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UNIVERSITÀ DEGLI STUDI DI SIENA

DIPARTIMENTO DI BIOTECNOLOGIE, CHIMICA E FARMACIA

DOTTORATO DI RICERCA IN

CHEMICAL AND PHARMACEUTICAL SCIENCES

XXXV° CICLO

Coordinatore del corso: Prof. Maurizio Taddei

ROLE OF LIPOOLIGOSACCHARIDE IN *NEISSERIA GONORRHOEAE* IMMUNE RESPONSE

Settore scientifico-disciplinare: CHIM/02

Candidata

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Abstract

In 2017, *Neisseria gonorrhoeae* (*Ng*) was listed by the World Health Organization (WHO) as a high-priority pathogen for which new therapeutics and prophylactic tools are urgently needed. In this panorama, renewed interest has been directed towards the development of GMMA- or OMV-based vaccines. GMMA (Generalized Module for Membrane Antigens) are outer membrane vesicles (OMV) shed from a strain genetically designed with different aims; among them, removing or reducing the expression of undesired antigens as in the case of lipopolysaccharide or lipooligosaccharide (LPS/LOS) removal to decrease the GMMA reactogenicity. Moreover, GMMA can be exploited for the expression new antigens and/or to enhance the expression of desired ones, as well as to increase the release of vesicles, thus improving the production yield.

Lipooligosaccharide (LOS) is the most abundant antigen on the gonococcal membrane and plays a crucial role in pathogenesis being involved in bacterial adhesion to human cells and serum resistance. Moreover, antibody responses to LOS can mediate complement activation and bactericidal and opsonic activity. Despite these promising properties, the heterogeneity of LOS structures remains a challenge. Indeed, the glycan extensions of LOS oligosaccharide chains are determined by the expression of glycosyltransferases, some of which encoded by phase variable genes (*lgtA, lgtC, lgtD,* and *lgtG*). Therefore, the impact of different LOS epitopes expressed on a GMMA-based vaccine on functional immunogenicity requires further examination.

In this study, GMMA derived from a mutant strain (FA1090 *ΔlpxL1 ΔlgtF*) engineered to express a highly truncated LOS have been tested *in vivo*. The resulting mouse sera showed low or negative bactericidal (hSBA) activity for the majority of tested strains, suggesting a role for anti-LOS antibodies in the hSBA functional response. Indeed, the corresponding GMMA from the parental isogenic strain having a functional *lgtF* gene, are able to elicit a functional response, thus confirming LOS truncation is abolishing the response. In addition, a library of 8 gonococcal MS11 isogenic mutant strains suitably engineered to express distinct LOS structures was used to dissect the contribution of the different LOS epitopes on GMMA immunogenicity. Competitive hSBA experiments and *in vivo* mice studies highlighted a direct correlation between the LOS α-chain structure and GMMA-mediated cross-functional activity.

Moreover, to investigate the role of LOS in the absence of other antigens, liposomes formulated *ad hoc* to mimic a GMMA membrane have been exploited as *naked* LOS carriers. The crucial role of anti-LOS antibodies in the functional response was confirmed by immunizing mice with the liposome-LOS vaccine and performing hSBA on the derived sera.

In conclusion, data indicated that GMMA-mediated cross-strain bactericidal antibody responses were referable to LOS epitopes contained in the long α-chain with respect to shorter α-chains, while the presence of a β-chain was shown to be dispensable. Furthermore, elicited functional activity was maintained by testing liposomes as LOS carriers, suggesting that LOS an important role in the elicitation of functional antibodies following GMMA immunization. Furthermore, this result underlines the importance of further exploring these lipid-based vesicles as carriers to deliver anti-gonococcal antigens and, hence, paves the way towards the design of new strategies to develop or improve vaccines against this concerning anti-microbial resistant pathogen.

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LIST OF ABBREVIATIONS

Ab	Antibody
Alum-OH	Alum hydroxide
AMR	Antimicrobial Resistance
BCA	Bicinchoninic Acid Assay
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control
CFU	Colony-Forming Unit
cmR	Chloramphenicol resistance
DLS	Dynamic Light Scattering
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPE-mPEG2000	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-methoxy-polyethylene glycol-2000
DMPG	1,2-dimyristoyl-sn-glycero-3-phosphoglycerol
DMSO	Dimethlyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
FFR	Flow Rate Ratio
GMMA	Generalized Modules for Membrane Antigens
Hep	Heptose
HPLC	High Performance Liquid Chromatography
hSBA	Human Serum Bactericidal Assay
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kanR	Kanamycin resistance cassette
KDO	2-keto-3-deoxy-mannooctulosonic acid
KO	Knock-out
LNnT	Lacto-*N*-neotetraose
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MADLS	Multi angle dynamic light scattering
MW	Molecular weight
Ng	*Neisseria gonorrhoeae*
NHS	Normal human serum
Nm	*Neisseria meningitidis*
OM	Outer membrane
OMV	Outer membrane vesicles
OS	Oligosaccharide
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PDI	Polydispersity index
PEG	Polyethylene glycol
PRR	Pattern recognition receptor
RMP	Reduction modifiable protein
SCA	Semicarbazide
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE-HPLC	Size exclusion high performance liquid chromatography
SUV or LUV	Small or large unilamellar vesicle
TEM	Transmission electron microscopy

TFR	Total flow rate
WB	Western blot
WHO	World Health Organization
WT	Wild-type
xg	Units of gravity

INTRODUCTION

I.1 Bacteria and immune system: general principles of vaccination

Bacterial pathogens are responsible for an enormous variety of infectious diseases. Bacteria are unicellular prokaryotic organisms of a few micrometers in length, composed of a plasma membrane and a semipermeable barrier consisting of phospholipids and proteins surrounding the cytoplasm. The cell wall forms a rigid structure around the plasma membrane, providing structural support and protection, and establishing the characteristic shape of the cell (rod, coccus, or spiral). The peculiarity of bacteria is the presence of an external dense carbohydrate coating called *glycocalyx*, which makes bacteria resistant to phagocytes and delays the protective action of specific immunological systems. Cell surface polysaccharides are polymers formed from a single monosaccharide unit (homopolymers) or more complex repetitions of oligosaccharides (heteropolymers). These polysaccharides can be charged or neutral, covalently linked to proteins or lipids to form *glycoproteins* and *glycolipids* respectively. [1]

Based on their ability to react to the staining method developed by Christian Gram in 1884, bacteria are divided into two classes: *Gram-positive* and *Gram-negative.* [2] Gram-positive and Gram-negative bacteria are characterized by different cell wall structures, as shown in **Figure I.1**. [3]

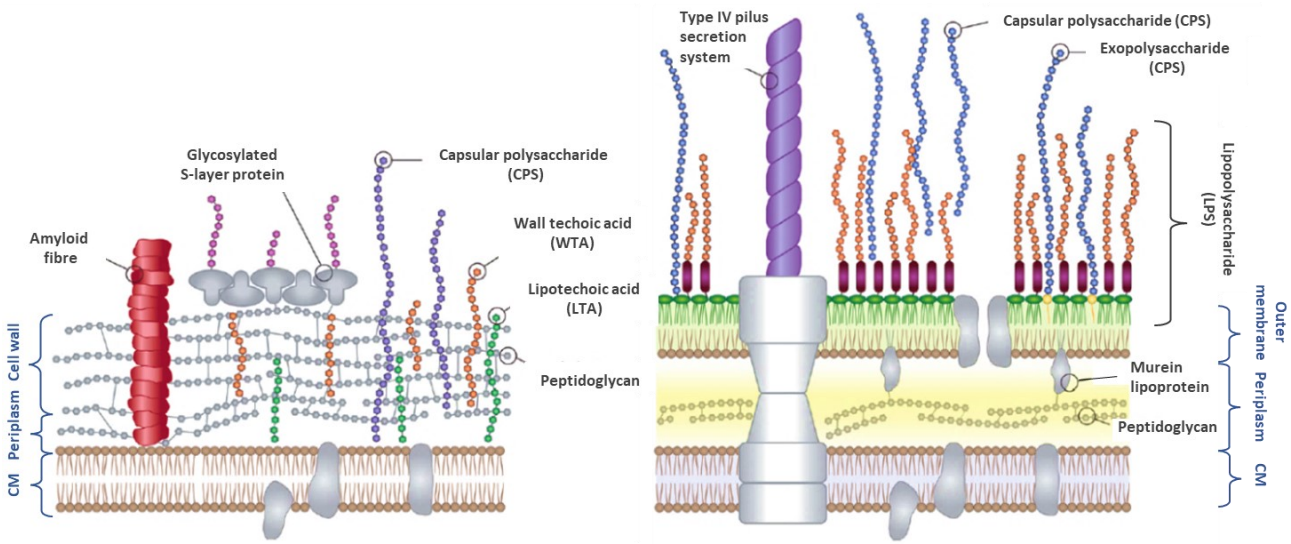


Figure I.1. Schematic representation of the cell envelope of gram-negative (*on the right*) and gram-positive (*on the left*) bacteria. [3]

The gram-negative bacterial envelope is composed of an outer membrane, containing lipopolysaccharides, lipoproteins and phospholipids; a peptidoglycan-containing compartment called *periplasm*; and an internal membrane. The peptidoglycan is a polymer consisting of alternate units of

N-acetylglucosamine and *N*-acetylmuramic acid. Gram-positive bacterial cell walls are characterized by the presence of a relatively thick carbohydrate outer surface layer, generally consisting of a capsule, which plays a key role in many molecular recognition processes.

The immune system is an important defense mechanism for our organism, capable of recognizing infected cells (*non-self*) from the healthy cells (*self*) and triggering an immune response against them. A foreign substance that is recognized as dangerous and capable of triggering an immune response is known as *antigen*. Immune responses can be distinguished into innate and adaptive immunity. The first recognizes pathogens in a rapid and non-specific manner and can be considered the first line of defense. The latter is delayed but directed against a specific antigen involving the participation of different types of cells called lymphocytes, which belong to leukocyte class.

Adaptive immunity is characterized by some advantages: specificity and diversity in the recognition of antigens, owing to the production of specialized lymphocytes and specific antibodies, and the ability to discriminate between *self* and *non-self* cells and to remember antigens that have already been encountered. The practice of vaccination is based on the potential of adaptive immunity. It is based on the principle that exposure to a disease-causing microorganism, or to a portion of it, creates pathogen-specific cells; when infections are resolved, some of these cells survive in the body to form immunological memory ready to attack the same pathogen rapidly and more efficiently when it is encountered a second time. Vaccination mimics natural infection without causing the related diseases. Vaccine-induced immune effectors are called *antibodies*; they are produced by B lymphocytes and are capable of recognizing and specifically binding to a toxin or a pathogen (or to a portion representative of it).

The main factors responsible for the immune response are B and T lymphocytes. Both have a plasma membrane surrounded by receptors recognizing specific antigens. When cell-antigen interactions occur, B lymphocytes generate plasma cells and secrete specific antibodies against a particular antigen, therefore B lymphocytes are responsible for the humoral response. T-lymphocytes produce T-cytotoxic and T-helper cells, which are involved in both humoral and cell-mediated immunity. These two lymphocyte types cooperate in a process that leads to the formation of memory B cells. When a second exposure to the same antigen occurs, memory B cells generate specific antibodies with combinatory sites capable of binding the structural-complementary antigens. They usually recognize only a small superficial part of the molecule, called *epitope.*

The activation of B cells to create memory B cells requires the participation of macrophages or T-helper cells, and it can be stimulated only by T-dependent antigens, such as proteins. Protein antigens interact with antigen-presenting cells (APCs), such as dendritic cells, macrophages, and B cells, and are then internalized. After being processed, they are re-exposed as small peptides and presented to

T lymphocytes in association with Major Histocompatibility Complex class II (MHC II) molecules. Interaction with T cells induces B cells to differentiate into plasma cells and memory B cells, thus initiating downstream adaptive immune responses. T-dependent antigens are immunogenic in infancy and the induced immune response can be boosted enhanced by adjuvants, and it is characterized by an antibody class switch with production of antigen-specific IgG.

On the other hand, the long polysaccharide chains found in the bacterial capsule are T-cell independent antigens and do not require T-cell activation for the induction of specific B-cell responses. T-independent antigens can provoke humoral immunological responses without the collaboration of macrophages or T helper cells; therefore, the memory B cells formation does not occur. In general, the antibody response to bacterial polysaccharides is poorly affected by adjuvants. IgM represents the major class of elicited antibodies and, since this type of immune response does not induce memory, it is not boosted by subsequent immunizations. Moreover, a pre-existing memory B-cell pool can be depleted by immunization with unconjugated polysaccharides, with the risk of hypo-responsiveness on subsequent immunizations. [4] However, polysaccharides can be covalently linked to carrier proteins that are used as sources of T-cell epitopes. The resulting glycoconjugates bind to the B-cell receptor specific to the polysaccharide and are transported into the endosomes. Once inside the cell, the protein portion is digested by proteases to release peptide epitopes, which are exposed on the surface in association with MHC II and presented to the αβ receptor of CD4+ T-cells. Peptide/MHC II-activated T-cells release cytokines to stimulate B-cell maturation into memory cells and induce immunoglobulin class switching from IgM to polysaccharide-specific IgG, resulting in the production of large amounts of high-affinity IgG antibodies upon exposure to the same carbohydrate antigen. Consequently, immunization with glycoconjugates induces long-lasting protection against encapsulated bacteria, even in infants and people in high-risk groups. [4] Furthermore, the immune response against glycoconjugates is boosted by subsequent vaccinations. (**Figure I.2**)

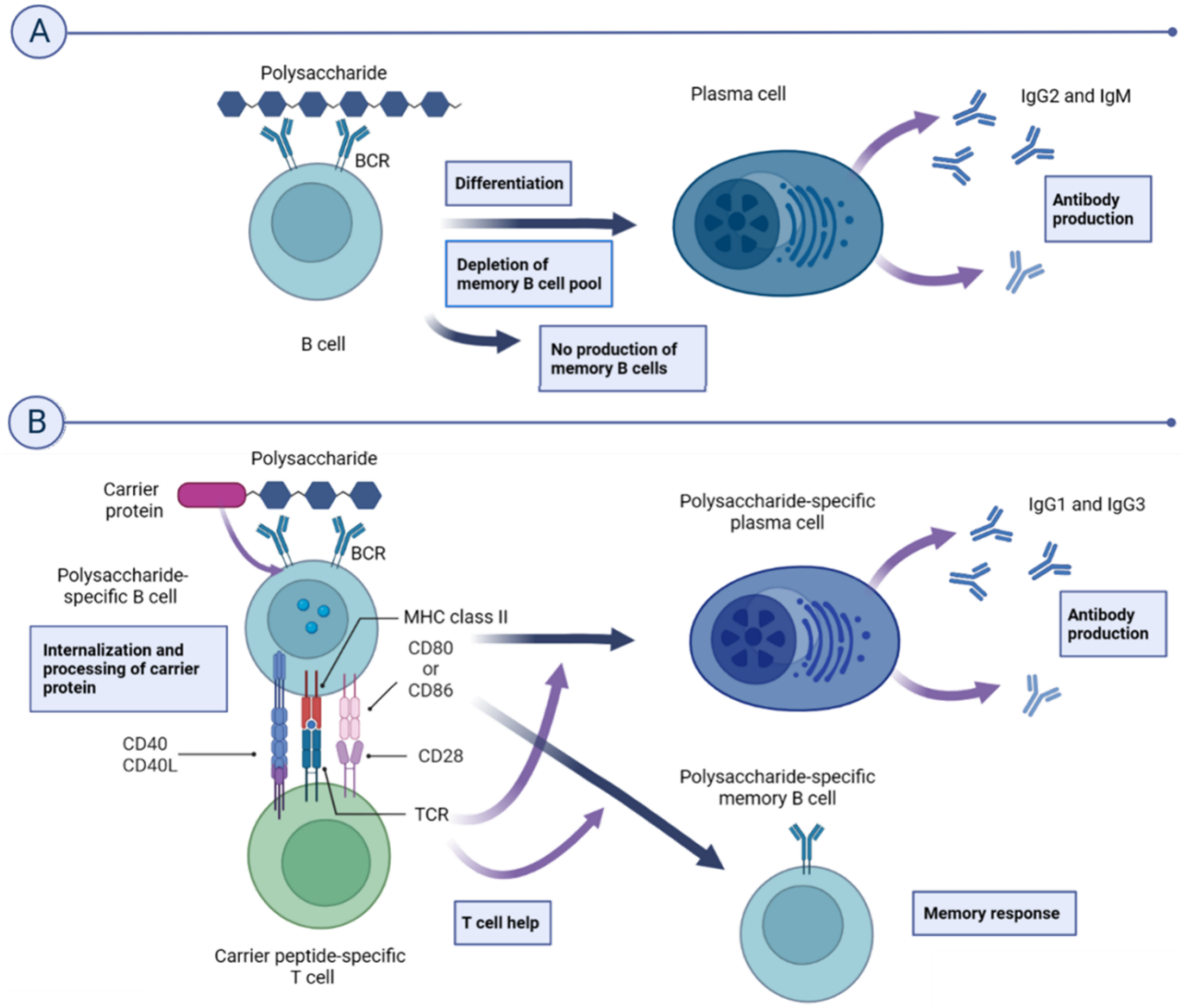


Figure I.2. The immune response to polysaccharide (A) and protein–polysaccharide conjugate (glycoconjugate) vaccines (B). Polysaccharides stimulate B cells by cross-linking the B-cell receptor (BCR) leading to production of antibodies. This process results in a lack of production of new memory B cells. On the other side, the protein in the glycoconjugate vaccines is processed by the polysaccharide-specific B cell, and peptides are presented to carrier-peptide-specific T cells, resulting in T-cell help for the production of both plasma cells and memory B cells (Figure created with Biorender.com, adapted from Pollard *et al*., 2009). [5]

Immunity to infectious agents can be achieved *via* active or passive immunization. This means that it can be acquired either by natural processes, such as the transfer from mother to fetus or previous infections, or by artificial processes, when there is a risk of infection and antibodies are administered because the body does not have time to develop its own immune response. Passive immunization is short-term because the antibodies are naturally decomposed. Active immunization results in immunological memory and can be achieved by infection with a microorganism or acquired by administration of a vaccine. Generally, this is a long-term response as leads to the formation of memory B cells.

I.2 Infectious diseases: AMR and the importance of vaccines

Despite enormous progress achieved in modern medicine, numerous diseases still have a profound impact on public health. Among them, infectious diseases are currently a leading cause of death worldwide.

They are generated by pathogenic microorganisms such as viruses, parasites, fungi or bacteria. These pathogens enter the host humans and animals, where they can produce disease symptoms through a variety of mechanisms.

Infectious diseases are communicable diseases, those diseases that can be spread directly or indirectly from one individual to another. Before the development and introduction of effective preventive and therapeutic strategies, life expectancy was estimated to be <50 years and infectious diseases were highly responsible for this limit. In the last century, this number has increased considerably, and this result has been achieved primarily by improving hygiene and with the use of antibiotics and vaccines. These measures have represented the most effective medical interventions to reduce death burden caused by infectious diseases. [6, 7]

The discovery in 1928 by Alexander Fleming of a substance isolated from the mold *Penicillium notatum*, defined as benzylpenicillin (penicillin G), gave rise to the "*golden era*" of antibiotics (1945-1960). [8] Since then, antimicrobials have enabled the treatment of potentially life-threatening infectious diseases, saving millions of lives every year and improving the health of many patients worldwide. Unfortunately, only a decade later, in 1940, the first case of a penicillin resistant *E. coli* strain was documented and by the late 1960s over 80% of *S. aureus* strains acquired the same resistance. [9-11] In fact, pathogens have shown to quickly acquire resistance phenotypes after only few years from the introduction of new antibiotics and the rising number and global distribution of drug-resistant pathogens is nowadays one of the major health challenges, compromising the ability to prevent and cure a wide range of infectious diseases that were once treatable. [12] The emergence of the antimicrobial resistance (AMR) results in drug inefficiency and persistent infections, leading to a high risk of severe disease and transmission. AMR is an ancient and natural mechanism developed by microorganisms as a defense against antimicrobials and chemicals present in the environment that are produced by other microorganisms, however, the overuse and misuse of antimicrobials in different areas, including the hospital and the agricultural settings, has greatly accelerated the spread of new resistance mechanisms. [12, 13]

AMR has become an endemic and widespread problem especially in low and medium income countries but also in high-income countries, with more than 2.8 million antibiotic-resistant infections

occurring in the U.S. each year and more than 35,000 deaths as a result, according to CDC's 2019 Antibiotic Resistance (AR) Threats Report. [14]

Moreover, according to the Centers for Disease Control and Prevention, this condition has worsened during the Covid-19 pandemic. Covid-19 has led to increased hospitalization, including mechanical ventilation, and extended duration of stay, thus highly increasing the risk of acquiring AMR infections. Bacterial coinfections and secondary infections following Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) disease have been reported and a recent study from India shows that co-infection rates are higher and are leading to increased mortality. [15] Moreover, many bacterial and fungal infections were potentially undiagnosed and/or untreated because of the Covid-19 pandemic, leading to a tremendous slowing in the detection and reporting of AMR data.

This may have been the case for sexually transmitted infections such as gonorrhea. This disease, if left undetected and untreated can cause serious health complications and can continue circulating in a community, thus increasing the chances of developing resistance to the available treatments. [16]

In the past few years, enormous efforts have been made to highlight AMR as an urgent global health threat. The Review on Antimicrobial Resistance, commissioned by the UK Government, has declared that, by 2050, 10 million people could die every year from drug-resistant infections, and World Health Organization (WHO), together with numerous researchers, agree that AMR burden is an urgent issue requiring a global action plan to tackle. [14, 17, 18]

The development of preventative therapies, including more efficient vaccines, novel antibiotics, and treatments, together with innovative diagnostic tools, is required to effectively combat the spread of AMR and all other associated drawbacks. In particular, vaccination plays a central role in the fight against AMR and is considered by the World Health Organization to be the most cost-effective strategy for controlling infectious diseases, as it should confer long-term protective immunity in the population. By acting as prophylactic tools, vaccines are able to prevent or reduce the number of infectious diseases, both mitigating severe consequences and also decreasing antibiotic use with the potential to diminish the emergence of AMR. Furthermore, if sufficient vaccine coverage is achieved in a population, vaccination may lead to indirect protection (*herd immunity*) further preventing the spread of resistant strains. In fact, vaccines have been used for decades with a much lower probability of resistance emergence compared with antibiotics, due to their intrinsic mechanism of action (**Figure I.3**). [19]

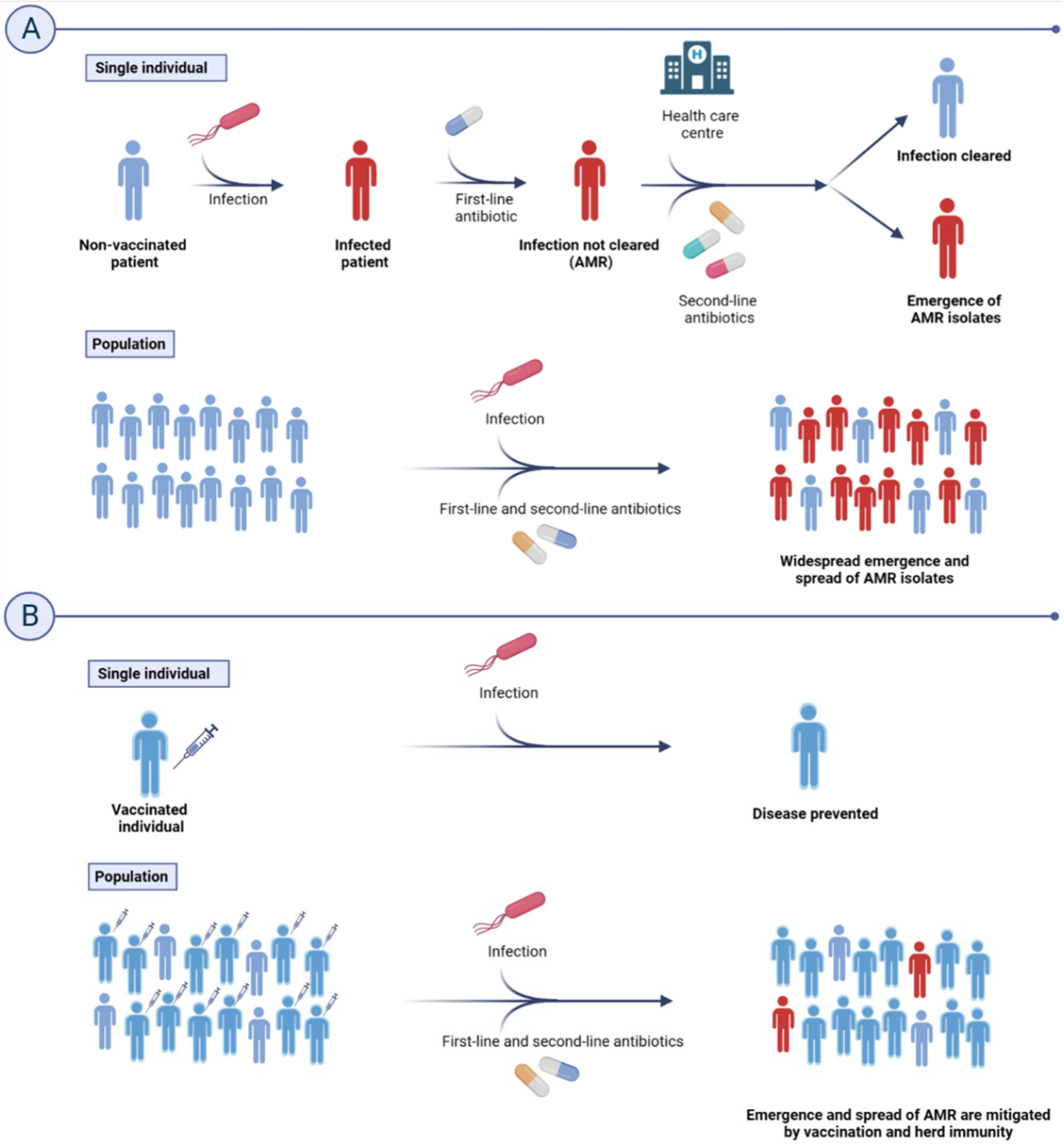


Figure I.3. Effects of vaccination on AMR. The spreading of AMR pathogens can lead to ineffectiveness of the treatment with first-line antibiotics. Second-line antibiotics are often required to resolve the infection, possibly leading to AMR isolates resistance to them (A). Vaccines could prevent or reduce the incidence of diseases, thus reducing the use of antibiotics (both first-line and second-line drugs). If sufficient vaccine coverage is achieved in a population, indirect protection (herd immunity) further prevents spread of AMR strains. (Figure created with Biorender.com, adapted from Micoli *et al*., 2021). [12]

In this panorama, although vaccination represents an unquestionable solution against disease, preventing 700 million of cases and more than 150 million of deaths during the last century [20], other tools such as monoclonal antibodies, bacteriophages, microbiota targeting and innovative diagnostic tools are emerging strategies that can complement vaccines in the fight against AMR.

I.3 Gonococcal disease: natural history, epidemiology and antimicrobial resistance

In AMR panorama, gonococcal disease represents one of the most serious challenges. In 2017, *Neisseria gonorrhoeae*, the etiological agent of the gonorrhea disease, have been listed by the WHO as high priority pathogen for the research and development of new antimicrobial and vaccines. [21] According to the WHO, about one million new cases of sexually transmitted infections occur every day worldwide and approximately 87 million of people were diagnosed with gonorrhea out of the 376 million globally reported cases of sexually transmitted infections (STIs; including chlamydia, gonorrhea, syphilis and trichomoniasis) that occurred among 15–49-year-olds in 2016 (**Figure I.4**). [22] Gonorrhea is the second most reported bacterial STI in the United States, just behind Chlamydia, and often these infections happen together. [23]

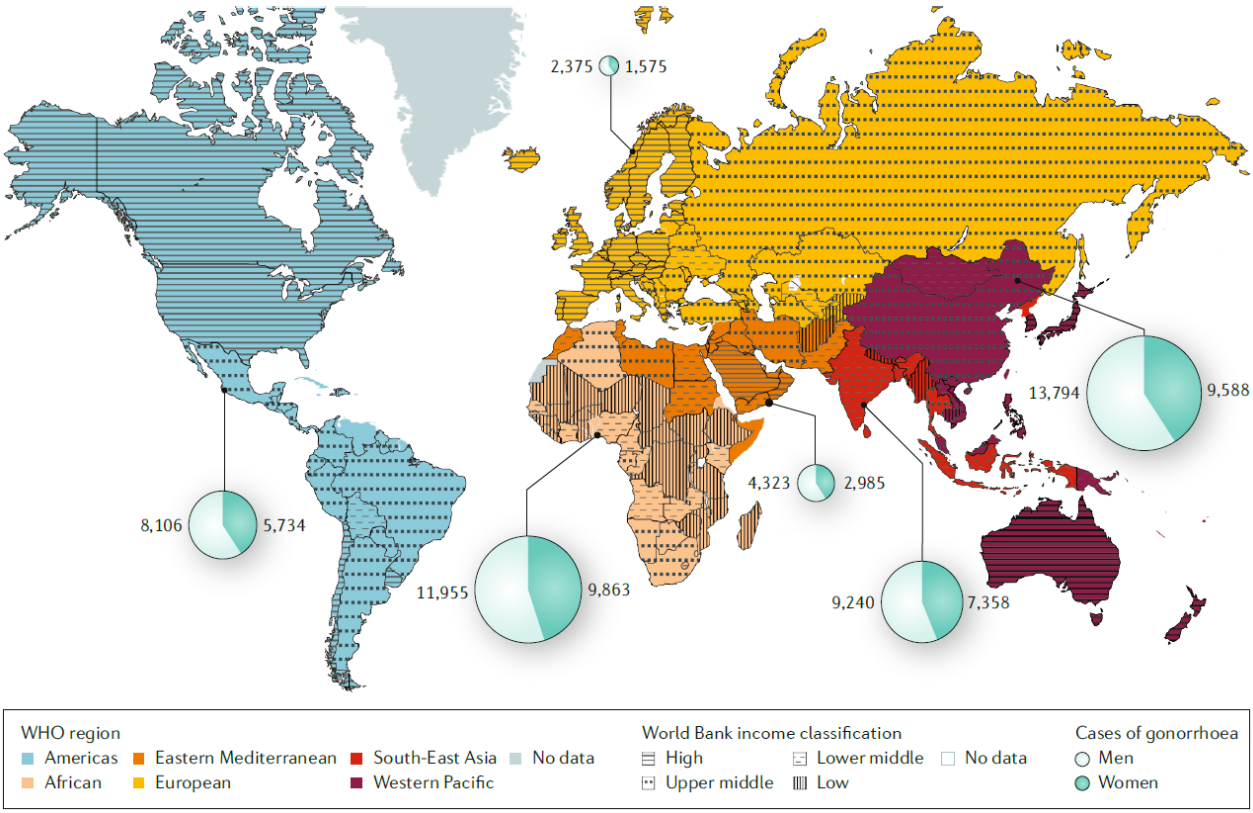


Figure I.4. Estimated new global cases (in millions) of gonorrhea in adults (15–49 years of age) in 2016 by WHO region. The highest incidence was in the African region, while the lower in the region of Europe. The World Bank Income Classification is also shown (Figure from Unemo *et al.,* 2019). [24]

The epidemiological surveillance of Gonococcus highlighted the variability of the geographical distribution in the rates of reported gonorrhea cases, with differences related to several factors,

including socioeconomic conditions, access to sexual education and prevention measures, but also gender, sexuality and ethnicity disparities. [24] Furthermore, it is important to consider that the rates of gonorrhea are likely to be underestimated due to the high frequency of asymptomatic infections and to incomplete reporting, especially during the Covid-19 pandemic. [15]

One of the main challenges related to *N. gonorrhoeae* is the rapid development of resistance to antibiotics. Indeed, antibiotics have been tremendously successful for treating gonorrhea, but treatment is now severely compromised by the emergence of strains resistant to all the available antibiotic treatments. In 2020, about half of all infections were estimated to be resistant to at least one antibiotic. [23] Based on isolates collected in the Gonococcal Isolate Surveillance Project (GISP), since 2010, almost all gonococcal circulating strains in the United States have remained susceptible to ceftriaxone, the primary treatment for gonorrhea; only 0.1% of isolates displayed elevated ceftriaxone minimum inhibitory concentrations (MICs) in 2020. In 2020, 5.8% of the isolates showed elevated azithromycin MICs (**Figure I.5**). [23]

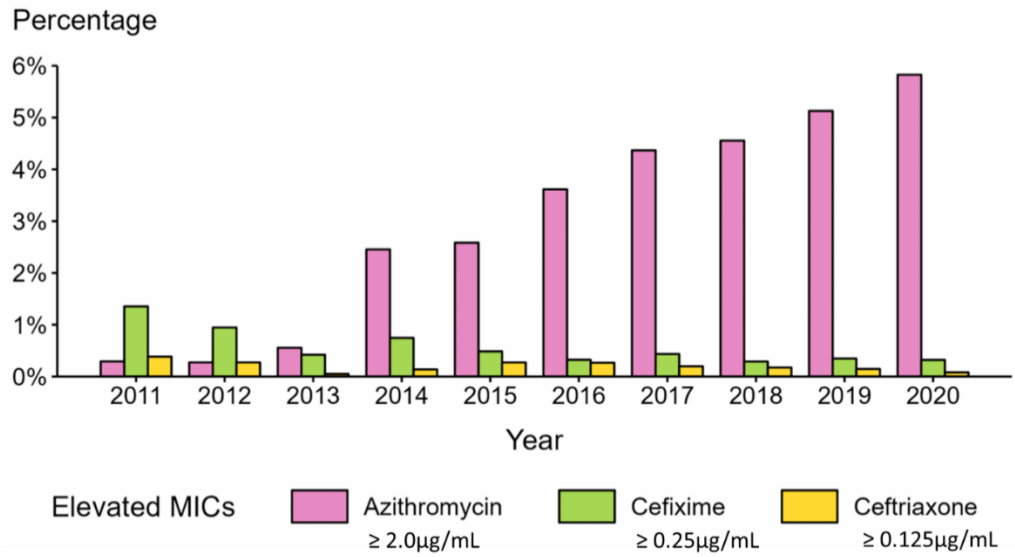


Figure I.5. *Neisseria gonorrhoeae* percentage of isolates with elevated Minimum Inhibitory Concentrations (MICs) to Azithromycin, Cefixime, and Ceftriaxone during 2011-2020 (Figure from Gonococcal Isolate Surveillance Project (GISP), 2011–2020). [23]

In 1990, the WHO established a worldwide surveillance laboratory network, the WHO Gonococcal Antimicrobial Surveillance Programme (GASP), which has been collecting data on gonococcal antimicrobial susceptibility since that time. However, since several factors limited the GASP data collection, such as variability in sampling strategies, laboratory techniques and interpretative criteria, recently an innovative project, the Enhanced Gonococcal Antimicrobial Surveillance Programme

(EGASP), was born under the Global Antimicrobial Resistance Surveillance System (GLASS). This program seeks to gather more detailed information to strengthen sentinel surveillance for gonococcal antimicrobial resistance (AMR) in selected countries. [25, 26]

Despite the positive intentions of global initiatives and funding for new treatments, the current pipeline is dismal. The rapid acquisition of antibiotic resistance has made *N. gonorrhoeae* an increasingly urgent global health threat, ushering in an era of potentially untreatable gonorrhea. Therefore, in recent years, the interest in developing a vaccine to tackle this AMR bacterium has been revived and prevention through vaccination is now a priority.

During the last decade, progress on gonorrhea vaccines has been discouraged for several reasons, including the antigenically variable nature, unclear mechanism of protection from infections and the ability to evade host immune defenses. Moreover, immune responses directed against conserved antigens fail to elicit protection and robust immune responses are not elicited during natural infections making repeated gonococcal infections common. These mechanisms represent a challenge for the identification of vaccine targets.

Nevertheless, a case control study conducted in New Zealand on epidemic of *Neisseria meningitidis* serogroup B reported that MeNZB® (Group B meningococcal outer membrane vesicle (OMV) vaccine) may give a 31% cross-protective efficacy against gonorrhea, thus making possible to think again that a vaccine against this pathogen is feasible. [27] Therefore, in the last years, optimism about the development of a gonococcal vaccine has been revived because of accumulating observational data and recent evidence of a cross-protective effect from Serogroup B Meningococcal Vaccines against *N. gonorrhoeae*, leading to a renewed hope for vaccine development. [28-34]

I.4 Neisseria *species and* Neisseria gonorrhoeae

The family Neisseriaceae comprises the genus *Neisseria* and other genera such as *Kingella* and *Eikenella*. [35-37] The *Neisseria* genus consists of 23 species, that can be commensal or pathogenic. Among them, half are human-restricted species and some are animal-restricted; a part of them can infect both the human and the animal. [37] The two human pathogenic *Neisseria* species, *Neisseria gonorrhoeae* and *Neisseria meningitidis* are genomically, morphologically and phenotypically closely related. *Neisseria meningitidis* is commonly found as a commensal in the nasopharynx of approximately 15% of the population, but it can sporadically cause fatal septicemia or meningitis. [35-38] These two pathogenic *Neisseria* species have evolved from commensal *Neisseria* species. Indeed, there is a strict correlation between the pathogenic and commensal *Neisseria*, and the latter also contain many of the pathogenic and virulence factors of *N. gonorrhoeae* and *N. meningitidis*, although they do not normally cause pathology, as they are unable to induce substantial polymorphonuclear leukocyte-based inflammation and lack several additional factors and mechanisms of interaction with host molecules, cells and tissues. [39, 40]

Gonorrhea is one of the oldest diseases known to humans. Evidence that gonococcal infections exist as sexually transmitted disease dates back to Egyptian papyrus (1500-3000 BC) [41], but the history is full of references to this venereal disease. The etiological agent of gonorrhea, *Neisseria gonorrhoeae* (also known as Gonococcus), is an obligate human pathogen transmitted through sexual contact or perinatally from the mother to the newborn during birth.

