen and host cells during infection.

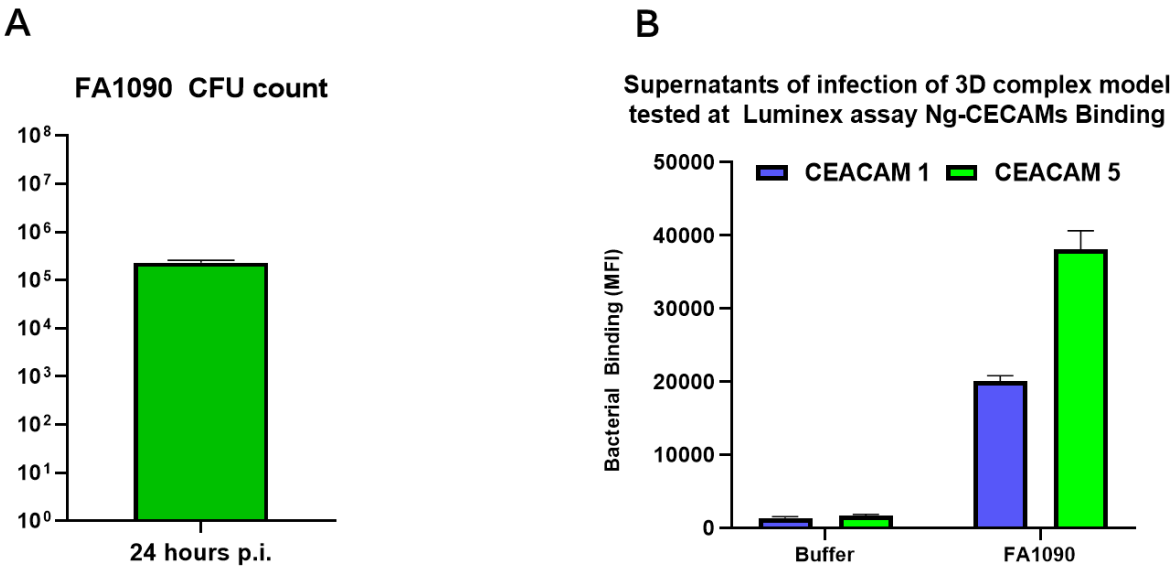

Figure 37 Ng strain effectively infects after 24h in liquid-liquid the 3D complex models: A) CFU count of homogenates of the infected models B) bacterial binding to CEACAM receptors: Binding of Ng strain, grown on a 3D complex model, to distinct CEACAM receptors was measured by Luminex assay as mean fluorescence intensity (MFI).

5.3.2. Bacterial Adhesion Inhibition on a Ecto-3D model

To confirm our findings, we designed a BAI experiment utilizing the complex model of the ectocervical tract with FA1090 strain and anti- Ng GMMA mice sera. (**Fig. 38**). The preliminary results showed an inhibition of adhesion at 1 h.p.i. The anti- Ng GMMA mice sera inhibited the adhesion of the homologous FA1090 strain, as shown in the graph in **Figure 38**. The mice sera achieved approximately 80% inhibition at sera dilution of 1:100 (step 3), maintaining the inhibitory capacity even at lower sera dilutions 1:900. Interestingly, the same inhibitory trend was observed testing Ect 1 E6/E7 with the same homologous strain FA1090 (**Fig. 27 A**). However, these data should be further confirmed performing a colony-forming unit (CFU) count of the models.

