Journal of Immunology Research

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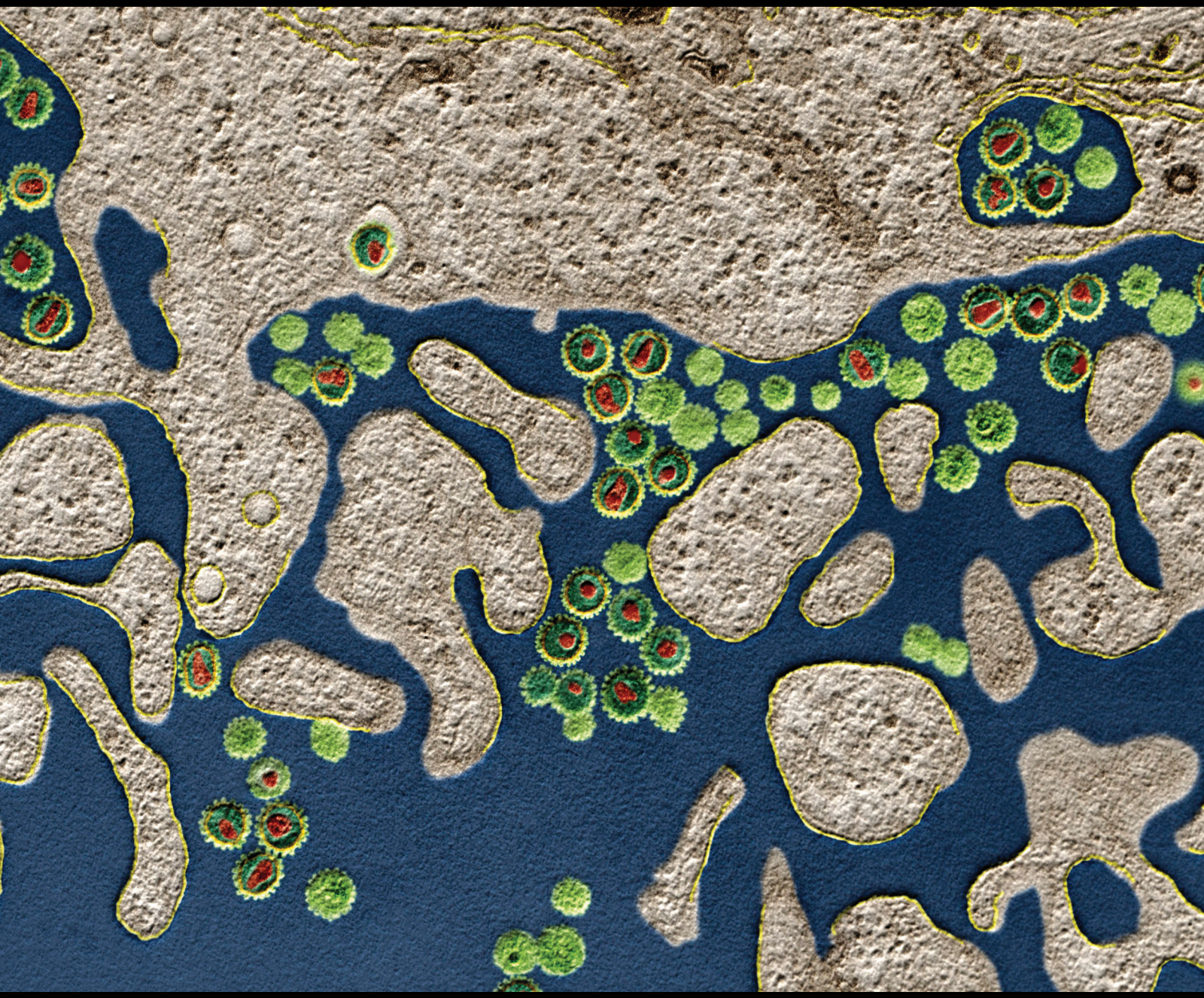
Guest Editors: Paolo Durando, Saul N. Faust, and Antoni Torres



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# Editorial Board

Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 934504, 3 pages
http://dx.doi.org/10.1155/2015/934504

*Editorial*

# Immunological Features and Clinical Benefits of Conjugate Vaccines against Bacteria

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Received 27 July 2015; Accepted 27 July 2015



Bacteria such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* are important pathogens that cause invasive and noninvasive diseases with a still high burden in terms of both morbidity and mortality worldwide [1–4].

The cornerstone for the prevention of these pathologies is by vaccination. In the last decades, significant advancement in the knowledge concerning both the mechanisms of pathogenicity of these pathogens, at a molecular level, and the features of the immune response to natural infection and vaccines have been achieved in humans, thanks to converging approaches of different disciplines, ranging from pathology to microbiology, immunology, vaccinology, and omics sciences (such as genomics and proteomics).

The acquisition of this knowledge is also of particular importance for public health policy makers, in order to establish new vaccines into clinical practice using well-designed immunization strategies.

First generation vaccines were based on bacterial capsular polysaccharides; yet, most of these antigens are considered T-independent antigens, showing significant gaps in terms of immunogenicity, particularly with respect to the generation of the immune memory [5–7].

The development of protein-polysaccharide conjugation technology in the 1980s allowed the availability of novel vaccines against *Haemophilus influenzae* type b (Hib) and different serogroups of *Neisseria meningitidis* [8, 9] that have demonstrated a very good safety and tolerability, together with the capability of eliciting a strong immunogenicity combined with the demonstration of the anamnestic antibody responses.

The main advantages of the conjugation technology used in bacterial vaccines, due to the generation of a T cell-dependent immune response, are briefly outlined:

(i) Improvement of the priming: immunogenic also in infants and young children (Ab-response, predominantly of the IgG1 isotype).

(ii) Capability of eliciting an immunogenic memory response (production of long-lived memory B-cells) and a booster effect upon new contact with the specific antigen (revaccination).

(iii) Capability of leading to affinity maturation of the Ab-response, with a consequent increased Ab-ag fit and improved opsonising function.

(iv) Generation of a mucosal immune response (secretory IgA and mucosally active IgG).

(v) Reduction of the mucosal carriage (a prerequisite of herd protection).

Since the implementation of the Hib conjugate vaccines [10] and their successful introduction into the paediatric immunization programme of some countries in the early 1990s, with the near elimination of Hib meningitis [11–13], it was clear that this was only the pivot of a series of successful experiences against other bacterial species relevant to public health globally.

The demonstration of the effectiveness of the immunization programs in children with these new generation vaccines was the direct consequence of their good immunological characteristics [14]. The implementation of safe and effective meningococcal type C (MenC) vaccines followed Hib vaccine programmes, with subsequent heptavalent pneumococcal conjugate vaccine (PCV7) from the mid 2000s, and further formulations expanding the antigens coverage (i.e., Men AC, Men ACW135Y, PCV10, and PCV13) [15–19].

These vaccines have proven effective for fighting not only invasive diseases, such as sepsis and meningitis, but also other important noninvasive diseases, such as community acquired pneumonia and acute otitis media in both children and adults, with new interesting perspectives for optimizing current prevention strategies in the future [20–22].

The herd protection observed among unimmunized populations living in countries where routine vaccination programs were initially implemented was due to the indirect effect of vaccination on nasopharyngeal carriage of the bacteria in healthy carriers. The radical change of their epidemiological and ecological pictures exemplified a further unanticipated positive impact of the wide use of these conjugate vaccines, further stressing how precious they were to obtain the control of the related diseases among the entire population [19, 23].

With respect to the very new and recently licensed meningococcal type B vaccine, a multicomponent approach to its development was used: efforts have been made to identify key-protein antigens capable of preventing Men B infection and associated invasive disease and possibly those sustained by other meningococcal serogroups too [24–27]. Whether new meningitis B vaccines can also provide population immunity remains to be seen.

Available evidence indicates that a majority of childhood meningitis mortality is preventable with existing Hib and PCV vaccines and these findings are consistent with the other empirical evidence and reviews [28]. The same can be extrapolated for the different available types of meningococcal vaccines (Men C, Men ACW135Y, and Men b) in Europe, depending on the different geographical area [29].

We hope that readers can appreciate the aim of this special issue of stimulating the continuing efforts within the scientific community in order to (i) understand the immunological interactions between conjugate and/or the other novel vaccine technology and the human host, (ii) develop novel immunization strategies for improving the prevention of *Streptococcus pneumoniae* and *Neisseria meningitidis* related conditions, and (iii) evaluate the conjugate vaccines use, particularly in terms of efficacy and effectiveness.

Immunologists, vaccinologists, microbiologists, together with paediatricians, infectious diseases specialists, and pulmonologists, general practitioners, public health experts, and policy makers could be mainly interested in the contents of the papers included in it.

## Acknowledgments

Guest editors wish to thank Cristiano Alicino, Ph.D., M.D., Andrea Orsi, M.D., and Cecilia Trucchi, M.D., Department of Health Sciences, School of Medical and Pharmaceutical Sciences of the University of Genoa, Italy, for the professional assistance.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 591580, 6 pages
http://dx.doi.org/10.1155/2015/591580

*Review Article*

# Impacts of the 13-Valent Pneumococcal Conjugate Vaccine in Children

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Received 30 September 2014; Revised 6 January 2015; Accepted 10 January 2015

Academic Editor: Antoni Torres



Applications of the heptavalent pneumococcal conjugate vaccine (PCV7) in the pediatric immunization schedule have dramatically reduced the incidence of pneumococcal diseases in both vaccinated children and unvaccinated individuals of all ages. However, increased infections caused by non-PCV7 serotypes have been reported by several groups. To overcome this problem, new vaccines covering more serotypes including the emerging serotypes have been developed. The 13-valent pneumococcal conjugate vaccine (PCV13) currently covers the 7 PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) and 6 additional serotypes 1, 3, 5, 6A, 7F, and 19A. After the first year of PCV13 applications in the immunization schedule in young children, global evaluation studies demonstrated that PCV13 provided a wider coverage and more effective prevention than PCV7 against invasive pneumococcal diseases (IPDs), mucosal pneumococcal diseases, and pneumococcal carriage. We reviewed the effects of PCV13 in the control of pneumococcal diseases in children based on previous studies.

