

Membrane Protein as Novel Targets for Vaccine Production in *Haemophilus influenzae* and *Neisseria meningitidis*

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Abstract

Haemophilus influenzae and *Neisseria meningitidis* are gram negative, commensal bacteria naturally present in the nasopharynx. They are also naturally competent and suffer genetic mutations. *H. influenzae* causes diseases such as otitis media and pneumonia. While *N. meningitidis* causes pneumonia, meningitis and sepsis. With the introduction of a vaccination program, a decrease in cases and deaths caused by these pathogens were observed over the following years. Especially, in countries where these vaccines were included in the vaccination schedule and in endemic regions, such as the meningitis belt in Africa. However, there are serotypes, biotypes and strains that these vaccines do not cover. Thus, these strains, biotypes and serotypes are emerging as pathogenic ones. Concerning the health authorities because the diagnosis of these diseases is most of the times unreliable and treatment needs to be immediate, due to the rapid evolution of the disease. For these emerging bacteria novel immunogenic targets are being researched as a way of trying to find and design new vaccines. These vaccines can be aimed in membrane proteins. Using these proteins as immunogenic targets with the help of adjuvants, to boost the immune system. The burden of a death or sequel to children and even adults that undergo meningitis infection and treatment is high. Therefore prevention is the best alternative. The aim of this review is to present this novel targets and their pros and cons. As a way of enlighten researches to view this new group of molecules and ligands as a possible target.

Keywords: *Haemophilus influenzae*; Membrane proteins; *Neisseria meningitidis*; Vaccine; Membrane vaccines

Introduction

Haemophilus influenzae and *Neisseria meningitidis* are gram negative commensal bacteria to the human upper respiratory tract [1].

Haemophilus influenzae is a pleomorphic bacillus that has been associated with localized and invasive infections, such as bronchitis, otitis, pneumonia, meningitis, septicemia, and epiglottitis [2,3].

H. influenzae is also responsible for most of the meningitis in children between 2 and 5 years old [4,5]. *H. influenzae* type b (Hib) is the most invasive type of six capsular serotypes (a-f) and is recognized as a major cause of meningitis [6].

The NTHi is typically associated with moderate disease from the upper respiratory tract in children and pneumonia in adults with cystic fibrosis or chronic obstructive disease [7]. The NTHi is a predominant bacterial agent of the prevalent pediatric disease otitis media (OM), and is also responsible for multiple diseases of the upper and lower respiratory tracts of both children and adults, although a commensal of the upper respiratory tracts of healthy persons, is an important cause of acute, recurrent, and persistent infections of the human respiratory tract [4].

In the United States, the overall annual incidence of Hib meningitis in children aged 0 to 4 years was about 50 to 60 per 100,000 (ranging from 19 to 69 per 100,000) prior to vaccine availability; the average was 54 per 100,000. This incidence was greater than twice the weighted average for pre vaccination Europe, 23 per 100,000 [3].

After the introduction of the Hib conjugate vaccine in the Netherlands in 1993, the incidence of Hib infections strongly decreased to very low levels in all age groups. However, NTHi infections have been reported to increase over time after Hib vaccine introduction [8]. There is no vaccine available for the NTHi and there is an emergence of this pathogen [9]. There are still related cases in the literature of Hib invasive disease post vaccination period [10,11].

N. meningitidis is a gram negative coccus that is a major cause of meningitis and septicemia globally, predominantly affecting children and adolescents. Meningococcal meningitis and sepsis are devastating diseases that kills children and young adults within hours despite the availability of effective antibiotics. Mortality and permanent disability rates are high, even under optimal health care conditions has also been associated with these infections [12].

Meningitis caused by bacterial infections is more likely to come with complications such as high mortality and morbidity levels. Among survivors, up to 20% have significant sequelae, including neurologic disability, amputation, and hearing loss [13]. The infections by meningococcal in the United States occur sporadically or in small clusters, and the most common *Neisseria meningitidis* serogroups involved are B, C, and Y. In some parts of the developing world, most notably across the center of Africa, serogroups A and, more recently, W135 cause severe epidemic disease [14].

The diagnosis of meningococcal disease presents challenges to the clinician because symptoms are similar to those of less serious illnesses, the symptoms have a sudden onset, and the disease may rapidly progress to permanent disability or death [15,16]. *Neisseria meningitidis* remains a major and insidious cause of death, even in industrialized countries

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[16]. Public concern is further heightened when cases occur in school or college settings, resulting in mass immunization efforts as part of outbreak control [17]. The introduction of a universal vaccination program in the United Kingdom resulted in a meningococcal serogroup C carriage reduction of 66% in 15- to 17-year-olds and herd immunity as evidenced by a 67% reduction of disease incidence among unvaccinated infants, children and adolescents [18].