Gonococcus causes infections principally at the mucosal epithelium of the male and female genitourinary tracts; however, anorectal, nasopharyngeal and ocular mucosal surfaces may be also infected. [42-46] In men, major symptoms of infection include urethral discharge and dysuria. Untreated infections may lead to acute epididymitis [47] and consequent infertility. In women, infection is frequently asymptomatic and half of all affected women do not develop clinical symptoms [48], hence leading to chronic complications. When symptomatic, infections of the ectocervix and endocervix lead to mucopurulent cervicitis. Asymptomatic gonorrhea in women is often associated with cervicitis accompanied by its subsequent spread to the upper reproductive tract (URT). [42, 49-51] Ascending gonococcal infection may result in pelvic inflammatory disease syndrome (PID), which consists of fallopian tube scarring leading to a condition of infertility, ectopic pregnancy and chronic pelvic pain. [52] Rectal or pharyngeal gonorrhea is often asymptomatic. If untreated, *N. gonorrhoeae* can enter the bloodstream and disseminate bringing to a condition of bacteremia, known as disseminated gonococcal infection (DGI). Although rare, it is associated with arthritis, endocarditis, and/or meningitis [53]. Moreover, neonates can be infected by vertical transmission

during birth, and this may cause the so called *ophthalmia neonatorum* (neonatal conjunctivitis), which may lead to irreparable corneal tissue damage and blindness; more rarely, abscesses, meningitis and sepsis are observed in infected newborns. Furthermore, septic abortion, premature delivery and low neonatal weight have been correlated with maternal gonorrhea.

I.4.1 Gonococcal antigens and major virulence factors

N. gonorrhoeae is a gram-negative β-proteobacterium, belonging to the Neisseriaceae family, firstly isolated by Albert Neisser in the gram-stained microscopy of urethral discharge in 1879. [54, 55]
Gonococcus is a facultative non-spore-forming and non-motile anaerobe [56], that is frequently encountered as diplococcus. Similar to other Gram-negative bacteria, its cell envelope consists of an inner cytoplasmic membrane, a middle space containing peptidoglycan and the periplasmic space and an outer membrane (OM). The gonococcal OM is constituted by an inner leaflet of phospholipids and an outer layer formed by a complex combination of phospholipids, lipooligosaccharide (LOS) and proteins, organizing an asymmetric bilayer.
The majority of the structures on this membrane represent virulence factors most involved in the pathogenesis of this bacterium (**Figure I.6**).

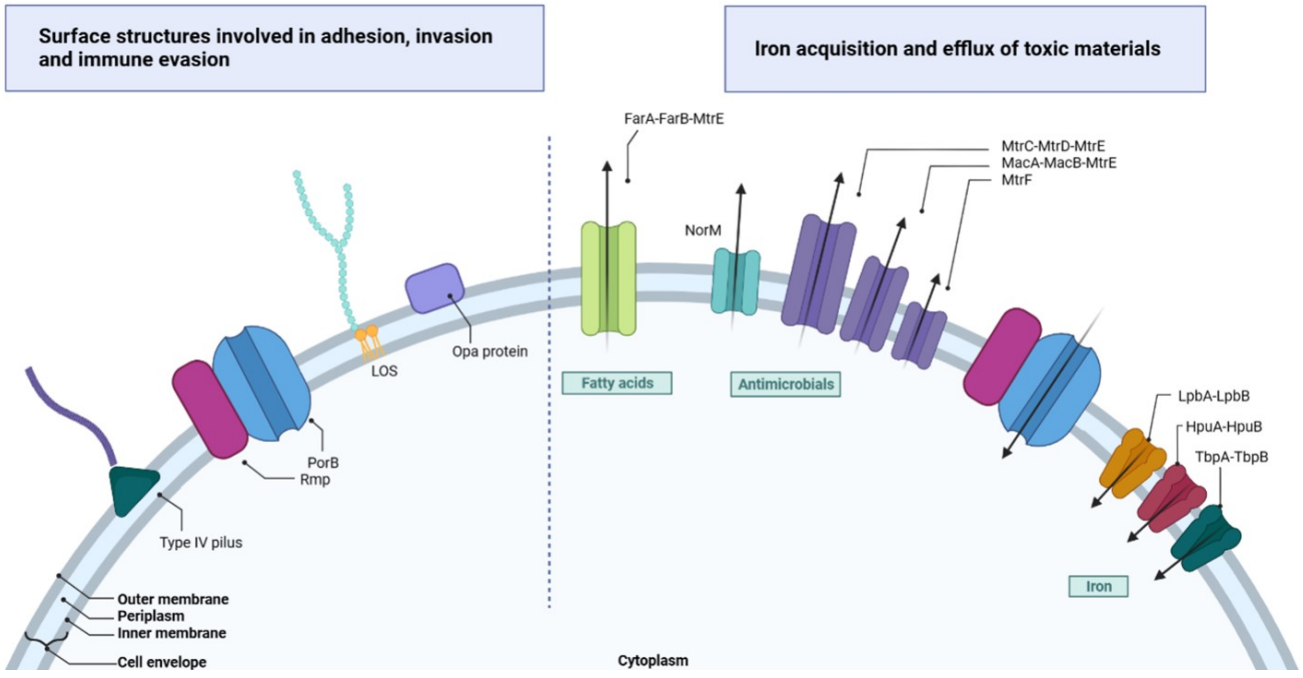


Figure I.6. *N. gonorrhoeae* cell envelope structure (Figure created with Biorender.com, adapted from Unemo *et al*., 2019). [24]

N. gonorrhoeae uses an array of virulence factors to adhere to and possibly invade host cells and to evade the immune system. Among them, type IV pili, opacity (Opa) proteins, lipooligosaccharide and porins play crucial roles.

The outer membrane-localized porin (PorB or Por or Protein I) represents the most abundant outer membrane protein (OMP), accounting for up to 60% of gonococcal OMPs. [57, 58] The porin channel is assembled as homotrimeric protein, in which each monomer is organized as β-barrel with eight surface-exposed loops varying in length with a molecular weight ranging between 32 and 35 kDa. [58, 59] PorB functions as an ion-channel, allowing the exchange of small nutrients and waste products between the bacteria and the environment and hence, it constitutes an essential factor for bacterial viability, contributing to the homeostasis of the bacterial energy metabolism. [60] Moreover, PorB is involved in bacterial pathogenesis through other mechanisms, including serum resistance *via* interaction with classical and alternative pathway regulators [61-65], induction or inhibition of apoptosis [66-69], invasion [70] and interference with the generation of reactive oxygen species (ROS) produced by neutrophils. [71, 72] PorB is highly heterogeneous between strains and this property makes it an excellent marker for strain classification. Immunological and biochemical data have shown that there are two main distinct structural variants of the porin protein, PorB.1A and PorB.1B [57], which are further subclassified into serovars according to their reactivity to a panel of anti-PorB monoclonal antibodies (mAbs) [73] able to recognize epitopes localized in the eight loops and encoded by PorB variable regions. [74] PorB does not undergo high frequency variation mechanisms; when low-frequency variations are present, these are largely restricted to surface-exposed regions of the porin protein.

Another important membrane component is the type IV pilus. Pili are long, thin filamentous appendages composed of the main subunit, Pilin (PilE), and the PilC portion. Gonococcal pili are grouped into type IV pili according to the presumed amino acid sequence from the gene sequence and the mechanism of assembly. Pili are required for efficient mucosal colonization: indeed, since they are dynamic structures that can be retracted, they mainly function as adhesins, mediating adherence to host cells and tissues, as well as self-adherence and adherence to other Gonococcus cells. Additional type IV pili functions include biofilm formation, DNA uptake from the extracellular milieu, thus increasing transformation frequency and therefore genetic adaptability [75] and finally, a flagella-independent system of bacterial movement over surfaces known as *twitching motility.* [76] These protein structures are subjected to phase and antigenic variation mechanisms, switching from a piliated to a non-piliated state under different *in vitro* conditions. [77] Interestingly, the presence of pilus allows Gonococcus to adhere better to mucosal cell surfaces, thus making these phenotypes more virulent with respect to non-piliated variants.

Among the surface components involved in pathogenesis, Opa proteins are a family of integral outer membrane proteins that can act as adhesins, binding a variety of receptors on many different cells and tissues, mediating a more intimate attachment in the first steps of colonization, after initial contact with type IV pili. Most Opa proteins bind to one or more *carcinoembryonic antigen-related cell adhesion molecules* (CEACAMs), a family of surface-exposed protein receptors present on epithelial cells, lymphocytes and PMNs, thus leading to cell signaling events. [78] Opa expression undergoes both phase and antigenic variation. Expression of Opa proteins is stochastically controlled [79] and each gonococcal isolate harbors 11 distinct *opa* genes [80], whose expression is controlled by independent molecular events that modulate the expression of each gene. A single gonococcal strain may express no Opa proteins, a single one, or a combination of several Opa proteins simultaneously. [81]. Changes in Opa expression pattern result in antigenic variation. In addition, despite the sequence of the 11 Opa proteins being 70% identical, conserved regions are not exposed on the membrane and some surface-exposed loops show sequence variations. [79, 80, 82] Opa proteins mediate self-adherence between gonococci by interaction with the lacto-*N*-neotetraose (Galactose β1-4-*N*-acetyl-glucosamine β1-3-galactose β1-4-glucose) saccharide portion of lipooligosaccharide on the opposing bacterium [83] and, in addition, Opa can promote adherence and invasion to host cells. Moreover, their expression is associated with different gonococcal pathogenesis: Opa^+ gonococci are often associated with localized infection in the urogenital, cervical and rectal mucosae, whereas Opa^- gonococci are mainly related with DGI. [84]

Furthermore, lipooligosaccharide (LOS) molecules account for the majority of the membrane components. These are complex glycolipids, localized to the outer leaflet of the OM and are similar in structure to other lipopolysaccharides, although they lack the O-antigen portion. LOS is subjected to a phase variation mechanism, that can enable a single cell of gonococci to produce up to six antigenically different structures simultaneously, enabling the bacteria to adapt to different host environmental conditions [85]. LOS mediates several mechanisms of gonococcal infection such as adhesion, invasion and escape from the host immune system when sialylated [86]. LOS structures and roles will be widely discussed further on in this work.

Another component of the gonococcal outer membrane is the reduction-modifiable protein (Rmp or Protein III). Rmp is a highly conserved surface protein non-covalently associated with PorB, which is present in pathogenic *Neisseria* spp [87-89]. Anti-Rmp antibodies are elicited after gonococcal infection and are able to block the binding of anti-PorB and anti-lipooligosaccharide bactericidal antibodies [90] present in the immune serum and, furthermore, anti-Rmp antibodies are able to enhance the susceptibility to repeat infection. [91] Natural antibodies to the carboxyl-terminal portion

of enterobacterial OmpA proteins cross-react with Rmp and also block the bactericidal action of normal human serum and human immune serum against gonococci. [92]

Moreover, since gonococci require iron as an essential nutrient, three membrane transport complexes (LpbA–LpbB, HpuA–HpuB and TbpA–TbpB) are present on the membrane and contribute to the iron-scavenging mechanism. Finally , five efflux pump systems (FarA–FarB–MtrE, NorM, MtrC–MtrD–MtrE, MacA–MacB–MtrE and MtrF) can protect gonococci from antimicrobials and fatty acid stress. [93]

I.4.2 Pathogenesis of gonococcal disease

The gonococcal pathogenesis is a complex mechanism, in which several components may take part (**Figure I.7**).

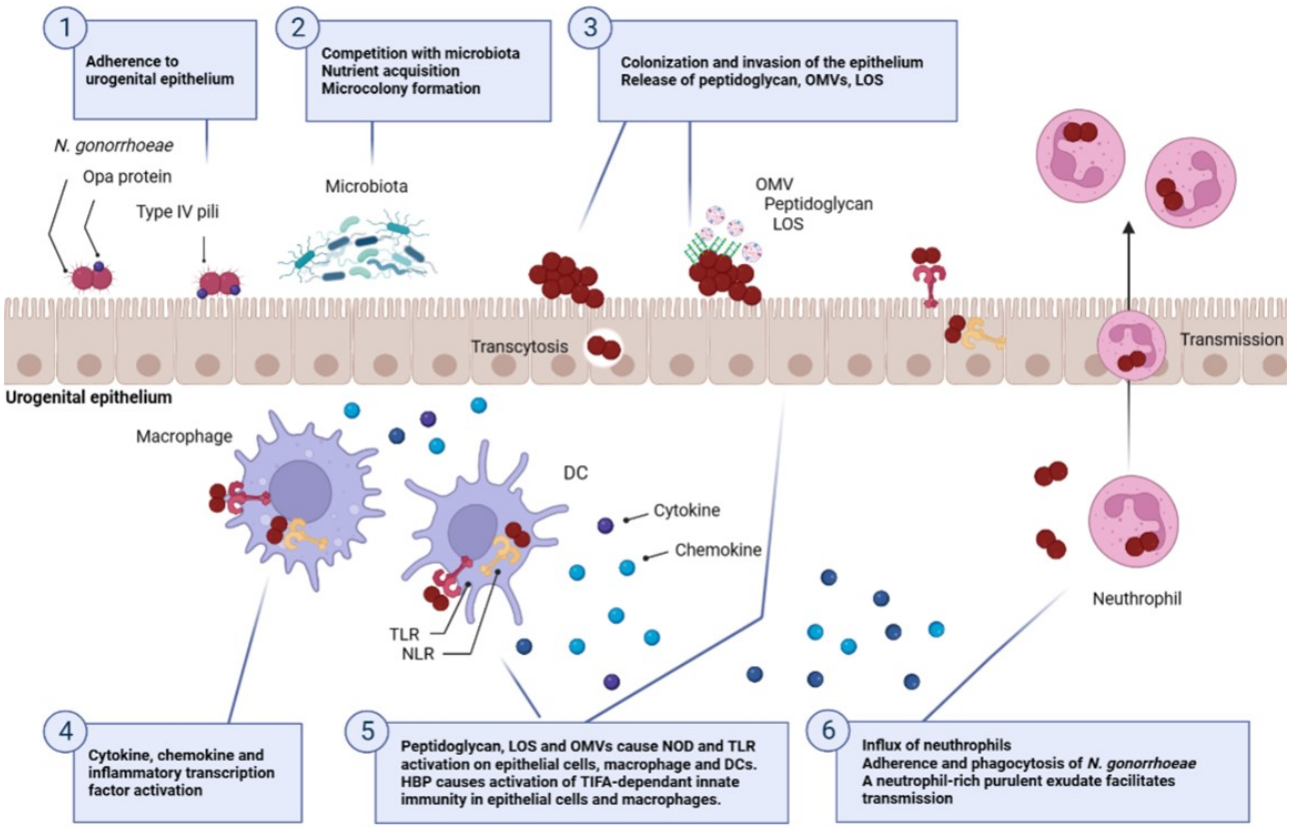


Figure I.7. *N. gonorrhoeae* infection and pathogenesis steps (Figure created with Biorender.com, adapted from Quillin and Seifert, 2018). [93]

The transmission can occur at different sites; indeed, *N. gonorrhoeae* infections mainly involve the mucosal epithelium of the urogenital tract, but they can also occur at the rectum, pharynx or conjunctiva mucosa. Gonococcal transmission occurs from an infected individual by direct contact between the mucosal membranes, usually through unprotected vaginal, anal or oral sexual activity,

with transmission rates that may vary: indeed, during vaginal sex, transmission rates are higher from men to women than the opposite. [94] It is known that *N. gonorrhoeae* is easily transmitted from infected men to their partners through ejaculates, that contains a high number of bacteria [93], however, the mechanism by which the organism is effectively transmitted from vaginal, rectal or pharyngeal locations is less understood. Of note, gonococcal infection may increase the risk of acquisition and transmission of other STIs, particularly HIV. [95, 96]

Moreover, *N. gonorrhoeae* can spread perinatally from mothers harboring gonococci to their child during birth (intrapartum), but not during pregnancy. Indeed, as reported before, the child's conjunctiva may be infected during transit of the birth canal and it may result in ophthalmia neonatorum, leading to childhood blindness.

Gonococci require mucous membranes for colonization and the transmission of the bacteria is limited by different host defense barriers, including the skin and ciliary action of some epithelia. However, peptidoglycan fragments and lipooligosaccharide released by gonococci can disrupt the ciliary action of the epithelium and may promote colonization. [97, 98]

After transmission, the initial step in establishing an infection is the bacterial adherence to the epithelium of the mucosa [99-102], mediated through distinct bacterial surface components. First, type IV pili mediate the initial cellular adhesion and subsequently retract and bring the bacteria close to the cell membrane. [103] After this step, Gonococcus replicates and forms microcolonies on the cell surface. (**Figure I.7**, step 1 and 2). Afterwards, adherence is mostly mediated by Opa proteins interacting with receptors like carcinoembryonic antigen-related cell adhesion molecule (CEACAMs), expressed by epithelial cells and immune cells such as neutrophils [104], and other molecules like polysaccharide-like heparin sulfate. [105] In addition, Opa proteins are involved in the subsequent colonization of the mucosal epithelium of the genital tract and other infection sites. Porin and lipooligosaccharide are other surface factors that affect colonization.

In addition to colonization, *N. gonorrhoeae* can also invade epithelial cells. Although this process is still unclear, it has been demonstrated that lipooligosaccharide needs to be desialylated to allow gonococci to invade non-ciliated cervical epithelial cells and the urethral epithelial cells. [42] Moreover, it has been reported that LOS interaction with the asialoglycoprotein receptors (ASGP-R) promotes invasion of urethral epithelial cells [106] and that the invasion in the lower cervical genital tract is mediated by complement receptor 3 (CR3). [107] This invasion and the resulting transcytosis of the epithelium can lead to disseminated gonococcal infection (DGI). The relevance of these mechanisms in uncomplicated infections remains unclear. Of note, the multitude of receptors that mediate gonococcal invasion highlights the complex, multifaceted nature of tissues lining the genital

tract. This represents one of the main difficulties in establishing appropriate animal and tissue culture models for studying this pathogen.

During these primary stages of infection, gonococci release peptidoglycan fragments, lipooligosaccharide and outer membrane vesicles (OMV), thereby activating Toll-like receptor (TLR) and nucleotide-binding oligomerization domain-containing protein (NOD) signaling in epithelial cells, macrophages, and dendritic cells (DCs) (Step 3). In response, these cells activate inflammatory transcription factors and release cytokines and chemokines (Step 4). *N. gonorrhoeae* also releases heptose-1,7-bisphosphate (HBP), which activates TRAF-interacting protein with FHA domain-containing protein A (TIFA) immunity (Step 5). The release of pro-inflammatory cytokines and chemokines recruits polymorphonuclear leukocytes (PMNLs), or neutrophils, to the site of infection, where they interact and phagocytose *N. gonorrhoeae*. The influx of neutrophils constitutes a purulent exudate that then facilitates transmission (Step 6). [93]

Therefore, once colonization is established, nutrient acquisition is required from the extracellular environment for bacterial growth and replication. *N. gonorrhoeae* requires uptake of nutrients from the host, such as iron, zinc, and manganese. At this level, a process of host defense against the pathogen called *nutritional immunity* limits the nutrients acquisition. [108, 109] However, during symptomatic colonization, the pathogen overcomes this host defense by relying on the influx of neutrophils, which promotes nutrient acquisition by causing leakage of serum components, thereby allowing the uptake of intracellular nutrients by the bacterium [110, 111].

I.4.3 Interactions with host innate immune system

The innate immune system comprises the complement system and phagocytic cells, whose combined action represents the first line of defense against infecting pathogens, including *N. gonorrhoeae*. However, this pathogen possesses a repertoire of pathogenic mechanisms to avoid the host immune response, as indicated by the recurrence of gonococcal infections along with long-term complications like DGI. [42, 112]

I.4.3.1 Interaction with complement system: *serum resistance*

One of the principal mechanisms of human host defense is represented by the complement-mediated bactericidal activity of human serum, which is involved in the clearance of both pathogenic *Neisseria* species. The fundamental role of the complement system is highlighted and confirmed by the increased susceptibility to *Neisseria* infections in patients affected by an overall deficiency in complement proteins expression [51, 113, 114].

The complement system is an essential component of the innate immune response comprising several fluid-phase and membrane-associated proteins that act both as enzymes, regulators or substrates and structured to trigger an extracellular proteolytic cascade. Depending on the different stimuli during pathogen infections, complement activation occurs *via* three distinct pathways. The classical pathway (CP) represents a mechanism triggered by the binding of the antibody to an antigen, forming complexes on the pathogenic cell surface recognized by hexameric C1q. In contrast, the lectin pathway (LP) is a process specifically activated by oligosaccharidic residues present on the microbial surface, whereas the alternative pathway (AP) represents a less specific system that can be activated by C3b binding to any type of external surface macromolecule, such as lipids, proteins, and carbohydrate structures of microorganisms. After the initial trigger, all the three complement pathways converge at the level of complement component 3 (C3) deposition. The formation of the C3 convertase enzymes cleaves the C3 producing the active complement component C3b. The C3b molecules deposit on the pathogen surface and act as opsonin, inducing recognition by phagocytic cells. Downstream activation of complement results in the formation of the complement component 5 (C5) convertase enzyme, which leads to the generation of the C5b-9 complex, also called the membrane-attack complex (MAC). MAC is a group of proteins formed by complement components C8 and C9 that form pores in the membrane of microorganisms and kill the bacteria. The activity of the complement system must be strictly modulated to avoid any accidental activation of the complement components. Under physiological conditions, the complement cascade is kept under control by inhibitors such as C4b-binding protein (C4BP), factor H (FH) and vitronectin [115]. In order to evade the host immune system, *N. gonorrhoeae* exploits different mechanisms targeting all the three complement pathways, involving LOS and the PorB (**Figure I.8**).

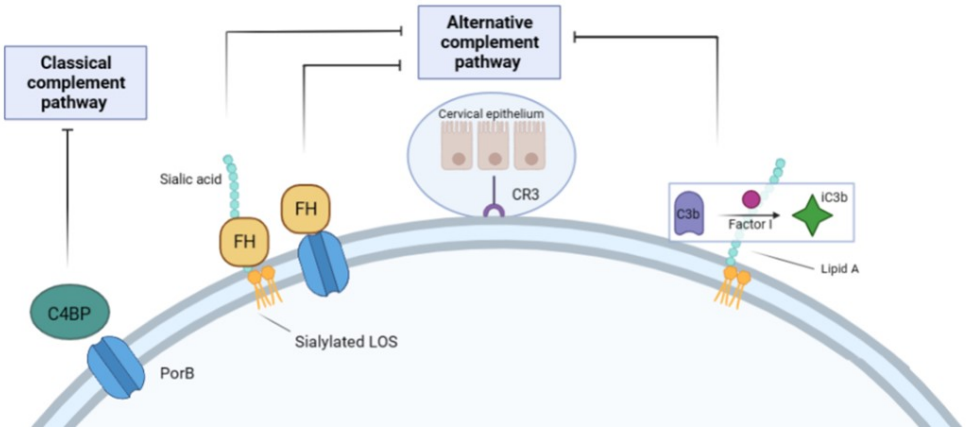


Figure I.8. During infection, *N. gonorrhoeae* can invade and modulate the innate immune system preventing complement activation, opsonization and bacterial killing. Indeed, *N. gonorrhoeae* bind host factors such as factor H (FH) and C4bp-binding protein (C4BP) blocking respectively the alternative and classical complement pathways (Figure created with Biorender.com, adapted from Quillin and Seifert, 2018). [93]

The ability to avoid complement recognition and killing permits effective colonization of the host by this bacterium, as shown by the observation that *N. gonorrhoeae* resists the action of the human complement system, but it is sensitive to animal complement systems. [65] *In vitro* studies have reported the ability of this bacterium to interact with several complement components. [116]

Complement-mediated killing evasion strategies implemented by *N. gonorrhoeae* mainly occur through two general processes: binding to and inactivating complement cascade components and mimicking the host molecules on the bacterial surface presenting itself as part of the host and binding to complement regulatory proteins.

The inactivation of the complement cascade by gonococci is mediated by the binding of C3b to the gonococcal LOS lipid A, followed by a rapid inactivation by factor I mediated- cleavage to iC3b. [117] Moreover, *N. gonorrhoeae* binds to the alternative complement pathway receptor CR3 and the receptor for iC3b in the cervical epithelium, possibly facilitating epithelial cell invasion (**Figure I.8**). [107, 118]

In addition, Gonococcus shields itself from complement recognition, suppressing complement activation in both the cervical epithelium and human serum.

In this process of immune system evasion, the importance of gonococcal LOS is demonstrated by several studies reporting that LOS undergoes to *in vivo* sialylation at the terminal galactose of LOS structures [119, 120] and this mechanism confers serum resistance by inhibiting all the three complement pathways [121]. Sialylated LOS has been associated with the so called *unstable serum resistance* [122]. When sub-cultured *in vitro,* some strains, mainly related to symptomatic pelvic inflammatory infection and classified as *serum sensitive* (SS), lose their ability to resist to the complement-mediated killing [123], which is restored after the supplementation in the growth media of exogenous cytidine monophospho-*N*-acetylneuraminic acid (CMP-NANA), a nucleotide sugar of sialic acid used as substrate for the enzymatic sialylation of glycans [124]. In the cervical epithelium, gonococci sialylated LOS shields itself from complement recognition, binding to the alternative complement pathway regulator factor H (FH**)** (**Figure I.8**).

Another important component in this resistance mechanism is the porin. *Stable serum resistance* is usually related to DGI and concerns *serum resistant* (SR) gonococcal strains which, when grown in *in vitro* conditions, remain resistant to the bactericidal activity of normal human serum (NHS), even in the absence of gonococcal LOS sialylation. [124] SR strains are the result of mechanisms that involve Por motifs that can bind to the complement pathway regulators factor H and C4-binding protein (C4BP), which downregulate the alternative and the classical complement pathways respectively. In particular, PorB.1A has the ability to bind factor H at the level of the surface-exposed loop 5 peptide, thus inhibiting the alternative pathway. [61] However, it can also block the classical

pathway by sequestering the C4b-binding protein [63], *via* loop 1 of the porin. [125, 126] This mechanism can also occur in PorB.1B bearing gonococci, that can bind the C4BP through loops 5 and 7, albeit to a lesser extent than the PorB.1A strains. [127] Nevertheless, these mentioned mechanisms are not exhaustive in explaining the gonococcal serum resistance, since some PorB.1B strains subvert complement-mediated killing even in the absence of FH and C4BP binding, and this remains unclarified so far. [127]

I.4.3.2 Resistance to neutrophil killing

During host invasion, *Neisseria gonorrhoeae* triggers a strong, local inflammatory response that is characterized by the recruitment of neutrophils to the site of infection. [128] Neutrophils, also known as polymorphonuclear leukocytes (PMN), are terminally differentiated phagocytic cells that, in response to chemotactic cues, extravasate from the bloodstream to the peripheral tissue to reach the target site. During gonococcal symptomatic infection, mucosal epithelial cells, dendritic cells and macrophages release proinflammatory cytokines and chemokines, including interleukin-8, interleukin-6, tumor necrosis factor-α, and interleukin-1, causing an influx of neutrophils. [129, 130] However, *N. gonorrhoeae* can rely on different strategies to survive the various functions of neutrophils, such as phagocytosis and the different antimicrobial activities like the release of reactive oxygen species, cationic peptides and antimicrobial enzymes.

Phagocytosis occurs through opsonic and non-opsonic uptake, both exploited by Gonococcus. [131] In the absence of both complement and antibodies, which usually mediate opsonic phagocytosis, *N. gonorrhoeae* can efficiently interact with neutrophils through pili and porins specifically binding to CR3 receptors. [107] In addition, Opa proteins can interact with the CEACAM family receptors driving non-opsonic phagocytosis. [104] Interestingly, although CEACAM1, CEACAM3 and CEACAM5 are expressed on neutrophils, it has been demonstrated that only the CEACAM3-Opa interaction can effectively stimulate a bactericidal oxidative burst. [78, 132, 133] Except for bacteria expressing Opa variants that engage CEACAM3, most bacteria are able to survive and replicate in association with human PMNs. Afterwards, once in the PMN, Gonococcus encounters the large variety of oxidative and non-oxidative antimicrobial components to which Gonococcus resists through different strategies [128], including LOS phosphoethanolamine (PEA) modification of lipid A and the active export of these components through the MtrCDE efflux pumps. [134-136]

In most cases, the mechanism exploited by Gonococcus to survive after exposure to PMNs remains to be clarified; however, it can be crucial to understand the mechanisms underlying the ability of gonococci to avoid the first line of defense in the human host.

I.5 Gonococcal lipooligosaccharide

Lipooligosaccharide is a major glycolipid component of the cell wall, that is responsible for several biological activities and plays an important role in pathogenesis, as already reported before. Moreover, LOS is target for bactericidal antibodies present in normal and convalescent human sera. [137] LOS is found in the outer membrane of some Gram-negative bacteria, including *Neisseria* spp., *Haemophilus* spp. and *Bordetella* species. Similar to rough lipopolysaccharide (LPS) from enteric bacteria, gonococcal LOS structure is composed by lipid A portion and an oligosaccharide moiety, but it has a lower molecular weight due to the absence of the O-polysaccharide repeating units (**Figure I.9**). [138] The saccharide portion of LOS is shorter and therefore, these structures are relatively more hydrophobic and more susceptible to cell surface-directed antibodies.

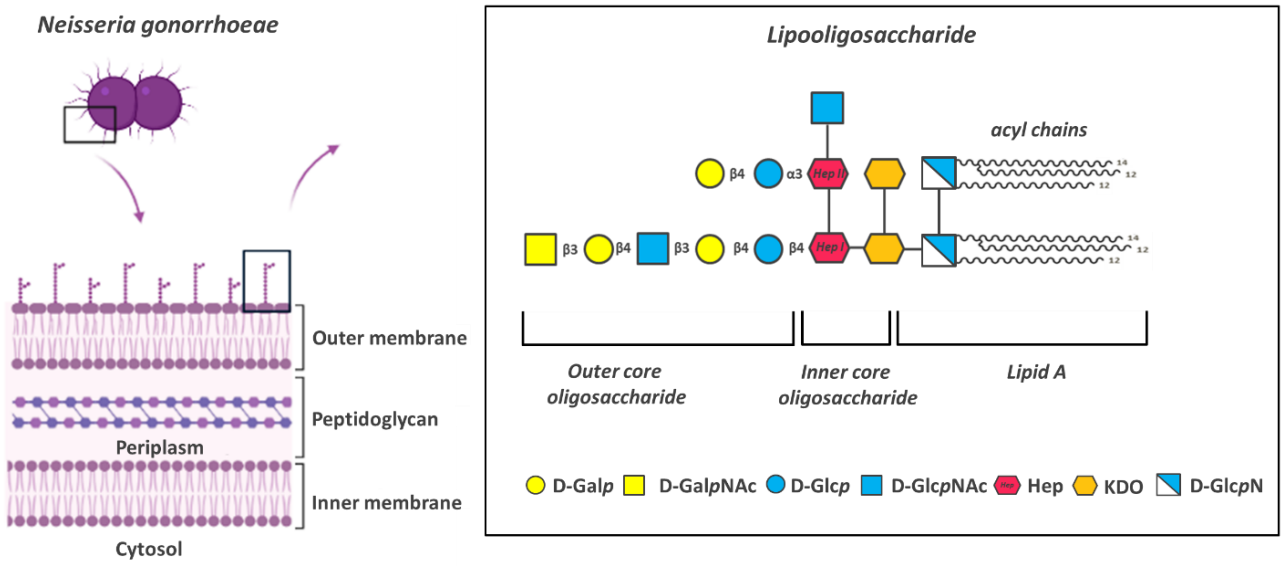


Figure I.9. Structure of gonococcal lipooligosaccharide. LOS is composed of a lipid A portion, anchored to the OM and an inner core oligosaccharide structure, to which are linked three oligosaccharide chain, composing the outer core moiety (Figure created with Biorender.com).

The LOS oligosaccharide portion consists of three oligosaccharide chains attached to a conserved tetrasaccharide core. The oligosaccharide chains branch from two heptoses (heptose I, or Hep I, and heptose II, or HepII) linked to lipid A via two 2-keto-3-deoxy-mannooctulosonic acid (KDO) molecules. The first oligosaccharide chain elongates from the HepI and is known as the α-chain, whereas the second and the third oligosaccharide chains, called the β- and γ- chains respectively, are connected to the second heptose (**Figure I.10**). The number of branches and the length of

oligosaccharides in each branch vary among gonococcal strains and, indeed, in the same strain during growth *in vitro* and *in vivo*.

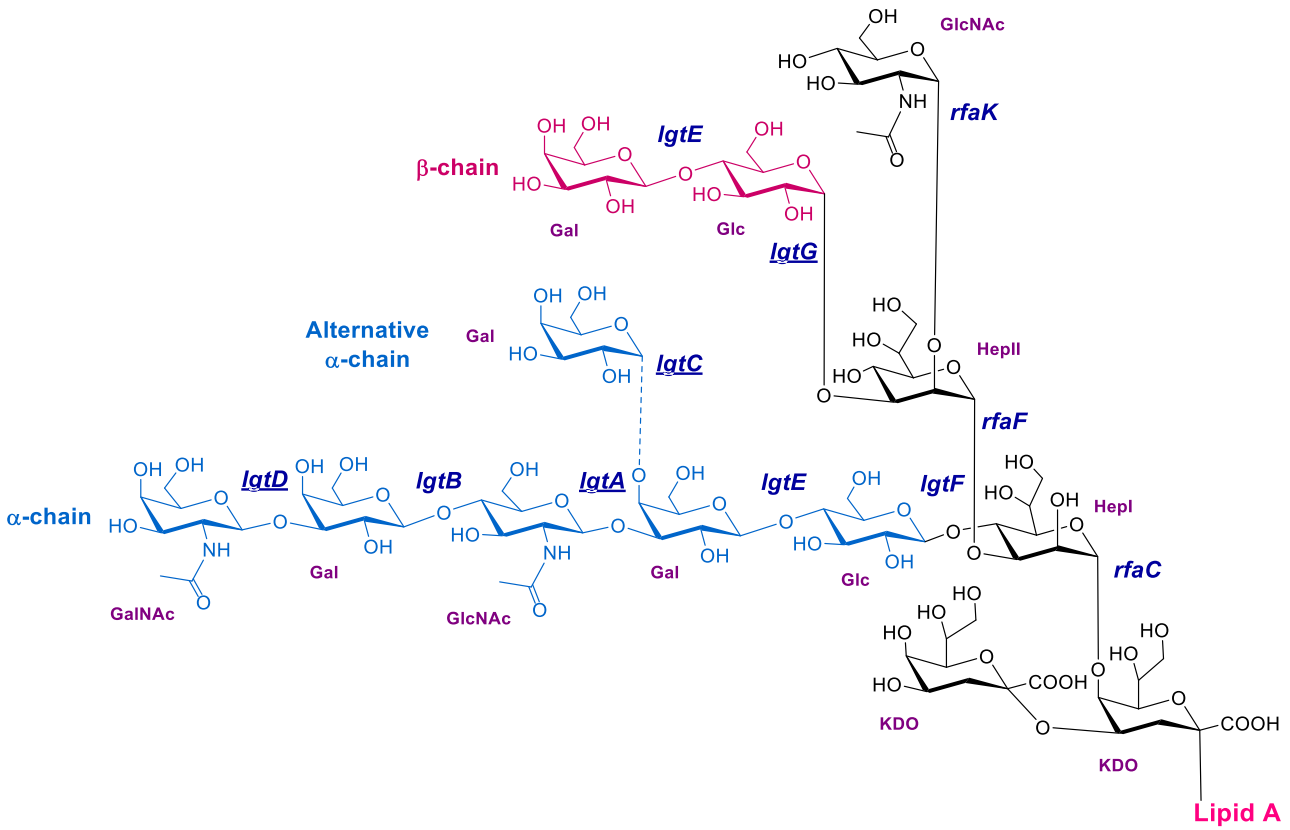


Figure I.10. One of the most complete chemical structures of the gonococcal LOS, in which the two possible α-chains and the β-chain are highlighted in blue and red, respectively. The gene responsible for the LOS biosynthesis are reported in blue and, among them, the underlined genes are the phase-variable ones.

The genes responsible for LOS biosynthesis have been identified and characterized. The *rfaC* and the *rfaF* encode for two different heptosyltransferases that are responsible for synthesizing the core heptoses moiety, linking the HepI to the KDO portion and the HepII to HepI respectively. [139-141] The *rfaK* gene product is required to attach a terminal GlcNAc to the HepII. Glycan extensions from the core heptoses are modulated by the expression of multiple saccharide transferases, products of *lgt* genes. The LOS glycosyltransferase genes *lgtF*, *lgtE*, *lgtA, lgtB* and *lgtD* are required for the stepwise addition of each hexose (or hexosamine) to synthesize the α-chain. [142, 143] In place of full extension of the α-chain, *lgtC* encodes for an α-galactosyl transferase responsible for adding a terminal galactose to the lactose linked to the α-chain creating the so-called P^K-like LOS structure, which is an alternative α-chain. (**Figure I.11**) [144] The *lgtG* gene product is required to add the first glucose to the HepI, thus determining the presence of the β-chain. The β-chain is usually composed

of a lactose, but in rare cases it can be constituted of a single glucose or a lactose with additional sugars.

N. gonorrhoeae synthesizes this variable portion by adding a glycose moiety sequentially on the conserved core tetrasaccharide to express different types of elongation: from the HepI only, from both HepI and HepII, or, occasionally, elongation from HepII only. [145] Gonococci can co-express up to six antigenically distinct LOS structures and this is mainly due to phase variation mechanism involving four *lgt* genes: *lgtA, lgtC, lgtD* and *lgtG* (**Figure 11**). [140, 146]

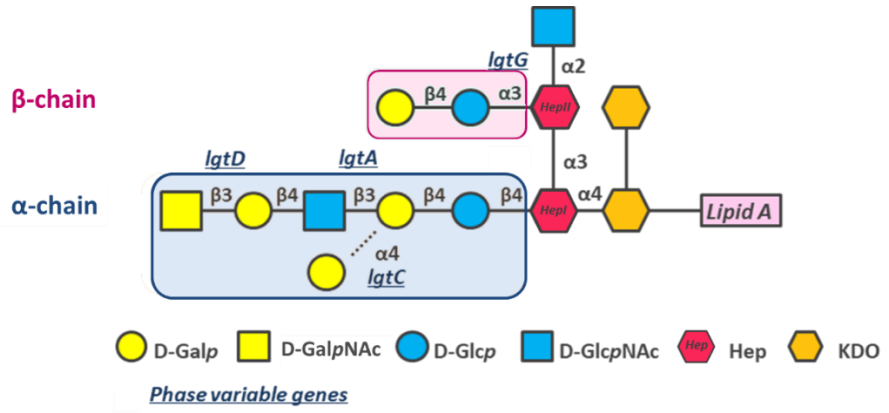


Figure I.11. Graphical structure of the possible structures of LOS in which are reported the phase variable genes. The two main variable oligosaccharide chains are highlighted in blue and red.