## 1. Introduction

The heptavalent pneumococcal conjugate vaccine (PCV7) schedule in the pediatric population has significantly reduced the incidence of pneumococcal diseases in both vaccinated children and unvaccinated individuals of all ages [1]. This led to the conclusion that PCV7 not only was highly effective in vaccinated children but also could induce herd immunity, which limited the spread of pneumococcal diseases in the population living in the same geographic areas as the vaccinated children. For example, the US CDC has reported up to about 90% reduction of the incidence of invasive pneumococcal diseases (IPDs) caused by *Streptococcus pneumoniae* in young children with the introduction of PCV7 [2]. After the applications of PCV7, a significant decline in pneumococcal mucosal diseases such as acute otitis media (AOM) and nonbacteremic pneumonia has also been reported worldwide in children and in adults, especially in the elderly [3, 4]. While the incidence of pneumococcal diseases caused by PCV7 serotypes continued to decline with the introduction of PCV7, increased incidence of infections caused by non-PCV7 serotypes, mainly serotypes 19A, 7F, 6A, and 6C, has been reported by several groups [1], which reduced the global efficacy of PCV7 against pneumococcal diseases [5–7]. To overcome this problem, a new vaccine covering more serotypes, especially the emerging serotypes, has been developed. The 13-valent pneumococcal conjugate vaccine (PCV13) currently covers the most serotypes, including 7 PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) and six additional serotypes 1, 3, 5, 6A, 7F, and 19A. The carrier protein used in PCV13 remains the same as that in PCV7. Thus, all the capsular polysaccharides of the 13 serotypes included in PCV13 were conjugated with the nontoxic mutant of diphtheria toxin CRM 197. PCV13 was licensed to replace PCV7 in 2010 for children between 6 weeks and 5 years of age in the United States of America and the European Union [8, 9]. PCV13 was licensed on the basis of immunogenicity data alone through a putative protection correlate derived from pooled immunogenicity data and results of pediatric efficacy trials on PCV7 [10]. Therefore, assessments of the protection

effectiveness of PCV13 have drawn great interest immediately after it was licensed. Given the limitations of immunogenicity data in predicting protective efficacy conferred by vaccines [11], postmarket surveillance and effectiveness studies are highlighted for evaluating newly developed vaccines, particularly those with additional serotypes such as PCV13. Therefore, a number of studies have monitored the incidence of pneumococcal diseases in PCV13-vaccinated and PCV13-unvaccinated population and the circulation of pneumococcal serotypes in both patients and healthy subjects. In this review, we evaluated the impacts of PCV13 in younger children based on previous studies on the effects of PCV13 on the incidence of invasive pneumococcal diseases, pneumonia, acute otitis media (AOM), and nasopharyngeal carriage. We searched PubMed for eligible studies published from January 2010 to August 2014 using the key words, "PCV13" or "13-valent pneumococcal conjugate vaccine" and "children" or "pediatric." Only articles published in English were included in the evaluation.

## 2. Invasive Pneumococcal Disease (IPD)

Most studies on the effectiveness of PCV13 against IPD have demonstrated that PCV13 introduction has significantly reduced the incidence of IPD in both vaccinated children and unvaccinated population compared to the previous PCV7 applications, and the results were independent of countries, the scheme of PCV13 administration [12–18]. However, different effectiveness regarding the 6 additional serotypes has been reported.

The traditional 3 + 1 scheme of administration at 2, 4, 6, and 12 months was used in the USA. Recently, the US Centers for Disease Control and Prevention (CDC) have evaluated the impacts of PCV13 on IPD incidence through an active population-based surveillance in 10 regions around the country [12]. Continuous reduction of IPD cases due to new serotypes included in PCV13 has been observed in all groups of different ages during the first three months after the introduction of PCV13 in 2010. Particularly, 93%, 75%, 72%, 62%, and 58% reductions of the incidence of IPD caused by serotypes 1, 3, 5, 7F, and 19A were observed for subjects of <5, 5–17, 18–49, 50–64, and >65 years of age, respectively, in 2012 and 2013. However, analysis of the incidence of IPD caused by non-PCV13 serotypes revealed a possible early evidence of serotype replacement among adults of 18–49 and 50–64 years. In these two groups, incidence of IPD caused by non-PCV13 serotypes in 2012-2013 was 13% and 26% that were higher than expected when PCV13 was not available. This had only a marginal impact on the global effectiveness of PCV13 because of a significant reduction (about 30,000 cases) in overall IPD incidence and mortality (3,000 deaths) in all groups of different ages. The most significant reduction of IPD incidence was observed in the group of children aged <5 years for whom the IPD incidence was reduced by 64% in 2012-2013 compared to the time when PCV13 is not available. On the contrary, reduction in mortality was more important in adults, particularly in those older than 50 years.

It has been reported that the protective effectiveness of each serotype included in PCV13 was different. For example, it was 100% for serotype 7F, 76–96% for serotype 19A, and 13–93% for serotype 3. In addition, PCV13 administration was significantly effective in a special population, the Alaska Native people who are under higher risk of IPD than the general population of the USA. Singleton et al. have followed a total of 3,714 Alaska Native children (<5 years of age) who received the PCV13 vaccination between January 2009 and August 2011 according to the US recommendations [13]. They found only 9 cases of IPD including 7 cases caused by PCV13 serotypes between 2009 and 2011 (106.7/100,000/year), whereas 52 IPD cases including 31 cases caused by PCV13 serotypes were diagnosed between 2005 and 2008 (399.0/100,000/year; $P < 0.001$). In addition, no IPD cases were reported among PCV13 recipients during 2009–2011.

The 3 + 1 schedule was also applied in Canada and led to highly protective effectiveness. After the introduction of PCV13 in Canada, the incidence of IPD in children <5 years of age declined from 18.0 to 14.2 cases per 100,000 population in the period of 2010–2012 [14]. Specifically, PCV13 serotypes declined significantly from 66% (224/339) to 41% (101/244; $P < 0.001$) in children <5 years of age and from 54% (1,262/2,360) to 43% (1,006/2,353; $P < 0.001$) in children ≥5 years of age. Serotypes 19A, 7F, 3, and 22F were the most common serotypes in IPD cases reported in 2012. The IPD caused by serotypes 19A and 7F declined from 19% to 14% ($P < 0.001$) and 7F from 14% to 12% ($P = 0.04$), respectively, during 2010–2012. IPD caused by serotypes 22F and 3 increased from 7% to 11% ($P < 0.001$) and 7% to 10% in children of <5 years of age ($P = 0.22$), respectively, during 2010–2012 [14].

The 2 + 1 schedule (PCV13 administration at 2, 4, and 12-13 months) was started in England, Wales, and Northern Ireland on April 1, 2010, to replace the previous PCV7 schedule. According to the epidemiological data in the Public Health of England, 706 IPD cases were reported during April 2010 and October 2013, including 30 caused by PCV7 serotypes, 292 caused by the additional 6 serotypes of PCV13 or serotype 6C, and 414 caused by non-PCV13 serotypes [15]. Regarding the vaccination status of the studied children, it was reported that the PCV13 effectiveness after 2 doses in the first year or one dose after 12 months was 75% (95% confidence interval [CI], 58%–84%). The effectiveness was 90% (95% CI, 34%–98%) for the PCV7 serotypes and 73% (95% CI, 55%–84%) for 4 of the 6 additional serotypes of PCV13. In addition, the vaccine effectiveness of serotype 3 was not significantly different and the vaccine effectiveness of serotype 5 could not be analyzed because no case of infection due to this serotype was observed during the studying period [15].

In Denmark, a nationwide population-based cohort study was conducted to evaluate the dynamic changes of IPD incidence during three periods, the baseline (2000–2007), the PCV7 period (2008–2010), and the PCV13 period (2011–2013) [16]. In the study, a 21% reduction (95% CI, 17%–25%) of IPD incidence in the whole population and a 71% reduction (95% CI, 62%–79%) of IPD incidence in children

aged <2 years were reported after PCV13 introduction. In addition, a 28% reduction (95% CI, 18%–37%) of the 30-day mortality due to IPD was observed, decreasing from 3.4 deaths (95% CI, 3.2%–3.6%) to 2.4 (95% CI, 2.2%–2.7%) per 100,000 population after PCV13 introduction. The decline of mortality was observed in all groups of different ages and mainly in the nonvaccinated population, which is consistent with the report from the USA [16]. However, for serotypes 1 and 3, there were no significant changes in incidence beyond what would be expected from the natural cyclical patterns [16]. On the contrary, for serotype 19A for which a significant increase in disease incidence following PCV7 has been reported, the introduction of PCV13 was followed by a significant decline in the number of the cases towards baseline pre-PCV7 levels.

The high effectiveness of PCV13 (the scheme 2 + 1) was also reported in Israel by Ben-Shimol et al. based on a prospective surveillance of IPD during July 2004 and June 2013 [17]. The incidence of IPD caused by PCV7 6A serotype during the PCV13 application period decreased by 95% (incidence rate ratio [IRR] = 0.05; 95% CI, 0.03%–0.09%) including 90% in the PCV7 period and further 5% in the PCV13 period among the 2,670 IPD cases compared to the pre-PCV13 period. The incidence of IPD caused by the 5 additional PCV13 serotypes 1, 3, 5, 7F, and 19A increased initially by 47% but subsequently decreased by 79%, resulting in an overall 70% reduction during the entire study period (IRR = 0.30; 95% CI, 0.21%–0.44%). In total, a 63% reduction of IPD caused by all serotypes was observed in children aged <5 years (69% and 48% in children <2 and 2–4 years of age, resp.). However, a certain degree of replacement was evidenced because a twofold increase of IPD caused by non-PCV13 serotypes was found (IRR = 2.43; 95% CI, 1.73%–3.66%). The most commonly increased serotypes were 12F, 15 B/C, and 33F [17].

On the contrary, negative replacement phenomenon was reported in a study conducted by Levy et al., in which 1,406 cases of pneumococcal meningitis were reported in 2001–2012 [18]. Three years after the PCV13 administration, the number of pneumococcal meningitis cases significantly decreased by 27.4% ($P = 0.041$). Specifically, a 28.2% ($P = 0.039$) reduction was reported for children <2 years of age. Pneumococcal meningitis caused by the 6 additional PCV13 types decreased by 66.7%, but the number of cases due to nonvaccine serotypes remained stable. In 2012, 67.6% of cases were caused by the non-PCV13 serotypes including 12F (15%), 24F (15%), 22F (7%), and 15B/C (7%).

## 3. Pneumonia

A marked reduction of incidence of community acquired pneumonia (CAP) mainly in vaccinated children <2 years after the implementation of PCV7 has been reported in a number of countries [19, 20]. However, a number of worldwide studies have reported a significant increase of the incidence of severe CAP cases (i.e., empyema) caused by PCV13 serotypes 1, 3, 5, 7F, and 19A during the large scale administration of PCV7 [21]. Therefore, it was hypothesized that PCV13 could reduce the incidence of CAP in both vaccinated children and unvaccinated subjects.

This hypothesis was confirmed with the implementation of PCV13 in a number of countries. In Uruguay, PCV7 (schedule 2 + 1) was applied in 2008 and replaced by PCV13 (schedule 2 + 1) in 2010, and a catch-up immunization with a single dose of PCV13 was offered to children born between 2005 and 2009. It has been shown that PCV7 and the PCV13 schedules have significantly reduced the incidence of CAP in children 0–14 years of age compared to the CAP incidence prior to the vaccination in 2003–2007 and the introduction of PCV13 had further reduced CAP compared to the CAP incidence during the PCV7 administration period [22]. Particularly, the global CAP hospitalization rate was reduced by 78.1% by PCV7 compared to prior vaccination period and increased to 92.4% after PCV13. In addition, PCV13 introduction was accompanied by a relevant reduction of the incidence of CAP due to serotypes 1 and 5, which, on the contrary, increased in the years of PCV7 administration. However, a significant increase of the total number of CAPs caused by non-PCV13 serotypes was observed during the period of 2009–2012 [22].