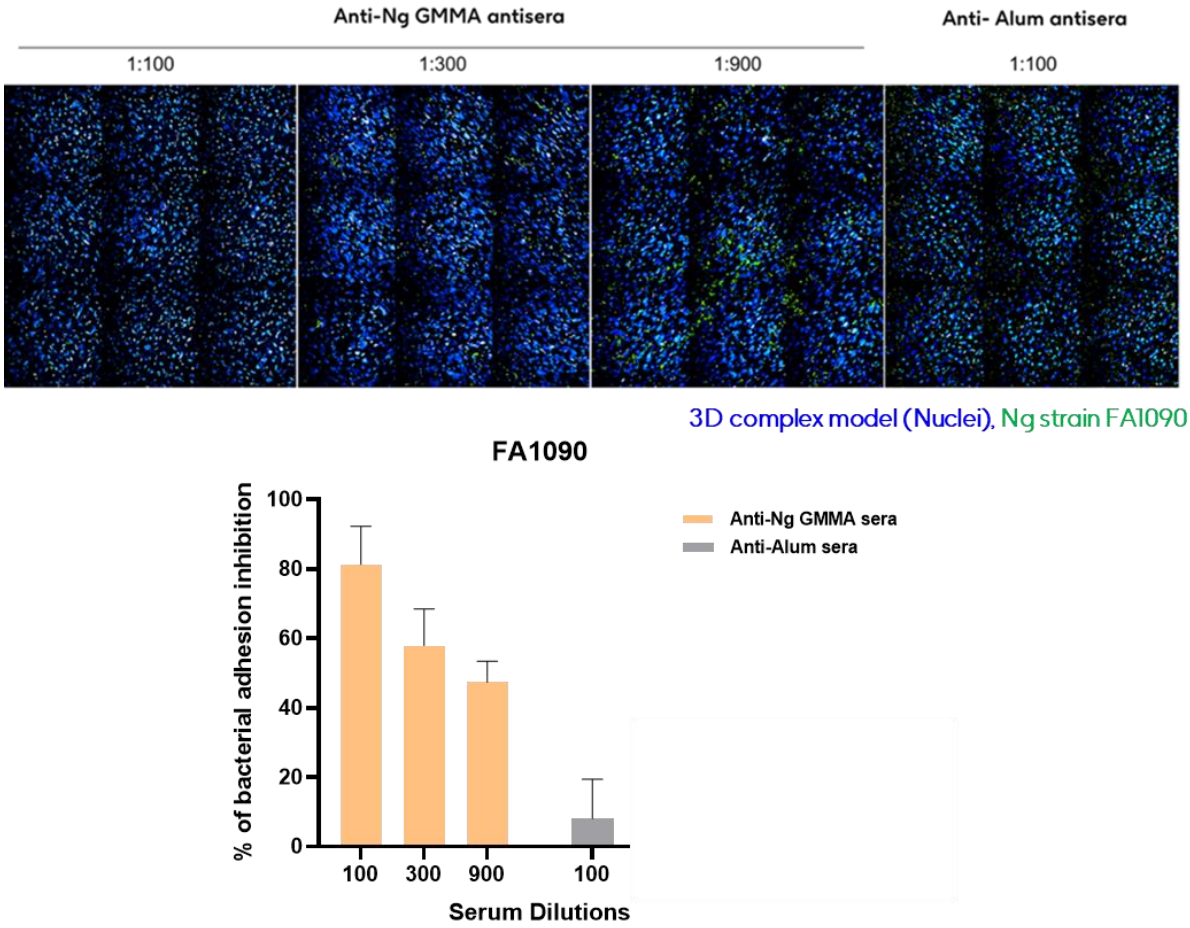

Figure 38 Bacterial adhesion inhibition assay. The ectocervical 3D tissue model was infected for 1h with Ng strain FA1090. DAPI (blue) nuclei, Ng strains Oregon green (green). Images taken with the 40x water microscope. Image analysis with Harmony software. Total fluorescence Area is used to quantify whole bacterial adhesion: the software uses green fluorescence to define a bi-dimensional space representative of bacterial biomass, the graph showed the % of bacterial adhesion inhibition, using harmony software.