Methods

This review was conducted using the following research database: Pubmed and for the author's site google scholar. The words: *Haemophilus influenzae* vaccine, *Neisseria meningitidis* vaccine, *Haemophilus influenzae* membrane protein, *Neisseria meningitidis* membrane protein, *Haemophilus influenzae* antibiotic resistance, *Neisseria meningitidis* antibiotic resistance were chosen for research purposes. The period investigated was from January 1980 to November 2011 due to the low number of articles published in the field of membrane protein. Foreigner articles in Spanish and Portuguese were also assembled and consulted.

Antibiotics Resistance

One reason for developing new vaccines is the constant raise of antibiotic resistant strains of these bacteria. *H. influenzae* resistance to β -lactam antibiotics is an increasing problem. The resistance to ampicillin in this organism varies from 10% to 60%, depending on the geographical region [19].

According to a study by Dimopoulou *H. influenzae* resistant strains collected in the UK and Greece were β -lactamase positive and ampicillin resistant. It is also known that biofilm formation and the production of β -lactamase both contribute to NTHi antibiotic resistance. It's been shown in vitro that, NTHi 86-028NP and NTHi 86-028NP bla biofilms are both resistant to amoxicillin killing at all concentrations tested, 0-2 mg/ml [20].

When it comes to *N. meningitidis* the literature has more frequent information about its resistance to penicillin [21]. Penicillin resistance in *Neisseria spp* is thought to be generated by the interspecies transfer of genetic material from naturally penicillin-resistant, commensal species. The well-known resistance mechanism is a decrease in penicillin affinity of penicillin-binding protein-2 (PBP-2), decreased expression of the class 3 porin as well as decreased affinity of PBP-1 may contribute to higher level resistance of *N. meningitidis* to penicillin G and other β -lactam compounds [22].

Penicillin-resistant (penr) clinical isolates of *Neisseria meningitidis*, which do not produce β -lactamase, were first identified in Spain in 1985. Serogrouping, determination of serotypes and subtypes, and multilocus enzyme electrophoresis of the penr strains showed an extensive diversity. Resistance is due, at least in part, to a decreased affinity of penicillin-binding protein (PBP) 2 for penicillin. Genetic transformation of an *N. meningitidis* type strain to low-level penicillin

resistance with DNA from resistant meningococci and other *Neisseria* species resulted in transformants that possessed low-affinity forms of PBP 2. These altered forms of PBP 2 have been shown to arise from recombinational events that replace parts of the PBP 2 gene with the corresponding regions from the PBP 2 genes of commensal *Neisseria* species [23].

The Existing Vaccines

There are three types of vaccines available for the *Neisseria Meningitidis* types:

1. Polysaccharide - Meningococcal polysaccharide vaccines are available in either bivalent (groups A and C), trivalent (groups A, C and W), or tetravalent (groups A, C, Y and W135) forms to control the disease.
2. Outer membrane protein OMP - for group B - polysaccharide vaccines cannot be developed, due to antigenic mimicry with polysaccharide in human neurologic tissues.
3. Meningococcal conjugate vaccines against group C - Tetravalent A, C, Y and W135 conjugate vaccine have been licensed since 2005 for use in children and adults in the United States and Canada [24].

In June 2007, Advisory Committee on Immunization Practices - ACIP - from the Center for Disease Control and Prevention (CDC) revised its' recommendation to include routine vaccination of all persons aged 11-18 years with 1 dose of MCV4 at the earliest opportunity. Persons aged 11-12 years should be routinely vaccinated at the 11-12 years health-care visit as recommended by ACIP. ACIP continues to recommend routine vaccination for persons aged 19-55 years who are at increased risk for meningococcal disease: college freshmen living in dormitories, microbiologists routinely exposed to isolates of *Neisseria meningitidis*, military recruits, travelers to or residents of countries in which *N. meningitidis* meningitis is hyperendemic or epidemic, persons with terminal complement component deficiencies, and persons with anatomic or functional asplenia [24]. The capsule of group B meningococci (MenB) is poorly immunogenic and may induce autoimmunity [25]. There is currently no licensed commercial vaccine against serogroup B meningococci available in Europe or the United States [26]. This is a crucial fact, as *Neisseria meningitidis* group B (NMB) is now a predominant cause of the disease in industrialized countries [16].