The genes that undergo phase variation contain homopolymeric tracts of guanine (poly-G tracts), in the case of *lgtA, lgtC* and *lgtD*, or cytosine, in the case of *lgtG*. These can lead to slipped strand mispairing during DNA replication, with consequent alteration in the coding sequences and premature termination of the corresponding gene; therefore, the encoded glycosyltransferase protein is not functional and leads to truncated LOS structures. [142, 146-149] This mechanism results in substantial LOS heterogenicity with changes in glycan composition of the predominant structures that are expressed both when gonococci grow *in vivo* or *in vitro.* The variation in these structures may alter the nature of antigenic epitopes recognized by anti-LOS antibodies as demonstrated by the characterization of gonococcal LOS from 20 different strains with a panel of monoclonal antibodies. [150] Moreover, this variability allows for alterations in the cell surface properties. Indeed, it may facilitate the exposure or protection of other constituents and amplify antigenic diversity that may modify attachment to cells and facilitate evasion of the host response. *N. gonorrhoeae* undergoes LOS phase variation at a frequency of $10^{-2}/10^{-3}$ per cell per generation when grown in culture [85, 151], even though the expressed LOS is determined by the LOS structure of the majority of the cells in the population.

Among the different possible predominant glycoforms, some share structures with the human glycosphingolipids [144, 145, 152]. The lacto-*N*-neotetraose structure, composed of four sugars extending from the first heptose (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-4- or L*N*nT) is identical to the human erythrocyte glycosphingolipid structure and the P^K LOS structure (Galα1-4Galβ1-4Glcβ1-4-) is similar to the human paraglobosides. [144, 150, 153, 154] Moreover, the pentasaccharide structure of the α-chain (GalNAcβ1-3-Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-4- or GalNAc-LNnT) has analogous structures to human asialo-G3 ganglioside. [154] This mimicry is an immune evasion strategy that may enable gonococci to avoid immune recognition. For several years, the role of this antigenic determinants in a possible vaccine has been discussed and therefore, these structures have been extensively studied for their role in pathogenesis, but some researchers didn't consider them suitable as possible vaccine antigens for the possibility of eliciting a response to this shared human antigen. Nevertheless, it is important to highlight that in the case of meningococcal LOS, which shares these structures with *N. gonorrhoeae* LOS, no safety issues related to the induction of antibodies cross-reactive with human blood cells have been reported following systemic disease or large-scale vaccination with OMV vaccines containing significant amounts of L3,7 LOS (lacto-*N*-neotetraose structure). [155, 156] However, to minimize this possibility, during the last years the majority of studies on LOS as possible vaccine target have focused on the two main LOS epitopes that do not cross-react with human glycosphingolipid antigens. In particular, the shorter alpha chain structures containing only lactose have been investigated since they were considered less likely to induce antibodies cross-reactive with human blood cells.

One example is the LOS epitope defined by the anti-meningococcal mAb L8, characterized by lactose β-linked to HepI with a PEA substitution at the 3-cyclic position on HepII (as occurs when *lgtG* is phase-off and HepII is not substituted at the 3- position with glucose). [157-159]

In 1996, Gulati and colleagues identified a highly conserved glycan epitope on LOS composed of two lactoses, one β-linked to HepI and the other α-linked to HepII, known as 2C7 epitope (**Figure I.12**). [160] This portion is the minimal epitope required for binding of mAb 2C7, even though *N*-linked fatty acids in lipid A are required for maximal expression. MAb 2C7 binding is permitted (although with lower affinity) even when the α-chain is extended beyond lactose; indeed, structures with lacto-*N*-neotetraose or a pentasaccharide in the α-chain are still bound by mAb 2C7 and the lowest binding is observed with the P^K LOS structures. [161]

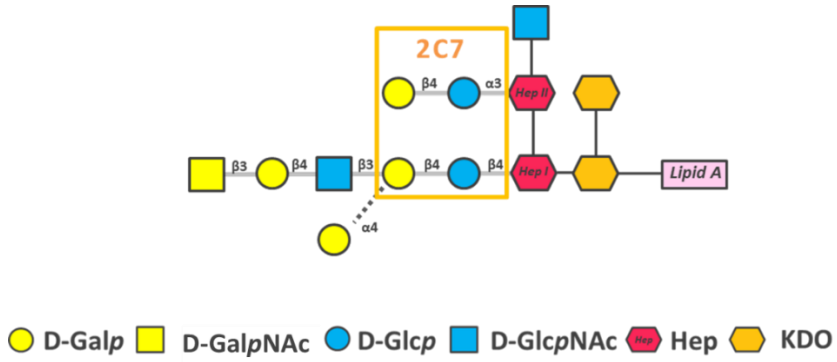


Figure I.12. The minimal oligosaccharide structures of lipooligosaccharide recognized by mAb 2C7.

The addition of an α-linked glucose residue to HepII, the first step in the synthesis of the β-chain lactose, required for binding of mAb 2C7, is mediated by the phase variable gene *lgtG*. Despite the phase variation of *lgtG*, the 2C7 epitope is widely shared and expressed by most gonococci *ex vivo* or after limited passages *in vivo*, including 100% of clinical isolates in Nanjing (China) and 95% in Boston (USA) [162]. Interestingly, *N. gonorrhoeae* is the only member of the genus *Neisseria* expressing a lactose extending from HepII; certain *N. meningitidis* strains possess *lgtG* and can add glucose to HepII (LOS immunotypes L2 and L4) [163, 164], but extensions beyond the proximal glucose in meningococci have not been described.

I.5.1 Relevance of LOS epitopes as potential antigenic target

The **2C7** epitope has been widely studied during the last decades either for its role in pathogenesis and colonization and as a possible antigenic target. It has been reported that the absence of HepII-linked lactose and subsequent loss of the 2C7 epitope significantly attenuates gonococcal infection in the experimental mouse cervicovaginal colonization model, indicating a potential role in pathogenesis and suggesting LOS as potential virulence factor required for survival and productive infection in humans. [162, 165] MAb 2C7 IgG exhibited broad complement-dependent bactericidal activity against a wide array of gonococcal isolates [160, 162, 166] and *in vivo* potency [167], and therefore it has been considered a promising candidate for the development of an antibody-based immunotherapeutic. A recombinant human IgG1 chimeric variant of mAb 2C7, designed to enhance complement activation, improved its efficacy both *in vitro* and *in vivo* in a mouse vaginal colonization model [167] and, recently, optimized 2C7-derived DMAbs (DNA-delivered monoclonal antibody) encoding this mAb 2C7 complement-enhancing Fc variant have been tested, showing long *in vivo* durability (up to 2 months of standard half-life), antibacterial activity and clearance of primary infection with sustained *in vivo* functionality and efficacy against subsequent rechallenges. [168] Moreover, in the last years, the 2C7 epitope has been also evaluated as a potential target for gonococcal candidate vaccines. A peptide mimic of the 2C7 epitope (a mimitope), configured as an

octameric multiantigen peptide (Octa-MAP) was developed as immunologic surrogate of the 2C7 epitope. Immunization of mice with the peptide vaccine elicited cross-reactive bactericidal IgG antibodies and the mimitope also attenuated gonococcal vaginal colonization in preclinical experimental model of infection. [169] Afterwards, an optimized version of the 2C7 mimitope called TMCP2 was developed. This experimental peptide vaccine, configured as a tetramer and adjuvanted with glucopyranosyl lipid adjuvant–stable emulsion, was shown to be able to induce bactericidal IgG antibodies and to accelerate clearance of gonococci in the mouse vaginal colonization model. Moreover, it was shown that the efficacy in the mouse colonization model of this mimitope and a passively administered chimeric mAb 2C7 requires an intact terminal complement pathway, evidenced by loss of activity in C9-/- mice. These results indicates that complement mediated killing of the bacteria is relevant to *in vivo* protection and, therefore, that serum bactericidal activity may serve as a correlate of protection. [167, 170]

Another possible LOS glycoform is a **P^K-like LOS** (or meningococcal L1 immunotype) structure composed of a trisaccharide (Galα1-4Galβ1-4Glcβ1-4-) extending from the α-chain. As previously reported, the P^K-LOS structures are comparable to the human paragloboside structure, providing this organism with an immune escape mechanism. [144, 150, 153, 154] Chakraborti *et al*. demonstrated that mutant strains genetically modified to express only this LOS glycoform are unable to colonize the mouse vaginal tract with a durable infection [161], reflecting the relatively low frequency of gonococcal clinical isolates expressing this LOS isoform. [171]

Several studies have focused on the pentasaccharide structure **GalNAc-LNnT** (GalNAcβ1-3-Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-4-). The GalNAc terminal sugar is the target of natural IgM present in the human serum. It is also able to interact with C-type lectin macrophage galactose-binding lectin (MGL), skewing the immunity towards a Th2 lineage and helping bacterial survival. [172] Studies by Balthazar and collaborators highlighted the role of this glycoform in the normal human serum resistance of gonococcal F62 strain, demonstrating that the length of the α-chain of the core oligosaccharide and 4′ lipid A phosphoethanolamine are important LOS structures that can determine the extent of gonococcal susceptibility to NHS. [173] Indeed, since *lgtD* is phase variable, those naturally NHS-sensitive gonococci that produce truncated LOS species due to spontaneous mutations, especially with a phase-off *lgtD*, might have a fitness advantage over those with a phase-on *lgtD,* since they would have an enhanced ability to escape NHS-killing mediated by complement at sites where complement components are at reduced levels, such as mucosal surfaces. [92] Moreover, NHS resistance expressed by gonococci also requires PEA modification of gonococcal lipid A (loss of 4’

PEA from lipid A with a phase-off *lptA*) and this decoration also enhances resistance to cationic antimicrobial peptides. [173]

Among the LOS glycan structures that have been studied for their role in pathogenesis and immune evasion, much attention has been directed to LOS that expresses the **lacto-*N*-neotetraose**, which consists of four sugars extending from the first heptose (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-4-HepI). LNnT is widely involved in immune evasion strategies, adhesion and pathogenesis. All the minimally passaged clinical isolates from Nanjing (China) tested by Sanjay Ram and colleagues have been found to express both the 2C7 epitope and the LNnT glycan portion, suggesting the importance of these two sugar substitutions in the pathogenesis of *N. gonorrhoeae.* [162]

As reported before, LNnT is identical to the terminal tetrasaccharide of paragloboside, a precursor of the major human blood group antigen and gonococci use this structure as mimicking strategies to evade the host immune response. Similar behaviors have also been encountered in other bacteria that can express the same structure, such as *N. meningitidis*, *Haemophilus influenzae* and *Campylobacter jejuni*. This form of molecular mimicry not only provides gonococci with a method of immune avoidance but also allows the bacterium to use host-derived molecules that normally associate with the mimicked structure. [42] *N. gonorrhoeae* invades non-ciliated cervical epithelial cells and urethral epithelial cells of men when LOS is not capped with sialic acid, thus expressing the unsialylated LNnT. It has been reported that LNnT interacts with the asialoglycoprotein receptor (ASGP-R) and facilitates adhesion and invasion of gonococci to male urethral epithelial cells [42, 106]. This interaction is abated in mutants expressing truncated LOS lacking the LNnT portion and in strains expressing terminal sialic acid. [106] LNnT tetrasaccharide is also directly involved in the gonococcal invasion into the cervical epidermoid carcinoma cell line, ME180. [174] A lectin-like interaction between the terminal lactosamine residue of LNnT and gonococcal Opa proteins plays a role in intergonococcal adhesion and the degree of colony opacity. [83]

A collection of studies by Schneider *et al.* addressed the impact of phase variation of α-chain glycans *in vivo* and of the presence of LNnT portions. First, the importance of lacto-*N*-neotetraose in pathogenesis and virulence was established in the human male volunteer infection model of gonorrhea. Human male volunteers were experimentally challenged with a gonococcal strain expressing a truncated 3.6kDa LOS species, in which the HepI chain was predominantly composed of a lactose (MS11 mkA). Sequential sampling of urethral contents demonstrated that the recovered bacteria transiently expressed this truncated structure until the onset of dysuria. However, with further progression of infection and development of urethritis, the HepI LOS phenotypes transitioned to predominantly tetrasaccharide and pentasaccharide HepI chain variants (MS11 mkC), indicating that positive selection occurred for bacteria that express these longer structures (approximately 4.5KDa).

[152] Similar behavior has already been reported for gonococcal strain 4505. [151] It is worth noting that it is not known whether these phenotypes were also capped by sialic acid or not. In another study, it has been showed that the required infectious dose of MS11 mkA (mostly composed of lactose in the α-chain) was higher than that of MS11 mkC (LNnT LOS). [175]

Notably, similar to what reported above, gonococci recovered from mkA-inoculated volunteers showed a transition from the mkA shorter α-chain phenotype to the mkC LOS LNnT-like phenotype. Taken together, these observations may suggest that small numbers of mkC gonococci would be sufficient to infect male volunteers and, moreover, that *N. gonorrhoeae* expressing predominantly truncated HepI glycan substitutions are less efficient in initiating and sustaining infection. The evidence of Schneider *et al.* in the human challenge model has been well mirrored in mice by the work of Chakraborti *et al.* where it was demonstrated that gonococci mutants displaying different surface glycans evade host immunity differentially, resulting in an infection that varies in duration. These studies confirmed that LNnT expression promotes vaginal colonization in mice, and it is necessary to establish a durable infection. [176] Interestingly, since the absence of the HepII chain significantly attenuates gonococcal infection in mice [162, 165], they analyzed four mutants with *lgtG* locked on and thus constitutionally expressing the β-chain, in which only the HepI glycan phase variation was permitted. They observed that mutants expressing LNnT colonized mice most effectively and phenotypes like the pentasaccharide GalNAc-LNnT, which also expresses LNnT in addition to the other structures, showed intermediate infectivity. Truncated mutants (lactose or Gal-lactose in the α-chain) gave a lower overall burden of infection. [176]

I.5.2 Gonococcal LOS sialylation

An important mechanism of immune escape shared among several bacteria, which largely contributes to pathogenesis and immune escape, is LOS sialylation. Sialylation consists of the ability of *N. gonorrhoeae* to add a 9-carbon amino sugar called sialic acid using a nucleotide sugar form, cytidine-5'-monophospho-N-acetylneuraminic acid (also referred to as CMP-Neu5Ac or CMP-NANA) to the terminal galactose portion of some LOS glycoforms. Gonococci use the organism's own endogenous sialyltransferase (Lst), an enzyme present in the outer membrane of the bacterium [177], and CMP-Neu5Ac substrate available from the host, since, unlike most serogroups of *N. meningitidis, N. gonorrhoeae* is not able to synthesize sialic acid substrates. *In vivo*, CMP-NANA substrate is present in mammalian genital secretions as well as in serum and in extracts of red or white blood cells [178-180]; whereas *in vitro* exogenous CMP-NANA is needed to obtain sialylated phenotypes. LOS sialylation has been reported to increase in presence of lactate and pyruvate in growth media with respect to glucose and in anaerobic conditions [181-184].

Sialylated LOS has been associated with the so called *unstable serum resistance* [122]; indeed, as reported above, it was observed that some gonococcal strains recovered from male urethral secretions and directly examined were able to fully resist killing by homologous normal serum and were therefore classified as serum resistant (SR) strains. These SR strains lost their ability to resist to the complement-mediated killing when tested directly *ex vivo* or sub cultured *in vitro.* [123] This evidence is corroborated by the restoration of serum resistance of these strains after supplementation in the culture media of exogenous CMP-Neu5Ac. [124] Hence, the ability of some gonococcal strains to resist complement only when tested directly *ex vivo,* but not after passage onto gonococcal media, is due to LOS sialylation mechanisms, suggesting that the gonococcal surface is likely to be modified *in vivo,* enabling the bacteria to resist complement-mediated killing.

Gonococci can enzymatically sialylate different structures, as reported in **Figure I.13.**

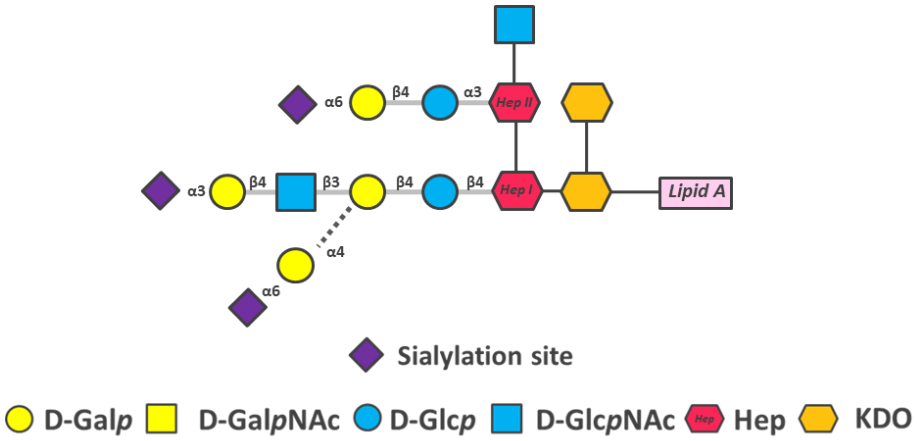


Figure I.13. *N. gonorrhoeae* LOS has three main sites of sialylation, here indicated with a purple box.

Among the sialylatable structures, the LNnT glycoforms have been widely studied since its sialylation have different effects on the gonococcal pathogenesis. However, other possible terminal galactoses can also be sialylated. One is the terminal Gal of the P^K-like α-chain which can be sialylated through an α2-6-linkage and, recently, an additional acceptor site for an α2-6 sialic acid was identified on HepII lactose, when HepI and HepII both bear only lactose extensions. [162]

During the last decades, several works have reported the impact of LOS LNnT sialylation on the inhibition of all the three complement pathways through several independent mechanisms (**Figure I.14**). In the case of the classical pathway, it is well known that an intact classical pathway for complement-dependent killing of *N. gonorrhoeae* is essential, as reported previously and confirmed by recent studies. [170, 185] NHS killing of serum sensitive strains is mediated mainly by IgM against LOS and its sialylation permits gonococci to inhibit the classical pathway by reducing the activity of

IgM antibodies present in non-immune normal human serum, without necessarily inhibiting IgM binding on the surface. In contrast, the binding of NHS IgG to the bacterial surface is inhibited by LNnT sialylation. [186] Moreover, the presence of Neu5Ac in LNnT LOS reduces the binding of IgG to selected targets. Indeed, sialylated LOS reduces binding of some anti-PorB, suggesting a possibile interference with the ability of antibodies to engage C1q, the first step of the classical pathway. No inhibition of IgG binding to Opa was observed. [187]
The activation of the lectin pathway is inhibited by reducing mannose-binding lectin (MBL) binding [188]; indeed, MBL binds to gonococcal LOS that terminates in GlcNAc [189], which is elongated with Gal and subsequently sialylated with Neu5Ac.

Lastly, sialylated LNnT LOS mediates the downregulation of the alternative pathway by enhancing factor H (FH) binding [62] with a mechanism in which gonococcal PorB, LOS α(2,3)-linked Neu5Ac and the FH portion are possibly involved. Sialylation of the P^K-like LOS [190], or lactose on HepII- [162] does not enhance FH binding. FH binds to sialylated gonococcal LOS through its C-terminus portion and this binding involves an interaction with PorB and sialylated LOS, similar to what happens with FH, C3 fragment and other components on the host cells. The FH binding acts as a cofactor in the factor I cleavage of C3b to iC3b and, also, irreversibly dissociates the C3 convertase, C3bBb (decay accelerating activity). It is worth noting that sialylation of meningococcal LOS LNnT is not capable of enhancing the direct interaction with FH. This observation may suggest that the PorB requirements is accounting for this difference among meningococcal and gonococcal LOS [64]. In fact, binding of FH is also dependent on the expression of PorB [64].

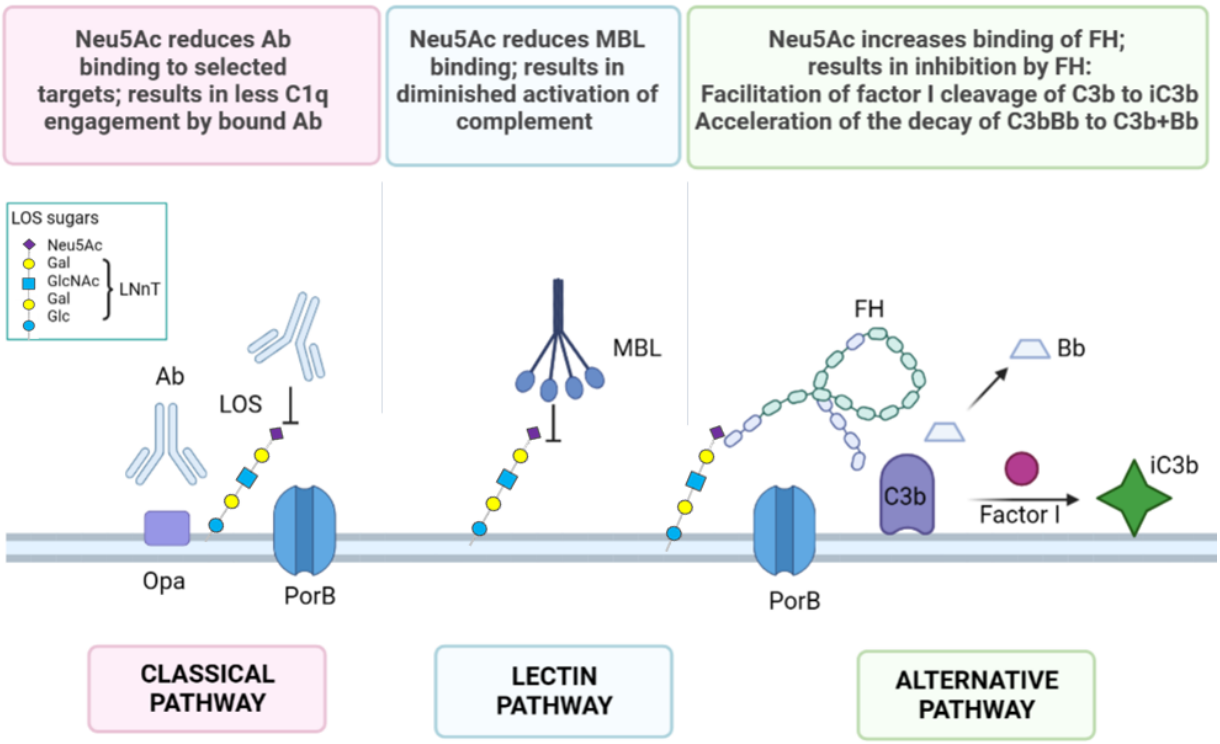


Figure I.14. The LOS sialylation inhibits all the three complement pathways through several independent mechanisms (Figure created with Biorender.com, adapted from Gulati *et al.,* 2019). [191]

Moreover, neisserial LOS also serves as a target for C4b and C3b [192]; it is likely that sialic acid obscure some targets for the deposition of C3 and/or C4 fragments present on LOS.

Sialylation of LOS has been demonstrated to have an impact also on opsonophagocytosis, inhibition of the invasion into epithelial cell and *in vivo*.

As previously discussed, gonococci interaction and killing by PMN may occur through opsonic or non-opsonic means. [193] The presence of Neu5Ac on LOS decreases the opsonic killing of gonococci [194-196], which may be in part because of reduced complement activation and C3 fragment deposition on the sialylated bacterial surface. [62, 197] Moreover, the presence of lactate on PMNs can further facilitate LOS sialylation and gonococcal survival. [182, 183]

As already discussed above, sialylated LOS can also inhibit the ability of Opa-positive bacteria to adhere to neutrophils and stimulate neutrophil oxidative burst, whereas killing by human PMNs of Opa-positive bacteria was not significantly affected. [195]

Although sialylation reduces Opa-mediated invasion of *N. gonorrhoeae* into human epithelial cell lines, no effect on the adherence of bacteria to epithelial cells was observed [198].

An electron microscopy study carried out by Apicella *at al.* confirmed that LNnT LOS sialylation occurs *in vivo.* [199] Wu and Jerse showed that a gonococcal mutant of strain MS11 that lacked sialyltransferase was less virulent than its wild-type counterpart in the mouse vaginal colonization

model of gonococcal infection, and these results in mice were also further verified with strain F62. [200, 201] It is likely that the amount of sialylation *in vivo* must be balanced for optimal colonization in humans: although on one side the complete absence of sialic acid may render the organism susceptible to eradication by host immunity, an excessive sialylation may block the invasion of bacteria into epithelial cells. In studies carried out by Schneider and co-workers, intra-urethral inoculation of male volunteers with *pre*-sialylated gonococci (strain MS11 mkC) infected only one of five (20%) subjects, whereas inoculation of the same number of unsialylated bacteria infected five of six (86%) individuals, suggesting a critical role of unsialylated LNnT in the first stage of infection. [202] Moreover, Ketterer *et al.* showed that cervical secretions obtained from women infected with gonorrhea contain sialidase in quantities sufficient to desialylate LOS, which may facilitate transmission of infection from women to men, suggesting that unsialylated LNnT may be important for the transmission and the first stages of infection, whereas sialylated LNnT has a critical role in the pathogenesis. [203]

In addition to inhibiting complement and enhancing resistance to opsonophagocytosis and cationic antimicrobial peptides, sialic acid engages sialic acid-binding immunoglobulin-type lectins (Siglecs), many of which in turn bind to an immunoreceptor tyrosine-based inhibition (ITIM) motif and prevent the inflammatory response. [204] Furthermore, sialic acid has been identified in gonococcal biofilms. [205]

Beyond lacto-N-neotetraose sialylation, it is worth highlighting that several studies have been published in recent years, studying the role of other possible sialylation sites. Indeed, as reported before, the Lst enzyme adds Neu5Ac to LNnT through an α2-3-linkage, while to P^K-like LOS, and to HepII-lactose through an α2-6-linkage. Sialylated P^K-like LOS can enhance gonococcal serum resistance, albeit to a lesser degree than sialylated LNnT. [190] Lactose on HepII have recently been found to be a possible site of sialylation, able to inhibit the complement activation. [162] Both of these sialylatable structures are characterized by low virulence profiles in the mouse vaginal colonization model and it is possible that the absence of a sialylable LNnT accounts for it. Whether LOS glycoforms with only a lactose on the HepI chain can incorporate Neu5Ac in their terminal galactose is still unclear. [162]

I.6 Current status of Neisseria gonorrhoeae *vaccines: feasibility, promising antigen and vaccine technologies approaches*

Despite during the last century several efforts have been made to develop a vaccine to protect against *N. gonorrhoeae* infection, only four candidate gonococcal vaccines have advanced to the stage of clinical trials or human challenge trials.

The first clinical evaluation was a gonococcal whole-cell vaccine; however, little information is available regarding this early 1900s trials. [206] The second candidate vaccine consisted of a crude, heat-killed, partially lysed whole-cell vaccine administered parenterally, which was evaluated in a double-blind placebo-controlled study conducted in a population of 62 volunteers in Canada. [207] Another attempt was an intradermally delivered single-antigen purified pilus that was examined in a double-blind placebo-controlled trial involving the randomization of 3250 US military personnel volunteers in Korea. [208] This trial failed to protect male volunteers against gonococcal urethritis and both killed whole-cell and purified pilin vaccines failed to induce protection against heterologous reinfection.

Eventually, the last attempt was a purified gonococcal PorB vaccine evaluated in a human challenge study conducted parenterally immunizing male subjects. Similar to the pilus-based vaccine, a significant antibody response was elicited, but offered no protection against intraurethral challenge in men with the homologous *N. gonorrhoeae* strain. [209] According to Rice and colleagues, it is worth highlight that the failure of this clinical trial could be possibly related to different reasons. Indeed, at the time of the vaccine trial in 1985, no reliable protocols to prepare pure PorB protein were available, with subsequent potential contamination of other immunogenic OM components such as LOS and Rmp. In particular, Rmp can elicit antibodies that block complement-dependent killing of *N. gonorrhoeae* by anti-PorB and anti-LOS antibodies. Another important consideration is related to the absence of stratification of volunteers to choose the cohorts according to the immunologic risk in order to better understand the protection from and the susceptibility to this infectious disease. [210] Beyond these four clinical trials, no other gonococcal vaccine candidates have progressed to human studies, highlighting the difficulties associated with gonococcal vaccine development. The product pipeline of gonococcal vaccines is still at preclinical level, focusing on the identification of possible vaccine targets and related immune correlates of protection.

In fact, the propensity of this bacterium to alter the antigen identity and expression levels of several gonococcal surface antigens has made the selection of promising vaccine candidate targets challenging. In addition, the lack of known correlates of immune protection against natural mucosal infections, the multiple mechanisms of immune evasion together with the host-restriction to humans

are among the main obstacles to vaccine development. [211, 212] Nevertheless, a proposed surrogate for vaccine efficacy has been identified in an increased level of complement-dependent bactericidal antibody activity against this pathogen. Although a high level of bactericidal antibodies has rarely been related to natural gonococcal infection, it is reasonably believed that a stronger and protective immune response can be forced by vaccination and it can possibly overcome factors like the presence of anti-Rmp blocking antibodies or the downregulation of soluble complement regulators such as FH and C4BP. [61, 62, 213, 214]

During the last decades, progress in antigen discovery has led to the identification of several stable and conserved gonococcal OM components that are being pursued as vaccine candidates. Even if no correlates of protection have been identified against *N. gonorrhoeae* in humans, in the preclinical investigation vaccine efficacy is usually measured by bactericidal and opsonophagocytic activity, blocking of target functions and coinfection in a female murine genital tract infection model. [210] In fact, most of these targets can elicit bactericidal antibodies against *N. gonorrhoeae* and, additionally, bactericidal antibody responses to several antigens may target important gonococcal physiological functions, including colonization and invasion, nutrient acquisition, and immune evasion (**Table I.1**). [210] Among them, the transferrin receptor (TbpA/B) or the methionine receptor (MetQ), have been reported, as well as some other targets identified by proteomic analysis of gonococcal surface proteins, including proteins involved in membrane biogenesis (BamA), LOS assembly (LptD), or translocation assembly (TamA). Vaccine targets that mediate evasion of host innate defenses comprise the outer membrane channel of the MtrCDE active efflux pump, MtrE, and the Neisseria adhesion complex protein (ACP). [210]

Other attractive targets are colonization factors, because of their potential to elicit antibodies that block the establishment of infection. An example is PilQ, the pilus secretin essential for pilus function or gonococcal OM proteins that mediate adherence and/or invasion of host cells, such as PorB, OpcA, OmpA and the opacity (Opa) proteins, that are able to induce bactericidal antibodies or to block interactions with host cells. [210]

Moreover, the LOS 2C7 epitope is an enticing vaccine target, against which a peptide mimic has been developed that induces highly bactericidal IgG antibodies able to promote opsonophagocytic killing of gonococci and *in vivo* protection against gonococcal challenge in mice. [169]

Table I.1. *N. gonorrhoeae* vaccine candidates that elicit bactericidal antibodies (Table adapted from Rice *et al.,* 2017). [210]

Antigen		**Function**	**Expression and variability**	**Immunogenicity** (Bactericidal antibodies)
Colonization and invasion	**PilQ**	OM channel for pilus extrusion	Stable; Conserved at C-terminus	Bactericidal Ab elicited by *N. meningitidis* homologs
	Opa	Adherence, invasion	Phase variable; Variable	Bactericidal Ab
	OpcA	Adherence, invasion	Stable; Conserved	Bactericidal Ab elicited by *N. meningitidis* homologs
	PorB	Adherence, invasion	Stable and essential; Variable	Bactericidal Ab
Nutrient acquisition	**TbpA**	Transferrin-binding protein	Induced in iron-limiting conditions; Semiconserved with hypervariable segments	Bactericidal Ab
	TbpB	Transferrin-binding protein	Induced in iron-limiting conditions; Variable with conserved segments	Bactericidal Ab
	LbpA	Lactoferrin-binding protein	Induced in iron-limiting conditions; Semiconserved	Bactericidal Ab elicited by *N. meningitidis* homologs (limited cross-reactivity)
	LbpB	Lactoferrin-binding protein	Induced in iron-limiting conditions; Variable	Bactericidal Ab elicited by *N. meningitidis* homologs (limited cross-reactivity)
	ZnuD	Zinc transporter	Induced by zinc limitations; Conserved	Bactericidal Ab elicited by *N. meningitidis* homolog
	MtrE	Surface-exposed channel of the active efflux pumps	Stable; Highly conserved	Bactericidal Ab
Immune evasion	**PorB**	Binds C4BP and FH; Critical for nutrient acquisition	Stable and essential; Variable	Bactericidal Ab elicited by *N. meningitidis* PorB cyclic loop peptides
	NspA	Binds FH	Stable; Highly conserved	Bactericidal Ab elicited by *N. meningitidis* homolog
	2C7 epitope	Promotes survival of *Ng* in humans and experimental mice	Under control of phase variable gene *lgtG*; Highly conserved (widely expressed in clinical isolates)	Bactericidal and opsonophagocytic antibodies (2C7 epitope mimics)

Proteomic analysis of *Ng* surface proteins	**BamA**	OM protein assembly factor	Stable and essential; Highly conserved	Bactericidal Ab
	LptD	LOS assembly	Stable and essential; Highly conserved	Bactericidal Ab
	TamA	Translocation assembly	Stable; Highly conserved	Bactericidal Ab
	MetQ	Methionine transport adhesin	Stable; Highly conserved	Bactericidal Ab
	NGO2054	Unknown	Stable; Highly conserved	Bactericidal Ab
Bioinformatic analysis	***Ng*-ACP**	Adhesin complex protein	Stable; Highly conserved	Bactericidal Ab

Together with the 2C7 peptide mimic, other antigens that showed protection against experimental murine infection include the gonococcal OMV given vaginally adjuvanted with microencapsulated IL-12 and a recombinant, refolded PorB (rrPorB) administered using a viral delivery system followed by rrPorB protein boosts.

Significant efforts have also been made in the development of innovative vaccine platforms for delivery and antigen presentation for vaccines. Although subunit vaccines, either alone or as a combination of multiple antigens, have shown the ability to raise broadly cross-protective immune responses and have been considered an effective approach for the development of gonococcal vaccines, other innovative and attractive methods have been evaluated during the last years. Among the newest promising approaches, immunotherapeutic vaccines are able to enhance the adaptive immune response using different strategies, such as the use of the inflammatory cytokine IL-12, which enhances humoral or antibody-mediated immunity. Preclinical studies using local administration of microencapsulated IL-12 have shown an increase in Th1-driven protective immunity and protection against reinfection in mice when administered either as a treatment for an on-set gonococcal infection or as an adjuvant with a gonococcal OMV vaccine. [215-217] Additionally, a new optimized version of the multi-antigenic peptide 2C7-epitope mimic in association with the toll-like receptor 4 agonist monophosphoryl lipid A tested immunizing mice, have been shown to shorten the time of *N. gonorrhoeae* clearance in a female murine genital infection model, showing the potential of peptide mimics of carbohydrate antigens. [165, 218]

Another strategy was reported by Gala *et al*, who developed a transdermal whole-cell-based inactivated gonococcal microparticle, characterized by several advantages, including the preservation of all immunogenic epitopes delivered in microparticles able to mimic the cocci shape of *N. gonorrhoeae* and the possibility of obtaining a slow and sustained release of the antigen over time. [219] Other alternative methods have been reported by Jiao *et al.,* who designed a *N. gonorrhoeae*

DNA vaccine delivered by *Salmonella enteritidis bacterial ghosts* (which are empty bacterial cell envelopes) with the idea of obtaining excellent DNA loading capacity with delivery to both professional and non-professional APCs, resulting in increased levels of gonococcal PorB-specific serum antibodies. [220] Lastly, Wang and colleagues employed *Helicobacter pylori* ferritin nanoparticles to present *N. gonorrhoeae* antigens, an approach already successfully applied by Kanekiyo *et al.* with the Influenza and Epstein-Barr viruses, resulting in more robust immune responses and protection against the viruses. [221-223]

Despite all these innovative approaches, OMV has always been considered one of the most promising. Indeed, OMV are self-adjuvanted systems that contain the majority of the gonococcal OM antigens, resembled on the vesicle membrane in their natural conformation. This approach is further supported by observational data related to an effective vaccine against the closely related *Neisseria meningitidis* serogroup B vaccines, suggesting that vaccination of humans with OMV of the related species might provide some protection against gonococci. In fact, despite the differences in the infectious pathologies caused by these two strictly human pathogens, *N. gonorrhoeae* and *N. meningitidis* share 80–90% genomic identity at the nucleotide sequence level and exhibit surface-exposed proteins shared at a high degree of sequence identity. [224, 225]

During the last years, several studies have suggested that outer membrane vesicle (OMV) *N. meningitidis* group B vaccines may reduce the incidence of gonorrhea. One of the first evidences of a protective effect was reported in Cuba, where a rapid decline in gonorrhea incidence was observed after a vaccine campaign with meningococcal VA-MENGOC-BC®, a proteoliposome OMV vaccine characterized by proteins from a hypervirulent meningococcal B strain and *N. meningitidis* serogroup C polysaccharide. [28] Interestingly, *N. meningitidis* and *N. gonorrhoeae* have been found to share many conserved proteins present in the OMV vaccine, which could elicit cross-reactive bactericidal antibodies against heterologous MenB strains and possibly against *N. gonorrhoeae.* Similarly, a 2016 analysis of gonorrhea rates in Norway showed a decrease in the incidence after a MenB vaccination campaign. [29]

Another important work was a case-control study by Petousis-Harris *et al.,* who reported the potential protective immunity against gonorrhea after a national immunization program in New Zealand with the MenB OMV vaccine MeNZB® (Novartis). Analyzing the reported gonorrhea cases from 2014 to 2016, it was found that people who received the MeNZB® vaccine had lower infection rates, with an estimated vaccine effectiveness of 31%. [27] Furthermore, a subsequent retrospective cohort study by Paynter and colleagues reported 24% effectiveness of MeNZB® against hospitalizations due to gonococcal infections, providing support of the vaccine's cross-protectivity. [30]

A recent investigation expanded the New Zealand findings by showing that immunization with the new recombinant protein based MenB vaccine (4CMenB) called Bexsero® elicits antibodies in humans that can specifically recognize *N. gonorrhoeae* proteins. [31, 32]

The Bexsero® vaccine contains the MeNZB® OMV antigens in addition to three recombinant meningococcal antigens (factor H-binding protein, FHbp; Neisseria adhesin A; NadA Neisserial heparin-binding antigen, NHBA). First, the possible Bexsero® cross-protection against gonorrhea was reported in a retrospective study in Canada, in which a 59% decline in gonorrhea was observed during the post-vaccination period. [33] Then, Semchenko *et al.* reported that anti-gonococcal antibodies elicited by Bexsero® vaccination cross-reacted with the highly conserved NHBA gonococcal protein, revealing that Bexsero® may provide additional protection with respect to MeNZB. [31]

These findings have been further validated by studies investigating the cross-protection offered by the vaccine, in which immunization of estrogen-treated mice with Bexsero® significantly accelerated clearance and reduced *N. gonorrhoeae* bacterial burden. [32] Additionally, Bexsero® induced antibodies recognizing several gonococcal outer membrane proteins including NHBA, PilQ, BamA, MtrE, PorB, and Opa. [32]

Eventually, a modelling study conducted in England, published by Looker *et al.,* highlighted the vaccination of 14-year-olds in England with 4CMenB against *N. gonorrhoeae* infection could lead to a substantial reduction in new gonorrhea infections, particularly among adolescents, with great advantages in preventing infection both in short and long-term protection. [34]

To date, there are three clinical trials to investigate the efficacy of Bexsero® vaccine against gonorrhea: the *MenGO* study, analyzing the incidence of *N. gonorrhoeae* infections in the MSM (men having sex with men) population, the *GoGoVax* clinical trial examining the efficacy of Bexsero® in the prevention of gonorrhea, determining any change in the incidence of symptomatic gonorrhea in MSM; and the *B part of it NT* observational study, which has different aims and among them to compare the rates of gonorrhea in vaccinated/unvaccinated participants. [226-228]

Collectively, these findings on the cross-protection of MenB vaccines against *N. gonorrhoeae* have recently renewed interest and optimism regarding the feasibility of an efficacious *N. gonorrhoeae* vaccine in the near future, highlighting the potential utility of OMV meningococcal serogroup B vaccines in decreasing the global incidence of this disease in the absence of a specific effective vaccine.