6. Discussion and conclusions

In this study we provide new evidence to understand the interactions between *Neisseria gonorrhoeae* (gonococcus) strains and CEACAM receptors, with particular attention to the Opa proteins expressed by the bacterium and their ability to modulate binding to different CEACAMs at sites of infection. CEACAM receptors, particularly CEACAM 1, 3, 5 and 6, play a crucial role in the adhesion of gonococcus to epithelial cells, thus determining its tropism for different tissues. Our main objective was to characterize these interactions in the context of the infection of the female cervix, an important colonization site for the pathogen (Epshita A. Islam et al. 2018; Jerse 1999). We have employed cell lines from key gonococcal infection sites, ecto/endo cervical, oropharyngeal, and male urethral epithelial cells, alongside a Luminex assay utilizing recombinant CEACAM receptors bound to beads. This methodology allowed us to quantify the binding interactions of different Ng strains with those receptors, shedding light on the specific dynamics of bacterial adhesion across these epithelial surfaces (**Fig. 20, 21, 23 and 24**). The integration of cellular models with Luminex assay Ng-CEACAMs binding (**Fig.20**) have provided a comprehensive framework to explore both the molecular mechanism of adhesion and cellular responses involved. Moreover, to better understand the complexity of the human female lower urogenital tract, we employed a physiological cervical complex model (**Fig. 34 and 36**). Several previous studies have shown that CEACAMs act as critical receptors for *N. gonorrhoeae* in human epithelial cells, modulating bacterial adhesion and invasion. In particular, the scientific contribution by Scott Gray-Owen and collaborators has shown that binding of Opa proteins to CEACAMs can facilitate the entry of gonococcus into host cells and influence the host's immune response.

Through binding analysis using the Luminex technique (**Fig. 20**), we have measured the ability of different gonococcal strains to bind to receptors CEACAM1, 3, 5 and 6. These receptors are expressed in various tissues, including those lining the female genital tract, making them potential targets for infection. Our results showed that gonococcal strains exhibited different binding profiles, suggesting that Opa proteins play a key role in the specificity of adhesion to these receptors. These results were also observed by Werner and colleagues using an experiment called Opa-CEACAM Pulldown (Werner et al. 2020), they have analyzed fluorescence intensity data from thousands of cells in a population using imaging flow cytometry to detect N-CEACAM bound to the surface of Opa-expressing Ng.

However, it is crucial to acknowledge that their study was based on the MS11 strain, which is a laboratory strain and may not accurately predict the real infection scenario (Werner et al. 2020). Furthermore, in the referenced study, they used Opa mutants of the MS11 strain to assess binding to CEACAMs. This strategy may lead to bias, as the specific Opa proteins expressed by the strain during actual pathogenic conditions remain unknown.

In contrast, in our experiments, we have used a panel that represents the diversity of Opa proteins but maintained the wild-type strains without any modifications, except for our negative control, the FA1090 *ΔOpa* strain. In particular, we have observed an high binding to CEACAM 1 and 5, suggesting that those receptors may have a predominant roles in the female genital tract, in line with what has been reported in the literature regarding their expressions in cervical tissues (Epshita A. Islam et al. 2018; Popp et al. 1999; Schmitter et al. 2004).

Moreover, our study shows that anti- Ng GMMA mice sera effectively inhibited the binding of several Ng strains to CEACAM receptors, as demonstrated by the Luminex assay results.

The sera significantly reduced adhesion of both the homologous FA1090 strain and heterologous strains WHO-M, SK92-679, and BG27 to CEACAM 1 and 5, indicating broad-spectrum inhibition (**Fig. 21**). Inhibition of binding to CEACAM 3 by strains like FA1090 and BG27 highlights the potential of these sera to disrupt key bacterial interactions with host tissues. Cross-referencing with Opa gene predictions confirms the role of specific Opa configurations in mediating the binding. These findings underscore the importance of targeting Opa-CEACAM interactions in novel therapeutics strategies.

The distinct interactions observed between Opa proteins and various CEACAM receptors are likely influenced by the specific amino acid sequences within the hypervariable regions HV1 and HV2 of the Opa proteins. These hypervariable domains contribute directly to receptor-binding specificity, as their structural variability enables selective recognition of particular CEACAM isoforms (Martin et al. 2016; Werner et al. 2020).

These results highlighted the considerable variability in Opa-CEACAM interactions among different strains and confirm that Opa proteins may undergo significant antigenic variability, which represents an immune evasion strategy employed by the pathogen to escape recognition by the host's immune system. Moreover, without a comprehensive repertoire of antibodies targeting these proteins, it becomes challenging to effectively inhibit adhesion and, subsequently, invasion.

Subsequently, we expanded our analyses by utilizing various cell lines that represent the primary sites of gonococcal infection. The use of these cellular models allowed us to examine both the adhesion of gonococcus and the ability of the sera to inhibit interaction with host cells. Confocal microscopy confirmed that ectocervical cells primarily express

CEACAM 5, as does the oropharyngeal cell line, while endocervical cells express both CEACAM 1 and 5 (**Fig. 23 and 24**).