Continuous studies have been developed, a novel tetravalent meningococcal glycoconjugate vaccine (MenACWY) included. Unlike the currently licensed vaccine, in which a chemically detoxified diphtheria toxoid is used as the carrier protein, MenACWY uses CRM-197, a natural mutant of the diphtheria toxin. The results of a randomized controlled multicenter trial of the safety, reactogenicity, and immunogenicity of this novel vaccine in infants, it was demonstrated that a primary immunization course of the novel

Existing Vaccines	Dosage	Advantages	Drawback
Menveo® (MCV4) (Meningococcal polysaccharide (Serogroups A, C, Y and W-135) Diphtheria CRM197 Conjugate Vaccine) - Novartis	0.5 ml - dose use for people between 2-55 years old a second dose after 2 months. (Intramuscular)	Prevents against serogroups A, C, Y and W-135.	Does not prevent <i>N. meningitidis</i> serogroup B infections. [27,28]
Menactra® (Meningococcal [Groups A, C, Y and W-135] Polysaccharide Diphtheria Toxoid Conjugate Vaccine - Aventis Pasteur	0.5 ml - 2 doses schedule for children between 9 to 23 months, 3 months apart. 11-12; 1 dose. (Intramuscular)	Prevents against serogroups A, C, Y and W-135. Can be used in infants.	Does not prevent <i>N. meningitidis</i> serogroup B infections [29].
Menomune®- A/C/Y/W-135 - Aventis Pasteur	0.5ml - 1 dose, 2 years of age and older. (Intramuscular)	Prevents against serogroups A, C, Y and W-135.	Does not prevent <i>N. meningitidis</i> serogroup B infections [30].

Table 1: Commercial available vaccines for *N. meningitidis* available in the USA.

tetravalent meningococcal glycoconjugate vaccine Men-ACWY was well tolerated and immunogenic for serogroups A, C, W-135, and Y when given to healthy infants at either 2, 3, and 4 months or 2, 4, and 6 months of age [13]. Immunological evaluation of the *N. meningitidis* 4 vaccines serogroups in children demonstrated it is less immunogenic in children than in adolescents [13]. In table 1 we can visualize the existing vaccines available for *N. meningitidis* today.

As for *H. influenzae* encapsulated strains, few vaccines in history have induced such a dramatic decline in incidence over such a short period as have the Hib conjugates vaccines [3]. Prior to the introduction of Haemophilus b Conjugate Vaccines, *Haemophilus influenzae* type b (Hib) was the most frequent cause of bacterial meningitis and a leading cause of serious, systemic bacterial disease in young children worldwide [31]. In addition to protecting against invasive infection, Hib conjugate vaccine prevents asymptomatic oropharyngeal (OP) Hib colonization or carriage. The lower prevalence of Hib carriage in the population decreases the risk of infection even among unvaccinated children through reduced transmission [32].

The World Health Organization recommends that Hib vaccine now be included in routine infant immunization programmes for all children, as appropriate to national capacities and priorities [24]. Several Hib conjugate vaccines are available from different manufacturers. All manufacturers use the capsular polysaccharide material of the bacteria and link it to tetanus toxoid, diphtheria toxoid, a diphtheria toxoid-like protein, or a mix of proteins from another bacterium. Each of these has been proven effective in the prevention of Hib disease [24].

There are three Hib conjugate vaccines licensed for use in infants <15 months old in the United States. The Hib conjugate vaccine polyribosylribitol phosphate *Neisseria meningitidis* outer membrane protein vaccine (PRP-OMP) (PedVaxHIB; Merck, Rahway, NJ) provides the earliest antibody levels thought to be protective against invasive disease. However, PRP-OMP vaccination does not achieve as high a peak antibody concentration after a full course as is seen after vaccination with either the Hib oligosaccharide CRM197 (HbOC) or polyribosylribitol phosphate tetanus toxoid (PRP-T) vaccines [11]. We can see the available commercial vaccines presented in the USA in table 2.

The Membrane Proteins in *H. influenzae*

A reasonable number of outer-membrane proteins in *H. influenzae* have been studied. Most of them came from isolates of *H. influenzae* type b that are usually examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [35].