A PCV13 schedule 3 + 0 (i.e., 3 doses at 2, 4, and 6 months without any booster) was introduced in Nicaragua in 2010. Becker-Dreps et al. compared the rates of pneumonia hospitalizations and ambulatory visits of CAP between the prevaccine (2008–2010) and vaccine (2011-2012) periods [23]. They found that the adjusted incidence ratio for all-cause CAP hospitalization between the vaccine and prevaccine periods was 0.67 (0.59–0.75) and 0.74 (0.67–0.81) among infants and 1-year-old children, respectively. The adjusted incidence ratio for ambulatory visits of CAP was 0.87 (0.75–1.01) and 0.84 (0.74–0.95) among infants and 1-year-old children, respectively. The low rates of health facility visits due to CAP among children of 2–4 years of age and 5–14 years of age were also observed, suggesting a significant herd immunity effect [23].

The pneumococcal serotypes 1, 3, 5, 7F, and 19A were the most frequently causes of CAP after the implementation of PCV7 and the switch from PCV7 to PCV13 in June 2010 in France, where an observational prospective study was conducted in 8 pediatric emergency departments between June 2009 and May 2012 CAP [24]. Children between 1 month and 15 years of age were included in this study and the CAP was confirmed through chest radiography examination. The incidence of CAP was compared among three periods (1 year for each period) including a pre-PCV13 (2009-2010), a transitional (2010-2011), and a post-PCV13 period (2011-2012). A total of 5,645 children with CAP including 365 and 136 cases of pleural effusion and pneumococcal laboratory-confirmed CAP, respectively, were analyzed. While no catch-up program was performed for children aged 2–5 years, all-cause CAP and pneumococcal cases decreased by 16% and 63%, respectively ($P < 0.001$), in the post-PCV13 periods compared to the pre-PCV13 period. Although the highest reduction was seen in vaccinated children, a significant decrease of CAP incidence was also observed in older unvaccinated children, confirming the herd effect induced by pneumococcal conjugate vaccines. In addition, the number of pleural effusion cases decreased

by 53% ($P < 0.001$) and the number of CAPs caused by the additional PCV13 serotypes decreased by 74% [24] in the post-PCV13 period.

## 4. Acute Otitis Media (AOM)

The introduction of PCV7 immediately reduced the office visits of AOM by 6%–7.8% and antibiotic prescriptions by 5.7% [25]. PCV7 vaccination had an even more significant impact on recurrent AOM by reducing tympanostomy tube placements by 20%–24% [26]. From the point of view of microbiologists, PCV7 initially induced a significant reduction of the incidence of pneumococcal AOM caused by the PCV7 serotypes. However, the incidence of AOM, caused by non-PCV7 serotypes, particularly serotypes 6A, 6C, and 19A, increased with the reduction of AOM cases caused by PCV7 serotypes [27, 28]. In addition, PCV13 could significantly reduce the nasopharyngeal colonization by emerging serotypes causing AOM. Therefore, it has been concluded that PCV13 provided better protective effects against AOM than PCV7 [29]. This conclusion was confirmed by the prospective study conducted in Southern Israel by Ben-Shimol et al. In this study, the effects of PCV7/PCV13 sequential introduction on pneumococcal and overall AOM necessitating middle ear fluid (MEF) culture in children <2 years of age were evaluated based on 6,122 AOM cases including 1,893 pneumococcal cases [30]. Compared to the prevaccination period, the incidence of AOM caused by serotypes PCV7 + 6A and the 5 additional PCV13 serotypes 1, 3, 5, 7F, and 19A decreased by 96% and 85%, respectively (IRR and 95% CI = 0.04, 0.02–0.08 and 0.15, 0.07–0.30, resp.). Specifically, in the PCV7 vaccination period, only the incidence of AOM caused by PCV7 + 6A serotypes decreased and in the PCV13 vaccination period the incidence of AOM caused by serotypes 1, 3, 5, 7F, and 19A decreased along with a further PCV7 + 6A AOM reduction. A nonsignificant increase of AOPM caused by non-PCV13 serotypes was observed (IRR = 1.07; 95% CI, 0.72–1.58). In total, the incidence of all-pneumococcal and all-cause AOM decreased by 77% and 60% reductions, respectively [30].

Zhao et al. have evaluated the microbiological characteristics of middle ear effusions of 118 pediatric patients (6 months to 12 years of age) undergoing pressure equalization tube (PET) placement between August 2012 and April 2013 [31]. A total of 39 middle ear cultures from 29 patients led to the growth of at least one bacterial pathogen. Among these patients, 7 only received PCV7, 18 only received PCV13, and 4 received a combination of PCV7 and PCV13. Only one culture from a child who has received PCV7 was positive of *S. pneumoniae* serotype 16. On the contrary, *Haemophilus influenzae* and *Moraxella catarrhalis* were isolated from 7 and 3 cases, respectively. The limited number of *S. pneumoniae* strains isolated from MEF in this study suggests that PCV13 was effective for preventing AOM caused by serotypes covered by the vaccine. One of the limitations of this study was that the studied population has previously received antibiotics treatment repeatedly; therefore, the bacterial strains isolated from the patients were not fully associated with the effectiveness of vaccination of PCV7 and PCV13.

## 5. Nasopharyngeal Carriage

Nasopharyngeal pneumococcal carriage is considered as a prerequisite for the development of pneumococcal disease; therefore, reduction of nasopharyngeal carriage through PCV7 is also useful for reducing the incidence of pneumococcal infections among vaccinated children, their families, and the community [32]. In addition, monitoring the changes of nasopharyngeal carriage induced by PCV13 vaccination is important for the evaluation of vaccine effectiveness and monitoring the development of replacement allows us to predict possible emergence of new serotypes causing pneumococcal diseases.

Cohen et al. have analyzed 943 nasopharyngeal swabs from children (6 to 24 months of age) with AOM between October 2010 and March 2011. Among the 943 children with AOM, 651 received at least 1 dose of PCV13 and 285 received only PCV7 [18]. The overall pneumococcal carriage and carriage of non-PCV7 serotypes in the PCV13-vaccinated children were significantly lower than those in children exclusively vaccinated with PCV7 (53.9% versus 64.6%, $P = 0.002$, and 9.5% versus 20.7%, $P < 0.0001$, resp.). For serotypes 19A, 7F, and 6C, the pneumococcal carriage rates were also significantly lower in PCV13-vaccinated patients than in patients vaccinated only with PCV7 (7.5% versus 15.4%, $P < 0.001$, 0.5% versus 2.8%, $P = 0.002$, and 3.7% versus 8.4%, $P = 0.003$, resp.). Analyses of the other new serotypes included in PCV13 were not available due to the low number of cases identified in the study.

A study on the surveillance of pneumococcal carriage in children <60 months was conducted in July 2010 at a pediatric center in Boston, USA [33]. A total of 1,050 *S. pneumoniae* strains were isolated from 1,042 children. Eighty-nine isolates (8.5%) were identified as one of the 6 additional serotypes included in PCV13. A fall/winter peak of pneumococcal carriage of PCV13 serotypes was observed in nonvaccinated children but was blunted in vaccinated children. The authors reported a 74% reduction of PCV13 serotype colonization in the vaccinated children compared to nonvaccinated children. Approximately >75% community children received PCV13 vaccination, and a >50% decline of PCV13 serotype carriage was observed in nonimmune children in the same community. Consequently, the differences of the PCV13 serotype colonization between nonvaccinated and vaccinated children became not significant [33]. In addition, no evidence of replacement has been observed to date.

In England, the pneumococcal carriage of a group of children and their families in 2012 and 2013 after the PCV13 implementation was studied and compared with that in two previous periods, 2001-2002 before the PCV7 introduction and 2008-2009 after the PCV7 introduction [34]. The prevalence of pneumococcal carriage in children <5 years was similar among these three periods, 47.7% (95% CI, 41.8–53.5), 51.0% (95% CI, 44.0–58.0), and 48.4% (95% CI, 44.1–52.7) in 2012-2013, 2008-2009, and 2001/2002, respectively.

The prevalence of pneumococcal carriage in children of 5–20-years of age was 22.3% (95% CI, 15.6–30.9) in 2012-2013 and most strains (22/25, 88.0%) were of non-PCV13 serotypes. Only 3.4% (95% CI, 1.9–6.1) children ≥20 years of age were positive of pneumococcal carriage in the period of 2012-2-013, which was lower than that of the last two periods. Compared to the pneumococcal carriage in 2001-2002 before the PCV7 introduction, the odds of PCV7 serotype carriage significantly decreased in both 2008-2009 and 2012/2013, while the odds of carriage of the additional six PCV13 serotypes increased after the PCV7 introduction but significantly declined after the PCV13 introduction. The case/carrier ratio (CCR) for the serotypes of the highest carriage was relatively low. The highest CCR was observed for serotypes 7F, 19A, 3, 8, and 33F. Across the three carriage studies, CCR estimates were stable for nearly all serotypes.

## 6. Conclusions

Despite the difficulties deriving from the differences in immunization programs, vaccination coverage, the timing of PCV13 introduction since previous PCV7 implementation, and the presence/absence of catch-up campaigns, global evaluation and comparison of the incidence of pneumococcal diseases in young children who received PCV13 and/or PCV7 suggest that PCV13 provides a wider and more optimal coverage against pneumococcal disease than PCV7. It has been reported that PCV13 vaccination resulted in significantly higher effectiveness against IPD, mucosal pneumococcal diseases, and pneumococcal carriage than PCV7. Given the high safety and tolerance, PCV13 is an effective conjugate vaccine for controlling the incidence of pneumococcal diseases. However, PCV13 has been implemented for a relatively short time and long-term surveillance should be conducted in the future to further evaluate the safety and effectiveness of this novel vaccine. First of all, effectiveness against the additional 6 serotypes covered by PCV13 should be better defined. If the effectiveness against serotypes 6A, 7F, and 19A is indisputable, the effectiveness against serotypes 1, 3, and 5 needs further evaluation. Due to the relatively low number of cases caused by serotypes 1 and 5, no conclusion was drawn on the effectiveness against these two serotypes between pre- and post-PCV13 implementation periods. However, the reduction of the incidence of CAP with empyema, a condition frequently caused by the most invasive serotypes, suggests that PCV13 was highly effective in preventing these pathogens [24]. It has been reported that the effectiveness of PCV13 against serotype 3 was not satisfactory [14]. Finally, the replacement phenomenon should be further studied due to inconsistent results.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

This review was supported by a grant from the Italian Ministry of Health (Bando Giovani Ricercatori 2009) and by an unrestricted grant from the Italian Society of Pediatric Infectious Diseases (SITIP).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 189153, 26 pages
http://dx.doi.org/10.1155/2015/189153

*Review Article*

# How the Knowledge of Interactions between Meningococcus and the Human Immune System Has Been Used to Prepare Effective *Neisseria meningitidis* Vaccines

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Received 21 January 2015; Accepted 9 June 2015

Academic Editor: Nejat K. Egilmez



In the last decades, tremendous advancement in dissecting the mechanisms of pathogenicity of *Neisseria meningitidis* at a molecular level has been achieved, exploiting converging approaches of different disciplines, ranging from pathology to microbiology, immunology, and omics sciences (such as genomics and proteomics). Here, we review the molecular biology of the infectious agent and, in particular, its interactions with the immune system, focusing on both the innate and the adaptive responses. Meningococci exploit different mechanisms and complex machineries in order to subvert the immune system and to avoid being killed. Capsular polysaccharide and lipooligosaccharide glycan composition, in particular, play a major role in circumventing immune response. The understanding of these mechanisms has opened new horizons in the field of vaccinology. Nowadays different licensed meningococcal vaccines are available and used: conjugate meningococcal C vaccines, tetravalent conjugate vaccines, an affordable conjugate vaccine against the *N. menigitidis* serogroup A, and universal vaccines based on multiple antigens each one with a different and peculiar function against meningococcal group B strains.