Interestingly, the urogenital cell line SV-HUC1 did not express CEACAMs. Receptors present on male urogenital tract cells might instead be HSPGs (heparan sulfate proteoglycans) though further investigations would be required to confirm this hypothesis (Barbosa, Biancardi, and Carvalho 2021).

Our bacterial adhesion inhibition (BAI) experiments demonstrated that anti- Ng GMMA mice sera inhibited the adhesion of the homologous strain FA1090, as well as the heterologous strains WHO-M and SK92-679, to ectocervical (Ect1 E6/E7), endocervical (End1 E6/E7), and oropharyngeal (Detroit 562) cell lines. However, consistent with the results from Luminex assay, BG8 strain was not inhibited by anti- Ng GMMA sera in either CEACAM Luminex analyses or ecto/endo-cervical cell lines. Interestingly, the anti- Ng GMMA mice sera did not inhibit the adhesion of the FA1090 *ΔOpa* mutant strain. This suggests a significant role for anti-Opa antibodies in the inhibition of gonococcal adhesion to ecto/endo cervical and oropharyngeal cell lines. Furthermore, using monoclonal antibodies specific to OpaB from the FA1090 strain (**Fig. 31 A-D**), we confirmed that bacteria expressing OpaB were inhibited to adhesion in our four representatives cell models: ecto/endo cervical, male urethral and oropharyngeal.

To evaluate the long-term infection dynamics and the influence of Opa-CEACAM proteins in a physiologically relevant context, we employed a 3D complex model of primary ectocervical tract (**Fig. 34-38**). This model allowed us to study the infection over a longer timeframe (1h, 24h, 48h), more accurately simulating in vivo conditions. We observed that the model expressed both CEACAM1 and CEACAM5, and that the adhesion of gonococcus

FA1090 strain was significantly inhibited by the mice sera. This result further confirms that the Opa-CEACAM interaction is an important mediator of gonococcal infection and that the ability of the sera to inhibit this interaction can be exploited to develop targeted therapeutic approaches.

At 24 hpi, besides to bacterial colonization, we observed a possible intracellular invasion, suggesting that the bacteria may also penetrate the host cells. However, this finding requires further validation through additional experiments, such as electron microscopy.

The interaction between Ng strain FA1090 and CEACAM1/5 seems to correlate with morphological changes in the infected cells. Specifically, the inhibition of apoptosis, inferred from the morphology of cells shown in **Figure 34 B**, points to a potential mechanism by which the bacteria may modulate host cell fate to favor its survival. This finding aligns with previous studies that have suggested *N. gonorrhoeae* can manipulate host signaling pathways to delay apoptosis, providing an intracellular niche for bacterial replication (Song et al. 2020; Walker et al. 2023).

However, the significant increase in the presence of gonococci on the apical surface of the cellular model 48 hpi suggests a rapid proliferation of the bacteria within this timeframe (**Fig. 34 C**). This proliferation appears to be directly linked to the progressive damage and eventual death of the host cells. It can be inferred that the infected cells likely died and detached from the cellular substrate between 24 and 48 hpi, indicating a high virulence of the bacteria.

Our results have shown a continuous expression of CEACAM 1/5 receptors by the cells, even at 48 hpi. Despite the progression of the infection, the bacteria maintained the ability

to interact with these receptors through the Opa-CEACAM interaction. This suggests a potential mechanism of host evasion or exploitation that persists even in the advanced stages of the infection (Gray-Owen, Lorenzen, et al. 1997; Epshita A. Islam et al. 2018; Sadarangani, Pollard, and Gray-Owen 2011). This could potentially indicate a survival strategy employed by the bacteria, which could have implications for treatment strategies.

In the **Figure 35 B**, the infection supernatants collected at 1-, 24-, and 48-hours post-infection were tested for binding to CEACAM 1 and 5 receptors. Interestingly, the Ng in the supernatants showed an increase of binding correlated to the hpi. This suggests a progressive interaction between the bacteria and the receptors over time, which could be indicative of the bacteria's adaptive mechanisms during infection.