When analyzed both encapsulated and non encapsulated *H. influenzae* strains showed protein components to contain up to 36

proteins of which 6 represent the major protein content. These major proteins have molecular weights between 50,000 and 15,000 and are labelled P1 to P6 in order of decreasing molecular mass. The proteins of the NTHi strains show greater variability in their migration patterns than do those of the *H. influenzae* type b strains. However, the general terminology of P1 to P6 has been transposed to the NTHi strains [36].

The P1 is a heatmodifiable surface exposed protein found in *H. influenzae* type b and NTHi. Significant variability in the primary protein sequence in the variable region of P1 and its ability to be modified by heat allowed it to be used as a form of subtyping for *H. influenzae* type b strains [35].

The P2 protein of different Hib strains was also studied, and it was indicated that some degree of antigenic heterogeneity existed among this protein from strain to strain. Being the P2 is a surface-exposed protein that functions as a porin in Hib and also represents the most abundant protein in the outer membrane of this pathogen [37,38].

According to a study by Tokudu et al. it is suggested that P2 molecules and surface antigens other than P2 are involved in the development of pulmonary defense against NTHi, and it also suggested that a host previously infected by a NTHi continues to be susceptible to infections by other strains of NTHi with different P2 epitopes [20]. The P4, protein, is a 28 to 30 kDa lipoprotein that is thought to be present in all encapsulated and nonencapsulated *H. influenzae* strains [36]. Figure 1 shows these important outer membrane proteins.

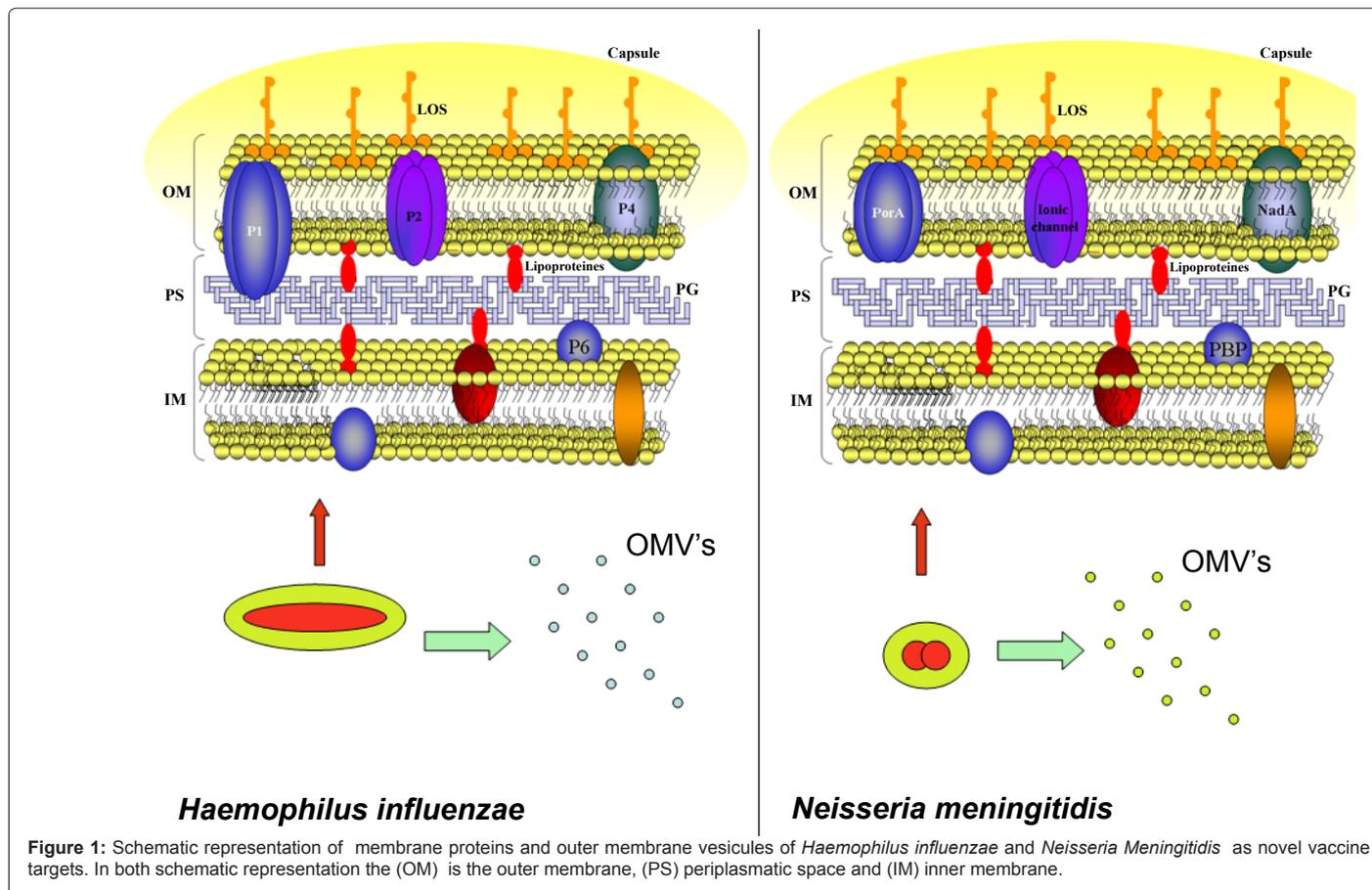
The Membrane Proteins in *N. meningitidis*