## 1. Introduction

The immune system protects humans from attack by microorganisms such as bacteria, viruses, protozoa, fungi, parasites, and organisms such as helminths. The skin is the first barrier and its protective action is enhanced by bodily secretions, such as sweat and sebum, which exert a broad antimicrobial activity [1, 2]. The mucous membranes are protected by external and internal secretions, such as tears, saliva, and mucus, which contain molecules that can neutralize bacteria. Tissues such as the skin and mucous membranes are populated by immune cells, which can act against the microorganisms that circumvent the first physical and biochemical barriers.

The immune system is very complex and its defensive response is subdivided into innate and adaptive responses [3]. The innate response triggers an immediate, nonspecific, general action and is activated by typical signs of infection. The adaptive response is able to develop a highly specific, extremely accurate action, which is stored in the so-called immune memory.

This paper provides an overview of the interaction between the immune system and Gram-negative bacteria with particular reference to *Neisseria meningitidis* in the perspective of developing new vaccines against this pathogen.

## 2. Gram-Negative Bacteria and Immunity

*2.1. Outer Membrane Components.* Over thousands of years, bacteria have developed several mechanisms whereby they can circumvent the immune system. Specifically, Gram-negative bacteria possess a complex of envelopes, which allow the selective passage of nutrients into the cell and the excretion of metabolic waste outside. Structurally, Gram-negative bacteria possess an outer membrane (OM), which,

together with the peptidoglycan and inner membrane (IM), delimits the periplasm and cytoplasm compartments. Many molecules of glycolipids, especially lipopolysaccharide (LPS), emerge from the outer leaflet of the OM, while, from the inner layer of the OM, lipoproteins reach the peptidoglycan, with which they engage. Moreover, proteins such as porins cross the OM; these are very important for the active, passive, and selective permeability of small molecules, ions, and water [4]. Most porins have a trimeric structure and an oval shape. The bacterial porins perform many functions; indeed, they help the microorganism to adhere to the cells of the host tissue and to evade the defence mechanisms of the human body, thereby favouring invasion of the host. They are also able to elicit both innate and adaptive immunity. Porins can inhibit phagocytic activity [5] and activate the complement system by means of both classic and alternative pathways [6]. For instance, Neisserial porins can activate the transport of NF-$\kappa$B into the nucleus of B and dendritic cells (DCs) [7]. The DNA/NF-$\kappa$B complex then recalls other proteins, such as coactivators and RNA polymerase, which transcribe the DNA into mRNA; finally, this mRNA is exported to the cytosol and translated into proteins. This leads to a change in the function of the cell; for example, the cell may begin to produce proinflammatory cytokines.

Porins are clearly involved in the induction of proinflammatory activity, although it is not known which toll-like receptors recognize them. By contrast, it is known that LPS stimulates toll-like receptors 2 and 4 [8]. Three distinct regions characterize LPS, namely, lipid A, which fixes the molecule to the outer leaflet of the OM, the core polysaccharide which binds to lipid A by means of a disaccharide phosphate bridge, and antigen O, which is the most distal portion. The general structure of LPS is fully conserved, while the core oligosaccharide is highly variable.

Toll-like receptors are a family of conserved signal transducers able to induce an innate immune response. To date, at least 11 mammalian TLRs have been identified. Their stimulation by bacterial components activates the innate immune response. TLR2 recognizes peptidoglycan, lipopeptides, and bacterial proteins. However, it is interesting that LPS can overstimulate the innate immune response, thereby eliciting inflammation. As a result, the normal defences may not function correctly. Furthermore, it should be borne in mind that TLR5 recognizes flagellin, which is the main component of bacterial flagella [9]. For example, mutations of the TLR4 gene contribute to development of severe meningococcal infections [10]. In addition, through the recognition of *N. meningitidis* DNA, TLR9 exerts strong protection against the microorganism [11].

*2.2. Innate and Adaptive Immune Responses.* The innate immune system is able to detect other conserved microbial components, called pathogen-associated molecular patterns (PAMPs), such as nucleic acid structures, lipoteichoic acid, and peptidoglycan [12]. The pattern recognition receptors (PRRs) of immune cells include, in addition to TLRs, the NOD-like receptors (NLRs) and the RIG-1-like receptors (RLRs), which are able to recognize microbial components in the cytosol [13]. TLRs, NLRs, and RLRs are able to activate mitogen-activated protein kinase (MAPK) and the transcription of NF-$\kappa$B factor. A different set of NLRs helps to activate caspase-1 and the consequent assembly of inflammasomes [14].

The granulocytes and macrophages are the first cells that participate in the activation of the innate immune response. Shortly afterwards, the DCs and natural killer cells are activated. Specifically, neutrophils produce antimicrobial proteins, such as LL37, alpha and beta defensins, enzymes [15], interferons (IFN) alpha, beta, and gamma, C-reactive protein, and chemokines, contribute to activating the complement cascade. Macrophages produce reactive oxygen species (ROS) (e.g., $H_2O_2$) and reactive nitrogen species (RNS). Subsequently, DCs, which can also be activated by TLR2 and TLR4, activate natural killer (NKs) cells [16] and induce maturation of CD4+ T cells [17–22].

Many bacterial components are able to stimulate the adaptive human immune response. Porins can activate the translation of NF-$\kappa$B in the nucleus of B and DCs, while class I Pilin E induces highly specific antibodies (Abs) and class II induces cross-reacting Abs. Furthermore, complement cascade activation, as well as particularly C3b activation, opsonizes antigens, thereby enabling APCs to activate the adaptive response.

## 3. *Neisseria meningitidis* and Immunity

*3.1. Meningococcal Genome.* Meningococci have developed several "immunoescape" strategies [23], the molecular bases of which can be understood by taking into account the nature of the Neisserial genome. Progress in the field of molecular biology and the introduction of high-throughput technologies (HTTs) have tremendously advanced our understanding of the complexity of the Neisserial machinery. By using sophisticated approaches such as whole-genome sequencing (WGS) and microarrays, functional genomics investigations have uncovered the mechanisms that facilitate or hinder *N. meningitidis* growth, colonization, and invasion and have helped to explain its extraordinary intrastrain variation and adaptation to the environment. Other techniques, such as genome-wide association studies (GWAS), have shed light on the pathogen-host interaction and the host's susceptibility to the microbe. Genomics and postgenomics have not only increased our knowledge of the biology and pathogenesis of *N. meningitidis* but proved to be extremely useful in discovering candidate antigens and in developing effective new vaccines [24].

Being a naturally competent pathogen, *N. meningitidis* has a highly dynamic, plastic, and flexible genome with a size range of only more than 2,000 kilobases [25]. This genome differs from other microbial genomes in that it lacks some of the typical two-component systems and sigma factors [26]. Despite being relatively small and compact, it has elaborated a variety of mechanisms that contribute to explaining its high adaptability both to host and to environment. Meningococcus is usually polyploid, containing up to 2–5 genomes—polyploidy being a sign of virulence—while *N. lactamica* is monoploid [27]. Neisserial pathogenicity is intrinsically polygenic [28] and is given by a variety of different pathogenic

islands (PAIs or genomic islands, GEIs), including gonococcal genetic islands (GGIs) [29] and a recently discovered meningococcal disease-associated (MDA) island [30].

The nature of the Neisserial chromosome and the presence of extrachromosomal material contribute to explaining an important immunoescape strategy, known as structural or antigenic variation, which consists of camouflage of the Neisserial repertoire expressed. Basically, it can involve horizontal or lateral gene transfer (HGT/LGT) (mainly via transformation and, to a lesser extent, via conjugation and phage transduction) and allelic exchange/rearrangement of genes or gene portions taken up from the environment (Table 1). In addition, as its genome contains multiple copies of certain genes, for example, opacity factor proteins and pilins [31], homologous intragenic recombination also results in frequent surface structural variation.

Moreover, the pathogen hosts a number of prophages, from the Mu-related family to the phage l-related group and the family of filamentous M13-like phages [27, 32]. The most widely studied sites for phage integration are known as duplicated repeat sequence 3 (dRS3) [26], which belong to the family of Neisserial intergenic mosaic elements (NIMEs). Plasmids, such as pJS-A and pJS-B, also play an important role [33].

Another surface modulation occurs via phase variation, a process involving the modulation of gene expression via a mechanism of on/off switching (transition from an expressed state of the gene to an unexpressed one or vice versa). Besides this kind of "functional" phase variation, *Neisseria* can also undergo a "structural" switch, namely, a transition between two forms of a gene product. The genes, which are involved in this strategy, are termed "contingency genes" [26] and can be coupled and interlinked in structures called phasevarions (phase-variable regulons) [34], which have a regulatory function. Phase variation includes a variety of sophisticated mechanisms [35], such as slipped strand mispairing (SSM) [36], microsatellite instability [37], and reversible insertion of minimal mobile elements (MMEs) [38]. Therefore, these mechanisms can involve single nucleotides (homopolymeric repeats) or complex nucleotidic structures (short tandem repeats), occurring either upstream of a gene in the promoter or within an open reading frame (ORF)/coding sequence (CDS). Changes upstream of a gene result in modulation of its transcriptional efficiency and therefore of its final protein concentration. This is, for example, the case of Opc, porin A (*por*A), and *fet*A genes. Alterations within a gene, which insert *de novo* stop codons, alter the full translation of the gene. An example of this mechanism is provided by the opa genes and the genes coding for adhesins, such as *nad*A [39]. Phase variation of opa genes has been extensively characterized: they occur in four distinct copies and code for similar, but not identical, proteins. Phase variation can thus involve one copy or another, independently of each other, and can result in eleven variants. In this case, phase variation is therefore equivalent to antigenic variation. Besides opacity factor proteins, phase variation can involve up to hundreds of genes [40]: from the genes coding for pilins [41] or for proteins involved in genome maintenance and DNA repair [42, 43] to genes encoding proteins involved in the cell cycle control and regulation [44], autotransporters [45, 46], or enzymes like the pilin phosphorylcholine transferase *ppt*A [47] or the glycosylase *mut*Y [48], among others [49, 50]; the reader is referred to Table 2, which provides a more detailed overview of the phase-variable genes. Moreover, new mechanisms leading to phase variations have been discovered [51].

The mechanism implying MMEs involves different kinds of genetic elements, such as the Correia repeats (CRs) and the Correia repeat-enclosed elements (CREEs), known also as the *Neisseria* miniature insertion sequences (NEMIS) [52, 53], which constitute about 2% of the Neisserial genome [54]. Other genetic elements are the insertion sequence (IS) elements, such as IS1016-like, IS1106, IS1301 [55, 56], and IS1655 [57].

It is worth noting that the number of genes involved in phase variation is enormously greater than for any other pathogen studies so far [58]. Some genes are "phasotypes"; that is to say, they play a role in carriage and are downregulated, favouring host persistence [59].