I.7 Promising vaccine technology for development of a Neisseria gonorrhoeae *vaccine*

I.7.1 OMV and GMMA technology

In the last years, the research on novel vaccine platforms that may accelerate vaccine development has received increased attention. Outer Membrane Vesicles (OMV) are a technology platform for vaccine delivery against bacterial pathogens, first explored as a vaccine platform against *Neisseria meningitidis* serogroup B disease. After the success of vaccination with the MeNZB® OMV-based vaccine in controlling an outbreak of meningitis in New Zealand, additional research and development resulted in the licensure of Bexsero®, with broader protection against multiple meningococcal B strains and possibly against the gonococcal disease.

Both pathogenic and commensal Gram-negative bacteria are able to naturally release vesicles during growth, known as OMV. These spherical vesicles of heterogeneous size (25-250nm) originate from the bacterial membrane and therefore resemble the composition of the OM surface, comprising externally exposed antigens required to induce a protective immune response (**Figure I.15**). Therefore, after their discovery, OMV have been proposed as vaccine platform.

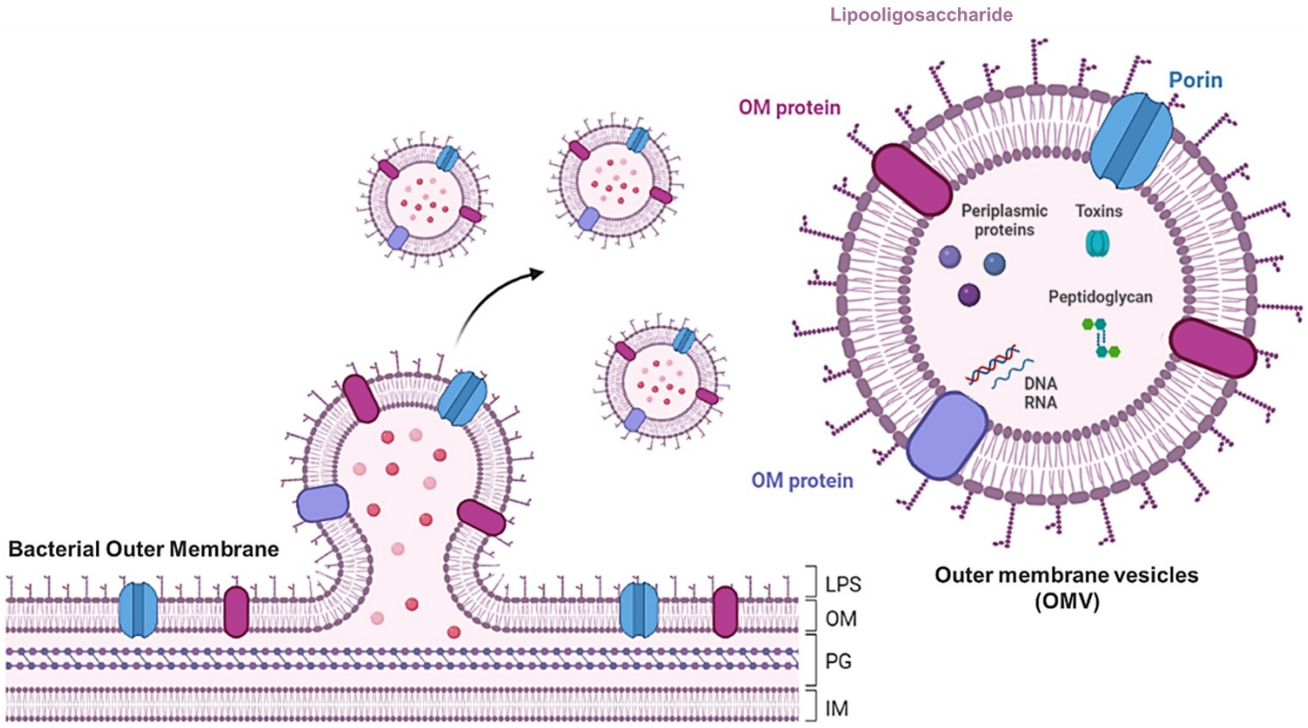


Figure I.15. OMV generation, composition structure and content (Figure created with Biorender.com).

OMV are constituted by a phospholipid bilayer with LPS or LOS exposed on the outside, together with membrane proteins in their native conformation; the lumen of the vesicle may contain various compounds from the periplasm or cytoplasm, such as proteins, RNA/DNA, and peptidoglycan (**Figure I.15**).

OMV have a fundamental role in microbial physiology and pathogenesis, including intracellular and extracellular communication, horizontal gene transfer, transfer of virulence factors to host cells and bacterial defense against antibiotics. [229]

The release of vesicles has been observed at all stages of growth and in a variety of growth environments, including liquid cultures, solid cultures and biofilms. [230]

Nevertheless, the natural release of vesicles from bacteria results in relatively low amounts of OMV, that contain endotoxins in their natural form, which might cause systemic reactogenicity in humans. Therefore, chemical extraction from whole bacteria using detergents has been used to obtain detergent extracted OMV (dOMV) in high yields. However, the use of chemical detergent treatment in the production process causes partial removal of LPS and lipoproteins and alters the general composition of the vesicles in terms of the protein profile, increasing the cytoplasmic and inner membrane protein content. [231]

Recently, in order to overcome issues of limited yield and to reduce the levels of endotoxicity, genetic modification of strains to increase outer membrane vesiculation [232] and reduce LPS/LOS endotoxicity [233] have been applied to obtain hyper-vesiculating strain with mutations in the LPS/LOS genes, known as Generalized Modules for Membrane Antigens (GMMA) (or mutant-derived or genetically detoxified OMV). [231, 234]

The production and purification of these vehicles is an easy and fast three-step process consisting of fermentation of the GMMA-producing strain coupled with two consecutive tangential flow filtration steps. In this way, GMMA can be produced at a high purification yield using a simple and economical process, thus supporting the development of affordable vaccines, that are particularly attractive for low and middle income countries. [235]

GMMA have been shown to be highly immunogenic in animal models when tested as vaccine candidates against *Shigella*, non-typhoidal *Salmonella* and *N. meningitidis*. [236-240] GMMA are systems capable of reflecting the outer membrane composition, but with the advantage of lacking the ability to cause the associated disease. Therefore, they present to immune system antigens in their natural conformation, facilitating uptake by immune cells and inducing a solid immune response. [241] GMMA have an optimal size for immune stimulation, and they naturally display several Pathogen-Associated Molecular Patterns (PAMPs), small molecular motifs well conserved in bacteria that are potent immunostimulatory molecules. PAMPs are recognized by receptors, called Pattern

Recognition Receptors (PRRs), that are predominantly expressed on innate immune cells such as dendritic cells, macrophages and neutrophils. [242] The interactions between PAMPs and PRRs rapidly elicit host immune responses *via* the activation of complex signaling pathways with the induction of pro-inflammatory responses mediated by various cytokines and chemokines, which subsequently facilitates the eradication of the pathogen. This may be the basis for the GMMA self-adjuvanticity.

An example of PAMPS expressed on the GMMA surface is LPS (or LOS). LPS Lipid A is the endotoxic component of LPS which is implicated in binding to Toll-Like Receptor (TLR) 4, inducing a strong stimulation of the innate immune response. Activation of the innate immune system can lead to high immune responses to an antigen; however, they may also induce unacceptable reactogenicity in human subjects. Therefore, a variety of strategies has been evaluated to avoid potential issues related to GMMA endotoxicity and to find a balance between immune stimulation and reactogenicity [242-244], which is the basis for obtaining an acceptable GMMA-based vaccine.

The main strategy involves the attenuation of LOS pyrogenicity, obtained by genetic manipulation of the GMMA-producing strain. A wide range of lipid A structures have been described and all possibly provide the bacteria with different abilities to activate TLR4 through different structures possessing different levels of agonist/antagonist abilities. The most reactogenic form of lipid A is bis-phosphorylated and hexa-acylated with 12 to 14 carbon acyl chains, whereas penta-acylated lipid A is much less active than the hexa-acylated form in activating human TLR4. [245-247]The number of lipid A acyl chains can be genetically modified to delete the acyltransferases involved in the late biosynthesis and secondary acylation of lipid A. In *Neisseria meningitidis* secondary acylation of lipid A is carried out by *lpxL1* and *lpxL2* [240, 248-253], whereas the genes that encode acyltransferases in Shigella are *htrB* or *msbB* and in Salmonella *msbB* and *pagP*. [233, 254-256] These genes have been mutated to generate GMMA with different penta-acylated lipid A forms with lower reactogenicity. Selecting the appropriate modified lipid A structure is important for obtaining GMMA-based vaccines without problems related to possible reactogenicity.

In conclusion, GMMA represents a flexible platform, as they can act as effective adjuvants and carriers thus having an intrinsic ability to improve the immunogenicity of protein and carbohydrate antigens. These vesicles are highly stable at different temperatures and treatments and they are non-replicative systems, hence, they are safe. The ability to activate the immune system provides these vesicles with a self-adjuvanticity, which is also related to their own nature. Indeed, as observed, they can enhance antibody and T-cell response and are also versatile systems that can be manipulated to reduce their intrinsic endotoxicity or bioengineered to express any desired antigen. Indeed, they can also be exploited as delivery systems for secondary antigens, decorating them with homologous or

heterologous proteins or polysaccharides, either through genetic manipulation or chemical conjugation, also supporting the development of multicomponent vaccines. [257]

I.7.2 Liposomes

Liposomes are spheres of phospholipid bilayers that enclose a central aqueous space, which are formed by self-assembly in water through hydrophobic interactions. They were created by Bangham *et al.* in 1965. [258]

Since the pioneering studies of Gregoriadis and coworkers in the mid-1970s, liposomes gained increasing attention as antigen delivery vehicles, particularly because of their intrinsic adjuvant activity and ease of production [259]. In fact, liposomes have been exploited as biocompatible carriers for vaccines against many viral, bacterial, and fungal infections of animals and humans with several liposomal vaccines that are currently licensed, namely Epaxal (Hepatitis A), Shingrix (Shingles), Mosquirix (Malaria) Inflexal V (Influenza) and m-RNA-1273 and BNT162b2 (COVID-19). [260]

The liposome technology offers many advantages. Indeed, liposomes are biodegradable and safe systems that can be easily produced, and they are capable of reducing antigen toxicity and potential allergic responses. They can also activate both innate and adaptive immune responses and modification to the liposome composition or physicochemical properties, such as size, lamellarity bilayer fluidity, surface charge, can influence interactions with the immune cells.

Liposomes have high loading and encapsulation capabilities, and their surfaces can be modified using different approaches to deliver the desired components. These aspects make possible to modulate these systems in order to transport both hydrophilic and hydrophobic molecules, also improving their stability, if needed.

Liposomes are phospholipid bilayer vesicles and, since phospholipids are amphipathic molecules characterized by a lipophilic tail and a hydrophilic head on the same molecule, these systems have a double nature. In liposome vesicles, the phospholipid polar heads orient themselves toward the aqueous medium and the hydrophobic tails are part of the inner region of the membrane. [261] (**Figure I.16**)

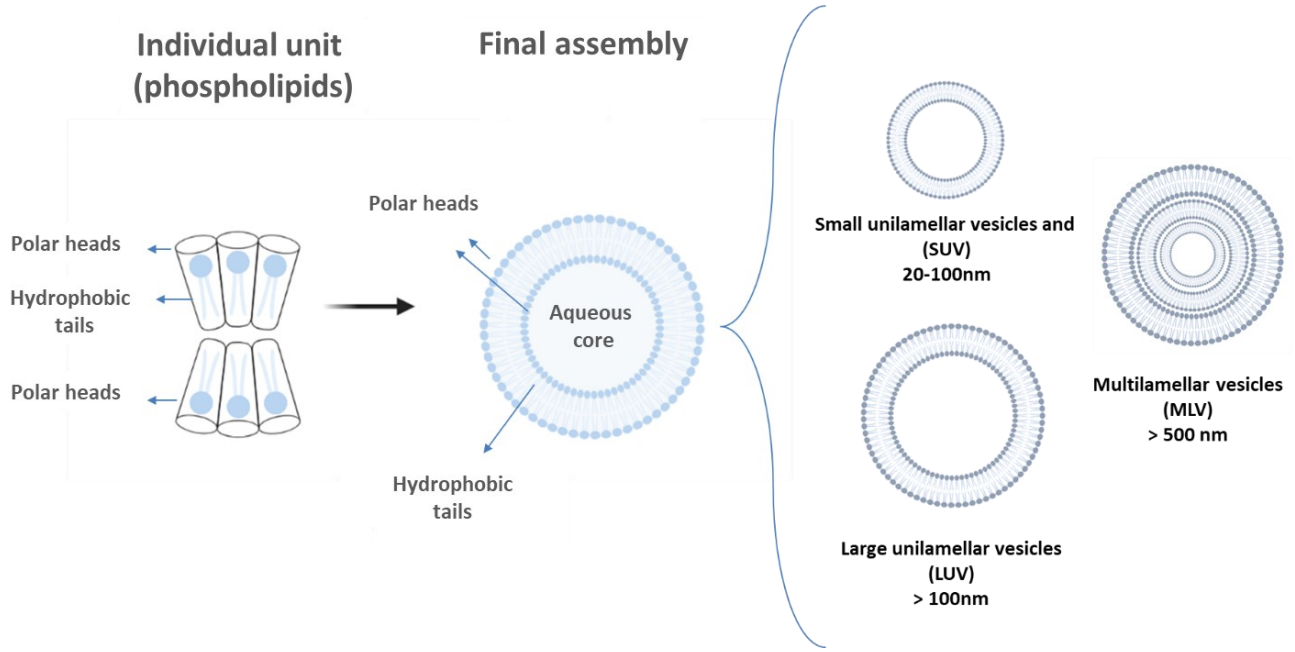


Figure I.16. Liposome composition, assembly and classification into SUV, LUV and MLV (Figure created with Biorender.com).

According to their morphology, liposomes can be classified as multilamellar vesicles (MLV), composed of several concentric bilayers separated by aqueous compartments and particularly useful for sustained antigen release, or unilamellar vesicles (ULV), an aqueous core surrounded by a single bilayer of phospholipids. ULVs are further classified in small unilamellar vehicles (SUVs) with size less than 100 nm and large unilamellar vehicles (LUVs) with sizes up to a micrometer (200nm-1µm). Lipid-based nanocarriers that are not characterized by a contiguous bilayer are classified as lipid nanoparticles (LNPs) which are micelle-like structures, encapsulating molecules in a non-aqueous core. LNPs represent a newly emerging strategy for nucleic acid vaccine delivery since they are more effective than classical lipid-based particles. [262]

The choice of the composition and the production methods can have a significant impact on the final product. Among the most used components for liposome-based vaccines (including also lipid nanoparticles) are neutral or charged phospholipids, which are responsible for the structures of the lipid bilayer, and cholesterol, which enhances membrane stability and rigidity. Negatively or positively charged lipids can be included in the formulation in order to modulate liposome structure and surface properties and to obtain highly stable surface-charged particles, owing to the electrostatic repulsion that prevents aggregation and flocculation phenomena.

Lipid formulations can be produced through various techniques. Among them, some of the conventional preparation methods are the thin-film hydration method, which is the simplest and oldest method, detergent depletion, solvent injection, reverse-phase evaporation, and emulsion methods. [263] Nevertheless, these techniques result in vesicle preparations with low encapsulation

efficiencies, high polydispersity and high variability in size and/or shape. Moreover, they are not scalable to industrial production.

To address these issues, microfluidic systems have recently been introduced as innovative techniques. Generally, microfluidics uses intersecting microchannels for highly controlled mixing of volumes of two or more miscible solvents (commonly an aqueous phase mixed with a water-miscible alcohol). During mixing, the change in polarity promotes nanoprecipitation and formation of lipid-based nanoparticles. As with other manufacturing techniques, these systems can be optimized by modulating some critical process parameters (flow rate, flow ratio and temperature) and material parameters (aqueous buffer selection and composition, solvent). The use of these techniques as part of the production process offers the advantages of robust particle size control and high reproducibility with no scalability problems. [264, 265]

Together with aluminum salts and oil-in-water emulsions, liposomes are a class of adjuvants that are currently included in licensed vaccines; however, an important advantage of these systems over the others is that they can be carriers of both hydrophilic and hydrophobic molecules such as antigens and immunopotentiators.

Liposome surfaces can be modified with appropriate ligands to alter their immunological profile. Their vaccine adjuvant activity is based on the ability to attract, interact and activate APCs (e.g., dendritic cells (DCs), macrophages, and B cells) based on their physicochemical (size and charge) and immunogenic properties (incorporation of other adjuvants and targeting ligands). [263] Indeed, liposomal formulations can be generated containing a wide variety of PAMPS to stimulate innate immune responses via specific PRRs and to preferentially interact with APCs, induce specific innate and adaptive immune responses and also reduce the possibility of simple clearance, without stimulation, by these cells.

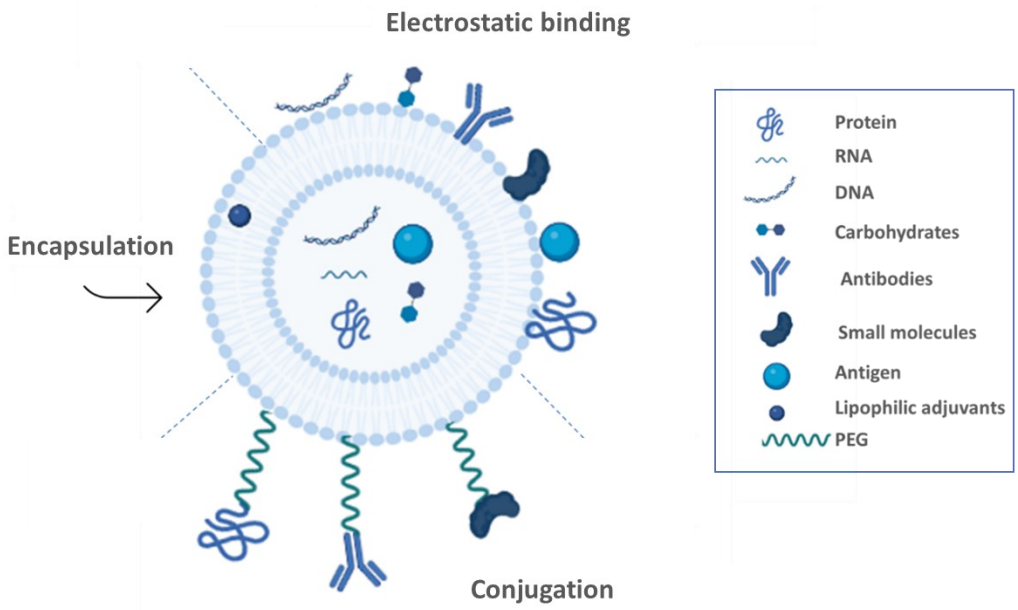


Figure I.17. Different methods for the incorporation of antigens, adjuvants, and targeting ligands into/onto liposomes: encapsulation into the aqueous core for hydrophilic molecules or incorporation into the lipid bilayer for hydrophobic one; electrostatic binding to the oppositely charged lipids on liposomes surface; conjugation to functionalized lipid membrane components (Figure created with Biorender.com, adapted from Chatzikleanthous *et al.,* 2021). [263]

The liposomal platform can also be exploited as carrier for antigens. Several combinations of surface functionalization and/or modification methods have been applied to prepare multifunctional liposomal formulations including simple co-administration, encapsulation, surface decoration through electrostatic complexation or covalent conjugation (**Figure I.17**). Moreover, the co-encapsulation of antigens and adjuvants into liposomes can also be applied in order to increase immunogenicity.

The use of SUV or LUVs liposome exposing recombinant OMP as vaccine carriers has been reported during the last decades, also for delivering *Neisseria* antigens. In this case, it is important to consider that liposome-delivered antigens need to mirror the native conformations of the proteins within the bacterial OM, and this can be obtained by refolding/intercalating them within the phospholipid bilayer of the liposome.

Furthermore, in this context, liposomes can also be used to incorporate antigens and study the binding and affinity/avidity of protective antibodies, in order to improve the conformational refolding of protein antigens and increase immunogenicity or to study the effect of a single antigen on the response. Indeed, liposomal vaccines have the advantage of displaying only the selected antigen, avoiding contamination of other possible problematic components and, moreover, decreasing the

toxicity and preventing the development of allergic reactions related to some components, such as LOS/LPS and lipid A of Gram-negative bacteria. [266]

In a panorama where the COVID-19 pandemic has demonstrated the urgency for vaccine technologies that are flexible and facilitate rapid development, production and upscaling, liposomal nanoparticles represent an innovative and promising vaccine platform.

Liposomes permit improved antigen delivery, ease and speed of production. This, together with the application of structural biology and immunological knowledge can improve our understanding of the induction of immune responses by vaccines, thereby aiding in enhanced antigen design and the discovery of better adjuvants to improve immunogenicity. Moreover, on the other side, liposomes represent enticing carrier for development of improved subunit-based vaccines.

CHAPTER 1

1.1 Aim of the study

Gonococcal surface molecules that may be appropriate vaccine targets are often antigenically variable through phase variation phenomena, making the bacterial surface a moving target, thus complicating vaccine development. [267] The ability to modify membrane determinants is advantageous for gonococci and results in evasion strategies to increase fitness and facilitate the adaptation of organisms to their environment. Furthermore, it has been demonstrated that adaptive immune responses that target highly conserved gonococcal antigens are not able to provide a satisfying protection. [268]

Among the surface components widely studied as potential vaccine antigens, LOS accounts for the majority of bacterial outer membrane constituents; therefore, it is an easily accessible target for adaptive immunity.

Results from earlier studies have provided important information on the role of anti-LOS antibodies in the engagement of complement, resulting in bactericidal [269, 270] and opsonophagocytic activities. [160] Likewise, LOS antibodies may promote protection against reinfection with the homologous strain in experimental infection studies in human male volunteers. [271]

Although extensive research has been carried out on the role of this antigen during the last few decades, no studies have adequately investigated the impact of LOS on gonococcal protection when used as a vaccine antigen in an outer membrane vesicle carrier, such as in the GMMA system. In this case, it is worth considering that LOS is co-presented with other OMPs that could influence the anti-LOS response. Moreover, there has been no reliable and clear evidence on the role of LOS in the GMMA-mediated immune response and to assign this role to a specific oligosaccharide structure.

Consequently, the key research question of this study was to establish whether the LOS-derived oligosaccharide moiety has an impact on the gonococcal GMMA immune response in terms of complement-mediated bactericidal killing and subsequently to determine the role of the different oligosaccharide moieties on the bactericidal response.

First, the effect of the oligosaccharide structure within LOS on the gonococcal GMMA immune response was investigated. To this end, mutants exposing a shorter oligosaccharide chain deprived of the principal saccharide epitopes have been instrumental in assessing the direct influence of gonococcal LOS on the bactericidal response.

The second main aim of this project was to verify the potential impact of the variable oligosaccharide portion of gonococcal lipooligosaccharide on the immune response induced in mice.

For this purpose, we leveraged on a panel of gonococcal mutants genetically engineered to express a distinct and invariant LOS structure, that was properly detoxified in order to produce GMMA. This approach made it possible to establish how different oligosaccharide lengths influence the immune response.

Taken together, these studies provide deeper insights into gonococcal LOS-derived oligosaccharides as immune targets and support the design of new vaccines against this antibiotic resistant human pathogen.

1.2 Materials and Methods

1.2.1 Bacterial strains

1.3.1.1 *Neisseria gonorrhoeae* strains for GMMA-producing strains and functional assays

Table 1.1 lists some of the features of the *N. gonorrhoeae* strain panel used for the functional assays and for the genetic engineering to generate GMMA producing strains (FA1090 and MS11). *Ad hoc* studies conducted internally allowed to select the strains used for the functional assays that are representative of the general population of *Neisseria gonorrhoeae* (internal unpublished data). The LOS glycoform was not considered as a parameter for the selection of relevant gonococcal strains, indeed, the rationale was based on the analysis of OMPs components with the aim of selecting strains with a wide genetic variability. A particular attention was paid to PorB, the most abundant protein in the gonococcal outer membrane that can be present in two variants named PorB.1A and PorB.1B.

Table 1.1. Characteristics of *N. gonorrhoeae* strains. Pili presence was assessed by Transmission Electron Microscopy (TEM). Genome sequences were retrieved from public databases (PubMLST or EMBL-EBI) or produced internally (INT).

Strain	PorB variant	Serum resistance	Pili presence	Country	Genome source
FA1090	PorB.1B	Yes	No	USA	PubMLST id #2855
F62	PorB.1B	No	Yes	N/A	PubMLST id #21070
MS11	PorB.1B	No	Yes	USA	PubMLST id #27228
BG27	PorB.1B	Yes	No	UK	INT
WHO-M	PorB.1B	No	Yes	Australia	EMBL-EBI (ENA) #LT591904
BG8	PorB.1B	Yes	No	UK	INT
SK92	PorB.1A	Yes	No	USA	PubMLST id #21071
WHO-F	PorB.1A	Yes	No	Canada	EMBL-EBI (ENA) #LT591897
WHO-G	PorB.1A	Yes	No	Thailand	EMBL-EBI (ENA) #LT91898
WHO-N	PorB.1A	Yes	Yes	Australia	EMBL-EBI (ENA) #LT591910

Strains were routinely cultured at 37°C in an atmosphere of 5% CO_2 on Gonococcus (GC) agar medium (Difco) plates enriched with 1% v/v of Isovitalex, a chemically defined supplement usually added to cultures of nutritionally fastidious microorganisms.

For liquid cultures, bacteria grown for at least 18h on the plates were diluted to an OD_{600nm} of 0.3-0.4 in liquid GC - 1% v/v Isovitalex, opportunely added of supplement if necessary, and incubated at 37°C at 160 rpm.

When required, to induce pilus expression and enable transformation, *Neisseria gonorrhoeae* strains were cultured on GC agar plates supplemented with 0.25 mM (IPTG). Chloramphenicol and Kanamycin were added to achieve a final concentration of 10 μg/ml for the selection of transformed clones.

1.3.1.2 *Neisseria gonorrhoeae* MS11 LOS *lgt* mutant strains

Neisseria gonorrhoeae MS11 LOS *lgt* mutant strains used in this work are described in **Table 1.2**. These strains were obtained from Prof. Sanjay Ram (Division of Infectious Diseases and Immunology, University of Massachusetts Medical School).

Table 1.2. *Neisseria gonorrhoeae* strains used in this study (adapted from Chakraborti *et al*., 2016). [161] These strains were all derived from *Neisseria gonorrhoeae* MS11 v.4/3/1 strain.

***N. gonorrhoeae* strain**	**LOS phenotype**	**Description**
2HexG-	Gal-Glc-HepI Unsubstituted HepII	*lgtA*-del *lgtC*-off *lgtG*::*kan*
2HexG+	Gal-Glc-**HepI** Gal-Glc-**HepII**	*lgtA*-del *lgtC*-off *lgtG*-on
3HexG-	Gal-Gal-Glc-**HepI** Unsubstituted **HepII**	*lgtA*-del *lgtC*-on *lgtG*::*kan*
3HexG+	Gal-Gal-Glc-**HepI** Gal-Glc-**HepII**	*lgtA*-del *lgtC*-on *lgtG*-on
4HexG-	Gal-GlcNAc-Gal-Glc-**HepI** Unsubstituted **HepII**	*lgtA*-on *lgtD*-del *lgtC*-off *lgtG*::*kan*
4HexG+	Gal-GlcNAc-Gal-Glc-**HepI** Gal-Glc-**HepII**	*lgtA*-on *lgtD*-del *lgtC*-off *lgtG*-on
5HexG-	GalNAc-Gal-GlcNAc-Gal-Glc-**HepI** Unsubstituted **HepII**	*lgtA*-on *lgtD*-on *lgtC*-off *lgtG*::*kan*
5HexG+	GalNAc-Gal-GlcNAc-Gal-Glc-**HepI** Gal-Glc-**HepII**	*lgtA*-on *lgtD*-on *lgtC*-off *lgtG*-on

The MS11 LOS *lgt* mutant strains were created in the background of *N. gonorrhoeae* MS11 4/3/1, a variant of MS11 VD300 with an isopropyl-D-thiogalactopyranoside (IPTG)–inducible *pilE* that controls pilus expression. [272] In these mutant strains the expression of the four phase-variable *lgt* genes (*lgtG, lgtA, lgtC* and *lgtD*, highlighted in **Figure 1.1**) was genetically fixed either on or off (or deleted) as described in Chakraborti *et al*., 2016. [161]

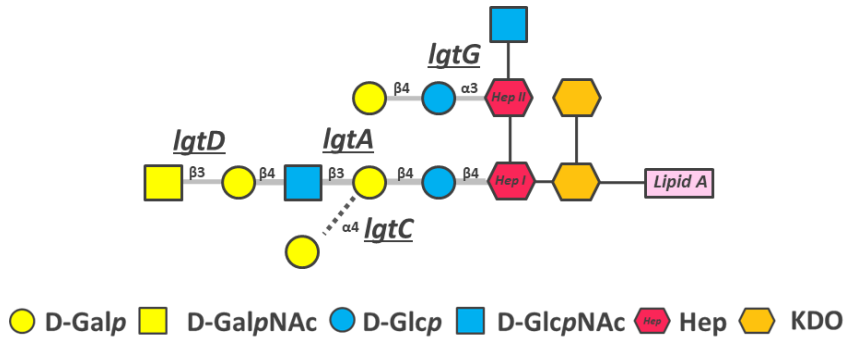


Figure 1.1. Schematic representation of gonococcal LOS. The four phase-variable *lgt* genes involved in glycan extensions are underlined.

1.3.1.3 Generation of *Neisseria gonorrhoeae* mutant strains

Generation of *lpxLI* knockout in *N. gonorrhoeae* FA1090 strain

DNA manipulations were carried out routinely as described for standard laboratory methods. [273] All DNA primers used in this study are reported in **Table 1.3**. The plasmid pBS-Δ*lpxL1 kanR*, which contains the kanamycin resistance gene and the upstream and downstream regions for the homologous recombination, was used as a template for the amplification of the DNA needed for the transformation. The PCR was performed using the primers Lpx UP Fwd and LpxL1 DO Rev and the KAPA Hifi 2X master mix (Roche), with reaction conditions as follow: 94°C for 5 min, 40 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 3 min, with a final step at 72°C for 5 min. DNA purifications were performed using the QIAquick PCR purification kit (QIAGEN) following the manufacturer's protocol.

Table 1.3. List of primers used for the generation of *lpxLI* knockout in *Ng* FA1090.

Name	Sequence 5'-3'	Use
Lpx UP Fwd	GGCATTTGTATTTTGCCGTCTG	Amplification of plasmid pBS-Δ*lpxL1 kanR*
LpxL1 DO Rev	CGCCATTTTCTACGCTTTGCCAAG	
NGO_lpxL1_wtcheck-Fw	CCGCGTTCGAGATGG	Check of wild-type locus
NGO_lpxL1_wtcheck-Rev	GCGGAACTGTTTGACGAG	
LpxL1 est FW	CCGCCAAACTCAATCCTTCG	Mutant screening
LpxL1 est REV	GCAAACTTTTGTTTCACCGTTTCCG	

The PCR product was used for the transformation of the FA1090 strains. Transformations were carried out by spotting a mixture of 30 µl of bacterial resuspension in PBS 1x and 30 µl of DNA onto a GC agar plate and by incubating it for 5-6 hours. Transformants were selected into GC agar plates with kanamycin 40 µg/mL. All transformants were tested by PCR analysis using Accuprime^{TM} Taq Polymerase (Thermo Scientific) and with external primers (primer couples LpxL1 est FW/LpxL1 est REV) to check the correct event of double recombination. Positive clones were streaked repeatedly onto selective agar plates; glycerol stock and DNA lysates were collected at each passage and tested for the presence of remaining wild-type population. PCR screenings were performed using Accuprime^{TM} Taq Polymerase (Thermo Scientific) and with internal primers, specific for the wild-type DNA (NGO_lpxL1_wtcheck-Fw/NGO_lpxL1_wtcheck-Rev).

Generation of *lgtF* knockout in *N. gonorrhoeae* FA1090 Δ*lpxL1* strain

DNA manipulations were carried out routinely as described for standard laboratory methods. The *lgtF* knockout was obtained by genomic recombination where the coding sequence of this enzyme was replaced with the chloramphenicol antibiotic resistance cassette (*lgtF::cmR*) with a double crossing over. The amplification of 515 bp upstream and 548 bp downstream regions of the *lgtF* gene was performed with the primer couples lgtF-UP-fw/rv and lgtF-DO-fw/rv respectively using as template 50 ng of genomic DNA purified from the FA1090 wild type strain and KapaHiFi DNA polymerase (Roche); the amplification of the chloramphenicol resistance gene (*cmR*) was done with the primer couple cloKOF/R using 10 ng of a synthetic DNA template (Geneart). Primer sequences are listed in **Table 1.4**. Primer tails indicated in italics were added to allow Polymerase Incomplete Primer Extension (PIPE) cloning [274] of PCR products into a modified pET15 vector, previously amplified with primer couple PIPEfw/pET15KOrv. According to PIPE method, PCR products were

transformed into *E. coli* MACH-1 competent cells (Thermo Scientific) immediately following amplification. Screening of transformants was done by colony PCR using T7prom/pETseqRv primers. PCR product from positive clone was purified using the QIAquick PCR purification kit (QIAGEN) following the manufacturer's protocol and used for Gonococcus transformation.

Table 1.4. List of primers used for the generation of *lgtF* knockout in *N. gonorrhoeae* FA1090 Δ*lpxL1* strain.

Name	Sequence 5'-3'	Use
lgtF-UP-fw	*ATAGGGGAATTGTGCTCGAG*ACGCACCACAACGACAGT ATGGAAAG	Amplification of *lgtF* upstream region
lgtF-UP-rv	*TCCTTCAGACGGCATTCCCGGG*GGTTTCTCAAAGCATTT TGGTTTC	
lgtF-DO-fw	*GGATCCCCATGGATACCCGGG*CAAACTATATTATCTGT ACAAATCC	Amplification of *lgtF* downstream region
lgtF-DO-rv	*AATTAAGTCGCGTTATCTAGA*GTATCGATACCGTTATGA ACAATCC	
cloKOF	*ATGCCGTCTGAAGGATCC*GTCAACCGTGATATAGATTG AAAAGTG	Amplification of *cmR* cassette
cloKOR	*TATCCATGGGGATCC*GATCCACGCGTCTTAAGGCGG	
PIPE-Fw	TAACGCGACTTAATTGGCCAGTGTGCCGGTCTCCG	Amplification of cloning vector
pET15KOrv	TCACAATTCCCCTATAGTGAGTC	
T7Prom	TAATACGACTCACTATAGGG	Screening for cloning
pETseqRv	GATATCCGGATATAGTTCCTC	
lgtF-ext-F	AAGACATCGGCCGGATTAATC	Mutant screening
lgtF-ext-R	GACGGTAAAAAAACGGCTGTCGG	
lgtF-wt-F	CTTCCATCTCGGCCGAGAAATACC	Check of wild type locus

N. gonorrhoeae FA1090 Δ*lpxL1* strain was previously generated as described above. Gonococcus transformation was carried out as previously described [275] and transformants were selected into GC agar plates + 1% Isovitalex with chloramphenicol 2 μg/ml. Transformants were tested by PCR analysis using Accuprime™ Taq Polymerase (Thermo Scientific) and with external lgtF-ext-F/R to check the correct event of double recombination. The expected band for the mutant strains with *cmR* (chloramphenicol resistance cassette) is about 1960bp while the wild-type locus is 1860bp long.

Positive clones were streaked repeatedly onto GC agar plates + 1% Isovitalex with chloramphenicol 3 µg/mL; glycerol stock and DNA lysates were collected at each passage and tested for the presence of remaining wild-type population. PCR screenings were performed using Accuprime™ Taq Polymerase (Thermo Scientific) with the internal primer lgtF-wt-F, specific for the wild-type DNA in combination with the external primer lgtF-ext-R. Clones presenting the *lgtF* deletion yielded no PCR products, while those expressing the wild-type gene gave a 767 bp long PCR product.

Generation of *lpxL1* knockout in *Neisseria gonorrhoeae* MS11 v.4/3/1 *lgt* mutant strains

DNA manipulations were carried out routinely as described for standard laboratory methods. [273] All DNA primers used in this study are reported in **Table 1.5**.

Table 1.5. List of primers used for the generation of *lpxL1* knockout mutants in *N. gonorrhoeae* MS11 v.4/3/1 LOS mutant strains.