There are several distinct adhesins in *N. meningitidis* between those the NadA, YadA and UspAs have proven to be the more immunogenic ones. NadA is a protein of 362 amino acids with a possible leader peptide of 23 amino acids [39]. The NadA - Neisseria Adhesin A - is a surface exposed trimeric protein inducing bactericidal response in vivo. The *nadA* gene is present in approximately 50% of pathogenic meningococcal isolates and cluster into three well-conserved genetic and antigenic cross-reactive variants (NadA1-3) [40]. The PorA that composes the new vaccine against *Neisseria meningitidis*. Penicillin-binding proteins (PBPs) are conserved proteins that play a major role in peptidoglycan biosynthesis. PBP2 shows a highly conserved N-terminal part as well as highly conserved catalytic motifs in its C-terminal part [18]. The results obtained in this work using convalescent sera demonstrate the immunogenicity of meningococcal PBP2.

PBP2 is associated with the membrane fraction in *N. meningitidis* and it is also accessible in these fractions for binding of radiolabelled penicillin G.16. Moreover, whole cell ELISA using anti- PBP2 IgG clearly showed a dose-dependent binding of anti-PBP2 antibodies to intact bacteria. These data cast light on the immunogenic/antigenic

Existing Vaccines	Dosage	Advantages	Drawbacks
Hib - <i>Haemophilus b</i> Conjugate Vaccine (Tetanus Toxoid Conjugate) ActHIB® - Sanofi-Pasteur	1 dose of 0.5ml contains 10 µg purified polysaccharide, 3 doses with 6 to 8 weeks of interval. (Intramuscular)	Can be used in children as young as 2 months old - achieve high peak antibody concentration	Only protects against type b, and does not offer coverage to emerging non-capsulated groups [11,33]
<i>Haemophilus b</i> conjugate Vaccine (Diphtheria CRM197 Protein Conjugate) HibTITER®- Wyeth Lederle	1 dose contains 10 µg of purified <i>Haemophilus b</i> saccharide and 25 µg of CRM197 protein. 3 doses with 2 months interval. (Intramuscular)	Achieve high peak antibody concentration, offer long term protection.	Only protects against type b, and does not offer coverage to emerging non-capsulated groups [11].
Liquid PedvaxHIB® [<i>Haemophilus b</i> Conjugate Vaccine (Meningococcal Protein Conjugate)]- Merck	1 dose contains 7.5 µg of <i>Haemophilus b</i> PRP (polysaccharide capsule), 125 µg of <i>N. meningitidis</i> OMPC and 225 µg of aluminum (Intramuscular)	Provides the earliest antibody levels thought to be protective against invasive disease.	Only protects against type b, and does not offer coverage to emerging non-capsulated groups. Does not achieve high peak antibody concentration as the other two vaccines [11,34].

Table 2: Commercial available vaccines for *H. influenzae* available in the USA.



properties of meningococcal PBP2 [25]. Figure 1 shows these membrane proteins.

The Nouvel Vaccine Targets in *Haemophilus influenzae*

Interest in the *H. influenzae* Outer Membrane Proteins has centered mainly on their antigenic qualities as potential vaccine candidates [36]. However, one important criterion that must be met so that an Hib outer membrane protein can be a successful vaccine is that the protein must possess surface-exposed and antibody accessible antigenic determinants that are common to most if not all strains of this pathogen. At least three Hib outer membrane proteins appear to satisfy this requirement. Data concerning the P6 protein indicate that this protein has at least one surface epitope that is common to all strains of this pathogen [41].