As already mentioned, in some cases antigenic/structural variation and phase variation, albeit conceptually two distinct mechanisms, cooperate in increasing the genetic complexity of the Neisserial genome. Antigenic variation of LPS, for example, can derive from phase variation of one or more enzymes involved in the synthesis of the oligosaccharide chain by SSM, or by modification of LPS, for example, by glycosylation [60–62], sialylation [63, 64], or acetylation [65, 66], which, moreover, confer resistance to neutrophil-mediated killing.

Thus, both antigenic and phase variations concur in enabling *Neisseria* to evade the immune system [26, 27].

*3.2. Meningococcal Capsule.* LPS and themeningococcal capsule (CP) are the two major virulence factors of *N. meningitidis*. Specifically, the capsule displays a large variability of surface antigens, on the basis of which 13 different *N. meningitidis* serogroups have been identified. The CP contributes in an important way to the camouflage of the microorganism, which thus can better circumvent the immune system's defences. The clearest expression of this phenomenon is given by the molecular mimicry [67]. This can be seen in the nature of the polysaccharide CP of serogroup B meningococcus, a homopolymer of $\alpha$2-8-linked sialic acid, which is identical to a neural cell adhesion molecule, NCAM-1 [68]. Moreover, lacto-N-neotetraose (L-NNT) in the lipopolysaccharide of virulent strains is similar to an antigen expressed on red blood cells [69–73]. Further mechanisms of molecular mimicry have been recently discovered and described [74].

During the first phase of infection, meningococcus has to avoid the surface defences of the nasopharynx, such as the peptides secreted at the mucosal surface [90] and IgA secretory Abs [90, 196]. To this end, the meningococcus can aggregate into clusters and produce abundant OM vesicles (OMVs), thus managing to hide its surface antigens and to deflect the action of the surface defences from the bacterial cell [196]. In addition, the CP protects *Neisseria* from

Table 1: An overview of the most important immunoescape strategies exploited by *Neisseria meningitidis*.

| Immunoescape mechanism | Details | References |
|---|---|---|
| Structural/antigenic variation | It consists in the modified expression of domains, which are antigenically different within a clonal population, by which the pathogen is able to escape the host immunity selection and circumvent the immune surveillance<br>It usually involves LOS/LPS, opacity, and pilin proteins<br>LOS/LPS and opacity factor structural/antigenic variation depends essentially on phase variation<br>Pili antigenic variations depend on RecA-mediated recombination | [31, 75, 76] |
| Autolysis | It is mediated by OMPLA | [77] |
| Blebbing and microvesicles formation | The blebs originate as evaginations of the outer layer | [78] |
| Capsule switching | Due to microevolution, there is shift from serogroup B to serogroup C, from serogroup C to W-135, from serogroup Y to W-135, and from serogroup Y to B; nanostructured materials such as MWNTs and mesoporous silica increase transformational capacity | [30, 79–87] |
| Capsule modification | For example, modification of lipid A of meningococcal LOS/LPS with phosphoethanolamine protects *Neisseria* from neutrophils-mediated killing<br>Another example is given by the O-acetylation of capsular antigens (LpxL2 gene mutants are indeed more virulent)<br>LpxL1 gene mutants activate TLR4 less efficiently | [88] |
| Genome plasticity | HGT/LGT (via conjugation, transduction, and transformation) and homologous intragenic recombination | [25, 27, 30, 89] |
| Host modification | *Neisseria* exploits a bacterial sialyltransferase scavenging available host CMP-NANA for modifying LOS/LPS | [70] |
| Molecular mimicry | CP of serogroup B strain is a homopolymer of $\alpha$2-8-linked sialic acid and is similar to NCAM-1<br>L-NNT in the lipopolysaccharide of virulent strains is similar to an antigen on red blood cells<br>DMP19 acts as a DNA-mimic protein | [67, 69, 71–74, 90, 91] |
| Metabolic pathways | Examples are iron, lactate, glutamate uptake, utilization, and avoidance of neutrophil oxidation burst, ROS, and RNS | [92, 93] |
| Molecular decoy | FprB has an antigenic subdomain for binding antibodies, which is not essential for the functioning of the autotransporter; it also blebs with OMPs and LPS/LOS distract the immune system, directing the response away from the microbe | [94] |
| Immunotype switch | LPS immunotype switches from L3 to L8/L1 by lgtA, lgtC phase variation<br>LOS immunotype can contribute to immunoescape | [95, 96] |
| Phages and prophages | The pathogen hosts a number of prophages, from the Mu-related family to the phage l-related group and the family of filamentous M13-like phages | [25, 30, 89] |
| Phase variation | High-frequency reversible changes can occur in the length of SSRs (of capsule, LOS, opacity factor, porin, adhesin, invasin, autotransporter, haemoglobin receptor, DNA mismatch repair, and pilin genes, termed as contingency genes and organized in modules called phasevarions)<br>Other repeat sequences can be REP2, CRs, CREEs, and NIMEs<br>Transposon-like elements can play a role<br>Phase variation mediates resistance to antibiotics<br>Phase variation mediates carriage persistence | [50, 52, 59] |
| Pilin conversion and modification | Pilin is posttranslationally modified by addition of a glycan, two phosphorylcholines, and a glyceramido acetamido trideoxyhexose residue | [97, 98] |
| Plasmid | Examples of plasmids that can contribute to *Neisseria* variability are pJS-A, pJS-B | [33] |
| Recruitment of human components of immune system | *Neisseria* escapes complement-mediated killing recruiting and sequestering fH to its surface | [91] |
| Temperature-regulated defence | RNA thermosensors finely tune the expression of CP components, LOS, and fHBP, thus protecting against human immune killing | [99] |

CMP-NANA: cytidine 5′-monophospho-N-acetylneuraminic acid; CP: capsule; CRs: Correia repeats; CREE: Correia repeat-enclosed element; DNA: deoxyribonucleic acid; fH: complement factor H; fHBP: fH binding protein; HGT: horizontal gene transfer; lgt: prolipoprotein diacylglyceryl transferase; L-NNT: lacto-N-neotetraose; LOS: lipooligosaccharide; LPS: lipopolysaccharide; LGT: lateral gene transfer; MWNTs: multiwalled nanotubes; NCAM-1: neural cell adhesion molecule 1; NIME: Neisserial intergenic mosaic element; OMPs: outer membrane proteins; OMPLA: outer membrane phospholipase A; RecA: recombinase A; REP2: repetitive extragenic palindromic sequence; RNA: ribonucleic acid; RNS: reactive nitrogen species; ROS: reactive oxygen species; SSRs: simple sequence repeats; TLR: toll-like receptor.

Table 2: An overview of the most important genes and gene products of *Neisseria meningitidis* involved in immunoescape mechanisms.

| *N. meningitidis* molecule | Immunological role | Reference |
|---|---|---|
| *ani*A | A nitrite reductase: it protects *Neisseria* from nitrosative stress during both colonization and invasion | [90, 100–102] |
| *App* | It is phase-variable | [103] |
| *ausI*/MspA | An autotransporter and a serine protease; it is phase-variable | [45, 46] |
| Biofilm (and molecules involved in the biofilm synthesis, such as *aut*A or *hrp*A, or optimizing pathogen survival in biofilm, such as the alpha-peptide of IgA1 protease, *adh*C, *est*D) | Biofilm protects from macrophages; *adh*C is involved in S-nitrosoglutathione metabolism and in glutathione-dependent detoxification system; EstD is involved also in *Neisseria* colonization | [104–108] |
| Blebs (with OMPs and LPS/LOS) and SOMVs | They protect from neutrophils-mediated killing and NETs; they divert the immune response away from the pathogen | [78] |
| Capsule and molecules involved in the capsule synthesis such as *kps*C, *kps*S | It activates TLR2 pathway, it increases serum resistance, and it inhibits the classical pathway of complement | [109–111] |
| Cas9 and the CRISPR-Cas system | CRISPR-Cas9-mediated repression of bacterial lipoprotein expression facilitates evasion of TLR2 by the pathogen; it is involved in gene expression and regulation | [112, 113] |
| *cbp*A | It mediates zinc piracy and protects from nutritional immunity | [93] |
| *Cps* | As a gene, it is involved in the capsule biosynthesis; as RNA, it acts as a thermosensor; Cps gene amplification protects the pathogen | [99, 114] |
| *CrgA* | It is involved in the regulation of pili and capsule expression; it plays a major role in the infectious cycle of *Neisseria* | [114–116] |
| *Css* | As a gene, it is involved in the capsule biosynthesis; as RNA, it acts as a thermosensor | [99] |
| *ctr*A, *ctr*D | As genes, they are involved in the capsule export; as RNAs, they act as thermosensors; IS1301 in the IGR between *sia* and *ctr* operons mediates resistance to Abs | [99, 117, 118] |
| *cyc*P | It is involved in denitrification metabolism and protects *Neisseria* from nitrosative stress | [90, 119, 120] |
| *dam* | It is involved in phase variation and modulation | [42] |
| *dca*C | It is phase-variable | [40] |
| *din*B | A DNA polymerase IV belonging to the SOS regulon: it is involved in phase variation and modulation | [42] |
| DNA mismatch repair genes (*fpg*, *mut*L, *mut*S, *mut*Y, *rec*A, *rec*N, *uvr*D) | They are phase-variables; they protect against oxidative stress | [42, 48, 51, 121] |
| *drg* | It is involved in phase variation and modulation | [42] |
| *far*A, *far*B, *far*R | They remove antimicrobial peptides, proteases, lysozyme, and acids from the bacterial cytosol and protect the pathogen | [122, 123] |
| *fbp*A, *fbp*B | They are involved in phase variation and modulation | [51] |
| *Feta* | It is involved in phase variation and modulation | [124–126] |
| fHbp (formerly known as GNA1870) | It is involved in phase variation and modulation; it protects *Neisseria* from complement-mediated killing, binding fH | [90, 127] |
| *frp*A, *frp*B, *frp*C | They are phase-variable; they can act as a molecular decoy | [124, 125, 128] |
| *fun*Z | It is a site of bacteriophage insertion; it is phase-variable | [49] |
| *fur* | It is involved in phase variation and modulation; it tunes the gene expression of virulence genes | [102, 129] |
| *ggt* | It regulates pathogen growth | [130] |
| *Ght* | It is involved in the capsule biosynthesis and in the resistance mechanisms of the pathogen | [131, 132] |
| *glt*T | It favours meningococcal internalization into human endothelial and epithelial cells; it regulates pathogen growth | [133, 134] |
| H.8 | AAEAP motifs are target for generation of blocking Abs | [135–138] |

Table 2: Continued.