Name	**Sequence 5'-3'**	**Use**
Lpx UP Fwd	GGCATTTGTATTTTGCCGTCTG	Amplification of ΔlpxL1-cmR-PheS
LpxL1 DO Rev	CGCCATTTTCTACGCTTTGCCAAG	
NGO_lpxL1_wtcheck-Fw	CCGCGTTCGAGATGG	Check wild type locus
NGO_lpxL1_wtcheck-Rev	GCGGAACTGTTTGACGAG	
LpxL1 est FW	CCGCCAAACTCAATCCTTCG	Mutant screening
LpxL1 est REV	GCAAACTTTTGTTTCACCGTTTCCG	

In order to reduce LOS endotoxin activity, the *lpxL1* gene (NGO0154) was genetically deleted in each *lgt* mutant strain. The plasmidic DNA containing the synthetic construct ΔLpxL1-cmR-PheS (construct3PHS) was received from Geneart. The linearized cassette ΔlpxL1-cmR-PheS, which contains the cloramphenicol resistance gene and the upstream and downstream regions for the homologous recombination, was used as a template for the amplification of the DNA needed for the transformation. The PCR was performed using the primers Lpx UP Fwd and LpxL1 DO Rev and the KAPA HIFI 2X master mix (Roche), with reaction conditions as follow: 95°C for 5 min, 30 cycles of 98°C for 30 s, 60°C for 30 s and 72°C for 3 min 30 s, with a final step at 72°C for 7 min. DNA purifications were performed using the KIT wizard SV Gel and PCR clean up system (Promega) following the manufacturer's protocol.

This PCR product, the linearized plasmidic DNA ΔLpxL1-cmR-PheS, was used for the transformation of all the previously generated MS11 LOS *lgt* mutant strains.

The *lpxL1* knockout was obtained by genomic recombination where the coding sequence of this enzyme was replaced with the chloramphenicol antibiotic resistance cassette (*lpxL1::cmR*) with a double crossing over. Transformations were carried out by spotting a mixture of bacterial resuspension in PBS 1x and DNA onto a GC agar plate and by incubating it for 5-6 hours. Transformants were selected into GC agar plates with cloramphenicol 10 µg/ml to select the Δ*lpxL1* mutants. All transformants were tested by PCR analysis using Accuprime^{TM} Taq Polymerase (Thermo Scientific) and with external primers (primer couples LpxL1 est FW/LpxL1 est REV) to check the correct event of double recombination. The expected band for the mutant strain with *cmR* (chloramphenicol resistance) is about 2986bp while the wild-type locus is about 1703bp long. Positive clones were streaked repeatedly onto selective agar plates; glycerol stock and DNA lysates were collected at each passage and tested for the presence of remaining wild-type population. PCR screenings were performed using Accuprime^{TM} Taq Polymerase (ThermoFisher) and with the external primer LpxL1 est FW in combination with the internal primer NGO_lpxL1_wtcheck-Rv, specific for the wild-type DNA. The desired Clones tested by PCR using the primer couples didn't yield any PCR product confirming the successful *lpxL1* deletion, while those expressing the wild-type *lpxL1* gene gave a PCR product of about 625 bp.

1.2.2 GMMA Production and Purification

Bacterial fermentation

For each batch, the production of biomass started from a frozen working bacterial stock by growth in plate for at least 12-16 h. After growth, this was sub-cultured to a pre-culture, that was subsequently used to inoculate the production medium into a shake flask in liquid GC - 1% v/v Isovitalex supplemented with 5 g/L Na-Lactate, 2.5 g/L Na-Glutammate, 0.5 g/L Serine, 0.3 g/L Cysteine. The culture was incubated at 37°C 180 rpm, starting from an optical density measured at 600 nm (OD_{600nm}) of 0.3-0.4. The bacterial culture was grown until it reached an OD_{600nm} equal to 1.5 ± 0.5, usually in 9 ± 2 hours.

OMV isolation and purification

The collected growth was centrifuged at 12000 xg for 30 min at 4°C in order to remove the bacteria and large debris from the solution. The supernatant, containing the vesicles released into the fermentation broth, was then carefully recovered and filtered with a 0.2µm PES filter to sterilize and further remove large debris. Then, 400U/L Benzonase (Merck) (2h, 37°C) was added to the crude

GMMA to digest DNA following the manufacturer's instructions and subsequently the supernatant was filtered again with a 0.2µm PES filter. Next, the solution was concentrated and diafiltrated using tangential flow filtration (TFF). The suspension was concentrated using a 200 cm2 300kDa cut-off PESU membrane (SARTOCON SLICE 200 Sartorius stedim polyethersulfone 300kDa), followed by 25 volume diafiltration with PBS 1x buffer to wash out the original buffer salts (or other low molecular weight species) in the retentate. The retentate was then concentrated again and then washed with 40 volumes of PBS 1x. When purity levels were below 80% (established by SE-HPLC), the sample was further purified by centrifugation at 150000 xg (Optima L90K, rotor SW31Ti S/N 15U4385, tubes Ultraclear 38.5mL P/N 344058) for 2h and solubilization in sterile PBS 1x buffer for 24h at 4°C in a laboratory tilting shaker. Lastly, the purified OMV were filtered using a 0.2 µm cut-off filter to obtain sterile samples.

1.2.3 GMMA characterization and LOS quantification

For all the analysis, the degree of purity of the water is "Milli-Q® Type 1 Ultrapure Water". It will be referred to as MilliQ® water or simply water.

Protein quantification by Lowry assay

Total protein quantification and protein concentration was performed with Pierce Modified Lowry Protein Assay from Thermo Scientific, diluting each sample in duplicate and preparing a 5-point calibration curve using a BSA starting solution of 100 µg/mL. Then, the two solutions of the Lowry kit are prepared and added to the sample, following the manufacturer's instructions. The samples are finally transferred to a plastic cuvette and are read at the spectrophotometer at 750 nm. The final protein content on the GMMA samples is quantified based on the BSA calibration curve.

GMMA purity and integrity by SE-HPLC

Size-Exclusion Chromatography (SEC) separates molecules on the basis of their hydrodynamic size. A column is filled with porous beads (polymeric gel or silica beads) that admit small molecules to enter their pores but not the large ones. When a mixture of molecules in solution is applied to the top of the column, the smaller molecules are distributed through a larger volume of solvent than is available to the large molecules. The large molecules move then more rapidly through the column, and then small and large molecules can be separated (fractionated). SE-HPLC was used to determine GMMA's purity using fluorescence (FLR) to monitor soluble protein contaminants and 260nm and 280nm wavelength for DNA content determination. 100 µL of GMMA samples were injected onto a TSKgel G6000PW column (Tosoh) equilibrated with PBS 1x (GMMA's storage buffer). An isocratic

gradient (1,5 CV; PBS 1x) was applied at 0,5 ml/min. Wavelengths of 260 nm, 280 nm, and FLR (ex277nm/em335nm) were selected to monitor the GMMA elution profile. For each peak, the peak start and end times is identified and the peak baseline is define as well. Then, peak areas, peak heights and peak retention times are calculated to have the final value of GMMA purity (express in %). Soluble proteins were estimated from the integration of FLR peaks since at this wavelength has been demonstrated that DNA is not visible.

Dynamic Light Scattering (DLS)

DLS provides information on size and size distribution of vesicles up to 1000 nm in radius. The Brownian motion of particles or molecules in suspension causes laser light to be scattered at different intensities. Analysis of these intensity fluctuations by a fast photon counter yields the velocity of the Brownian motion and hence hydrodynamic radius (*Rh*) using the Stokes-Einstein relationship:

$$D = \frac{kT}{6\pi\eta R}$$

The fluctuations are directly related to the rate of diffusion of the molecule through the solvent, which is related in turn to the particle's *Rh*. Larger size particles have higher influence in terms of light intensity. Samples were vortexed to provide a homogeneous solution and then 0,1 mL or 0,5 mL were transferred to a cuvette. The Zetasizer software (Malvern Panalytical) collects and interprets measurement data to assign the mean particle radius, calculated by the software from the particle distributions measured, and the polydispersity index (PDI) of the size ranges present in the solution.

1.2.4 LOS extraction, physicochemical and immunochemical characterization

LOS extraction

LOS cwas extracted using a general method based on the Westphal hot phenol extraction process to purify whole LOS from *N. gonorrhoeae* bacterial pellet or directly from GMMA/OMV samples. Bacterial pellets were obtained after centrifugation of the *Ng* strain grown in liquid culture to exponential phase. The pellet was suspended in a buffer (6mM tris-base 10mM EDTA 2% SDS, pH 6.8) containing 50µg/ml of proteinase K and stirred at 65°C for 2 hours and it was then placed overnight at 37°C. A solution of sodium acetate (1/10 of the sample volume) was added together with 3 volumes of cold ethanol. After centrifugation at 12000xg for 10min at 4°C, the supernatant was removed, and the pellet suspended in sodium acetate buffer. Cold ethanol precipitation was repeated 3 times, and, at the end, the pellet was suspended in a buffer containing 10mM Tris base, 100mM

NaCl, 5 mM $CaCl_2$, pH 7.3, $MgCl_2$ 10mM and 100µL of benzonase (Merk, 250U/µL). The sample was stirred overnight at 37°C and 50rpm. LOS was extracted with a hot phenol/water procedure from bacterial pellets treated as described above or from OMV/GMMA particles. In detail, the bacterial suspension or GMMA were stirred at 65°C until the temperature equilibrated. An equal volume of 90% (w/v) phenol which had been preheated to 65°C was added and thoroughly mixed for 30 minutes. The resulting mixture was rapidly cooled by stirring for 30 minutes in an ice-water bath. The phenol mixture was then centrifuged at 4°C at 4000xg for 10 minutes. A sharp interface occurred between the aqueous, phenol, and interface layers. The aqueous and phenol layers were removed by aspiration. The aqueous layer containing the lipopolysaccharide was retained while the phenol layer was temporarily discarded, to be treated again to remove the remaining LOS. Cold ethanol precipitation was performed 3-4 times on the aqueous phase and the final pellet was suspended in distilled water and ultracentrifuged at 175000xg for 3 hours. After ultracentrifugation, the pellet containing extracted LOS was suspended in distilled water.

Silver stained SDS-PAGE

The samples of LOS or GMMA were titrated in previous silver staining experiments to check the optimal quantity of samples that can permit a clear LOS profiling with well separated LOS bands that correspond to different LOS structure populations (data not shown). Loading of GMMA/OMV or LOS on each well of SDS-PAGE gel was normalized based on LOS quantity obtained from semicarbazide-HPLC method (described below): 0.12 $nmol_{LOS}$/well. The SDS-PAGE gel is a 16% Tris-glycine gel (Invitrogen) and samples are run with Tris-glycine 1x buffer (Invitrogen). The marker is Ultra-Low range and consists of the following protein markers: Bradykinin (1060 Da), Insulin Chain B (3496 Da), Aprotinin (6500 Da), α-Lactalbumin (14200 Da), Myoglobin (17000 Da) and Triosephosphate Isomerase (26600 Da). After running, gels are fixed with fixation solution (40% ethanol, 5% acetic acid, 55% water) for 5 minutes after 30 seconds in microwave oven (700W). Gels were then oxidized with 0.07% $NaIO_4$ in the same fixation solution left for 5 minutes in the darkness. Gels were washed with a solution of 30% ethanol for 5 minutes after 30 seconds in microwave oven then are stained using SilverQuest™ Staining kit (Thermo Fisher), according to manufacturers' recommendations.

LOS quantification by SCA-SE-HPLC assay

The content analysis of the LOS present in the GMMA samples is performed by the quantification of the reactive carbonyl groups of the saccharide moiety, generated after acid hydrolysis to remove the Lipid A and derivatized with semicarbazide (SCA), by SE-HPLC analysis. The SCA reaction coupled with SE-HPLC analysis has been already reported in literature [276, 277]. In the first step, the

purified GMMA samples were treated with Acetic Acid, 1% final concentration, and hydrolyzed for 2 hours at 100°C to remove the Lipid A. After the hydrolysis, the sample is centrifuged for 10 minutes at 14000xg to separate pellet (Lipid A) and supernatant (OS). The supernatant was dried in a SpeedVac system to remove the Acetic Acid, and then dissolved with water. To obtain UV detectable samples, the supernatants are derivatized with semicarbazide. A stock SCA solution was prepared dissolving 100 ± 2 mg of SCA Hydrochloride and 90.5 ± 2 mg of Sodium Acetate in 10 mL of water. Equal volumes of sample and SCA solution were transferred into clean vials (e.g., 100µL sample + 100µL SCA solution) and incubated in a pre-heated water bath at 50°C for 50 minutes. Samples were chilled at 2-8°C for 15 minutes and then filtered into HPLC vials. The KDO content of the GMMA samples is quantified based on a calibration curve prepared starting with standard KDO ammonium salt solution. The LOS content is expressed in nmol/mL of KDO, which matches the nmol/mL of OS, and it is lastly reported as $nmol_{LOS}$/mg protein, to normalize on the protein content.

SE-HPLC settings:

- TSKgel PW-XL guard with TSKgel G3000 PW-XL columns (Tosoh) connected in series.
- Column compartment temperature at 30°C
- Autosampler temperature at 10°C
- UV detection at 252 nm
- Isocratic elution in 100 mM sodium phosphate buffer pH 7.0, 100 mM sodium sulfate, 5% acetonitrile
- Flow rate at 0.5 mL/min
- Total run time 40 min
- Injection volume of 80 µL

Anti-LOS Antibodies

Anti-LOS mAb 17-1-L1 (henceforth referred to as mAb L1) [278], 4C4 [279], L3,7,9 [278] and 2C7 [160] have been described previously. The tissue culture supernatant containing anti-LOS mAbs L1 was obtained by Professor Sanjay Ram (Division of Infectious Diseases and Immunology, University of Massachusetts Medical School). Mouse mAb 2C7 has been produced as recombinant mAb internally. Purified mAb 4C4 and supernatant of mAb L3,7,9 are commercially available (Novusbio).

Western blotting

The samples of LOS or GMMA were titrated in previous WB experiments to check the optimal quantity of samples that can permit a clear LOS profiling with well separated LOS bands that

correspond to different LOS structure populations (data not shown). Loading of GMMA/OMV or LOS on each well of SDS-PAGE gel was normalized based on LOS quantity obtained from semicarbazide derivatization/SE-HPLC method: 0.008 $nmol_{LOS}$/well. Sample were run on a 16% Tris-glycine SDS-PAGE gel using a Tris-glycine 1x buffer. The marker was Ultra-Low range and consists of the following protein markers: Bradykinin (1060 Da), Insulin Chain B (3496 Da), Aprotinin (6500 Da), α-Lactalbumin (14200 Da), Myoglobin (17000 Da) and Triosephosphate Isomerase (26600 Da). LOS was transferred to nitrocellulose membranes (The iBlot™ Kit Thermofisher) and membranes were blocked with with PBS 1x + BSA 3% + Tween20 0.05% for 1h at RT. Anti-LOS mAbs (diluted 1:1000 in PBS 1x + Tween20 0.05%) were incubated with membranes for 1h at RT. mAb-reactive LOS bands were visualized with anti-mouse IgG alkaline phosphatase (diluted 1:2000 in PBS 1x + Tween20 0.05%) incubated for 30 minutes at RT, followed by AP Conjugate Substrate kit (Biorad) for 5 minutes at RT.

1.2.5 In *vivo* studies and functional assays

Immunization study

Female seven weeks old CD1 outbred mice (10/group) were immunized intraperitoneally two times on days 1 and day 29 with different GMMA at a 10μg protein-based dose in 200μL adsorbed to Alum hydroxide (3 mg/mL). Mice sera collected two weeks after the second dose (day 43) were analyzed in pools in hSBA assay and/or competitive hSBA as described below.

In the second *in vivo* immunization study, female seven weeks old CD1 outbred mice (10/group) were immunized intraperitoneally 2 times on days 1 and day 29. Differently from the other study, GMMA were normalized based on LOS content at a dose of 1,5 $nmol_{LOS}$ in 200μL, adsorbed to Alum hydroxide (3 mg/mL). Mice sera were collected two weeks after the second dose (day 43) and analyzed in pools in hSBA assay and/or competitive hSBA as described below.

Functional antibodies measured by Serum Bactericidal Assay

Functional antibodies were measured by human Serum Bactericidal Activity assay (hSBA) on the strains FA1090, F62, SK92, MS11, BG27, WHO-M, BG8, WHO-F, WHO-G and WHO-N using normal human serum as complement source. The hSBA was performed on sera from mice immunized with different GMMA preparations and collected 2 weeks after the second immunization.
Bacteria were plated on a round GC+1% Isovitalex agar plate and incubated 16 (±2) hours at 37°C with 5% CO_2 in humid atmosphere. The day after, single colonies were inoculated in GC + 1% Isovitalex medium (CMP-NANA was added to the broth medium for serum sensitive strains: 0.5 ug/mL for F62 strain, 0.2 ug/mL for WHO-M and MS11 strains) and incubated at 37°C at 180rpm

until the culture reached OD_{600nm}=0.4-0.5. After that, bacteria were diluted 1:10000 in SBA buffer (DPBS, 1% BSA, 0.1% Glucose) except for BG27 bacteria that were diluted 1:2500.
Mouse sera, previously heat inactivated at 56°C for 30 minutes, were serially diluted (ten 2-fold dilution steps) in SBA buffer. The assay was assembled in a sterile 96 flat bottom well microplate in a final volume of 32 µl/well. The serial dilutions of each test sample were let to react with bacteria and human complement. The volumes and concentrations of each reaction component were added in the order reported in **Table 1.6.**

Table 1.6. Reaction mixture volumes for each component used in hSBA.

Reaction mixture Volumes (µL/well)	**Serum resistant strains** (FA1090, BG27, BG8, SK92, WHO-F, WHO-G, WHO-N) **µL/well**	**Serum sensitive strains** (F62, MS11, WHO-M) **µL/well**
Test sample	16 µL	16 µL
Bacteria	11 µL	9.5 µL
Human complement	5 µL (final concentration 16%/well)	6.5 µL (pre-diluted 1:2 in SBA buffer, final concentration 10%/well)

Each plate also includes the following controls:
- Heat inactivated complement (HIC) controls: all serum samples tested in the presence of bacteria and HIC. This control allows to exclude serum toxicity.
- Without (w/o) serum control (8 wells): bacteria with active human complement (AC) in absence of serum sample. This control is used to exclude complement toxicity and to determine 100% bacterial growth.
- A hyper-immune serum: a mouse pooled serum included as positive control serum.
The reaction mixture was incubated at 37°C for 60 minutes at 160 rpm. After 60 minutes of incubation (T60), 100µl/well of agar overlay medium were added in each well. After agar addition, the microplates were incubated overnight at 37C° with 5% CO_2 in humid atmosphere. The day after the plates were automatically acquired with the image analysis system DISCOVERY V12 AXIOLAB. The CFUs in each 96-well of plate were counted using the image analysis system (Reading AxioVison). The bactericidal titer for each test sample was calculated as the reciprocal of the serum dilution giving a killing >50% respect to the average number of CFU calculated on the 8 replicates

of without serum control at T60 (average CFU without serum control). Where more than one serum dilution gives 50<killing<55%, the lowest dilution is chosen to calculate the hSBA titer.

Competitive human Serum Bactericidal Assay

The competitive human Serum Bactericidal Assay (hSBA) was used to measure the serum bactericidal activity of a pool of mice sera immunized known to give high hSBA titers after incubation with different competitors, such as GMMA or extracted LOS. These experiments allowed to understand which is the role of anti-LOS antibodies in SBA functional response elicited by a GMMA-based vaccine. A fixed dilution of the tested pool (sera obtained from mice immunized with OMV/GMMA vaccine) was incubated 1:1 (vol/vol) with 3 different concentrations (in terms of $nmol_{LOS}$/mL) of each competitor for 1h at 37°C, 180 rpm. The same pool was also incubated 1:1 (vol/vol) with SBA buffer to measure the hSBA titer of "NOT INHIBITED" sample. After 1 hour of incubation, the mixture serum-competitor was dispensed in plate, diluted 1:2 for eleven dilution-steps and then bacteria and human complement were added following the hSBA assay protocol (see section *Materials and Methods > In vivo studies and functional assays > Functional antibodies measured by Serum Bactericidal Assay*).

The "without serum" control (8 wells) was included in each plate: bacteria with active human complement (AC) in absence of serum sample. This control is used to exclude complement toxicity and to determine 100% bacterial growth.

1.3 Results

1.3.1 Development of physicochemical and immunochemical techniques for LOS characterization

One of the main aims of this PhD project was the set-up of a combination of physicochemical and immunochemical methods for a deep characterization of gonococcal LOS. Although the majority of these techniques can be applied to different types of samples, particular attention has been paid to GMMA and OMV characterization.

GMMA are complex systems, but a large panel of analytical methods have been developed to allow their full characterization [280] (**Table 1.7**). Many of these methods are generic and can be applied to GMMA from different pathogens.

Table 1.7. General methods used for GMMA characterization.

Aims	Methods
Size and aggregation status	SE-HPLC
Total protein quantification	BCA assay, Lowry assay
Purity (*e.g.,*from soluble proteins and/or DNA)	SE-HPLC
Size and polydispersity and particle quantity	DLS, MADLS
Vesicles Integrity	EM

However, as previously mentioned, *N. gonorrhoeae* produces a lipopolysaccharide consisting of a branched oligosaccharide linked to lipid A through 2-keto-3-deoxy-octonic acid (KDO), with no O-polysaccharide chains, hence it is referred to as *lipooligosaccharide* (LOS). Therefore, because of this different nature, it was necessary to set up or adequately improve the available characterization techniques for LOS.

In this study, according to what had already been reported in literature, we developed an improved method for *N. gonorrhoeae* LOS-derived oligosaccharide epitopes identification through a panel of specific anti-LOS monoclonal antibodies in combination to a classical silver stained SDS-PAGE procedure properly optimized to detect the oligosaccharide-derived bands. These techniques are applicable to different types of samples such as bacterial pellets, OMV, GMMA as well as bacterial and GMMA extracted and purified LOS samples.

First, the sample is analyzed on a 16% Tris-glycine gel using Tris-glycine 1x as running buffer and then, after the fixation step using acetic acid and ethanol, an additional step of oxidation of the sugar with sodium metaperiodate is performed, followed by the silver-staining classical procedure. This analysis enables visualization and dissection of all the predominant LOS structures in the sample on the basis of their molecular weight, close to the *Insulin Chain B* (3496 Da) band of the marker (either below and/or above) which is therefore considered a reference for the identification of LOS bands (**Figure 1.2**).

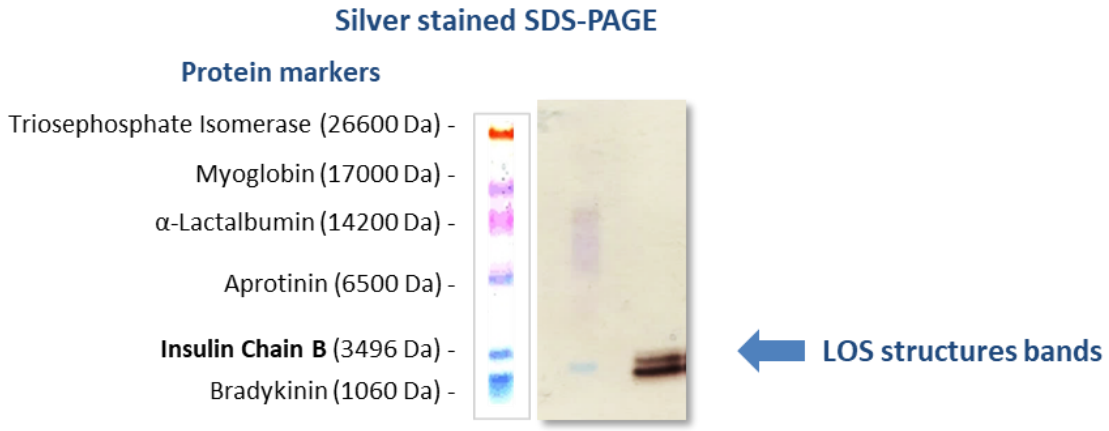


Figure 1.2. LOS structures are visualized in a 16%Tris-glycine SDS-PAGE, after an optimized procedure of silver-staining to highlight the LOS-derived bands.

Using Western blot analysis with mAbs that specifically react with different terminal sugar components of LOS, it was possible to define the LOS structures expressed in each strain.
The epitope recognized by the anti-lipooligosaccharide mAbs selected for this characterization are depicted by colored boxes in **Figure 1.3**.

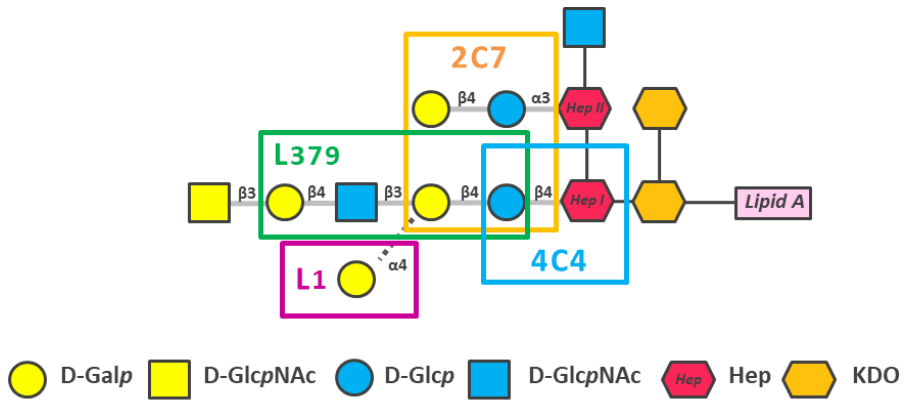


Figure 1.3. The 4 anti-lipooligosaccharide mAbs targeting different saccharide epitopes on LOS.

By exploiting anti-meningococcal mAbs targeting LOS saccharide epitopes, we were able to identify OS epitopes shared by the two *Neisseria* sp., such as the lacto-*N*-neotetraose structure, composed of four sugars extending from Heptose-I (Galβ1-4GlcNAcβ1-3Galβ1-4Glc**, Figure 1.3, green box**), targeted by mAb L3,7,9 [142], and the alternative Heptose-I structure (Galα1-4Gal), recognized by mAb L1 [142], which binds to the L1 meningococcal serotype, also known as the P^K structure (Galα1-4Galβ1-4Glc as α-chain, **Figure 1.3, purple box**). Furthermore, anti-gonococcal mAb 2C7 was used to detect the presence the respective epitope involving the β-chain. Indeed, this mAb, widely described and characterized by Ram and coworkers [160, 281], was valuable for detecting the presence of a lactose in the β-chain linked to Heptose-II (**Figure 1.3, yellow box**), in concomitance with a lactose in the α-chain linked to Heptose-I, both in absence or presence ofα-chain sugar extensions beyond lactose. The last mAb required to complete this panel was the mAb 4C4 [279], a commercially available mAb that targets highly truncated structures of LOS (Glcβ1-4Hep), as reported in **Figure 1.3, blue box.**

Content analysis of the LOS present in *N. gonorrhoeae* GMMA was performed by quantification of the reactive carbonyl groups of the saccharide moiety, generated after acid hydrolysis to remove the Lipid A portion and derivatized with semicarbazide, by SE-HPLC analysis. The SCA reaction coupled with SE-HPLC has already been reported in the literature [276, 277] to determine the reactive carbonyl groups of different molecular size populations in O-Antigen polysaccharide chains. The procedure is based on the strong molar absorbance at 252 nm of the semicarbazone derivative (an imine) formed in the reaction between KDO and SCA (**Figure 1.4**).

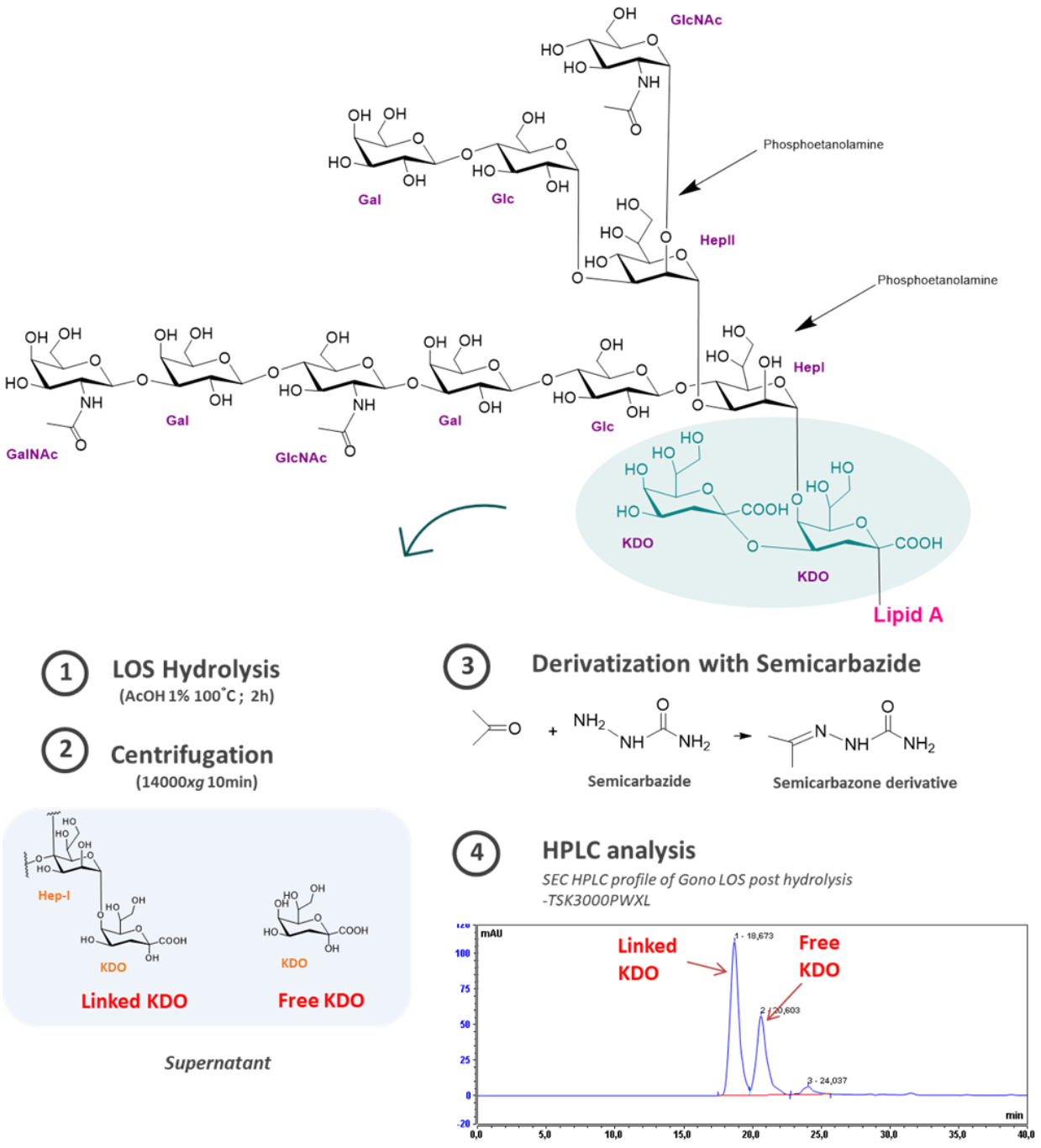


Figure 1.4. Workflow for LOS quantification by SE-HPLC after derivatization of the KDO with semicarbazide.

The *N. gonorrhoeae* LOS structure comprises two keto-deoxyoctanoate (KDO) residues and, during the acid treatment of LOS to remove lipid A, both KDO are hydrolyzed. SCA derivatization occurs on both the KDO bound to the OS chain and the KDO free in solution, resulting in two separate peaks in the SE-HPLC chromatogram recorded at 252 nm.

The KDO content of the *N. gonorrhoeae* GMMA LOS samples is quantified based on a calibration curve built with a standard KDO ammonium salt solution. The LOS content is expressed in nmol/mL

of KDO, which matches the nmol/mL of OS, and it is lastly reported as $nmol_{LOS}$/mg protein, to normalize based on the GMMA protein content.

1.3.1.4 Characterization of LOS in a panel of *N. gonorrhoeae* mutants

To set up the immunochemical characterization, a library of eight mutant strains expressing single defined LOS structures was instrumental in confirming the specific recognition of LOS bands by the four different mAbs. The Western blot results were then combined with silver-stained SDS-PAGE analysis to gain a precise picture of the predominant LOS structures produced by each isogenic mutant strain.

As described in the introduction (see *Introduction > I.5 Gonococcal lipooligosaccharide*), glycan extensions from LOS core heptoses (Heptose-I and Heptose-II) are controlled by the expression of phase variable LOS glycosyltransferase (*lgt*) genes. A panel of *N. gonorrhoeae* LOS *lgt* mutant strains, kindly provided by Professor Sanjay Ram from University of Massachusetts, was obtained by modulating the phase variable expression of the genes *lgtA, lgtC* and *lgtD*, responsible for the variation in the Heptose-I glycan extensions, and of the *lgtG* gene, which controls the expression of the β-chain (a lactose on the Heptose-II). This series of mutants was produced in the background of *N. gonorrhoeae* MS11 v.4/3/1, a variant of MS11 VD300 with an isopropyl β-D-1-thiogalactopyranoside (IPTG)–inducible *pilE* that controls pilus expression. Since the phase variation of LOS glycan extensions is mediated by slipped-strand mispairing at homopolymeric tracts of guanine (G) or cytosine (C) within the coding regions of the *lgt* genes, this series of mutants were obtained by modifying the expression of the phase variable *lgt* loci, changing the stretch of repeating G or Cs. The *lgt* genes were genetically fixed on or off (or deleted) in different combinations to obtain eight different isogenic gonococcal strains (**Figure 1.5**). [161] The description of the genetic modifications is reported in **Table 1.8**. As example, to overcome *lgtG* variability, the C11 homopolymer had been changed to the non-phase variable sequence CCCCTCCGCCA. The mutants are referred to by their Heptose-I structure (2Hex, 3Hex, 4Hex or 5Hex as mutants with respectively two, three, four or five sugars on the α-chain) and as G+ or G- according to the status of the *lgtG* gene (on or off mean presence or absence of the β-chain, respectively).

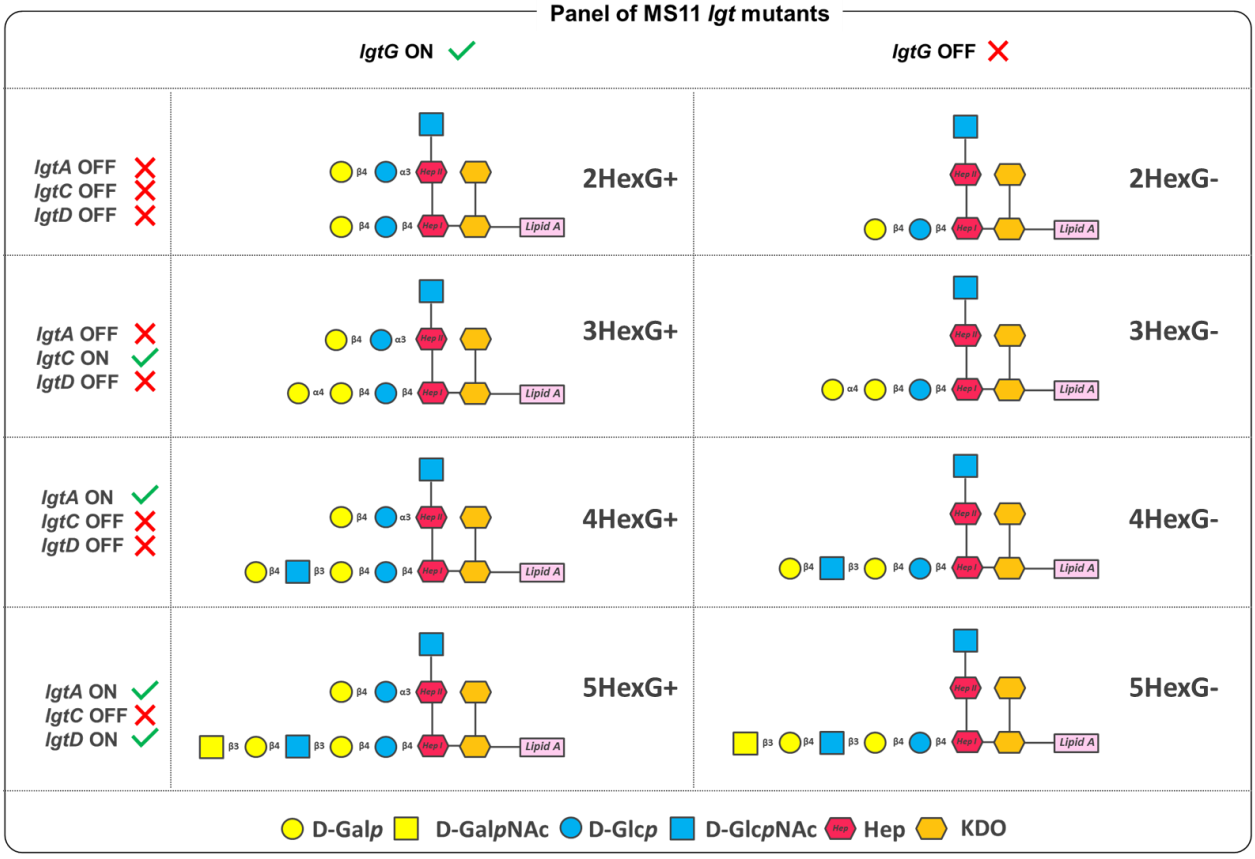


Figure 1.5. Schematic representation of the different LOS structures potentially exposed on the outer membrane of the library of *Neisseria gonorrhoeae* MS11 *lgt* mutant strains received from Sanjay Ram, highlighting the combination of genes that are locked on (√) or off (x). [161]

Table 1.8 Genetic modification approaches of *lgt* genes of the library of *Neisseria gonorrhoeae* MS11 *lgt* mutant strains received from Sanjay Ram. (Table adapted from Chakraborti *et al.,* 2016) [161]

Genetic modification of *lgt* genes	Description
lgtA ON	G12 → GGGCGGAGGTGG
lgtC ON	G14 → GGGGCGGAGG
lgtC OFF	G14 → GGTGAGGGGGGGG
lgtD ON	G13 → GGGCGGAGGTG
lgtG ON	C11 → CCCCTCCGCCA
lgtA del	417 base pair (50-467 of coding sequence) deletion from *lgtA*
lgtD del	44 base pair (64 – 808 of coding sequence) deletion from *lgtD*

We were able to define and confirm the different structures displayed on the surface by immuno-characterization of the bacterial pellet of these strains using the panel of selected LOS-specific mAbs in a Western blot analysis. A summary of the results is presented in **Figure 1.6**.