P2 and P6 have generated the most interest to date as potential vaccine candidates against nontypeable *H. influenzae* [35]. P4 has one surface-exposed epitope was conserved across the 28 clinical isolates tested, while P5 is a heat-modifiable 27 kDa OMP. This protein is the lower-molecular-weight OMP, both of them, after in vitro and vivo trials did not demonstrated effective antigenicity [36].

Monoclonal antibodies raised against 8 epitopes of P1 protein from *H. influenzae* type b demonstrated significant areas of conservation among typeable and nontypeable strains. The potential for P1 as a vaccine candidate against NTHi shows mixed results at this stage. Antisera raised in rabbits to any of the eight conserved epitopes showed no bactericidal activity against NTHi [42].

The P2 major outer membrane protein of Hib and NTHi represents

one of the four protein antigens the most dominant band on SDS-PAGE OMP preparations. Purified P2 has been shown to induce the synthesis of antibodies protective against experimental Hib disease. Analysis of sequences of P2 from different strains reveals the presence of both heterogeneous and conserved surface-exposed loops of the P2 molecule among strains [43,44].

Bactericidal antibodies to P2 are present in normal human sera. Immunization with a P2-LOS complex in mice and purified P2 preparation in a rat model showed enhanced pulmonary clearance of a homologous strain of NTHi. Immunization with P2, however, did not enhance clearance as much as did immunization with P1 or P6 [36,44].

The study by Neary et al shows that antibody specificity to P2 loop 6 is high. With rabbits immunized as follows: 50 µg of loop 6 Multiple Antigenic Peptide (MAP) in complete Freund's adjuvant was administered subcutaneously on day 0, and 50 µg of loop 6 MAP in incomplete Freund's adjuvant was administered subcutaneously on days 14 and 28. Blood was obtained on day 35. And the antibodies were recovered by affinity chromatography demonstrating that P2 is a possible candidate for vaccination produce [22].

P6 (PAL) is a 16 kDa lipoprotein found in all *H. influenzae* type b and NTHi strains. It elicits bactericidal antibody [45]. Since this protein is highly conserved among encapsulated and non encapsulated *H. influenzae* strains [44], surface exposed, and immunogenic, its' as a potential vaccine candidate is highly explored. Experiments demonstrating enhanced pulmonary clearance of homologous and heterologous strains of NTHi, after gut immunization with the purified protein are encouraging. Mice and rabbits have been shown to produce

Target	Advantages	Drawbacks	Preliminary data
Outer Membrane Protein - P1	Significant areas of conservation among typeable and nontypeable strains	Only 1 epitope in common between 28 strains tested	Rabbit antisera presented bactericidal activity against NTHi [42].
Outer Membrane Protein - P2	reveals the presence of both heterogeneous and conserved surface-exposed loops of the P2 molecule among strains Bactericidal antibodies to P2 are present in normal human sera	Immunization with P2, did not enhance clearance as much as did immunization with P1 or P6.	Induce the synthesis of antibodies protective against experimental Hib disease. Immunization with a P2-LOS complex in mice and purified P2 preparation in a rat model showed enhanced pulmonary clearance of a homologous strain of NTHi [22,43,44].
Outer Membrane Protein - P4	One surface-exposed epitope was conserved	Did not presented effective antigenicity	After in vitro and vivo trials did not demonstrated effective antigenicity [36]
Outer Membrane Protein - P6	1 surface epitope common to all strains, conserved among encapsulated and nonencapsulated <i>H. influenzae</i> strains	Cannot be a transmembrane protein and is not conserved in all NTHi strains [49].	Mice and rabbits have been shown to produce bactericidal antibodies after systemic immunization with P6 and recombinant P6 [44-48].
lipooligosaccharide (LOS)-tetanus toxoid (TT)	Protective against NTHi strains	Not yet tested in children	Well tolerated in adults [49]
NTHi OMV	Protective against NTHi strains. Various heterologous antigens in the vesicles	Not yet tested in humans	Tests in mice demonstrate a cross-protective immunization [50].

Table 3: Novel Membrane proteins target in *Haemophilus influenzae*.