| *N. meningitidis* molecule | Immunological role | Reference |
|---|---|---|
| Haemoglobin-linked iron receptors (*hpuA*, *hpuB*, *hmbR*) | They are involved in phase variation and modulation | [43, 139–141] |
| *Hfq* | A RNA chaperone: it is involved in stress response and virulence and is a pleiotropic regulator of protein expression | [142] |
| *hsd*S | It is phase-variable | [49] |
| IgA protease | It cleaves secretory IgA, hinders Ab binding and function, and may play role in biofilm formation; it cleaves lysosomal LAMP1 in epithelial cells; moreover, it is phase-variable | [122, 142, 143] |
| *kat*A | It confers resistance to RNS, including peroxynitrite (PN), protects against ROS, and detoxifies $H_2O_2$ | [90, 102, 122, 144] |
| Laz | A lipid-modified azurin: it protects against hydrogen peroxide and copper toxicity; it promotes *Neisseria* growth and survival | [135, 138, 145] |
| *lbpA*, *lbpB* | They are involved in iron acquisition and metabolism; they are phase-variable; moreover, the release of LbpB enables *Neisseria* to escape from complement-mediated killing | [90, 122, 146] |
| *lct*P | Its inactivation results in C3-mediated cell lysis | [102, 147, 148] |
| *lgt*A, *lgt*B, *lgt*C, *lgt*D, *lgt*E, *lgt*G | They are involved in LOS biosynthesis and are phase-variable; for example, *lgt*A or *lgt*C phase variation mediates LPS immunotype switch from L3 to L8/L1 | [60] |
| LOS/LPS | It protects from macrophages; strains of the same species produce different LOS glycoforms | [122] |
| *lpt*A | It adds a phosphoethanolamine group to lipid A and confers resistance to defensins and cathelicidins | [90, 149] |
| *Lst* | LOS sialylation (by the enzyme Lst) prevents complement deposition and phagocytosis by neutrophils | [122, 150] |
| *mes*J | It is phase-variable | [49] |
| *Msf* | It binds to vitronectin; it increases serum resistance | [151] |
| Mip | It tunes gene expression | [102, 152, 153] |
| *mis*R, *mis*S | They are phase-variable; they are involved in capsule regulation and modification | [114, 154] |
| *mlt*A (formerly known as GNA33) | It tunes gene expression | [155] |
| *mnt*A, *mnt*B, *mnt*C | They protect against oxidative stress | [122, 156] |
| *mod*A, *mod*B | They are phase-variable | [34] |
| *msr*A, *msr*B | They are involved in the methionine sulfoxide reduction and they repair oxidized proteins | [122, 157] |
| *mtr*C, *mtr*D, *mtr*E | They protect against cationic antimicrobial peptides and toxic hydrophobic molecules | [122, 158, 159] |
| *nadA* and its regulator *nad*R | It binds to Hsp90, recruits ARF6 and Rab11, and activates human monocytes and macrophages, triggering IFN-gamma and R-848 dependent pathways; it interacts with beta1 integrins; it is phase-variable | [39, 160–165] |
| *nal*P | An autotransporter protease: it cleaves C3, facilitates degradation of C3b, and enhances Neisserial survival in human serum; it stabilizes the biofilm; moreover, it is involved in the processing of other proteases, such as the proteases which release LbpB, whose release enables *Neisseria* to escape from complement-mediated killing; NalP processes also App and IgA1 protease; it has an important role in the virulence of the pathogen | [24, 102, 166] |
| Nhba (formerly known as GNA2132) | It tunes gene expression | [167] |
| *nhhA* | It activates TLR4-dependent and independent pathways; it triggers apoptosis in macrophages; it increases serum resistance and protects from phagocytosis and complement attack; it is essential for colonization | [168, 169] |

Table 2: Continued.

| *N. meningitidis* molecule | Immunological role | Reference |
|---|---|---|
| *nif*S | It is phase-variable | [49] |
| *nir*K | It protects *Neisseria* from nitrosative stress during colonization and invasion | [170, 171] |
| *nor*B | It favours the pathogen growth, enabling utilization and consumption of NO during microaerobic respiration, enhances pathogen survival, protects *Neisseria* from nitrosative stress during colonization and invasion, decreases and downregulates the production of NO-regulated cytokines, such as TNF-alpha, IL-12, IL-10, CCL5 (RANTES), and CXCL8 (IL-8), and prevents host cell S-nitrosothiol formation | [100, 119, 120, 170, 172] |
| *nsp*A | It binds to factor H and inhibits AP | [122, 173–175] |
| *nsr*R | It is involved in denitrification metabolism and protects *Neisseria* against nitrosative stress | [176, 177] |
| *oat*W, *oat*Y | They tune gene expression | [178] |
| Opa | It interacts with CEACAM, promoting endothelial cell attachment and upregulating endoglin (CD105) and cooperation with $\beta$1 integrins; it elicits innate host defences and actively suppresses adaptive immune responses that would eliminate the pathogen | [179–184] |
| Opc | It binds to vitronectin, it inhibits AP, and it increases serum resistance; it elicits innate host defences and actively suppresses adaptive immune responses that would eliminate the pathogen | [179–182, 184] |
| *oxy*R | It regulates catalase expression and is involved in the protection from oxidative stress | [185, 186] |
| P36 | It is involved in Neisserial adhesion. | [187] |
| *pac*A, *pac*B | They are involved in the composition and regulation of peptidoglycan membrane | [188] |
| *pgl*A, *pgl*B, *pgl*G, *pgl*H | They are phase-variable | [60–62] |
| Pili | They alter the expression levels of human genes known to regulate apoptosis, cell proliferation, inflammatory response, adhesion, and genes for signaling pathway proteins such as TGF-beta/Smad, Wnt/beta-catenin, and Notch/Jagged | [189] |
| *pil*C1 | It interacts with mucosal surface and mediates the crossing of the BBB | [41, 169] |
| P*ilE*, *pil*S | They are involved in nonreciprocal recombination-based antigenic variation | [76] |
| P*ilE*, *pil*V | They bind to CD147 for vascular colonization; they mediate also *Neisseria* internalization | [190, 191] |
| *pil*P, *pil*Q | They are involved in pilus biogenesis and outer membrane stabilization | [51, 192] |
| *porA* | It binds to fH, C3b, C4b, and C4bp (more strongly under hypotonic conditions); it increases serum resistance; it is involved in phase variation | [122, 139, 173] |
| *por*B | It inhibits factor H-dependent AP; it interacts with TLR1 and TLR2 and activates I$\kappa$B, MAPK/MAPKK, and PTK pathways, leading to CD86 upregulation, to IL-6, IL-12, and TNF secretion in B cells and DCs, and to IgB secretion in B cells | [122, 173] |
| *ppt*A | It is phase-variable | [47] |
| *Ppx* | It is an exopolyphosphatase whose mutation protects *Neisseria* from complement-mediated killing; it interacts with the AP of the complement activation | [64] |
| *rmp*M | It is involved in phase variation and modulation | [193, 194] |

Table 2: Continued.

| *N. meningitidis* molecule | Immunological role | Reference |
|---|---|---|
| Sialic acid synthase (*neu*B, *sia*A, *sia*B, *sia*C, *syn*C) | They are phase-variable | [102] |
| *sod*B, *sod*C | They protect from phagocytosis by human monocytes/macrophages | [102] |
| *tbpA*, *tbpB* (also known as *tbp*1, *tbp*2) | They are involved in nutritional immunity | [121] |
| *TdfF* | It is involved in intracellular iron acquisition and is found only in genomes of pathogen strains | [28] |
| Temperature sensors (such as RNA thermosensors located in the 5′ UTRs of genes necessary for capsule biosynthesis, the expression of fHbp, and sialylation of LOS/LPS) | Activated by coinfecting pathogens, they recruit mechanisms of resistance and immunity escape | [99] |
| *ton*B | It is involved in nutritional immunity, supplying energy to the pathogen | [93] |
| Uncharacterized proteins (NGO1686, NMB0741, NMB1436, NMB1437, NMB1438, and NMB1828) | They protect from nonoxidative factors, but their mechanisms are still not understood; NMB1436, NMB1437, and NMB1438 are putatively involved in iron metabolism | [122, 195] |
| Uncharacterized factor (NMA1233) | It is involved in phase variation and modulation | [26, 51] |
| *xse*B | It is involved in phase variation | [26] |
| *znuD* | It protects from neutrophils and nutritional immunity | [92] |

Ab: antibody; AP: Alternative Pathway; ARF6: ADP-ribosylation factor 6; App: adhesion and penetration protein; BBB: blood-brain barrier; cbp: calprotectin binding protein; CEACAMs: carcinoembryonic antigen-related cell adhesion molecules; CRISPR: clustered regularly interspaced short palindromic repeats; ctr: capsule transport apparatus; dam: DNA adenine methyltransferase; drg: dam replacing gene; fur: ferric uptake regulator; ggt: gamma-glutamyl aminopeptidase; hsp: heat-shock protein; IgA: immunoglobulin A; lbp: lactoferrin binding protein; lct: lactate permease; LOS: lipooligosaccharide; Mip: macrophage infectivity potentiator; mltA: membrane-bound lytic transglycosylase A; IGR: intergenic region; Msf: meningococcal surface fibril; Msr: methionine sulfoxide reductase; NadA: *Neisseria* adhesion A; NhhA: *Neisseria* hia homologue A; oat: O-acetyltransferase; OMV: outer membrane vesicle; opa: opacity-associated protein a; opc: opacity-associated protein c; pac: peptidoglycan O-acyltransferase; pil: pilin; por: porin; RNA: ribonucleic acid; RNS: reactive nitrogen species; Sod: superoxide dismutase; SOMVs: spontaneously released OMVs; Tbp: transferrin-binding protein; TLR: toll-like receptor; UTRs: untranslated regions; uvr: ultraviolet resistant.

cationic antimicrobial peptides (CAMPs), including cathelicidin [196]. Conversely, the presence of capsular polysaccharide restrains the invasion and colonization of the nasopharyngeal barrier by hiding the adhesins and invasins of the meningococcus [143, 158]. On the other hand, the presence of the capsule may allow the microorganism to pass unharmed through the intracellular environment by exploiting the system of cell microtubules, at least in the case of serogroup B *N. meningitidis* [197]. Moreover, the CP is essential for meningococcal growth and survival in the bloodstream and cerebrospinal fluid, increasing serum resistance. During the different stages of infection, the capsule may hinder or promote the survival of *N. meningitidis* in the human host; indeed, the microorganism can modulate the production of capsule components, which depends on 4 genes, three of which—*sia*A, *sia*B, and *sia*C—are situated in the *cps* locus. The *sia*D gene induces the production of polysialyltransferase, which allows the polymerization of sialic acid. For instance, in the early stages of infection, the production and assembly of sialic acid are downregulated [198]. Another example of polysialyltransferase system is given by *oat*WY [178].

In addition to the above-mentioned actions, the most important virulence activity of the CP is probably the significant impairment of both Neisserial adherence to DCs and the phagocytic killing of bacteria; indeed, the CP inhibits both the opsonic and the nonopsonic phagocytosis of *N. meningitidis* [199]. It prevents the formations of effective Abs against *N. meningitidis*.

CP downregulates both classical and alternative complement pathways and prevents the proper insertion of the membrane attack complex (MAC) [200, 201]. LPS also contributes to inhibiting MAC deposition [201, 202].

Moreover, CP switching contributes to escaping detection and killing. This is a complex phenomenon due to microevolution and usually involves Neisserial strains expressing sialic acid (e.g., the shift from serogroup B to C, from serogroup C to W-135, from serogroup Y to W-135, and from serogroup Y to B) [79–82, 203]. The molecular basis is provided by the allelic replacement of the sialic acid CP polymerase.

Surprisingly, nanostructured materials such as multiwalled carbon nanotubes (MWNTs) and mesoporous silica have been found to increase *Neisseria*'s transformational capacity [83, 84].