Lysates of gonococcal MS11 *lgt* mutant strains		2HexG+	3HexG+	4HexG+	5HexG+	2HexG-	3HexG-	4HexG-	5HexG-
Western Blot with anti-LOS mAbs	mAb 2C7	✓	✗	✓	✓	✗	✗	✗	✗
	mAb L3,7,9	✗	✗	✗	✗	✗	✗	✓	✗
	mAb 4C4	✗	✗	✗	✗	✓	✗	✗	✗
	mAb L1	✗	✓	✗	✗	✗	✓	✗	✗

Figure 1.6. Summary of results obtained from Western blot with four different anti-LOS specific mAbs testing bacterial lysates from *Neisseria gonorrhoeae* MS11 *lgt* mutant strains. As reported in the text, the name of the strains reported at the top are referred to the hypothetical structures, also reported in Figure 1.5. The mutants are referred to by their Heptose-I structure (the first number corresponds to the number of sugars on the α-chain) and as G+ or G- according to the presence or absence of the β-chain, respectively.

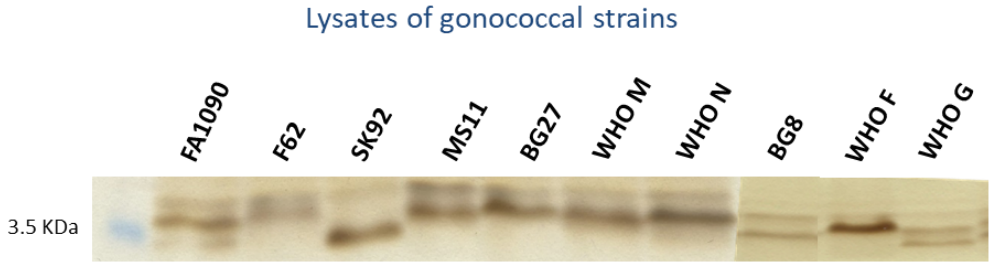
Bacterial lysates of mutants expressing highly truncated LOS structures, such as 2HexG- like mutants, are recognized by mAb 4C4. Mab L1 binds to the P^K LOS structure expressed by 3Hex mutants. The lacto-*N*-neotetraose LOS structure, present in the 4HexG- mutants, is targeted by mAb L3,7,9.
Moreover, the bands below the Insulin Chain B band (3496 Da) of the marker (see **Figure 1.2**), recognized by mAb 2C7, correspond to LOS structures with a short α-chain (2HexG+). In contrast, bands at the same height or above the Insulin Chain B band of the marker and recognized by mAb 2C7 match with a long α-chain HepI glycan extension (*e.g.* 4HexG+ and 5HexG+).
It is important to highlight that mAb 2C7 recognizes the 2HexG+, 3HexG+, 4HexG+ and 5HexG+ structures. However, it binds the 3HexG+ structure with low affinity [161] and therefore this structure is not properly detected in Western blot.

1.3.1.5 Immunochemical characterization of LOS glycoforms in bacterial lysates from gonococcal strains selected for functional assays

In order to determine the prevalent epitopes of LOS expressed by the gonococcal strains selected for functional assays, the LOS immunochemical characterization was performed on total bacterial lysates after growth in hSBA-like conditions (see section *Materials and Methods > 1.2.5 In vivo studies and functional assays > Functional antibodies measured by Serum Bactericidal Assay*). This phenotypic characterization of LOS is useful for correlating functional responses with the specific LOS structures exposed on the bacterial membrane of the strain tested in the hSBA assay.

Heat-inactivated whole bacterial cell lysates were separated on a 16% Tris-glycine gel and subsequently either stained with silver or transferred to a nitrocellulose membrane for Western blotting, probing with the panel of specific anti-LOS mAbs.

The **Figure 1.7** below summarizes the data obtained from the Western blot and Silver stain SDS-PAGE analyses of the bacterial lysates from the library of *Ng* strains. From the interpretation of these data and combination of the two analyses we were able to assign the prevalent LOS structures expressed by each strain, as reported in **Table 1.9.**



Silver-stained SDS-PAGE	Number of LOS bands (isoforms)	3	2	1	3	2	2	2	2	3	2
Western Blot with anti-LOS mAbs	mAb 2C7	✓	✗	✓	✓	✓	✗	✓	✗	✓	✗
	mAb L3,7,9	✗	✓	✗	✓	✗	✓	✗	✓	✓	✓
	mAb 4C4	✗	✗	✗	✗	✗	✗	✗	✗	✗	✓
	mAb L1	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗

Figure 1.7. Summary of results obtained from Silver-stained SDS-PAGE and Western Blot with four different anti-LOS mAbs of whole bacterial cell lysates from gonococcal strains used in functional assays.

Table 1.9. Prevalent LOS structures expressed by the *Ng* panel of strains.

Strain	LOS phenotypes					
	2HexG-	4HexG-	5HexG-	2HexG+	4HexG+	5HexG+
FA1090				✓	✓	✓
F62		✓	✓			
SK92				✓		
MS11		✓	✓		✓	
BG27					✓	✓
WHO-M		✓	✓			
WHO-N					✓	✓
BG8		✓	✓			
WHO-F		✓			✓	✓
WHO-G	✓	✓				

What emerges from the interpretation of results reported in **Figure 1.7** and **Table 1.9** is that six out of twelve strains in this panel express the saccharide epitope recognized by mAb 2C7. Gulati and coworkers [160, 162] reported this epitope as widely shared and expressed in *Ng* isolates, thus highlighting the importance to include some 2C7-like strains in this panel. Interestingly, among the strains recognized by mAb 2C7, it is worth highlighting the peculiarity of SK92 strain, which expresses a single 2C7-like LOS structure.

Another epitope shared among six of these gonococcal strains is the one recognized by meningococcal mAb L3,7,9, targeting lacto-*N*-neotetraose (LNnT) structures. As already widely discussed in the introduction, Schneider *et al*. demonstrated that these long α-chain LOS immunotypes are selected during urethral infection in men [152] and, similar to *N. meningitidis,* Lacto-*N*-neotetraose has been reported to be essential for LOS-mediated adherence and invasion into male urethral epithelial cells [106] and immune evasion strategies as well. Therefore, this epitope remains of primary importance for the selection of representative gonococcal strains.

Notably, most of these strains are targeted by more than one mAb, giving multiple bands in the silver-stained SDS-PAGE analysis, enabling a more in-depth analysis of the LOS isoform combinations.

Some strains express shorter and truncated structures; in particular, WHO-G is the only strain expressing a 2HexG- structure, which is the minimal OS structure, characterized by a lactose (Galβ1-4Glc) extending from the first heptose.

The P^K structure (Galα1-4Galβ1-4Glc) recognized by mAb L1 is not present in any of these strains. However, it has been reported that gonococcal strains expressing the P^K epitope are rare or absent *in vivo* [171, 186] and therefore, they are considered less representative of circulating gonococcal strains.

In light of these considerations, these results confirm that there is a large variability in LOS structures among the hSBA-selected *Neisseria gonorrhoeae* strains, and that the selected panel of strains can be considered representative of the variety of LOS structures that can be found in naturally occurring infections.

Accordingly, the role of LOS as primary antigen in the GMMA-induced immune response was firstly evaluated against the whole library of strains and then, a subset of these gonococcal strains was selected based on their LOS phenotype to assess the impact of the different LOS glycoforms. Indeed, since some of these strains showed similar pattern of oligosaccharide structures, this selection facilitated to draw attention to a possible correlation between the observed data and the LOS pattern phenotypes.

1.3.2 Importance of the variable oligosaccharide moiety on the gonococcal GMMA immunogenicity

To investigate the role of the LOS-derived oligosaccharide outer core in the GMMA-induced immune response, an attractive approach is to exploit the genetic engineering of the bacterial strains to create mutants expressing a defined LOS-derived glycan structure to overcome the variability and assess the correlation between glycan composition and immune response.

1.3.2.1 Genetic engineering of bacteria to generate GMMA expressing LOS without α-chain

With the aim of investigating the role of the oligosaccharide moiety in the immune response induced by anti-gonococcal GMMA-based vaccines, a strain genetically designed to express a LOS structure fully lacking the α-chain was generated in the background of a FA1090 strain. This strain was chosen since it expresses two saccharide epitopes, the 2C7 and the LNnT structures, that are widely shared and expressed in *Ng* isolates, as demonstrated by Gulati and coworkers. [160, 162]

The first step in the generation of a GMMA-producing strain is the reduction of lipid A endotoxic activity to avoid side effects, following a genetic approach widely reported in the literature for GMMA detoxification [282-284]. Indeed, it is well-known that the hexa-acylated lipid A molecule is responsible for LOS endotoxin activity and GMMA reactogenicity. The *N. gonorrhoeae* FA1090 detoxified strain was obtained by genetic deletion of the *lpxL1* gene (NGO0154), homologous to the *E. coli* gene *htrB*, which encodes for a lateacyltransferase of lipid A biosynthesis. Its deletion results in penta-acylated lipid A structures with a strongly attenuated reactogenicity, as shown in **Figure 1.8**.

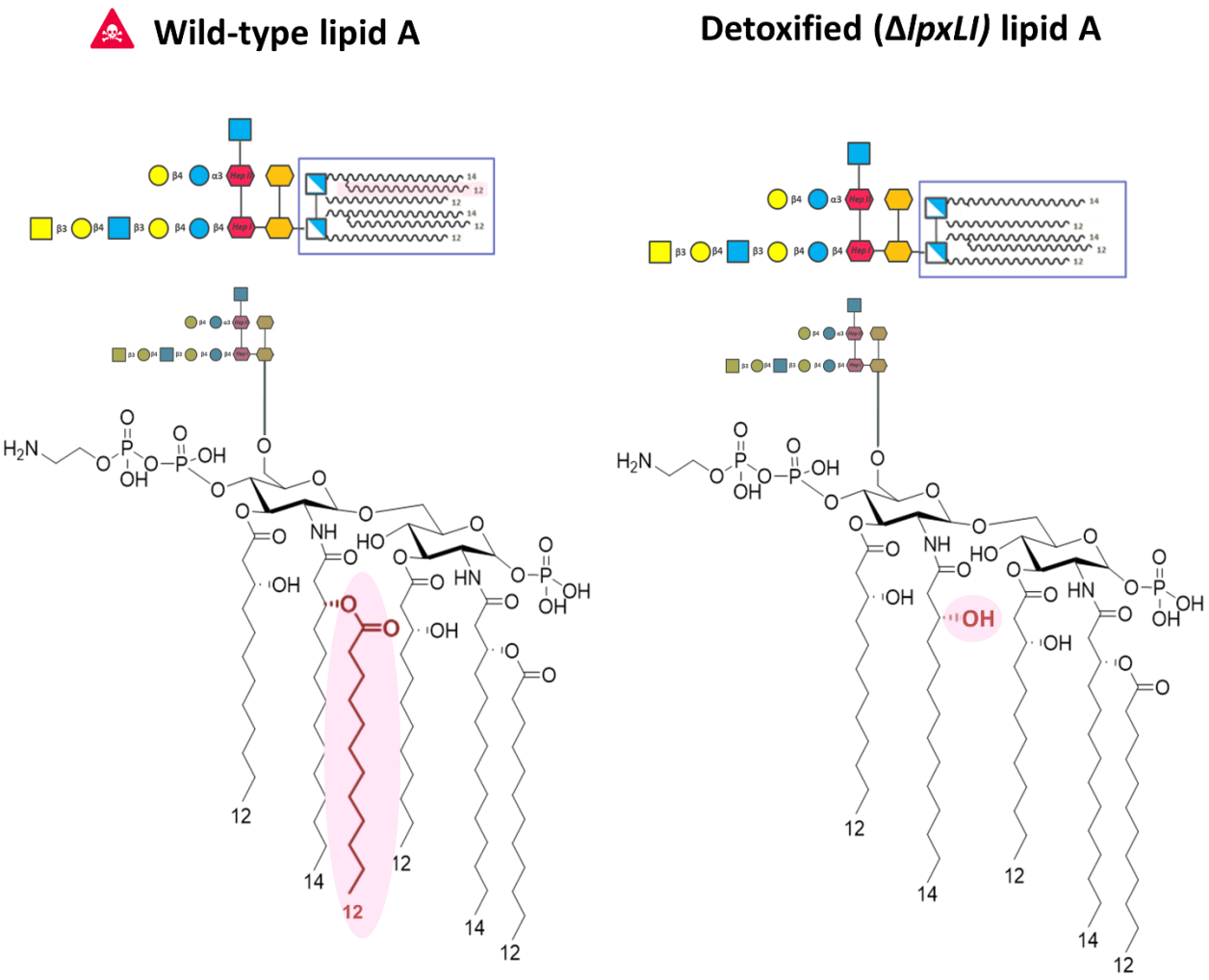


Figure 1.8. The genetical detoxification of the LOS Lipid A consists in the deletion of the *lpxLI* gene, responsible for the late acyltransferase of lipid A biosynthesis. This genetic modification results in a penta-acylated one *(reported on the right)* in place of a hexa-acylated lipid A molecule *(on the left),* with a strong reduction in the endotoxic activity. PEA is reported attached to 4' position of lipid A in this representation, but variable phosphoforms have been identified. [134, 285]

A region of the coding sequence of the *lpxL1* gene was replaced with a kanamycin resistance cassette (*kanR*) by double homologous recombination and the correct occurrence of this recombination was checked with the appropriate controls (**Figure 1.9**).

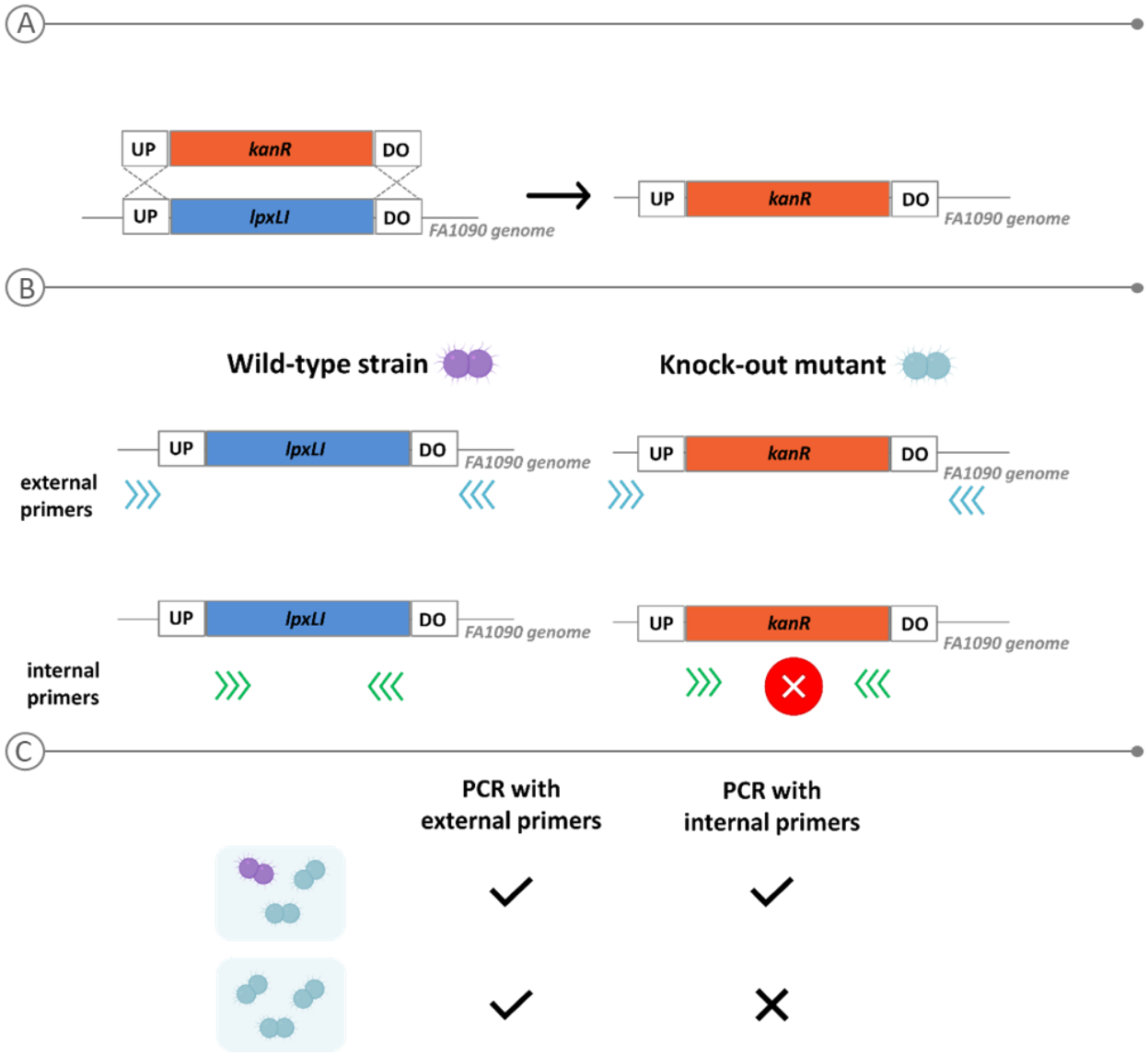


Figure 1.9. Schematic representation of the homologous recombination mechanism in which the *lpxLI* gene is replaced with the *kanR* cassette (**A**). PCR primers used as controls to check the occurrence of the double homologous recombination and the generation of the mutant clone: a pair of primers external to the deletion region were designed as depicted (**B**). The presence of wild-type cells mixed in the total mutant population was investigated with primers that pair specifically to the wild-type genome but not to the mutant (**C**).

In order to evaluate the impact of the oligosaccharide chains on the immune response, the GMMA-producing strain FA1090 Δ*lpxL1* was further genetically mutated to remove the α-chain by deleting the *lgtF* gene, by replacing the coding sequence of this gene with a chloramphenicol resistance cassette (*cmR*) by double homologous recombination (**Figure 1.10, A)**. The *lgtF* gene encodes for a glucosyltransferase responsible for adding a glucose, the first sugar on the α-chain, to Heptose-I. Therefore, the deletion of this gene generates a strain fully lacking the α-chain, since its synthesis is

inhibited upstream (**Figure 1.10, B**). The complete removal of α-chain leads to a mutant strain in which all OS epitopes targeted by the majority of anti-LOS mAbs are incomplete or absent.

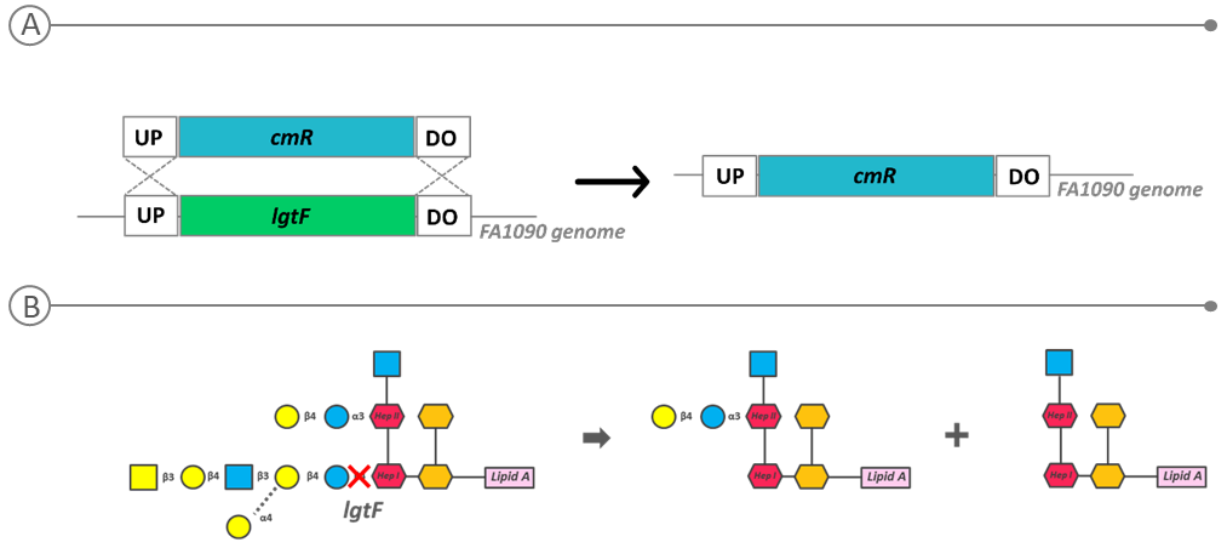


Figure 1.10. Schematic representation of the homologous recombination mechanism in which the *lgtF* gene is replaced with the *cmR* cassette (**A**). After the *lgtF* genetic deletion, the resulting phenotypes are reported on the left. Mass Spectrometry analyses confirmed the presence of the structure with neither the α-chain nor the β-chain (**B**).

The bacterial pellets of these two strains were immunocharacterized by Western blot with the four anti-LOS mAbs and silver-stained SDS-PAGE. As expected, the resulting phenotype was characterized by the complete absence of hexoses linked to the α-chain, although, also the β-chain was not detectable (data not shown).

Hence, it is likely that this genetic modification led to a reduced expression of the phase variable *lgtG* gene encoding for the corresponding glycosyltransferase, required for initiating the synthesis of the β-chain, thus making possible to obtain a gonococcal mutant completely devoid of the two variable oligosaccharide chains.

1.3.2.2 Production, purification and characterization of GMMA from *N. gonorrhoeae* FA1090 Δ*lpxL1* Δ*lgtF*

GMMA from the mutated gonococcal strains FA1090 Δ*lpxL1* Δ*lgtF* were produced and purified by ultracentrifugation and/or tangential flow filtration (TFF). Therefore, the resulting GMMA were characterized using different physicochemical techniques (**Table 1.10**).

GMMA purity level was estimated by SE-HPLC by comparing chromatograms of absorbance at 260 nm, 280 nm and fluorescence (excitation at 277 nm and emission at 335 nm) and radius dimension was obtained by DLS. All the produced GMMA had a purity higher than or around 90% and comparable hydrodynamic radius dimensions measured by dynamic light scattering (DLS) in a range of 40-50 nm.

The total protein concentration was established by modified Lowry protein assay. The content analysis of the LOS present in GMMA was performed by quantification of the reactive carbonyl groups of the saccharide moiety, generated after acid hydrolysis to remove the Lipid A and derivatized with SCA, by SE-HPLC analysis. Notably, the final LOS content in the FA1090 Δ*lpxL1* Δ*lgtF* GMMA preparation is about four fold higher than the FA1090 *Δlpxl1 Δrmp* GMMA used as control. This has been taken into consideration, especially in the interpretation of the following functional assay results, normalizing in terms of protein or LOS quantity.

From a qualitative perspective, GMMA were analyzed by SDS-PAGE and stained with Coomassie Blue to check protein patterns. The absence of the oligosaccharide portion on the FA1090 Δ*lpxL1* Δ*lgtF* GMMA did not impact the overall protein profile, which remained comparable to the one of FA1090 *Δlpxl1 Δrmp* GMMA (data not shown).

A combination of Western Blot analyses using the panel of anti-LOS mAbs confirmed the desired LOS phenotype, showing no binding of FA1090 Δ*lpxL1* Δ*lgtF* GMMA. This result was also confirmed by silver-stained SDS-PAGE which showed a band at lower relative masses. This phenotype was further verified by Mass Spectrometry (data not shown).

Table 1.10. Recap of the GMMA from FA1090 *Δlpxl1 Δlgtf* characterization through different techniques, together with the FA1090 *Δlpxl1 Δrmp* GMMA used as control in the following assays.

Analysis		**GMMA FA1090**	
		Δ*lpxL1* Δ*lgtF*	**Δ*lpxL1 Δrmp***
Purity level by HPLC	**% 260**	92	90
	% 280	94	94
	% FLD	98	97
DLS radius dimension (d.nm) and polydispersity index (PDI)		84.3 (PDI: 0,38)	67.8
LOS $nmol_{LOS}/mg_{protein}$ (SCA SE-HPLC/Lowry assay)		483	124

1.3.2.3 Investigation of the impact of LOS-derived oligosaccharide chain on the cross-bactericidal immune response elicited by gonococcal GMMA

To understand the role of anti-LOS antibodies in the serum bactericidal assay functional response, a competitive human Serum Bactericidal Assay (hSBA) was performed. This assay can measure the functional antibodies that remain available after incubation with a specific competitor.

The serum bactericidal activity of a pool of sera from mice immunized with FA1090 Δ*lpxL1* Δ*rmp* GMMA, that are known to elicit cross-bactericidal antibodies, was measured after 1-hour incubation with increasing concentrations (expressed as quantity of LOS in terms of $nmol_{LOS}$/mL) of the competitor. This experiment allowed to understand which is the role of anti-LOS antibodies in hSBA functional response elicited by a GMMA-based vaccine.

The experiment was performed using as competitors the LOS extracted from wild-type FA1090 bacterial cells and FA1090 Δ*lpxL1* Δ*lgtF* GMMA, deprived of LOS epitopes, as described above. Furthermore, FA1090 Δ*lpxL1* Δ*rmp* GMMA, from which the tested sera were derived, were used as control.

The competitive hSBA experiment was conducted against four *N. gonorrhoeae* heterologous strains: MS11, WHO-N, WHO-G and F62. These strains were chosen *ad hoc* for this assay based on their LOS phenotype in order to assess the impact of different LOS forms expressed by the tested heterologous strain on the observed hSBA data. Indeed, as shown in **Table 1.11**, MS11 predominant LOS structures are characterized by long α-chains composed of four or five sugars, with or without the β-chain. In contrast, F62 expresses a similar composition of LOS with long α-chains, but all the structures lack lactose in the β-chain. The WHO-N strain presents an opposite phenotype, with four/five sugars in the α-chains and a lactose in the β-chain. Lastly, WHO-G was chosen because it is interesting for its phenotype in which short and long α-chains are mixed in the absence of the β-chain. This combination of LOS glycoforms allows on one side to dissect the differences between short and long α-chains and on the other side to assess the role of the β-chain.

Table 1.11. LOS Phenotypes of the selected *N. gonorrhoeae* strains.

Strain	LOS phenotypes					
	2HexG-	4HexG-	5HexG-	2HexG+	4HexG+	5HexG+
FA1090				✓	✓	✓
MS11		✓	✓		✓	
F62		✓	✓			
WHO-N					✓	✓
WHO-G	✓	✓				

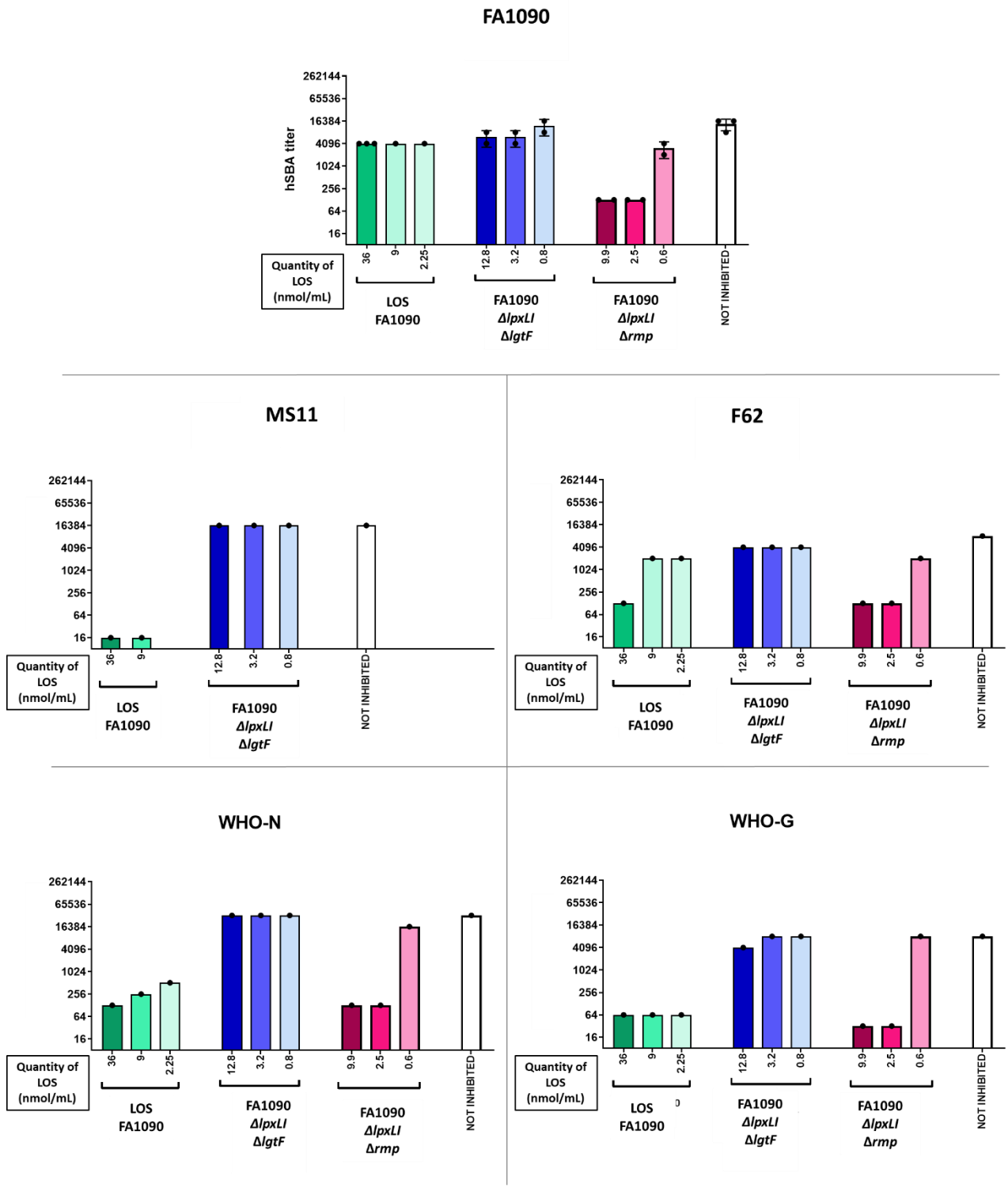


Figure 1.11. hSBA titers measured against the indicated Gonococcus strains using a pool of sera from mice immunized twice with FA1090 Δ*lpxL1* Δ*rmp* GMMA after incubation with three concentrations of each competitor: extracted LOS from FA1090 wild-type strain, FA1090 Δ*lpxL1* Δ*lgtF* GMMA, FA1090 Δ*lpxL1* Δ*rmp* GMMA and hSBA buffer (white bar) that represent the "not inhibited" sample (negative control).

The extracted LOS was able to strongly reduce the hSBA titer, at least at the highest concentration tested, against all the four heterologous strains, as well as the homologous GMMA (FA1090 Δ*lpxL1*

Δ*rmp* GMMA), whereas FA1090 Δ*lpxL1* Δ*lgtF* GMMA did not compete with functional antibodies elicited by FA1090 Δ*lpxL1* Δ*rmp* GMMA (**Figure 1.11**). Interestingly, analyzing the homologous strain FA1090, the hSBA titer was not inhibited neither by incubation with the extracted LOS nor with FA1090 Δ*lpxL1* Δ*lgtF* GMMA, while it is possible to observe a strong decrease in the bactericidal titer when the homologous GMMA (FA1090 Δ*lpxL1* Δ*rmp* GMMA) were tested as competitors. This may suggest the bactericidal titer against the homologous strain is mediated by the combined presence of antibodies targeting LOS and protein antigens.

These data confirm a major role of anti-LOS antibodies in the bactericidal activity against heterologous strains of the sera obtained following the administration of a gonococcal GMMA-based vaccine.

To confirm these results, FA1090 Δ*lpxL1* Δ*lgtF* GMMA was tested *in vivo* in comparison with FA1090 Δ*lpxL1* Δ*rmp* GMMA vaccine.

Mice sera collected two weeks after the second dose were analyzed in pools in hSBA assay. The serum antibody functionality was tested using a panel of *N. gonorrhoeae* strains, including the homologous FA1090 strain and ten heterologous strains representative of the *N. gonorrhoeae* population, based on the comparative genome analysis of a wide panel of circulating strains.

The hSBA assay results are reported in **Figure 1.12.**

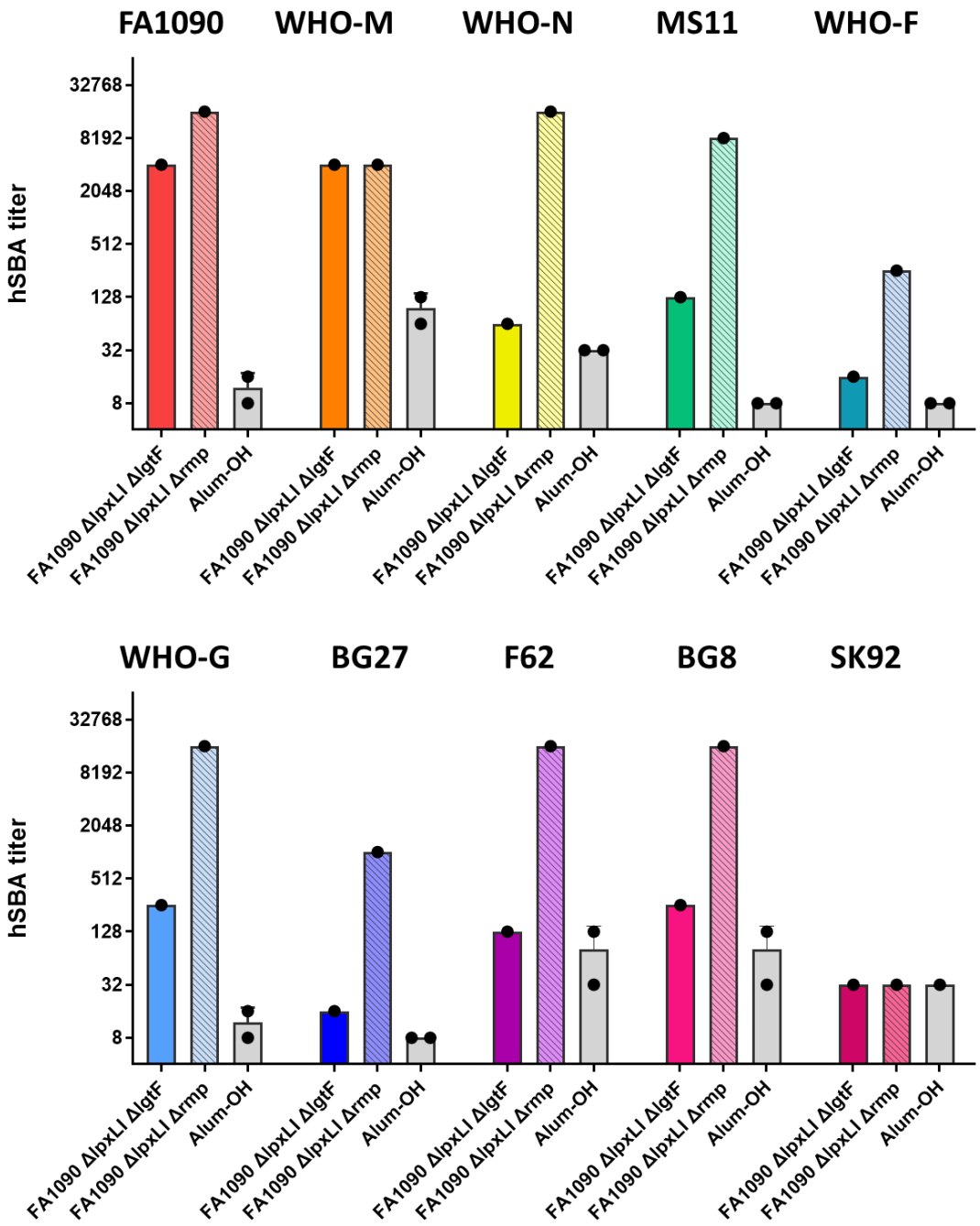


Figure 1.12. hSBA titers measured against the indicated Gonococcus strains on pooled sera from CD1 mice immunized twice with FA1090 Δ*lpxL1* Δ*lgtF* GMMA and FA1090 Δ*lpxL1* Δ*rmp* GMMA as positive control. A pooled mouse serum from immunization with alum hydroxide was used as negative control. Each hSBA titer is the mean of two or three different analyses.

As expected, higher hSBA titers were obtained for sera from mice immunized with FA1090 Δ*lpxL1* Δ*rmp* GMMA, with respect to FA1090 Δ*lpxL1* Δ*lgtF* GMMA and alum hydroxide, against nine out of ten Gonococcus strains.

On the other side, FA1090 Δ*lpxL1* Δ*lgtF* GMMA was able to elicit bactericidal antibodies comparable to FA1090 Δ*lpxL1* Δ*rmp* GMMA against FA1090 and WHO-M strains, while at least five fold lower hSBA titers were detected for the other heterologous strains. In fact, similarly to what was observed in the competitive hSBA experiment described above, in the case of the homologous FA1090 and the heterologous WHO-M strain, there are no main differences between the hSBA titers of the two tested sera, with the same titer or a variation of two fold decrease; indeed, the value remains high with respect to the negative control even testing the FA1090 Δ*lpxL1* Δ*lgtF* GMMA serum. In this case, it could conceivably be hypothesized that high bactericidal titer are related to a possible synergistic effect of LOS and protein antigens.

Of note, the hSBA on SK92 strain resulted in an outlier behaviour with lower hSBA titers, comparable to the alum hydroxide negative control titers, when testing both the FA1090 Δ*lpxL1* Δ*lgtF* GMMA and FA1090 Δ*lpxL1* Δ*rmp* GMMA sera.

A further support of these results is also given by the observation of similar trend when testing the FA1090 Δ*lpxL1* Δ*lgtF* Δ*rmp* GMMA (data not shown). This control was done in order to exclude any possible influence of the Δ*rmp* mutation on this trend.

These results clearly demonstrated that anti-LOS antibodies play a major role in SBA functional response against the majority of the heterologous strains.

1.3.3 Evaluation of the impact of α-chain length on the elicitation of functional bactericidal titers by gonococcal GMMA

To further understand the correlation between LOS and cross-bactericidal activity, a direct comparison between hSBA titers and the LOS phenotype of the tested GMMA was needed. Indeed, producing different lots of GMMA from a FA1090 Δ*lpxL1* mutant, a certain variability was observed in the LOS phenotypes. In particular, some lots presented LOS glycan structures that were unusually highly truncated (2HexG-/3HexG- like **Figure 1.13**, *left*), presumably because of phase variation of the *lgtG* and *lgtA* genes. In fact, it has been reported that LOS may undergo phase variation at a frequency of $10^{-2}/10^{-3}$ when bacteria are grown in culture. [85, 151] Consequently, analysis of the derived mouse sera in hSBA against different heterologous strains showed that titers of these GMMA were lower than those of GMMA obtained from a FA1090 Δ*lpxL1* Δ*rmp* strain containing both the β-chain and a higher percentage of structures with a long α-chain (2HexG+, 4HexG+ and 5HexG+ LOS structures, **Figure 1.13**, *right*). This observation led to the hypothesis that the various LOS oligosaccharide moieties could have different influences on hSBA activity.