Target	Advantages	Drawbacks	Preliminary data
Neisseria Adhesin A- NadA	Predicted molecular structure strikingly similar to the known virulence-associated.	Based only in one antigen.	Is capable of generating local and systemic cellular and humoral immunity when coadministered with mucosal adjuvants [26,51,52].
Surface Porin - PorA	<i>N. meningitidis</i> strains express usually only one kind of porin class.	Vaccines based on this porin are effective only against clonal epidemics	Effective only against clonal epidemics [25,53].
Outer membrane vesicle - OMV MenBvac	vaccine consists of several recently discovered, relatively conserved surface antigens, NadA, fHBP, and NHBA, and a PorA	Protected only against homologous meningococcal strains.	All four doses of MenBvac are safe, no serious adverse events occurred [57,58].
rMenB-OMV -recombinant MenB with OMV	vaccine consists of several recently discovered, relatively conserved surface antigens, NadA, fHBP, and NHBA, and a PorA	Protected only against homologous meningococcal strains.	Clinical trials revealed that vaccinated individuals produce bactericidal antibodies, which protect against infection with homologous meningococcal strains [54,55].
Intranasal Neisseria OMVB	Intranasal vaccination with OMV, presenting complex antigens mixture.	Still in trials.	Immunogenicity and safety of a group B vaccine proved. [59].

Table 4: Novel membrane proteins target in *Neisseria meningitidis*.

bactericidal antibodies after systemic immunization with P6 and recombinant P6 [46]. P6 has been a vaccine candidate for nontypable *Haemophilus influenzae* (NTHi) based on its location on the outer membrane and immunogenicity. Because P6 is attached to the inner peptidoglycan layer of NTHi, and is putatively surface exposed, it should be a transmembrane protein. The study of Michel et al. examined the P6 structure using computational modeling, site-directed mutagenesis, and nuclear magnetic resonance spectroscopy and it was found that P6 cannot be a transmembrane protein, and therefore may not be surface exposed. A conclusion that there may be another protein on the surface of NTHi that has epitopes similar if not identical to P6 [47]. And in a study by Chang et al. [48] proved that P6 is not conserved in all NTHi strains. In table 3 we can see a comparison between these proteins and see which one would be more effective.

The Nouvel Vaccine Targets in *Neisseria meningitidis*

According to Magagnoli et al. [49] proteins from *N. meningitidis* were found to be capable of inducing bactericidal antibodies in mice, and were recognized by sera of meningitis patients. Among these proteins NadA (*Neisseria* Adhesin A) had a predicted molecular structure strikingly similar to the known virulence-associated adhesins YadA and UspA2. This led to the discovery of a number of relatively well conserved and cross-reactive surface proteins, of which factor H-binding protein (fHBP), neisserial adhesion A (NadA), and neisserial heparin-binding antigen in particular, warranted further investigation as potential components of a broadly cross- protective MenB vaccine [50].

In a study performed by Bowe et al. intranasal immunization of mice with 0, 3 and 6 weeks old, with NadA (10 µg), a conserved, putative adhesin found in serogroup B strains of *N. meningitidis*, is capable of generating local and systemic cellular and humoral immunity when coadministered with mucosal adjuvants in an intranasal via. Moreover, the antibodies induced are bactericidal, a correlate of protection against serogroup B meningococci [26]. One of the most promising among the alternatives being investigated while waiting for a definitive solution is vaccines based on outer membrane proteins (OMPs), especially those that use meningococcal class 1 OMP or porin A (PorA) [51]. The porin, proteins from pathogenic neisseria strains, are important for the serotyping of the antigen, and also for the vaccine development. The majority of the *Neisseria* specimen express only one kind of porin class, know as Por. These porins are targets for studies of serotyping and the development of vaccines [21].

Vaccines based on the major immunodominant surface porin, PorA, are effective against clonal epidemics but, thus far, have a limited scope of coverage against the wider MenB population at large [25]. Immunity against PorA tends to be highly subtype specific (specifically, for variable region 2 [VR2]), however, and so a single PorA vaccine component would achieve limited coverage against the more diverse MenB populations endemic to many countries and regions [50].

When it comes to the rMenB-OMV vaccine consists of several recently discovered, relatively conserved surface antigens, NadA, fHBP, and NHBA, and a PorA-containing OMV component [52]. This vaccine has met with some success. Clinical trials have revealed that vaccinated individuals produce bactericidal antibodies, which protect

against infection with homologous meningococcal strains, but since *N. meningitidis* species are subject to antigenic variation, they offer no protection against infection with heterologous strains [53].