*3.3. Major and Minor Adhesion Mechanisms of N. meningitidis.* *N. meningitidis* possesses a multifaceted system of adhesins, such as pili and other systems of adhesion (i.e., opacity-associated proteins OpA and OpC). Adhesion is probably a coordinated process mediated first by pili, which are composed of several proteins; the most important of these is Pilin E (PilE) [204], but Pilin C (PilC) [205] and the secretin Pilin Q (PilQ) [206] have also been described. PilE is the main constituent of the *Neisseria* type IV pilus. In 1984, Diaz et al. identified proteins I and II as the main components of the type IV pili and noted that Abs against protein I were highly specific [207]. Subsequently, Pilin E was classified as

belonging to class I and class II. Class I Pilin E is highly variable, while class II Pilin E is highly conserved [208, 209]. For this reason, class II Pilin E has been suggested as a candidate antigen for a vaccine against meningococci [147]. The regulation of pilin genes is quite complex [97, 115, 116, 190].

Other components are as follows: *pil*V [210], *pil*P, *pil*D (a prepilin-processing leader peptidase), *pil*F and *pil*T (traffic NTPases), *pil*G (involved in the pilus assembly), *pil*M (pilus biogenesis protein), and *pil*W (involved in the pilus stabilization) [148], among others.

Although the interactions between type IV pili and cellular receptors are not completely known, they may interact with a membrane cofactor protein, named CD46 receptor, and with alpha 1 and alpha 2 integrins [211]. However, it is known that pili contribute to the aggregation of Neisserial cells [212]. This fact, associated with the ability of pili to act as a signalling protein by interacting directly with the $\beta$2-adrenergic receptor, contributes to the depletion of junction proteins, thus helping meningococcus to pass through the epithelial and endothelial cells and, subsequently, to cross the blood-brain barrier (BBB) [75, 213].

Although pili are essential to the first phase of Neisserial adhesion to the cells of the airways, other adhesion molecules, such as LPS and porin A, intervene to strengthen the microbial bond. In particular, OpcA and OpcC appear to be very important; indeed, OpcA binds carcinoembryonic antigen cell adhesion molecules (CEACAMs), heparan sulphate proteoglycan (HSPG) and integrins [179–183, 214]. Opc proteins can combine with HSPGs and, through vitronectin and fibronectin, with their integrin targets. Furthermore, Opa proteins are able to elicit innate human defences that favour the survival of *N. meningitidis*, while actively suppressing adaptive immune responses that would eliminate the pathogen [184]. The variability of the expression of different Opa proteins could play a major role in prolonging the state of infection by circumventing the humoral host immune response [215].

The adhesion and penetration protein (*app*) [103], which is a member of the autotransported family of secreted proteins, owes its name to its ability to adhere to human cells, thereby favouring the entry of Neisseriae. To circumvent the immune system, meningococci possess formidable machineries that allow them to secrete proteins in different manners; in particular, Neisseriae mainly use the autotransporter pathway (also known as type V secretion system) [216]. The first-described protein belonging to the autotransporter superfamily was an IgA protease from *N. gonorrhoeae* [217]. MspA (meningococcal serine protease A) is another putative autotransporter protein. Not all strains of *Neisseria gonorrhoeae*/*meningitidis* possess the gene for MspA/AusI (also known as NMB1998). However, Turner et al. [218] demonstrated that convalescent sera from subjects affected by serogroup B invasive disease recognized the MspA antigen. NhhA (*Neisseria* hia/hsf homologue A, also known as Hsf) and Neisserial adhesin A (NadA) also belong to the autotransporters. Nhha contributes to bacterial adhesion by binding heparin sulphate and laminin. In addition, through the activation of caspase, NhhA increases the rate of macrophage apoptosis [168, 219]. NadA, which is expressed by 50% of virulent strains [160], but only by 5% of the strains isolated from carriers, is of particular interest because it is one of the components of the 4CMenB (Bexsero) vaccine and binds beta1 integrins [220].

*3.4. N. meningitidis: Avoidance Mechanisms against Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), and Antimicrobial Peptides (AMPs).* When in contact with the mucosa of the nasopharynx, *N. meningitidis* can implement several strategies of adhesion, but it must overcome many barriers of innate immunity. We have already mentioned how the capsule allows bacteria to mitigate the activity of DCs. However, it must elude other substances, such as the reactive oxygen species and reactive nitrogen species produced by macrophages and the antimicrobial peptides produced by activated neutrophils. As already mentioned, the capsule protects *N. meningitidis* from LL-37 cathelicidin, but LPS also contributes to the resistance of the bacterium against this cathelicidin [156].

Furthermore, the toxic action of ROS is neutralized by the secretion of enzymatic proteins, such as catalase and superoxide dismutase [144, 149, 221]. The gene that codes for catalase is katA and is regulated by OxyR [185, 186, 222], while SodB and SodC code for superoxide dismutase [223]. Laz, a lipid-modified azurin, protects the pathogen against $H_2O_2$ and cupper toxicity [135–138, 145, 176].

In addition, *N. meningitidis* possesses genes, which encode enzymes able to exert a denitrification action, such as aniA, CycP, nirK, nsrR, and norB. They favour the growth of the pathogen, enabling utilization and consumption of NO during microaerobic respiration, enhance pathogen survival, and protect *Neisseria* from nitrosative stress during colonization and invasion by preventing host cell S-nitrosothiol formation. Moreover, they reduce and downregulate the production of NO-dependent cytokines, such as TNF-alpha, IL-12, IL-10, CCL5 (RANTES), and CXCL8 (IL-8) [100, 101, 119, 120, 130, 157, 170, 172, 177, 224].

Contemporarily, already at the level of the mucosa, the microorganism must resist the complement system.

Another interesting mechanism is the strategy whereby *N. meningitidis* escapes the attempts of the host to sequester nutrients essential for growth and survival of the pathogen. This process has been termed "nutritional immunity" [131, 139]. The microbe is endowed with OM receptors (such as HmbR, HpuA or HpuB, TbpA or TbpB, and TdfF) for acquiring iron and other important metals [93, 124, 125, 128, 129, 140, 195, 225]. ZnuD is a high-affinity zinc uptake receptor, which plays an important role in enabling the pathogen to evade neutrophil-mediated killing [226, 227]. CbpA, a receptor for calprotectin, a protein released by neutrophils during inflammatory processes, is upregulated when *N. meningitidis* suffers from zinc limitation [226, 227]. Further examples of metabolic enzymes involved in nutritional immunity are glutamate transporters or molecules taking part in the carbon cycle [132–134, 228–230].

*3.5. How Meningococcus Circumvents the Complement System.* Three pathways can activate the complement functions, namely, the classic pathway, the alternative pathway, and

the lectin pathway. All three of these pathways contribute to the transformation of C3 to C3b [231].

The alternative pathway acts by comparing self- with non-self-antigens and is activated by anything that differs from the markers of host cells. Specifically, factor H recognizes host-associated molecular patterns (HAMPs). Properdin, first identified in 1959, is another protein that can directly activate the alternative pathway of the complement system [232]. It has been demonstrated that properdin deficiency favours recurrent episodes of *N. meningitidis* infection [233].

Meningococci produce three different variants (1, 2, and 3) of a protein that binds factor H. This protein, named fHbp (factor H binding protein) or GNA1870, blocks activation of the alternative pathway of the complement system. Indeed, by surrounding themselves with fHbp, *N. meningitidis* cells capture and inactivate factor H. Thus, it is easy for the microorganism to survive and reproduce, especially in the bloodstream and cerebrospinal fluid. Hence, it is important that the 4CMenB (Bexsero) vaccine contains variant 1 of this protein, which is often expressed by virulent meningococcal strains [228]. Another vaccine (Trumenba), recently licensed in the USA, contains recombinant variants 1 and 2 of fHbp from *N. meningitidis* serogroup B, A and B subfamilies (A05 and B01, resp.) [234, 235]. The proteins are produced by exploiting an advanced genetic engineering technique, using *E. coli* as a vector.

*3.6. The Adaptive Immune Response against Neisseria meningitidis.* Microorganisms such as *N. meningitidis* are able to change many exposed surface proteins, while the polysaccharides, which constitute the capsule, are T-independent (TI-2) antigens and can activate B cells directly, without the intervention of the MHC. However, TI-2 antigens do not induce an efficient secondary response and do not induce the production of avid immunoglobulins. Rather, they induce the production of short-lived Abs belonging to the IgM class [236].

In addition, it is important to consider that, in infants and children, the development of the immune system is a dynamic process, which begins *in utero* and continues for months and even years after birth. This explains why many components of the immune system are inefficient or partially efficient in infancy and early childhood [237–239]. For this reason, most cases of meningitis and sepsis from *N. meningitidis* occur under the age of 4 years and particularly in the first year of life.

The critical role of bactericidal Abs against the exposed surface antigens of *N. meningitidis* has been demonstrated by several studies. Indeed, Goldschneider et al. [240] showed that only individuals without bactericidal Abs contracted the clinical disease. In addition, the successful therapeutic use of immune sera, which markedly reduced lethality when first implemented by Flexner [241], has shown the central role of these Abs in protecting against invasive disease. The opsonic activity of Abs is also very important in the protection against and the recovery from meningococcal disease, as is demonstrated by the role of neutrophils, macrophages, and DCs in combating *N. meningitidis*. It is also well known that the cerebrospinal fluid of patients contains large numbers of neutrophils full of microorganisms. These clusterings of neutrophils are known as neutrophil extracellular traps (NETs) and massively release cathepsin G. *N. meningitidis* circumvents these traps by blebbing spontaneously released OMVs (SOMVs). Other strategies that the pathogen exploits are modification of lipid A of LPS with phosphoethanolamine protected and upregulation of ZnuD [92].

The adaptive immune response has been studied in carriers and during both the invasive period leading to clinical meningitis and the convalescence period. T cell response is "two-faced"; while proinflammatory T cells may indeed blunt the invasive power of the pathogen, the induction of the Treg response, which is able to limit virulence, carries the price of the reduced effectiveness of the protective response, especially in children [242]. During infection, increased meningococcal antibody titres can be detected from the 4th day, peaking at the end of the third week or the beginning of the fourth week and showing a correlation with the severity of the disease and the age of the patient. In the acute period of the disease, the number of T cells generally drops, while that of B cells increases; by the end of the second week, IgG levels decline and IgM levels rise [243]. In particular, abnormalities in T cell response can be detected, such as an elevated percentage of CD25+ and HLA-DR+ T cells, an increase in CD4+ CD45R+ (suppressor-inducer) cells, with subsequent expression of activation antigens, and a decrease in CD4+ CDw29+ (helper-inducer) cells [244]. During convalescence, an age-associated Th response can be observed: specifically, a Th1 response (low IL-10/IFN-$\gamma$ ratio) and a highly proliferative Th2 response (higher IL-10/IFN-$\gamma$ ratio) can be detected in younger and older patients, respectively [245]. Generally, a significant CD4+ T central memory response, with serum bactericidal antibodies, a marker of protective immunity, can be found [246]. However, the above-mentioned age-related mucosal T effector/memory cell response may also be present without bactericidal antibodies [247].