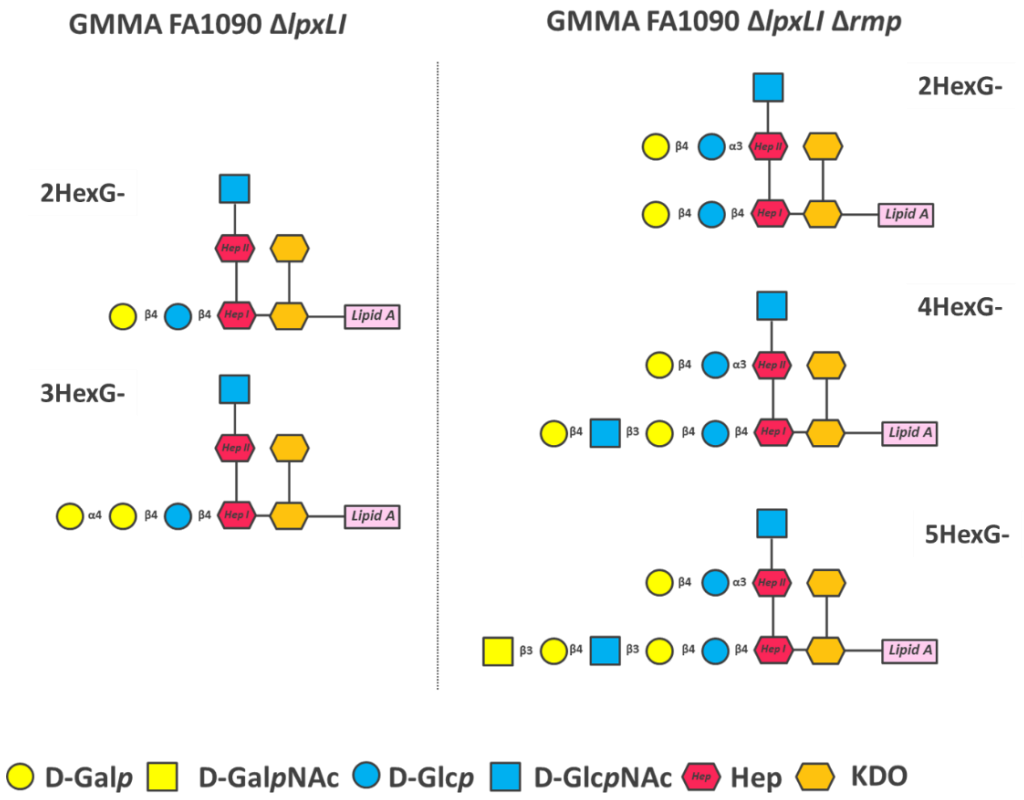


Figure 1.13. The prevalent LOS phenotypes detected in the FA1090 Δ*lpxL1* GMMA (*left*) and FA1090 Δ*lpxL1* Δ*rmp* GMMA (*right*).

To date, research has not focused on the role of LOS on OMV/GMMA immune response and therefore the impact of the different oligosaccharide portions on the GMMA-induced functional bactericidal activity has yet to be clearly established. Therefore, one additional purpose of this study was to assess the extent to which oligosaccharide epitopes affect the bactericidal response induced after GMMA-based immunization. By leveraging on a panel of *N. gonorrhoeae* mutants each mainly expressing one different oligosaccharide structures that can be co-present on the bacterial surface of wild-type bacteria, it was possible to investigate the antibody response in mice after immunization with GMMA exposing different LOS structures. In this way, this section seeks to study a possible correlation between LOS structures, focusing on the α-chain different lengths, and the GMMA-induced immune response by analyzing the functional hSBA activity.

1.3.3.1 Genetic engineering of bacteria to generate detoxified MS11 *lgt* mutant GMMA producing strains

The effect of Heptose-I glycan extensions on the immune response was investigated and compared using the eight MS11 LOS *lgt* mutant strains received from Professor Sanjay Ram (see details paragraph *1.3.1.4 Characterization of LOS in a panel of N. gonorrhoeae mutants*).
A schematic representation of the genetic manipulation and the final Heptose-I glycan composition of these mutants with a simplified designation is provided in **Table 1.2**.

The phase variable mechanism involving the *lgt* genes *lgtA, lgtC* and *lgtD* leads to the modification of the Heptose-I glycan extension of the α-chain, whereas the phase variable expression of *lgtG* controls the presence of lactose linked to Heptose-II (β-chain).
The structures expressed from all these mutants were characterized by Western blotting with anti-LOS mAbs and the relative masses were determined by silver-stained SDS-PAGE, confirming the desired phenotype.
It is worth noting that even though the expression of the *lgt* genes is fixed as on or off, this genetic manipulation does not guarantee that all the LOS structures displayed on the bacterial outer membrane will be effectively substituted with the glycan added by the encoded *lgt* enzyme(s). This is related to the possible transport of incomplete LOS molecules to the surface from the site of assembly on the cytoplasmic side of the inner membrane, which may occur prior to the addition of a glycan by all *lgt*s that are set on, despite the expression of the relative LOS glycosyltransferase. The ratio of complete to incomplete LOS expression is regulated by the amount and efficiency of each *lgt* enzyme. [286]
This means that, although the simultaneous production of multiple LOS glycoforms expressed by a particular strain is defined by on-off strand slippage of homopolymeric tracts of *lgtA*, *lgtC*, *lgtD*, and *lgtG* genes [147, 149], this event can also be mediated by the production of limiting amounts of LgtA, LgtD, or LgtE, occurring via several different mechanism. [286-288]
An example is the 5HexG- strains, in which the *lgtD* gene has been locked on, that showed two bands in silver-stained SDS-PAGE. One of these bands reacts with mAb L3,7,9 and represents the corresponding 4Hex structure with a terminal lactosamine. Indeed, despite the expression of *lgtD*, most LOS in these mutants is exported to the surface prior to the addition of the terminal GalNAc to LOS. This is observed also for the 5HexG+ strain, as demonstrated by Chakraborti and coworkers using mAb 3F11. [161]
Similarly, MS11 2HexG+ expresses two different structures that can be clearly identified using silver-stained SDS-PAGE. One is recognized by mAb 2C7 and the other is a LOS species with only

Heptose-I-linked lactose, similar to the LOS expressed by MS11 2HexG-. This is related to the export of LOS to the outer membrane prior to the addition of glucose to Heptose-II by the LgtG glycosyltransferase.

Likewise, mAb L8 specifically binds to structures that contain lactose on Heptose-I and no glycan from the 3-position of Heptose-II. In this case, as previously demonstrated [161], this mAb reacts with all the three mutants that had *lgtA* off and *lgtC* and/or *lgtG* on, indicating the export of LOS prior to the addition of glucose to Heptose-II by *lgtG* or the distal α1-4-linked galactose on Heptose-I (*lgtC*).

In contrast, fixing *lgtA* on did not result in any detectable short LOS structures, suggesting in this case that the *lgtA* enzyme efficiently adds the GlcNAc to the proximal lactose of Heptose-I. **Table 1.12** represents a summary of the LOS structures detected and theoretically present in each mutant.

Table 1.12. Structures detected by immunocharacterization of the *Ng* bacterial lysates of the *lgt* mutants compared to the theoretical structures present according to the genetic manipulation.

		Detected structures present in *N. gonorrhoeae* MS11 *lgt* mutants bacterial lysates and GMMA							
		2HexG+	3HexG+	4HexG+	5HexG+	2HexG-	3HexG-	4HexG-	5HexG-
Single theoretical structures present in *N.gonorrhoeae* MS11 *lgt* mutants	2HexG+	+++				+			
	3HexG+	++	+++			+	+		
	4HexG+			+++				+	
	5HexG+			++	+++			+	+
	2HexG-					+++			
	3HexG-					++	+++		
	4HexG-							+++	
	5HexG-							++	+++

\+ structure present as impurity in low quantity
++ structure present as impurity in high quantity
+++ prevalent structure

As already described for the detoxification of the other strains selected for GMMA production in order to reduce LOS endotoxin activity, this library of eight *N. gonorrhoeae* MS11 strains was

detoxified by genetic deletion of the *lpxL1* gene (NGO0154), resulting in penta-acylated lipid A structures with strongly attenuated reactogenicity (see **Figure 1.8**). The *lpxL1* knockout mutants were obtained by genomic recombination where a region of the coding sequence of this gene was replaced with the chloramphenicol antibiotic resistance cassette (*lpxL1*::*cmR*) with a double crossing over (**Figure 1.14**). Immunochemical characterization through Western blot and Silver-staining SDS-PAGE of the bacterial pellet confirmed that the desired LOS phenotype was maintained after genetic manipulation. A graphical representation of the resulting LOS structure phenotypes for each strain was represented in **Figure 1.5** and **Figure 1.6**.

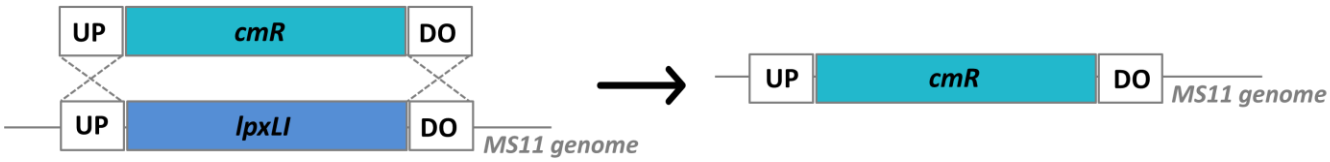


Figure 1.14. Schematic representation of the homologous recombination mechanism in which the *lpxLI* gene is replaced with the *cmR* cassette.

1.3.3.2 Production, purification and characterization of GMMA from *N. gonorrhoeae* MS11 Δ*lpxL1 lgt* mutants

GMMA derived from the detoxified (Δ*lpxL1*) MS11 *lgt* mutant strains were produced and purified by ultracentrifugation and tangential flow filtration (TFF). Therefore, the resulting GMMA were characterized using different physicochemical techniques.
Characterization confirmed a good purity level of all GMMA preparations estimated by SE-HPLC, with similar radius dimensions (measured by DLS).
Silver-stained SDS-PAGE and corresponding Western blot analysis with the different LOS-specific mAbs for GMMA of MS11 mutant strains are reported in **Figure 1.15**.

Notably, the expression of LOS in these GMMA resembled perfectly the one present on the respective non-detoxified strain. Indeed, the absence of the Lipid A acyl chain did not affect the overall LOS phenotype on GMMA. Western blot analysis performed using the four anti-LOS mAbs and silver-stained SDS-PAGE confirmed what has been already reported previously for these mutant strains. [161]

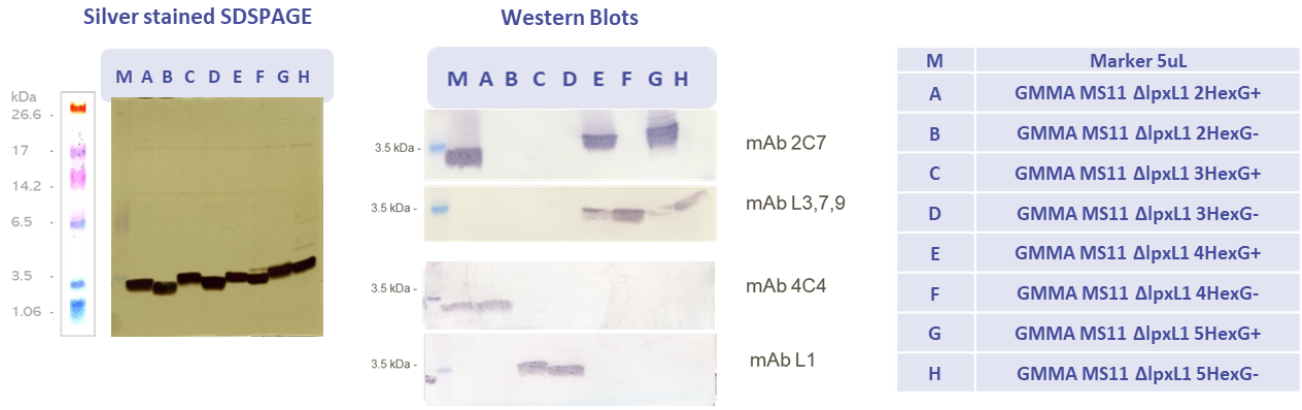


Figure 1.15. Silver stained SDS-PAGE and WB with four different LOS specific mAbs of GMMA from MS11 Δ*lpxL1 lgt* mutant strains.

The different oligosaccharide moieties of these GMMA did not affect the overall protein profile, as confirmed by SDS-PAGE, in which the band patterns were similar among the different samples (data not shown).

On the contrary, the sugar/protein ratio was more variable, ranging from 156,4 $nmol_{LOS}/mg_{protein}$ to 386,9 $nmol_{LOS}/mg_{protein}$. A summary of the analysis is reported in **Table 1.13**.

Table 1.13. Recap of the MS11 *ΔlpxL1* LOS *lgt* mutant strain GMMA characterization through different techniques.

Analysis		**GMMA MS11 Δ*lpxLI***							
		2HexG+	**2HexG-**	**3HexG+**	**3HexG-**	**4HexG+**	**4HexG-**	**5HexG+**	**5HexG-**
Purity level by SE-HPLC	**% 260**	98,6	96,4	97,6	90,1	81,2	89,1	98,4	95,8
	% 280	99,6	97,6	99,1	90,9	86,8	86,6	98,2	97,6
	% FLD	97,1	99,8	100	88,7	88,6	85,1	100	95,5
DLS radius dimension (d.nm) and polydispersity index (PDI)		82,34 (PDI: 0,242)	93,72 (PDI: 0,236)	76,57 (PDI: 0,232)	69,41 (PDI: 0,217)	81,60 (PDI: 0,222)	76,65 (PDI: 0,233)	80,92 (PDI: 0,183)	88,92 (PDI: 0,161)
LOS $nmol_{LOS}/mg_{protein}$ (SCA SE-HPLC/Lowry assay)		194,8	250,4	247,0	226,0	238,4	386,9	208,1	156,4

1.3.3.3 Investigation of the impact of LOS-derived α-chain length on the cross-bactericidal immune response elicited by gonococcal GMMA

To compare the impact of the LOS-derived variable oligosaccharide portions on the bactericidal response, the GMMA from these eight *N. gonorrhoeae* MS11 isogenic mutant strains were tested *in vivo*.Differently from the other study, GMMA were normalized based on LOS content at a dose of 1,5 $nmol_{LOS}$ in 200µL, adsorbed to Alum hydroxide (3 mg/mL). Doses in terms of GMMA protein quantity are reported in **Table 1.14** and ranged from 3,9 µg up to 9,6 µg.

Table 1.14. Dose of each MS11 Δ*lpxLI* LOS *lgt* mutant GMMA in terms of proteins. All doses were below 10 µg of protein, which is the dose selected after dose ranging studies for mouse immunization with gonococcal GMMA, obtaining a relevant bactericidal response (data not shown).

GMMA MS11 Δ*lpxLI*	**LOS/Protein ($nmol_{KDO}/mg_{protein}$)**	**Dose ($µg_{protein}$)**
2HexG+	194,8	7,7 µg
2HexG-	250,4	5,9 µg
3HexG+	247,0	6,1 µg
3HexG-	226,0	6,6 µg
4HexG+	238,4	6,3 µg
4HexG-	386,9	3,9 µg
5HexG+	208,1	7,2 µg
5HexG-	156,4	9,6 µg

Sera were collected two weeks after the second dose and analyzed in pools. The serum antibody functional activity was tested in duplicate or triplicate by human Serum Bactericidal Assay (hSBA) using a panel of *N. gonorrhoeae* strains including the homologous MS11 strain and five heterologous strains, appropriately selected in order to have a combination of strains expressing a heterogeneous population based on LOS structures exposed on the surface (FA1090, WHO-N, F62, WHO-G and SK92, **Table 1.15**).

Table 1.15. Prevalent LOS phenotypes expressed by the selected *Ng* strains.

Strain	LOS phenotypes					
	2HexG-	4HexG-	5HexG-	2HexG+	4HexG+	5HexG+
MS11		✓	✓		✓	
FA1090				✓	✓	✓
WHO-N					✓	✓
F62		✓	✓			
WHO-G	✓	✓				
SK92				✓		

The results, reported in **Figure 1.16,** highlighted that the pooled sera derived from mice immunized with GMMA with long α-chain LOS structures (4HexG+, 4HexG-; 5HexG+, 5HexG-) showed higher hSBA titers compared to the sera derived from mice immunized with the GMMA exposing shorter LOS structures (2HexG+, 2HexG-; 3HexG+, 3HexG-) for five out of six tested *N. gonorrhoeae* strains.

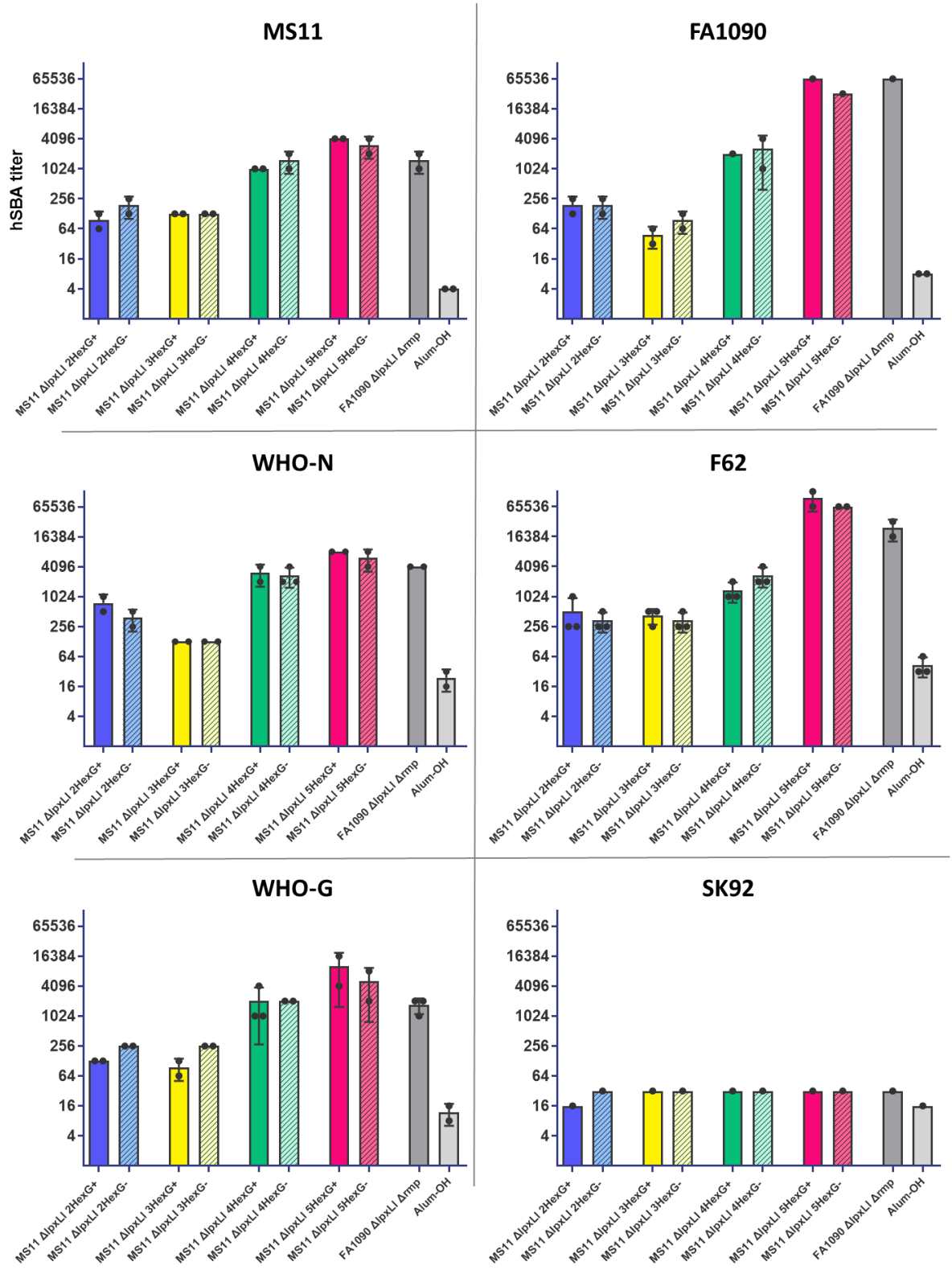


Figure 1.16. hSBA titers measured against the indicated Gonococcus strains on pooled sera from CD1 mice immunized twice with the GMMA MS11 Δ*lpxL1* LOS *lgt* mutants and FA1090 Δ*lpxL1* Δ*rmp* GMMA. A pooled mouse serum from immunization with alum hydroxide was used as negative control. Each hSBA titer is the mean of two or three different analyses.

These results underline the higher impact of anti-LOS antibodies elicited by GMMA with longer LOS structures (long α-chain) on the SBA functional response against the majority of tested strains. The presence of the β-chain, particularly of the 2C7 epitope (2HexG+ mutant), was clearly dispensable for elicitation of bactericidal antibodies, whereas the crucial parameter responsible for an increased hSBA titer was the length of the α-chain. Interestingly, this trend is not influenced by the different LOS isotypes of the tested gonococcal strains and it is maintained even when there is no correspondence between the LOS expressed by the tested strains and the glycoform of the GMMA. An example is the WHO-G strain, which is very well killed by sera generated with a 5-HexG+ GMMA, while it expresses a 2HexG- truncated structure.

To confirm that a long α-chain LOS epitope is important for eliciting functional responses in gonococcal FA1090 GMMA-based vaccines, a competitive hSBA was performed using pooled sera from mice immunized with the FA1090 Δ*lpxL1* Δ*rmp* GMMA, that are known to induce functional cross-bactericidal antibody titers. The sera were preincubated with GMMA from MS11 *lgt* mutant strains used as competitors and hSBA was subsequently performed testing the sera against four heterologous gonococcal strains.

The hSBA titers against three different heterologous strains (MS11, WHO-N and WHO-G) were drastically inhibited by GMMA with a long α-chain (four or five sugar residues, corresponding to structures 4HexG+, 5HexG- and 5HexG+) beyond the presence of the β-chain and therefore of the 2C7 epitope. The hSBA titers were less inhibited when the experiment was conducted against the F62 strain but with a comparable trend. In contrast, GMMA with structures containing only short α-chains, such as 2HexG+, the structure recognized by the 2C7 mAb with the highest affinity, did not inhibit functional activity. Therefore, the results, reported in **Figure 1.17**, showed that antibodies against 4HexG+, 5HexG- and 5HexG+ LOS structures were bactericidal in hSBA for three out of four heterologous strains. These data confirm that antibodies against LOS structures with a long α-chain have a majore role in the bactericidal activity of the sera obtained following administration of GMMA from the FA1090 Δ*lpxL1* Δ*rmp* strain.

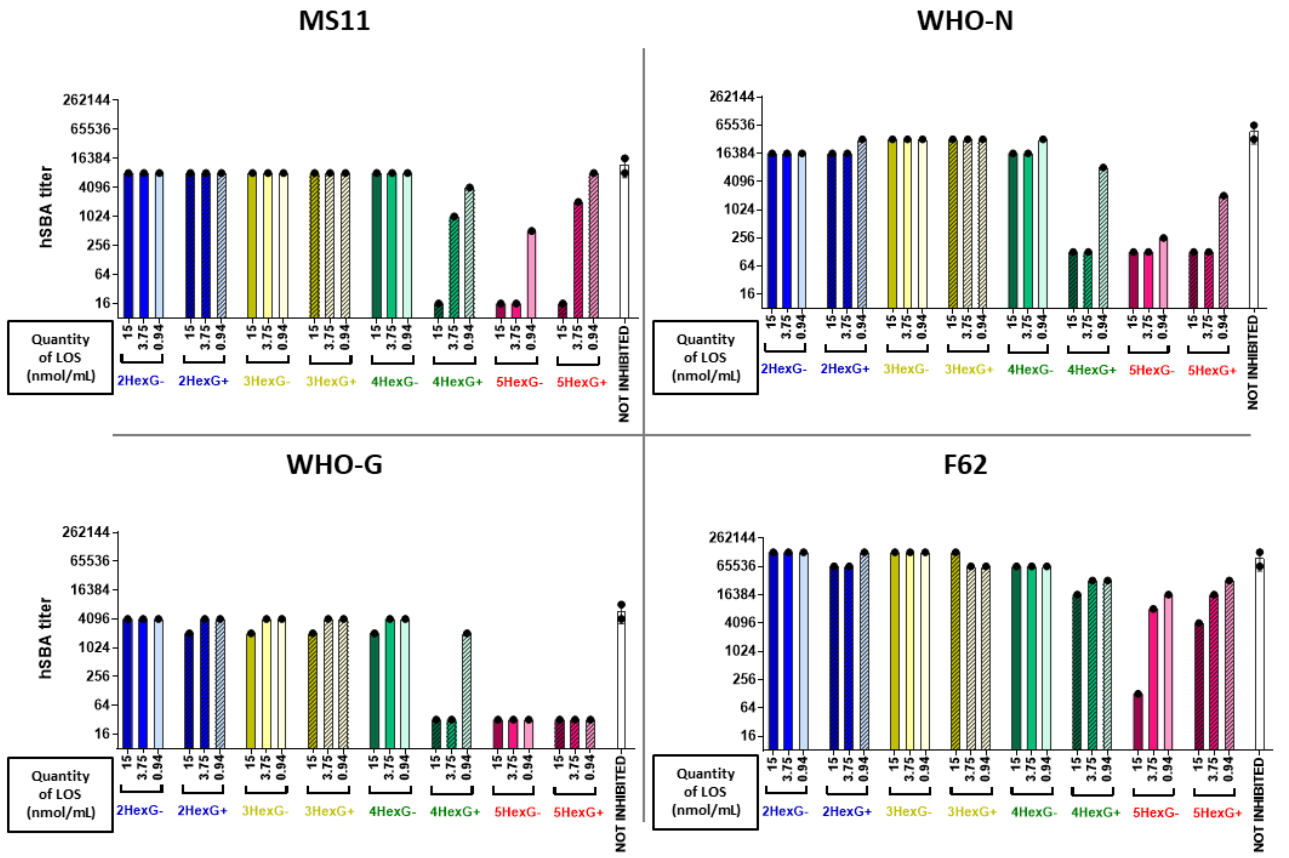


Figure 1.17. hSBA titers measured against the indicated Gonococcus strains using a pool of sera from mice immunized twice with FA1090 Δ*lpxL1* Δ*rmp* GMMA after incubation with three concentrations of each competitor: FA1090 Δ*lpxL1* Δ*rmp* GMMA, the eight MS11 Δ*lpxL1* LOS *lgt* mutant GMMA and hSBA buffer (white bar) that represent the "not inhibited" sample (negative control).

It is worth drawing attention to the behaviour of GMMA 4HexG-, which did not inhibit hSBA titers against all the four tested strains. If we consider the LOS phenotype of GMMA used in the mouse immunization of this tested serum, characterized by LOS glycoforms with two, four or five sugars in the α-chain, together with the β-chain, it could plausibly be hypothesized that the antibodies elicited by these GMMA are mainly directed against structures also expressing the β-chain, thus explaining the strong inhibition of the titer after incubation with GMMA 4HexG+ and 5HexG+.

The data reported here appear to support the assumption that the difference in the cross-recognition of sera from GMMA 4HexG- and GMMA 4HexG+, could be possibly related not only to the presence of β-chain, but probably to the concomitant presence of β-chain and long α-chain. Considering that, another possible consideration is that in the GMMA expressing the β-chain it is often present a small LOS population without this chain; indeed, the genetical manipulation of the *lgt* genes does not ensure that all the expressed LOS structures will be effectively substituted due to the possible transport of incomplete LOS molecules to the surface which may occur before the addition of a glycan by all *lgt*s that are fixed on. For this reason, GMMA 4HexG+ could also express a small population of 4HexG-

LOS structures and this could be the explanation for the role of anti-LOS antibodies generated by a G+ GMMA on the killing of a strain like WHO-G, which expressed only LOS without β-chain.
Moreover, the absence of a decrease in the titer after incubation with GMMA 2HexG+ suggests the importance of expressing a long α-chain to elicit functional antibodies, as sequestering the antibodies against 2HexG+ structures does not have an impact on the bactericidal titer.Furthermore, from this analysis, we observed that the incubation of this functional serum with GMMA 5HexG- resulted in a marked decrease in the bactericidal titer. In this case, it was theorized that in the immunization, functional bactericidal antibodies against epitopes present in such structures were elicited. These antibodies could possibly target an epitope involving the LOS terminal GalNAc portion, similar to the anti-LOS bactericidal antibodies that are present in the normal human serum, and this binding is not influenced by the presence of the β-chain. It should also be pointed out that this effect is certainly related to antibodies induced by the immunization since it is not observed in the unrelated mouse sera used as control.
These data confirmed that the key attribute for GMMA-induced functional antibodies is strictly related to the length of the α-chain, beyond the presence of a β-chain.

1.4 Discussion

Recently, several evidence have suggested that prevention of gonorrhoea infection might be possible with a multivalent OMV based vaccine. Accordingly, our research was based on applying the Generalized Modules for Membrane Antigens (GMMA) platform strategy for *N. gonorrhoeae* vaccine development. Among the main membrane surface components evaluated as potential vaccine candidate antigens, extensive research has been carried out on the role of the gonococcal outer membrane lipooligosaccharide, primarly focusing on the impact of this antigen on the pathogenicity, host-adaptation and protection against gonococcal infection. Nevertheless, the heterogeneicity of LOS related to phase variation mechanisms involving some *lgt* genes (*lgtA*, *lgtC*, *lgtD*, and *lgtG*) responsible for the synthesis of the oligosaccharide chains, may represent a challenge. Whilst several studies have shown the role of the LOS epitopes on the immune response, little scientific work has been done to investigate the effect of each of these glycoforms on the immune response induced by a GMMA or OMV-based vaccine.

In the first evaluation that was made to select an ideal GMMA-producing strain, several GMMA have been produced in the background of different gonococcal strains expressing different LOS pattern. Among the panel of strains, GMMA from detoxified SK92 and F62 were produced and tested *in vivo.* As reported in **Table 1.9**, SK92 strain (and, hence, the corresponding detoxified GMMA) were characterized by a single prevalent LOS structure, composed of a lactose on both the α-chain and the β-chain, representing the minimal structure required for mAb 2C7 binding. On the other side, F62 GMMA have LOS glycoforms characterized by long α-chains, composed of four or five sugars, and the β-chain is not expressed. Interestingly, a significant difference between the two samples was observed testing in hSBA the pooled sera from mice immunized with the detoxified SK92 and F62 GMMA, resulting in F62 GMMA eliciting high SBA titers if compared to the low hSBA titers obtained from SK92 GMMA (data not shown).

Beyond this first evidence, another important finding was that sera from mice immunized with FA1090 Δ*lpxL1* GMMA exposing highly truncated LOS glycan structures (2HexG-/3HexG- structures, **Figure 1.13**), presented low hSBA titers towards the majority of the tested heterologous strains; on the other side, FA1090 Δ*lpxL1* Δ*rmp* GMMA displaying both the β-chain and long α-chain structures (2HexG+, 4HexG+ and 5HexG+ structures, **Figure 1.13**) gave good bactericidal titers against the same tested strains. The observed variability in LOS isoforms expressed by these two samples could be attributed to the naturally occurring phase variation mechanism to which some of the *lgt* genes may undergo. These results provided further support for our hypothesis on a possible

relationship between the structures exposed on the GMMA surface and the cross-bactericidal activity of the derived sera.

In light of these data, this study was set out with the aim of assessing the role of the LOS oligosaccharide epitopes in a GMMA-based vaccine and, moreover, investigating the contribution of the different antigenically distinct LOS isoforms to the functional immune response elicited by a GMMA-based vaccine.

In order to understand whether and how LOS influences the GMMA immune response, GMMA from a *N. gonorrhoeae* FA1090 strain genetically engineered to expressing a highly truncated LOS deprived of the α-chain (*Ng* FA1090 Δ*lpxLI* Δ*lgtF*), were tested in competitive hSBA studies using functional sera from mice immunized with *Ng* FA1090 Δ*lpxLI* Δ*rmp*. A strong decrease in the bactericidal titers was observed incubating the sera with the Δ*lgtF* GMMA and subsequently testing it against four different heterologous strains, while no effect on the bactericidal titers were observed using *Ng* FA1090 Δ*lpxLI* Δ*lgtF* GMMA as competitors. These data suggested that antibodies directed against LOS oligosaccharide portion are relevant for the bactericidal activity against *Ng* heterologous strains of the sera derived from immunization with a gonococcal GMMA-based vaccine. Afterwards, *Ng* FA1090 Δ*lpxLI* Δ*lgtF* GMMA were tested *in vivo*. The corresponding mouse sera showed a lower bactericidal activity for the majority of tested strains, with at least a five-fold decrease in the titer with respect to the *Ng* FA1090 Δ*lpxLI* Δ*rmp* used as positive control. These results further confirmed the role of anti-LOS antibodies in hSBA functional response.

Subsequently, a library of gonococcal detoxified MS11 isogenic mutant strains suitably enginereed to express a distinct LOS structure was used to dissect the contribution of the different LOS epitopes on GMMA immunogenicity. Firstly, GMMA from these strains were tested *in vivo* immunizing mice with a LOS-based dose. The analysis of the corresponding sera highlighted the fundamental role of antibodies elicited by the GMMA where LOS α-chain is composed of at least four monosaccharides (LNnT structures or GalNAc-LNnT), that directly correlated with higher bactericidal titers. Further support to these results was provided by the competitive hSBA studies, that showed a drastic inhibition in the functional activity of *Ng* FA1090 Δ*lpxLI* Δ*rmp* GMMA-based vaccine derived sera after incubation with GMMA expressing a long α-chain, irrespectively of the presence of the β-chain. In contrast no inhibition was observed by GMMA expressing a short α-chain and the β-chain.

In conclusion, this research work made possible to establish the crucial role of LOS oligosaccharide portion on the gonococcal GMMA immune response and subsequently, to dissect the influence of the different oligosaccharide moieties on the complement-mediated bactericidal killing, thus highlighting the importance of exposing preferably LOS glycoforms with longer α-chain structures to have a

promising gonococcal GMMA-based candidate vaccine, able to elicit cross-protective bactericidal antibodies against this pathogen.

In this study the serum bactericidal activity was used as a correlate of protection for the development of an effective gonococcal vaccine. As reported in the introduction, the extensive work of Gulati and colleagues on the efficacy of the 2C7 mimitope and the chimeric mAb 2C7, showing the importance of an intact terminal complement pathway to have a LOS-mediated protection, clearly highlight that complement mediated killing of the bacteria is relevant to *in vivo* protection, thus further supporting the relevance of the hSBA assay to test the functional activity. [167, 170, 218]

Nevertheless, since a potential implication of LOS in eliciting antibodies able to inhibit invasion was reported [174], a further study with more focus on the role of this antigen in the adhesion and invasion of host epithelial cells will be needed to design a full picture of the potential of LOS for the development of an effective gonococcal vaccine.

CHAPTER 2

2.1 Aim of the study

After assessing the key role of lipooligosaccharide in GMMA-based immune response, the next step was to dissect the influence of this promising antigen in the absence of other potentially contributing membrane components and without the support of possible synergistic activity related to them.
Hence, the purpose of this study was to select a delivery system capable of mimicking GMMA, either for membrane composition or for physicochemical characteristics such as size, lamellarity, bilayer fluidity, and antigen loading. In this context, liposomes provide several advantages.
The fundamental tenet in producing these *Neisseria* liposome-based vaccines is to mimic the native-like conformation of the antigen within bacterial or GMMA/OMV membranes by intercalating the antigen within the phospholipid bilayer of the liposome.
In the last few decades, liposomes have been widely explored as vehicles for the delivery of *Neisseria* antigens, particularly because of their adjuvant activity and ease of production. Nevertheless, studies focusing on liposomes carrying *Neisseria* LOSs as antigens have been reported only in the context of *Neisseria meningitidis.* [289-291] In 1992, Petrov and colleagues [289] described the incorporation of LOS into liposomal vesicles to obtain non-toxic and highly immunogenic LPS for immunization; therefore, they demonstrated changes in the immunomodulatory activity of LOS after incorporation into liposomes. Indeed, it is well known that these safe and biodegradable systems are able to reduce antigen toxicity and to activate both innate and adaptive immune responses.
The similarity between liposomes and GMMA resides on several characteristics, such as particle size and antigen loading per particle, making these two platforms easily comparable. However, liposomes have the advantage of carrying defined antigen vaccines, whereas GMMA have a more complex antigenic heterogeneity. Another aspect related to liposomes is that they can be used as carrier exposing solely the selected antigen, thus on one side avoiding the contamination of antigens that can be potentially problematic, such as, in the case of *N. gonorrhoeae,* the Rmp protein, an antigenically invariable membrane component capable of inducing antibodies that can block bactericidal activity of other protective antigens [268], and, on the other side, reducing the toxicity related to LOS, as demonstrated by Mistretta *et al.* [266]
Accordingly, the lipid composition was chosen to create vesicles as similar as possible to the GMMA membrane, taking into consideration the possible different effects of each constituent. Moreover, the physicochemical properties of the final particles can be modified by modulating the reaction conditions in the NanoAssemblr® system.

Therefore, liposomes have been prepared by inserting detoxified LOS into the lipid mixture composition, leveraging their amphiphilic properties, and thus obtaining liposomes bearing LOS, called *liposomal LOS* (or *LOS-liposomes*).

An essential step in investigating liposomes as antigen carriers is the set-up of techniques that can be easily applied to either GMMA or liposomes, allowing an easy and direct comparison between the two systems.

Eventually, in order to consider liposomes as potential carriers for LOS, it became critical to evaluate the impact of liposomal LOS on the induction of bactericidal antibodies. Both gonococcal lipooligosaccharide alone or incorporated in liposome carriers were tested *in vivo* in mice, using the corresponding GMMA as controls. Interestingly, the promising results highlighted the ability of liposomes to display gonococcal LOS in an optimal manner, also confirming the role of this antigen in eliciting a protective immune response, especially when presented in multiplicity on particles and even in the absence of other protein antigens that could contribute to the immune response.