A novel antigen that induces cross-reactive bactericidal antibodies against a number of *Neisseria meningitidis* strains, a 28 kDa lipoprotein called LP2086, was first observed within a complex mixture of soluble outer membrane proteins (sOMPs) following a series of fractionation, protein purification, and proteomics steps. Approximately 95 different neisserial isolates tested positive by Western blotting and PCR screening methods for the presence of the protein and the gene encoding LP2086. A gene encoding one variant of LP2086 was identified in our analysis of the Sanger Institute *N. meningitidis* serogroup A Z2491 early release of genomic sequence in contig form [54]. In table 4 we can visualize the targets and the advantages and drawbacks from them.

Membrane Antigens Distinct from Polysaccharides

An alternative approach to vaccine development is based on surface exposed proteins contained in outer membrane vesicles. These vaccines have been shown both to elicit serum bactericidal antibody responses and to protect against meningococcal disease in clinical trials [39].

The prediction of efficacy of *Neisseria meningitidis* serogroup B (MenB) vaccines is currently hindered due to the lack of an appropriate correlate of protection. For outer membrane vesicle (OMV) vaccines, immunogenicity has primarily been determined by the serum bactericidal antibody (SBA) assay and OMV enzyme-linked immunosorbent assay (ELISA) [55]. In another study by Feiring et al. [56] a MenBvac that is an outer membrane vesicle vaccine against systemic meningococcal disease caused by serogroup B *Neisseria meningitidis* showed that all four doses of MenBvac are safe, the MenBvac and the placebo had reactogenicity profiles of mild to moderate local and systemic reactions. Pain was the most common side effect, no serious adverse events occurred. This study confirmed the good immunogenicity of the primary course of MenBvac and demonstrated prolonged persistence and increased cross-reactivity of functional antibodies elicited by a booster dose [56].

Another type of OMV was tested to evaluate the safety and immunogenicity of an intranasal native outer membrane vesicle (NOMV) vaccine prepared from a capsule negative strain of group B of *Neisseria meningitidis*. In this study all volunteers received the same dose of vaccine, intranasal and serum vaccine-specific antibodies were measured as well as serum bactericidal activity. The vaccine was well tolerated without evidence of inflammation on nasal cytology. The group receiving the extra vaccine dose showed the maximum increase in bactericidal activity. We have demonstrated the immunogenicity and safety of a group B lipopolysaccharide-containing, intranasal, NOMV vaccine [57].

OMVs from *H. influenzae* NTHi were also made and tested in a study by Roier et al, the mice were vaccinated with 25 µg of OMV from NTHi intranasally or 2 µg of OMV from NTHi intraperitoneally. Antibody titers in serum to OMVs were monitored at four time points before (day 0), during (day 14 and 28), and after (day 39) the immunization period by using an indirect ELISA. This study has demonstrated an induction of cross-protective immunization [58].

Based upon sero-epidemiological data in humans and immunochemical data in laboratory animals, a lipooligosaccharide (LOS)-tetanus toxoid (TT) conjugate was prepared and evaluated for its safety and immunogenicity for phase I for NTHi *Haemophilus*

influenzae The LOS-TT conjugate is well tolerant in adults and a Phase II evaluation of the conjugate in children is planned [59].

Conclusions

Therefore there is still no ideal vaccine for *N. meningitidis* B or NTHi. These microorganisms suffer modifications from time to time so that they manage to evolve and escape the hosts' defense mechanism. Hence continuous research in the area is needed. *H. influenzae* and *N. meningitidis* are commensal bacteria; however once they manage to mutate or exchange DNA material, such as resistance plasmids - in case of *H. influenzae* - they can become pathogens and cause diseases. Vaccines targets are always going to be needed in order to accompany their evolution. Based in the research we can conclude that the most prominent vaccine candidates for *H. influenzae* would be the porin proteins P2 and the OMV strategy. Because the OMV carries more antigens and enables for the organism to be more exposed to them rather than just a single antigen (as verified in the figure 1). As for the P2 porin, it is probably one of the most conserved outer membrane protein in between strains of typable and non typable *H. influenzae*. The *N. meningitidis* OMV based vaccines will be probably be soon in the market for use after trials, since it would be the only vaccine against emerging serogroup B. In both cases a new via of administration for these vaccines is being studied and well tolerated: intranasal. Since both bacteria are upper respiratory tract commensal, the immunity response is enhance in this via at this moment. However, the antibodies production by intranasal administration via is not verified by long term (immunologic memory by years for example), which rise in doubt the real efficiency of this procedure. To sum up additional targets are available and being explored in order to bring new vaccines.

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