A key insight from this data is the apparent greater involvement of CEACAM 5 in the binding process compared to CEACAM 1, particularly at 48 hpi. This could suggest a preferential or more efficient interaction with CEACAM 5 by the Ng strain FA1090, which may have implications for the bacteria's survival or pathogenicity.

At 24 hours post infection in liquid-liquid conditions, there was a notable colonization of the FA1090 strain on the apical surface cells and also signs of bacterial cell invasion. Interestingly, despite the bacterial invasion, the cells within the model did not exhibit any apparent signs of morphological stress (Walker et al. 2023). These observations suggest that the cells were able to preserve their structural integrity and did not display significant morphological alterations, indicative of stress or damage. This could suggest a potential resistance or tolerance mechanism employed by the host cells against the bacterial invasion.

Of note, we observed the release of CEACAM 5 into the supernatant and bacterial aggregates bound to CEACAMs located at a distance from the cellular membranes (**Fig. 36**). This could suggest a potential mechanism of bacterial evasion, which warrants further investigation (Quillin and Seifert 2018; Simms and Jerse 2006). It has been suggested that soluble CEACAM 5 may promote angiogenesis by activating endothelial cells, which plays a role in tumor vascularization. In the cancer setting, soluble CEACAM 5 has also been shown to interact with integrins, leading to pro-angiogenic signaling through pathways like PI3K/Akt and MAPK/ERK (Nicole Beauchemin and Arabzadeh 2013; Holmer et al. 2015). Further research also highlights that CEACAM 5, while normally tethered to the cell membrane, can be cleaved and released into circulation, which is a significant factor in its function in the tumor microenvironment. This release can influence not just angiogenesis but also immune response modulation, making it relevant for further investigation in both cancer and potentially other infections where CEACAM 5 expression is involved (Bramswig et al. 2013) .

The presence of CEACAMs on bacterial aggregates located far from cells could indicate a bacterial masking mechanism that allows gonococci to avoid recognition by the immune system or to maintain a protective microenvironment to promote colonization. These results therefore provide valuable information on the potential mechanisms by which gonococci modulate interaction with host cells during infection.

Our hypothesis is that the up-regulation of interleukin-6 (IL-6) in response to *N. gonorrhoeae* infection leads to an increased expression of CEACAM 5, thereby facilitating bacterial colonization in the female reproductive tract. Studies in colorectal cancer have shown that IL-6 trans-signaling can elevate the expression of CEACAM 5 and CEACAM 6 (Holmer et al. 2015). Although this mechanism has not been directly investigated in the context of

gonococcal infections, the known increase in IL-6 during such infections and the expression of CEACAM 5 in ectocervical epithelium suggest a plausible link. CEACAMs, including CEACAM 5, interact with the opacity-associated proteins (Opa) of *N. gonorrhoeae*, enhancing bacterial adhesion and invasion. Additionally, *N. gonorrhoeae* can manipulate the host immune response by suppressing Th1 and Th17 responses and promoting regulatory T cell (Treg) expansion, creating an environment conducive to chronic infection. Hormonal influences, such as estrogen and progesterone, may also modulate CEACAM expression and immune response in the reproductive tract. Future studies should directly examine the interplay between IL-6 signaling, CEACAM 5 expression, and gonococcal infection to confirm this hypothesis and develop new therapeutic strategies.

In conclusion, our results indicate that the Opa proteins of gonococcus modulate adhesion to CEACAM receptors, and that these interactions vary depending on the strain and specific receptor involved. The preference for CEACAM 5 in cervical tissues suggests that this receptor may be a prime target for infection in the female genital tract. The variability in adhesion inhibition mediated by antisera raises important questions about the role of the antigenic diversity of Opa proteins in pathogenesis and immune evasion. Overall, these data provide new insights into the molecular mechanisms of gonococcal infection and offer clues for the development of new therapeutic and vaccine strategies.

Future research should investigate the dynamics of Opa phase variation, particularly how gonococci regulate or silence Opa expression and its interaction with CEACAMs, especially those present in the urogenital and pharyngeal tracts.

7. References

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