2.2 Materials and Methods

2.2.1 LOS extraction and Western Blot

LOS was extracted from FA1090 Δ*lpxLI Δrmp* GMMA and analysed following the procedures reported in paragraph *Chap 1 – Materials and Methods - LOS extraction and physicochemical and immunochemical characterization.*

2.2.2 Preparation of LOS-liposomes

The preparation of DMPC:DMPG:Cholesterol:DMPE-mPEG2000:LOS liposomes was achieved via microfluidics (NanoAssemblr®, Precision NanoSystems Inc) processes based on previously developed methods [292-294]. Briefly, DMPC:DMPG:Cholesterol:DMPE-mPEG2000 lipid stock mixture was prepared in ethanol at 20,7:2,2:55:1 molar ratio. Another solution of LOS extracted from FA1090 Δ*lpxLI* Δ*rmp* GMMA was prepared solubilizing it in dimethyl sulfoxide (DMSO) at 1140 $nmol_{LOS}$/mL. Then, the two preparations were heated up to 65°C to equilibrate and then mixed. This lipid/LOS mixture and an aqueous phase (MilliQ® water) were injected simultaneously in the micromixer. The volume of lipid (organic) and aqueous phase injected depends on the manufacturing conditions that have been selected. Herein, all formulations were prepared at 13 mg/mL initial lipid concentration (LOS excluded), 2:1 v/v aqueous:organic flow rate ratio (FRR) and 12 mL/min total flow rate (TFR) (**Table 2.1**). All newly formed liposomes (3 mL) were then subjected to buffer exchange with PBS 1x *via* PD10 desalting column.

Table 2.1 Details of the liposome formulation and NanoAssemblr® conditions.

Liposome composition

	Component	Molar ratio
Liposome	DMPC	20.7
	DMPG	2.2
	Cholesterol	55.4
	DMPE-mPEG2000	1
LOS – Liposome	DMPC	20.7
	DMPG	2.2
	Cholesterol	55.4
	DMPE-mPEG2000	1
	LOS from GMMA FA1090 Δ*LpxL1* Δ*rmp* in DMSO (65°C/10min)	1.4

NanoAssemblr® conditions

Solvents	Water:Ethanol
Final Volume (µL)	2136 (2 mL)
TFR	12 mL/min
FRR	**2:1 (Aq:Lip)**
Temperature	RT

Solvents	Water:Ethanol/DMSO
Final Volume (µL)	3060 (3 mL)
TFR	12 mL/min
FRR	**2:1 (Aq:Lip)**
Temperature	RT

2.2.3 Liposome characterization

Cryoscopic Osmometer

To measure the osmolality of formulations, an Osmomat 030 osmometer (Gonotec GmbH) was used, according to manufacturer's instructions, to evaluate the correct buffer exchange and the final osmolality of the formulation. A volume of 50 µL/formulate was used for the measurements, the accepted osmolality range was in a range of ± 60 mOsm/Kg. The osmolality was 0.275 mOsm/Kg confirming the complete buffer exchange.

Negative Staining Electron Microscopy (NS-EM)

Transmission electron microscopy is a technique that allows to obtain resolution images of a biological sample and to analyze its integrity, dimension, state of aggregation, providing detailed structures. Morphological analysis of Liposome and LOS liposome was performed by Negative Staining Electron Microscopy (NS-EM). In NS-EM, heavy metal salt contrast which allows the visualization of the objects, is not applied to the object itself but to its environment. The electron microscope beam, consisting of high energy electrons, can cross biological material easier than the surrounding space, thus defining the structure of the sample.

Negative Staining Method

A volume 5 µL of samples in PBS 1X was loaded onto a copper 200-square mesh grid of carbon/formvar rendered hydrophilic by glow discharge (Quorum Q150R S). The excess solution was blotted off after 30 seconds using Whatman filter Paper No.1 and then the grids were negatively stained with NanoW (Nanoprobes) for 30 seconds and then blotted using Whatman filter Paper No.1 and let air dry. After air drying, the specimen is ready to be introduced into microscope. Micrographs were acquired using a Tecnai G2 Spirit Transmission Electron Microscope equipped with a CCD 2kx4k camera. The images were acquired using a Tvips TemCam-F216 (EM-Menu software).

Dinamic light scattering (DLS) and multi-angle dynamic light scattering (MADLS)

Size determinations including Z-average and polydispersity index (PDI) were determined using methods based on dynamic light scattering. This technique measures the time-dependent fluctuations in the intensity of scattered light which occur because the particles are undergoing Brownian motion. Analysis of these intensity fluctuations enables the determination of the diffusion coefficients of the particles which are converted into a size distribution. Measurements were made at 25 °C using a Zetasizer Ultra (Malvern Panalytical Ltd., Malvern, UK) and all measurements were repeated tree times. The Z average was used to determine the hydrodynamic diameter (nm) of particles where PDI

indicates the width of size distribution and the homogeneity of the particles in the system. Dispersions with a PDI of less than 0.2–0.3 can be considered as a homogenous system, where PDI 1 shows a heterogeneous dispersion. The instrument settings were 101ppose101ed automatically by means of the ZS XPLORER software (Malvern Panalytical Ltd., UK).

The zeta potentials of the liposomes were measured on a Zetasizer Nano (Malvern Panalytical Ltd., Malvern, UK). The zeta potential of a particle is the overall charge that the particle acquires in a particular medium. Measurement of the zeta potential of samples in the Zetasizer Nano is done using the technique of laser Doppler velocimetry. In this technique, a voltage is applied across a pair of electrodes at either end of a cell containing the particle dispersion. Charged particles are attracted to the101pposetely charged electrode and their velocity is measured and expressed in unit field strength as their electrophoretic mobility.

The MADLS technology was exploited to have a precise measurement even with less material or lower concentrations. This was possible through an automated series of single-angle measurements: backscatter (173°), side scatter (90°) and forward scatter (17°), that are combined to provide a single particle size distribution. Each particle concentration result reported is the average of three or more repeat of MADLS measurements, performed with an acquisition time of approximately 200s each on a Zetasizer Ultra (Malvern Panalytical Ltd., Malvern, UK). Refractive index values were automatically assigned by ZS XPLORER software. This analysis gave also the D10, D90 and D50 measurements value that are statistical parameters that can be read directly from the cumulative particle size distribution and indicate the size below which 10%, 50% or 90% of all particles are found.

2.2.4 In *vivo* studies and functional assays

Immunization study

Female six weeks old CD1 outbred mice were immunized intramuscularly 2 times at a day 1 and day 22 at a 1 $nmol_{LOS}$-based dose in 50µL, either adsorbed to Alum hydroxide (3 mg/mL) or without Alum. Mice sera collected three weeks after the second dose (day 42) were analyzed in pools in hSBA assay as described in *Chapter 1 > 1.2 Materials and Methods > 1.2.5 in vivo studies and functional assays.*

2.3 Results

2.3.1 Liposome as *naked* carrier: LOS exposed in an antigen-free delivery system

2.3.1.1 Liposome formulation: composition and preparation

Over the years, liposomes have been widely studied for their potential as drug or antigen carriers in formulations or as model systems for studying the properties of biological membranes with different lipid components. [295]

As our aim was to prepare liposomes as similar as possible to an OMV membrane, the first step was to properly select the lipid composition. Cholesterol was inserted to stabilize the membrane and avoid phase transition effects caused by phospholipids, together with two different phospholipids, dimyristoyl-phosphatidylglycerol and dimyristoyl-phosphatidylcholine. The choice of liposomal phospholipids depends on different factors, that need to be balanced to obtain an efficient liposome formulation for the delivery of the antigen under consideration. Among the variables, one important consideration is to keep the fatty acid chain length constant for all phospholipid components. In our case, since LOS is an amphipathic lipid, it should be considered as an integral part of the liposome partially inserted in the liposome bilayer (Lipid A portion) and in part expressed on the surface (oligosaccharide portion); therefore, starting from this consideration, the other lipid components should have approximately 14-C or 12-C aliphatic saturated chains in the fatty acid residues of their hydrophobic tails, to be as similar as possible to LOS.

Moreover, liposome formulations can bear a PEGylated surface to extend the circulation half-life while in the body [263] and for this reason a PEGylated lipid, dimyristoyl-phosphatidylethanolamine-PEG2000, has been included in the formulation. In general, even if pegylation increases liposome circulation time, the immunological profile of the liposome needs to be balanced relative to the ligand/PEG content in order to obtain particle stability, high encapsulation, biodistribution, and presentation of the attached ligands. Herein, some of these characteristics do not represent a problem. Nevertheless, Goswami et al. observed that reduction of PEG from 2% to 0.3% in terms of molar ratio, resulted in increased mannose exposure on the LNPs. [296] For this reason, PEG in the formulation was added in a molar ratio of 1.2% with respect to the other components to obtain particles with a less probability of a PEG shield effect on the LOS oligosaccharide epitopes exposed on the surface.

The detoxified gonococcal lipooligosaccharide selected for liposome preparation was derived from GMMA of *Neisseria gonorrhoeae* FA1090 Δ*lpxLI* Δ*rmp*, obtained following an optimized water-phenol method for LOS extraction. Genetic detoxification of the lipooligosaccharide obtained by

knocking out the *lpxLI* gene ensures the formation of vesicles with a low reactogenicity profile. [248-253, 297]

Moreover, we should consider that the endotoxic activity of LOS can also be reduced by its incorporation into liposomal vesicles, while its adjuvant potential and, consequently, improvement of the elicited immune response are retained. [289, 298-301]

Figure 2.1 shows the composition and structure of the components selected for the LOS-liposome formulation.

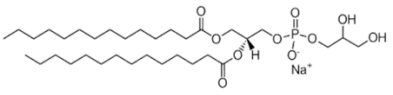
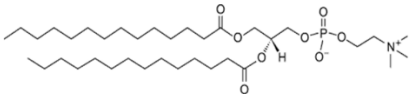
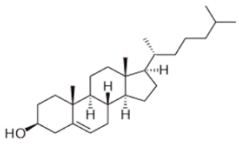
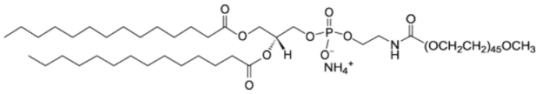
Component	Structure
DMPC 1,2-dimyristoyl-sn-glycero-3-phosphocholine 14:0 PC	
DMPG 1,2-dimyristoyl-sn-glycero-3-phospho-(1'rac-glycerol)(sodium salt) 14:0 PG	
Cholesterol	
DMPE-mPEG2000 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt)	

Figure 2.1 Structures and features of the components selected for the liposome formulations.

Liposomes were prepared by leveraging microfluidic techniques using the NanoAssemblr® Benchtop microfluidic instrument. NanoAssemblr® technology uses laminar flow microfluidic system to achieve rapid and consistent mixing to control the conditions of solvent/antisolvent nanoprecipitation, and hence, the physicochemical properties of the desired nanoparticles with high reproducibility. The mechanism involves nanoprecipitation induced by a polarity change in the solvent environment, wherein the solvent phase containing the starting materials is rapidly merged in a NanoAssemblr® microfluidic mixer with an aqueous solution, which drives liposome formation *via* nanoprecipitation. Among the parameters that may influence physicochemical characteristics, solvent and anti-solvent selection play important roles in successful formulation. In our case, according to a study by Joshi *et al*. [302] reporting how changes in the solvent and aqueous buffer can directly modulate liposome size, we chose ethanol and water as combinations of organic and aqueous phases.

Another important factor is the solubility of the starting material in the solvent, at or above the concentrations and temperatures used in the NanoAssemblr® process. In our case, we must consider that lipids with saturated tail groups have stronger intramolecular interactions, that make them difficult to dissolve in some solvents, and they are also usually characterized by higher phase transition temperatures ™.

The maximum concentration of solutes (solubility limit) is also affected by temperature; therefore, both concentration and temperature require consideration when developing these types of formulations; indeed, operating within the limits of solubility contributes to consistent results. Since formulating below the T_m of a lipid can lead to much larger particles and generally poor polydispersity or a multimodal population, we overcame this problem by heating the lipid mixture at temperatures of at least 10 °C above the T_m of the lipid species with the highest T_m.

Additionally, it is important to consider the poor solubility of LOS in ethanol, which may represent a challenge. Therefore, according to the manufacturer's instructions, we used the co-solvent method, introducing a second organic solvent in small quantities to enhance the solvent power of the primary one. Herein, the selected solvent is dimethyl sulfoxide (DMSO), following the manufacturer's guidelines, in which it was reported a similar approach to prepare liposome composed of phosphatidylcholine, phosphatidylglycerol and monophosphoryl lipid A, a compound with a nature similar to LOS. Considering the quantity of LOS in GMMA, the corresponding LOS amount in terms of $nmol_{LOS}$ was solubilized in dimethyl sulfoxide (DMSO) and heated at 65°C to fully dissolve. This solution was then mixed with the other lipids, previously resuspended in ethanol and equilibrated at 65°C.

These lipid mixtures were used as organic phase in the NanoAssemblr® process to successfully produce the desired liposomes. The selected liposome preparations and their corresponding reaction conditions are listed in **Figure 2.2**.

	Component	Molar ratio
Liposome	DMPC	20.7
	DMPG	2.2
	Cholesterol	55.4
	DMPE-mPEG2000	1

	Component	Molar ratio
LOS – Liposome	DMPC	20.7
	DMPG	2.2
	Cholesterol	55.4
	DMPE-mPEG2000	1
	LOS from GMMA FA1090 Δ*LpxL1* Δ*rmp* in DMSO (65°C/10min)	1.4

Figure 2.2 Composition and molar ratio of the different liposome formulations.

After assembling the liposomal vesicles, the buffer in the preparations was exchanged to PBS 1x using desalting size-exclusion chromatography pre-packed columns (PD10 desalting column), and the samples were analyzed using a cryoscopic osmometer to verify the correct occurrence of the buffer exchange.

2.3.1.2 Development of techniques for the characterization of GMMA and Liposome

Liposomes have been characterized using different physicochemical techniques.

Firstly, negative staining transmission electron microscopy (NS-EM) was performed on all the samples in order to verify the correct assembly of these vesicles and to characterize their integrity, size and shape. By analyzing these liposome preparations, it was possible to confirm the presence of round-shaped unilamellar vesicles with diameter of 70-75 nm, as shown in **Figure 2.3**. Moreover, comparing the two liposome preparations, it was observed that liposomes maintained their integrity in the presence of LOS.

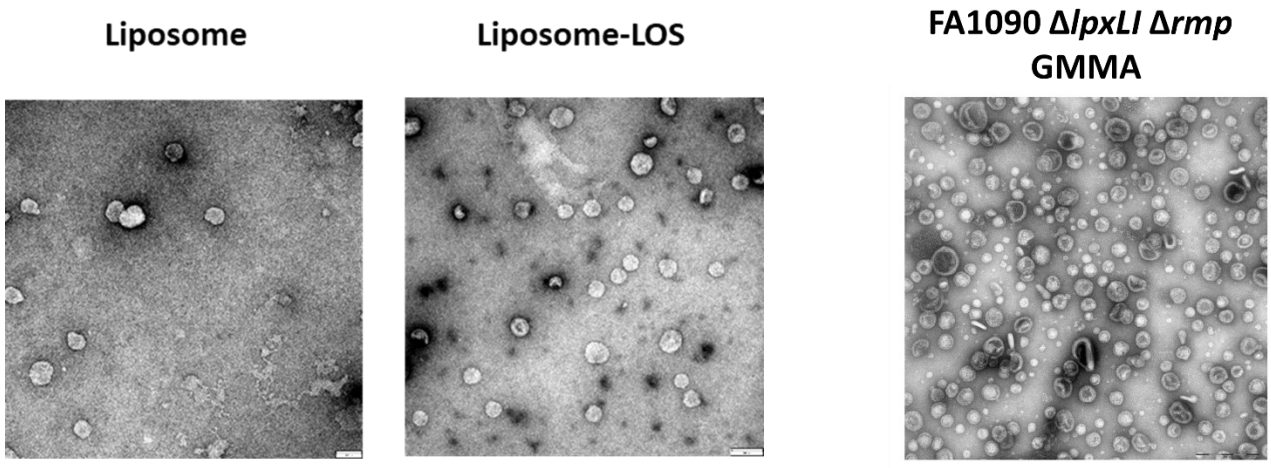


Figure 2.3 NS-EM negative staining analyses of liposome and LOS-liposome samples (*on the left*). GMMA NS-EM analysis was reported to compare the vesicles size and integrity. NS-EM confirmed the similarity between size of liposomes and GMMA, as further verified by DLS.

To further confirm the size and to analyze the different liposome formulations, DLS and MADLS analyses were performed using Zetasizer Ultra, proving the presence of monodisperse small vesicles. The hydrodynamic radius dimension measured by dynamic light scattering (DLS) was comparable between GMMA and liposomes, with a range of values between 70 and 75 nm. Moreover, the value of polydispersity index (PDI) of the two sample of liposome is even lower than the GMMA, indicating that these vesicles are in a monodisperse suspension.

A more in-depth characterization of these vesicles was performed by multi-angle dynamic light scattering (MADLS). MADLS is a fully automated process that measures the correlation function in

three scattering directions – backscatter (173°), side scatter (90°) and forward scatter (17°) – and uses that data from three angles to create an angle-independent result. This gives a particle size distribution with a higher resolution, beyond that attainable with a single-angle DLS experiment alone. The additional value is related to an extension to MADLS that affords the ability to directly test particle concentration. The measurement of particle concentration combined with the LOS quantity by SE-HPLC analysis allowed to obtain the LOS quantity/particles value, indispensable for comparing GMMA and LOS-liposome. Moreover, the D10-50-90 indicates the size below which 10%, 50% or 90% of all particles are found. The difference among the three measurements needs to be similar to have monodisperse samples. Herein, this value reflects what seen with the PDI index results, confirming the monodispersity of the sample.

The Z-potential of the liposome preparation was estimated using Zetasizer Ultra in order to verify the stability of this formulation. The zeta potential of a particle is the overall charge that the particle acquires in a particular medium. The magnitude of the zeta potential of particles in a colloidal system indicates system stability. Here, the value was around – 5mV, corresponding to what was expected according to literature data for liposomes with PEG in their formulation [303]. Indeed, starting from a value of approximately -40 mV for liposomes with PEG, by increasing the concentration of PEG in the formulation, the absolute value of Z-potential became lower until a plateau around -5 mV. This trend was confirmed by analyzing the corresponding formulation of liposomes prepared without PEG, showing a higher Z-potential value, around -11,07 mV.

As for the GMMA preparation, the analysis of the LOS content is performed by the quantification by SE-HPLC analysis of the reactive carbonyl groups of the oligosaccharide portion, derivatized with SCA after acid hydrolysis to remove the Lipid A.

The recap of the characterization of liposome, LOS-liposome and GMMA are reported in **Table 2.2.**

Table 2.2 Characterization of the liposome samples and GMMA through different techniques.

Sample	DLS		MADLS		Z-potential		LOS quantity by HPLC analysis	LOS quantity/ particles
	Z-average (d.nm)	PDI	D10-D50-D90 (nm)	Number particles (particles/mL)	Measures (mV)	Average Z-pot	LOS quantity ($nmol_{LOS}$/mL)	LOS quantity ($pmol_{LOS}$/ particles)
Liposome	69.49	0.10	-	2.30 E+11	-3.62 -3.50 -4.91	-4.01	-	-
LOS-Liposome	72.01	0.15	68.87 70.85 72.83	3.33 E+11	-4.89 -5.11 -5.99	-5.33	38 $nmol_{LOS}$/mL	0.2 $pmol_{LOS}$/ particles
GMMA FA1090 *ΔlpxLI Δrmp*	73.34	0.28	57.20 58.85 60.49	1.16 E+12	-		302.6 $nmol_{LOS}$/mL	1.6 $pmol_{LOS}$/ particles

From a qualitative perspective, liposomes were analyzed by Dot Blot, spotting our liposome and the corresponding GMMA and using anti-LOS mAb 2C7, which is able to recognize a saccharide epitope of LOS (data not shown). Dot blot confirmed that at least a certain percentage of the oligosaccharide fraction of LOS is oriented outward and therefore allows binding by the mAb.

In addition, Western blot was performed on the sample. Firstly, a 4-12% polyacrylamide gels was run in a MES buffer to perform a Western blot using anti-LOS mAb 2C7, able to recognize all the LOS structures of the FA1090 LOS. This allowed a fast screening of the LOS presence, confirming that it is present only in the liposomal LOS and extracted LOS alone, and as expected, no bands appeared in the liposome alone line. Afterwards, the sample were tested in a 16% tris-glycine gel - Western Blot with mAb 2C7 to check if all the three structures of LOS were incorporated into the liposome, besides the differences in their oligosaccharide portion (**Figure 2.4**).

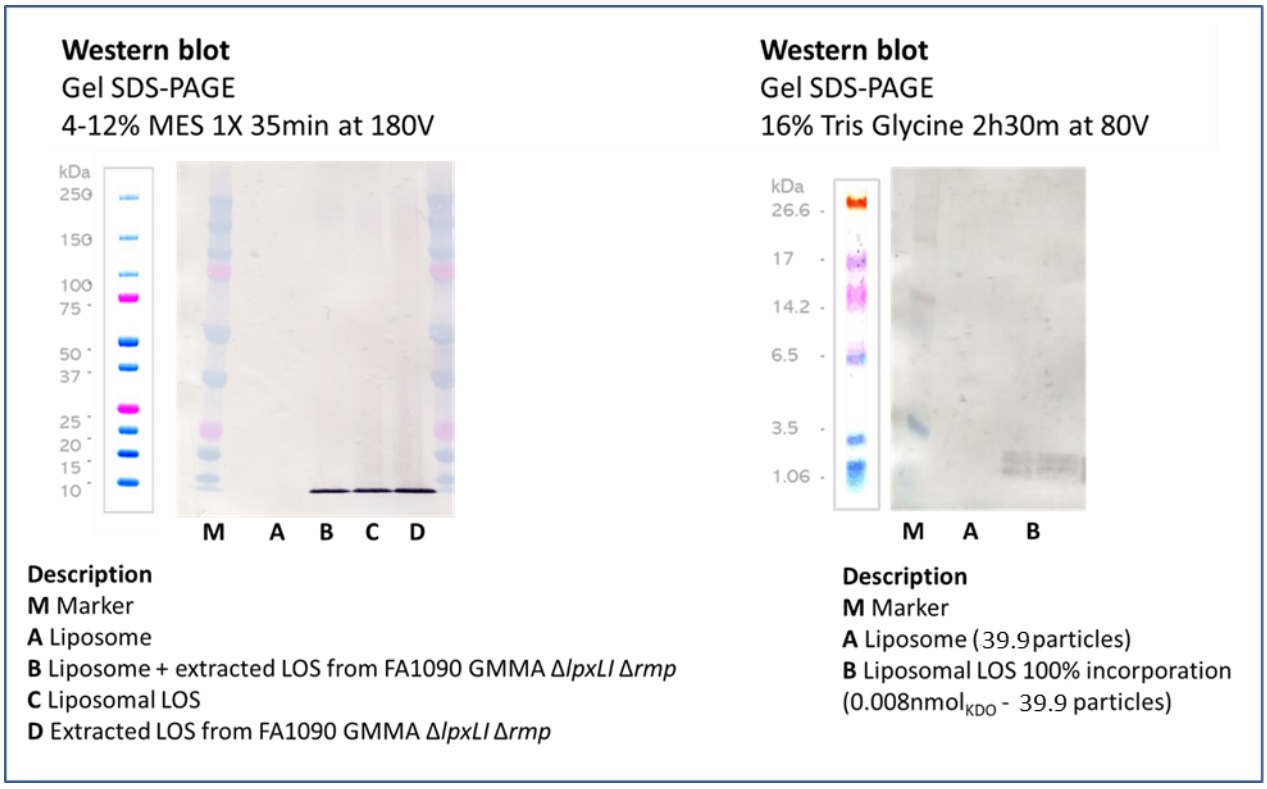


Figure 2.4 Western Blot analyses performed on the liposome sample to check the presence of LOS and the presence of the three glycoforms recognized by mAb 2C7.

The stability of these formulations under different storage conditions (4°C, -20°C and -80°C) and at different time point (24h, 72h, a week) was evaluated analyzing the sample by DLS (Zetasizer Ultra). A slight increase in size, from 70.9 d.nm up to 75.1 d.nm, was observed for LOS-liposome after a week at 4°C, while its PDI remained similar (from 0.15 to 0.17). Storage at 4°C was the only temperature condition that allows to preserve the samples. As reported in literature, for long-term storage of these preparations storage at -20°C and -80°C of liposome might be possible using proper cryoprotectors or leveraging on freeze-drying techniques [304].

The impact of sterile filtration (0.22 μm) was also assessed. DLS analysis of the sample before and after this step showed that the formulation maintained the same size and PDI characteristics.

2.3.1.3 Investigation of the immunogenicity of LOS exposed in liposome carrier on the bactericidal immune response in mice

The two vaccine delivery systems were compared for their ability to induce bactericidal antibodies against different *Neisseria gonorrhoeae* strains.

Liposomes with and without LOS were tested together with the corresponding GMMA and free LOS as controls, by immunizing mice at a 1 $nmol_{LOS}$-based dose, either adsorbed to Alum hydroxide (3 mg/mL) or without Alum (**Table 2.3**). In the case of GMMA, the dose in term of protein quantity

corresponds to 3,7µg. In the case of the liposome scaffold, the dose matches to the corresponding number of particles of LOS Liposome, measured by MADLS.

Table 2.3 Doses and details of the samples test in this *in vivo* immunization study.

Antigen	Dose ($nmol_{LOS}$)	Adjuvant
Extracted LOS from GMMA FA1090 *ΔlpxLI Δrmp*	1	-
Extracted LOS from GMMA FA1090 *ΔlpxLI Δrmp*	1	Alum hydroxide 3mg/mL
Liposome	Corresponding number of particles	Alum hydroxide 3mg/mL
LOS Liposome	1	-
LOS Liposome	1	Alum hydroxide 3mg/mL
GMMA FA1090 *ΔlpxLI Δrmp*	1	-
GMMA FA1090 *ΔlpxLI Δrmp*	1	Alum hydroxide 3mg/mL

Sera were collected three weeks after the second dose (day 42) and analyzed in pools in hSBA assay. The serum antibody functionality was tested using a panel of *N. gonorrhoeae* strains, including the homologous FA1090 strainand three different heterologous gonococcal strains, F62, WHO-N and WHO-G, all displaying different LOS structure (**Table 2.4**)

Table 2.4. Prevalent LOS phenotypes expressed by the selected *Ng* strains.

Strain	LOS phenotypes					
	2HexG-	4HexG-	5HexG-	2HexG+	4HexG+	5HexG+
FA1090				✓	✓	✓
F62		✓	✓			
WHO-N					✓	✓
WHO-G	✓	✓				

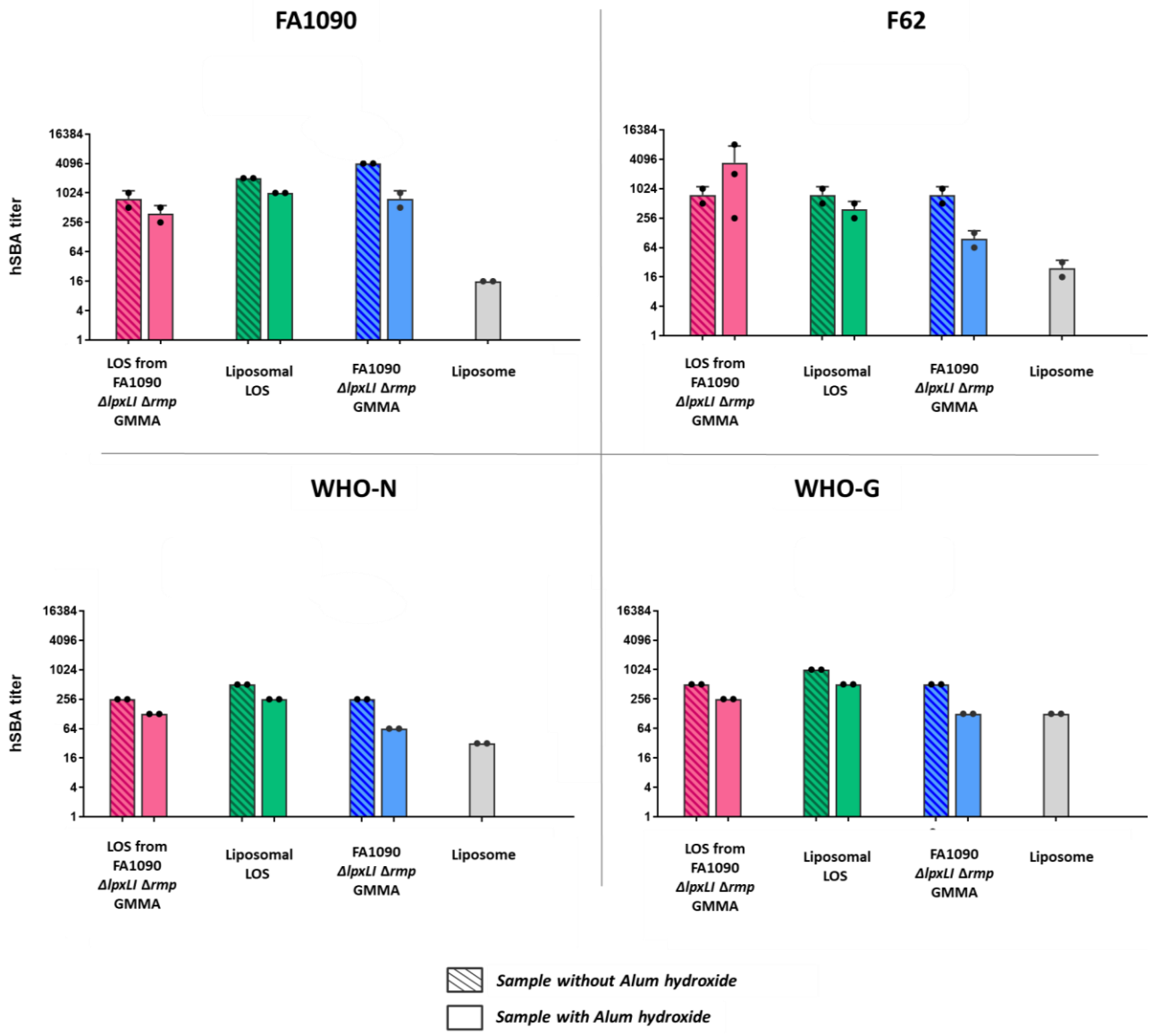


Figure 2.5 hSBA titers measured against the indicated Gonococcus strains on pooled sera from CD1 mice immunized twice with LOS extracted from FA1090 Δ*lpxL1* Δ*rmp* GMMA, liposomal LOS and the corresponding FA1090 Δ*lpxL1* Δ*rmp* GMMA. All the samples were tested either with or without Alum hydroxide (3 mg/mL) as adjuvant. Each hSBA titer is the mean of two or three different analyses.

A comparison of the results (**Figure 2.5**) revealed that LOS-liposomes induced slightly higher bactericidal responses against both the homologous FA1090 strain and the majority of the heterologous strains with respect to the LOS alone and the liposome scaffold (used as negative control). These results suggest that liposomes could be optimal carrier to be further exploited for the delivery of LOS, exposing this antigen in an ideal manner and possibly improving the response with respect to the antigen alone, similarly to what was observed in other previous studies with meningococcal LOS. [155, 289] However, in the case of the heterologous F62 strain, this effect was not observed, but the LOS alone and the liposomal LOS hSBA titers remains still comparable.

Another interesting observation that emerged from the data was that the bactericidal antibodies elicited by liposomal-LOS derived sera gave hSBA titers against the homologous FA1090 strain that are comparable to the GMMA-derived sera results. This observation is relevant especially if we consider that, in liposomes, we do not have an abundant protein component that might be able to induce an effective immune response by themselves. Moreover, when looking at the results in hSBA against the 3 heterologous strains, the liposomal LOS group reported slightly higher titers than the GMMA group. This results also indicated that the high response of the GMMA in hSBA against the gonococcal heterologous strains could be mediated by bactericidal anti-LOS antibodies.

In summary, these results provide important insights into the role of LOS in the hSBA response after immunization with a liposome or OMV/GMMA vaccine, suggesting that LOS could be a key antigen to be taken into consideration for the development of a gonococcal vaccine.

2.4 Discussion

Once established the role of LOS in the GMMA-induced immune response against several gonococcal heterologous strains, the elucidation of the impact of the LOS oligosaccharide portion without any other potentially contributing OM component deserved a deep examination.
LOS-derived oligosaccharide epitopes have been considered as potential gonococcal candidates and several attempts were made to explore them as vaccine antigen. It is well-known that carbohydrate immunogens evoke T-cell independent responses, stimulating the production of low affinity IgM antibodies predominantly, without affinity maturation. For this reason, an approach that has been applied to overcome this issue is to chemically conjugate the saccharides to a carrier protein, thus switching the immune response towards a T-cell dependent one.

However, in the case of gonococcal LOS, this strategy has proved difficult to apply since the purification of the oligosaccharide portion from LOS may result in a change in configuration, and hence in its antigenicity, thereby modify immunogenicity. On the other side, the precise configurations of OS structures within intact LOSs are not known, thus making the synthetic approach difficult to be exploited and, moreover, optimizing the production of the correct isomers may not be possible without advance structural knowledge. [305]

To circumvent the potential limitations of LOS-derived oligosaccharide as vaccine antigen, we embarked on a different strategy, taking advantage of liposome as *naked* carrier. The idea was to have functionalized lipid vesicles, able to mimic GMMA, thus enabling a direct comparison of the LOS-induced hSBA response when this antigen is exposed on vesicles alone or in presence of the other OM components. This strategy allows, on one side, to confirm the impact of LOS on the hSBA response and on the other side to understand whether this effect is due entirely to this antigen or if the influence of other components may lead to a synergistic effect.
In addition, this study permits a direct comparison among these two vaccine platforms. Liposome turned out to be an interesting technology to produce vesicles exposing single and defined antigens, thus bypassing the heterogenicity related to GMMA or OMV vesicles and avoiding the presence of disadvantageous components. Moreover, in this case, another important benefit derived from the exposure in liposomal vesicles is the reduction of the toxicity of LOS [266], although the LOS that was used here for the liposome preparation was extracted from GMMA vesicles and, hence, already genetically detoxified. On the other side, GMMA constitute a straightforward platform combining the low-cost of production, high purification yields and ease of production, circumventing the steps of antigen purification and the assembly of the liposomal vesicle.

The results reported in this chapter indicated that LOS is a crucial constituent to elicit a bactericidal response against Gonococcus, especially against heterologous strains. Nevertheless, the bactericidal activity against the homologous strains was maintained testing liposomal LOS. LOS exposed in liposomes confirmed the importance of the multi-exposure of this saccharide antigen on a vesicle, showing a slightly positive trend in hSBA if compared to the hSBA titers of LOS alone.

Therefore, it was confirmed that liposomes are a promising vaccine platform to develop a LOS-based gonococcal vaccine. For a full picture, additional studies will be needed to optimize this platform for the delivery of this type of antigen playing with different parameters, such as the lipid formulation or the combination with other adjuvants. A more in-depth study of the immune response may include the comparison of antibody persistence of GMMA and LOS-Liposome and the antibody subclasses elicited and their relationship with functionality.

CONCLUSIONS

The retrospective epidemiological study of Petousis-Harris *et al.* in 2017, reporting the potential protective immunity against gonorrhoea after a national immunization program in New Zealand with the MenB OMV vaccine MeNZB® (Novartis), was one of the first analysis leading to a renewed interest regarding the feasibility of a vaccine against *N. gonorrhoeae.* In light of this, OMV and GMMA platform represents an attractive platform to be exploited.

Among the components that turned out to be interesting as major antigens for a gonococcal vaccine, lipooligosaccharide represent one of the most studied and debated. Several studies have been focusing on the variety of effects attributable to the different LOS glycoforms, both on pathogenesis, by inducing host inflammatory responses, and on evasion of host innate immunity, through sialylation. Epitopes within LOS are also potential vaccine candidates. Indeed, a promising peptide mimic of the 2C7 epitope have been reported to elicit cross-reactive bactericidal antibodies in mice and to attenuate gonococcal vaginal colonization in a mouse model of infection. [169]

Nevertheless, little was known about the factors which are thought to contribute to elicit a protective immune response after immunization with gonococcal GMMA-based vaccine and, moreover, the influence of LOS on a gonococcal GMMA-based immune response had not been thoroughly investigated.

The present study was initially designed to determine the effect of this antigen on the immune response after vaccination with a GMMA-based vaccine. Generation of a mutant lacking the variable oligosaccharide moiety was generated to produce GMMA used to immunize mice. The findings clearly indicated that LOS has a role in promoting protection against gonococcal infection, eliciting antibodies that are bactericidal against the majority of the tested gonococcal strains. The second aim of this study was to investigate the effects of the different LOS glycoforms on the elicitation of bactericidal antibodies to assess which of them are crucial to have protection in hSBA. Data from immunization with GMMA from detoxified MS11 mutants, expressing defined lipooligosaccharide portions, highlighted the importance of long α-chains LOS structures to elicit a functional response, suggesting that having these structures can lead to the development of an effective gonococcal GMMA-based vaccine.

Lastly, liposome vesicles exposing LOS have been exploited as *naked* carrier. These vesicles, formulated in order to mimic GMMA composition but exposing solely LOS as antigen, have been instrumental to establish the impact of LOS in absence of other components, that may contribute to

the immune response. The LOS-liposome results offered us a key to understand the importance of this antigen in eliciting a functional response after an immunization with vesicles.

The insights gained from this study may provide a basis to support the development of novel GMMA-based vaccine to fight *Neisseria gonorrhoeae*. Nevertheless, underlining the importance to further explore the use of lipid-based vesicles, this research paves the way towards design of new strategy to develop or improve vaccine against this concerning anti-microbial resistant pathogen.

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DISCLAIMER

TRANSPARENCY STATEMENT

This work was sponsored by GlaxoSmithKline Biologicals SA. Rossella Cuffaro is a PhD student at the University of Siena and partecipates in a post graduate studentship program at GSK, Siena, Italy.

ANIMAL SAMPLES

All animal sera used in this study derived from mouse immunization experiments performed at the GSK Animal Facility in Siena, Italy, in compliance with the ARRIVE guidelines, the current Italian legislation on the care and use of animals in experimentation (Italian Legislative Decree 116/92) and consecutive ministerial newsletter (Circolare Ministeriale n.8 del 22 Aprile 1994), and with the GSK Animal Welfare Policy and Standards. The animal protocol was approved by the Animal Welfare Body of GSK Vaccines, Siena, Italy, and by the Italian Ministry of Health.

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