*3.7. Other Immunoescape Strategies.* Temperature fluctuation plays an important, although underscored, role in microbial pathogenesis, colonization, invasion, and host evasion. In contrast to mammals that maintain constant body temperature, pathogens' and other animals' temperature oscillates on a daily basis. Loh and collaborators [99] have identified the molecular bases of this temperature-dependent strategy. They have studied three RNA thermosensors located in the $5'$ untranslated regions (UTRs) of genes involved in the CP biosynthesis, the expression of fHbp, and sialylation of LOS/LPS. Increased temperature (e.g., during inflammation by coinfecting pathogens, such as influenza virus) "alarms" the meningococcus and triggers its defence mechanisms against human immune killing. This could be a key determinant for the transformation of a symbiont pathogen into a virulent one. However, the precise nature of this mechanism remains elusive.

Clustered regularly interspaced short palindromic repeats- (CRISPR-) Cas9 is another molecular tool that *Neisseria* can use in order to divert immune surveillance. It is an intrinsically ambivalent device, since, on one hand, being involved in gene expression and regulation, it restricts the possibility of editing the *Neisseria* genome via HGT/LGT or the insertion of exogenous nucleic material, and therefore it

limits the microbial variability. On the other hand, CRISPR-Cas9-mediated repression of bacterial lipoprotein expression facilitates evasion of TLR2 by the pathogen [112].

Another mechanism is the molecular decoy, with which the microbe deceives the human immune system. For example, *fpr*B has an antigenic subdomain for binding antibodies, which is not essential for the functioning the autotransporter [94].

The example of *fpr*B is useful to show how *Neisseria* can use concurrently the previously described immunoescape strategies: *fpr*B is subject to a high degree of antigenic variation, is a phase-variable gene, is involved in nutritional immunity, and moreover exploits a molecular decoy. Neisserial carbohydrates, mimicking host carbohydrates, circumvent immune system and at the same time exploit their mimicry to recruit fH [91].

## 4. Meningococcal Vaccines

*4.1. Polysaccharide Vaccines.* As at least six meningococci are pathogenic in humans, the development of meningococcal vaccines has been a challenge. The first step towards solving the problem was to find a common denominator among antigens that showed high variability. In 1969, Gotschlich et al. [248] correctly put the first imperfect piece of the puzzle in place by demonstrating the possibility to extract the capsule polysaccharide. However, although high molecular weight groups A and C meningococcal polysaccharides proved immunogenic in adults [249–251], important limitations of this vaccine emerged in subsequent years. Indeed, while both group A and group C vaccines proved effective in USA and Italian recruits [252, 253] after one administration, they displayed only short-term efficacy in older children and adults. Moreover, vaccines against serogroup C did not prevent disease in infants, and the efficacy of group A vaccines in children under 1 year of age was unclear. The immune response occurred 10 days after vaccination. In schoolchildren and adults, one dose of these vaccines seemed to provide protection for at least 3 years [254]. These findings are explained by the characteristics of the antigens contained in the vaccines. Indeed, polysaccharides with repeating epitopes induce an immune response with the following characteristics [255, 256]:

(i) The response occurs between the ages of 3 and 18 months but is variable; in children less than 2 years of age, the response is usually poor;

the affinity maturation of the Abs does not occur.

(ii) The immunologic memory is not stimulated.

(iii) Almost all (>90%) Abs produced belong to the IgM class and are produced in the spleen [257, 258]. Furthermore, several studies have suggested that vaccination with large amounts of polysaccharides induces immune tolerance towards these antigens [259].

*4.2. Polysaccharide Conjugate Vaccines.* Three immunogenic carrier proteins are generally used inpolysaccharide conjugate vaccines, namely, diphtheria or tetanus toxoid, CRM197 (a nontoxic variant of diphtheria toxin obtained by molecular biology), and a complex of outer membrane protein (OMP) mixture from *N. meningitidis*. The toxoids were selected as carriers, firstly because of their immunological potency and secondly because if the recipient had previously been immunized with the toxoid, a booster effect was predictable. Moreover, under particular conditions, suppressive effects can also occur. The conjugate vaccine against *N. meningitidis* serogroup C had great success in several countries (UK, Netherlands, Spain, Italy, etc.) [260]. Today, tetravalent conjugate vaccines ACWY are also available in both the USA and Europe [260–262].

*4.3. Vaccines against N. meningitidis Serogroup B.* The most difficult problem was to prepare a vaccine against *N. meningitidis* serogroup B strains. Indeed, the maximum expression of camouflage is found in the capsule of these strains. Specifically, the polysaccharide of serogroup B meningococcal strains is a homopolymer of sialic acid residues and has structural similarities to brain glycoproteins. For this reason, it was impossible to prepare a polysaccharide antimeningococcus B vaccine. To overcome this obstacle, OMVs-vaccines were developed and used during epidemics caused by *N. meningitidis* serogroup B strains. However, given the high variability of the proteins, such as porins, present in OMVs, these vaccines were effective only against very specific epidemic hypervirulent strains [263, 264]. In order to develop a vaccine against meningococcus B, other strategies were therefore implemented.

The most promising results were obtained through reverse vaccinology. This involves identifying the antigens for the vaccine not in the classic way—that is, from the components of the bacterium—but instead from the genes that express the proteins with the best characteristics to be good antigens for the vaccine. To obtain a universal vaccine against serogroup B meningococcal strains, complex bioinformatics software was also applied. Following the complete sequencing of the meningococcus B genome [265], researchers at Novartis Vaccines and Diagnostics identified 600 ORFs, which expressed proteins that are exposed on the surface of the bacterial cell. Subsequently, 350 proteins were successfully expressed in *E. coli*, purified, and used to immunize mice. Later, 28 novel protein antigens able to elicit Abs with bactericidal activity were identified. Finally, three of these 28 proteins were selected, namely, NHBA (GNA2132, fused with GNA1030 protein), fHbp (fused with GNA2091 protein), and *nad*A. NHBA (*Neisseria* heparin binding antigen), which is present in virtually all strains, binds heparin, which may increase the serum resistance of bacteria. fHbp (factor H binding protein) binds factor H, which enables the bacterium to survive in theblood [266, 267], thereby blocking the alternative pathway of the complement system. *nad*A (Neisserial adhesin A) promotes adherence to and invasion of human epithelial cells [161, 162]. In addition, the vaccine developed by Novartis Vaccines and Diagnostics contains a fourth component, namely, the vesicle of the OM from the New

Zealand strains, which contain porin 1.4. Theoretically, this vaccine should elicit bactericidal Abs against the following:

(i) NHBA, thus increasing the bactericidal activity of the serum.

(ii) fHbp, thus exposing *N. meningitidis* to the alternative pathway of the complement system.

(iii) NadA, thus hindering the adherence of *N. meningitidis* to epithelial cells.

(iv) Porin A 1.4 and other components of the mixture of proteins contained in OMVs; indeed, OMVs can enhance the immune response by functioning as a conjugate complex of proteins or, rather, a complex of adjuvants.

Several clinical trials have demonstrated the immunogenicity and safety of this vaccine, also named 4CMenB, in infants, children, adolescents, and adults [268, 269]. Consequently the vaccine has been approved by the EMA and named Bexsero [270]. It has also been approved by several national drug agencies (such as FDA), including the Agenzia Italiana del Farmaco (AIFA, Italian National Agency for Drugs) [271].

Owing to the wide variability of Neisserial antigens, a particular laboratory test (MATS) has been developed to estimate the potential effectiveness of the vaccine. Studies conducted worldwide have shown the potential effectiveness of Bexsero, which has been estimated at 87% in Italy [85].

Trumenba was approved for individuals aged 10 to 25 years in the USA in October 2014 [85]. The potential of the vaccine antigen was tested in the laboratory [272] and on a murine model [273, 274]. The immunogenicity, safety, and tolerability of this vaccine were investigated in a randomized controlled trial in infants aged 18–36 months, who were subdivided into three cohorts (receiving 20-, 60-, and 200-$\mu$g rLP2086 dose, resp.) and matched with two control groups: one vaccinated against hepatitis A virus (HAV) and the other with a saline placebo. After the vaccination cycle, seroconversions against Neisserial strains expressing LP2086 variants homologous to the vaccine antigens were found in 61.1–88.9% of toddlers and against strains expressing heterologous LP2086 variants in 11.1–44.4%. Adverse reaction rates were negligible and the vaccine proved to be safe and well tolerated [275]. However, another randomized phase 1/2 clinical study found high fever rates in toddlers receiving one 20- or 60-$\mu$g rLP2086 dose (64% and 90%, resp.) [276].

In a randomized study performed in the USA and Europe in a sample of adolescents (11–18 years of age), Trumenba proved to be highly immunogenic. The proportion of vaccinees with human serum bactericidal activity (h-SBA) titres with a ≥4-fold rise against hypervirulent Neisserial strains with different variants of fHbp was in the range of 75–100%. In another randomized clinical study, carried out in Australia, the safety and immunogenicity of the vaccine were assessed in 60 healthy adults (18–40 years of age) who received 120 $\mu$g doses at 0, 1, and 6 months. The percentage of seroprotected vaccinees was 94.3% against the homologous strains and 70–94.7% against the heterologous strains. The vaccine was well tolerated [277, 278].

As fHbp is also expressed by Neisserial serogroups other than B, the anti-fHbp Abs elicited by rLP2086 might exert a bactericidal effect on meningococci, such as those against *N. meningitidis* serogroup C, as proved by Harris and collaborators [279] and by Konar and colleagues [280]. Moreover, there is some evidence that Trumenba could, at least in part, have an effect on carriage and reduce the risk of acquiring some hypervirulent strains [281].

*4.4. New Vaccines.* The currently available *Neisseria* vaccines, described in the previous paragraphs, are reported and summarized in Table 3.

The elucidation of immunoescape strategies and genomics have enabled scholars to discover new potential vaccine candidates, like NMB0928 [282] or NMB1468 [283], FrpB/fetA [125], LbpA and LbpB [284], adhesin complex protein (ACP) [285], NspA [286, 287], MIP [152], ZnuD [93], PilE [76] and PilQ [288], IgA protease [289], T cell stimulating protein A (*tsp*A) [289], or the CP polymerase of *Neisseria* serogroup X [290], among others [291].

Reverse vaccinology has proved to be a promising approach, enabling researchers to develop the effective vaccine Bexsero. New highly integrated approaches, which combine genomics with postgenomics, are leading to next-generation vaccines. A combination of reverse and forward vaccinology techniques, such as immunoproteome investigation via combined cell surface immunoprecipitation and mass spectrometry (MS) [153], and new bioinformatics strategies, such as the protectome approach [292], are promising in identifying highly conserved motifs in known bacterial protective antigens and using them for the design of effective universal vaccines [293, 294].

## 5. Conclusions

The development of effective vaccines against meningococcal disease has been a long and hard struggle. Early efforts yielded only partial results, with the creation of polysaccharide vaccines [295]. Subsequent research, however, led to the production of the conjugate vaccines [296]. Today, we have the conjugate meningococcal C vaccines [297], an affordable conjugate vaccine against *N. meningitidis* serogroup A (MenAfriVac) [298, 299], the tetravalent conjugate vaccines [270], and, finally, two "universal" vaccines against meningococcal group B strains [300, 301]. The critical rate of coverage required in order to eliminate the disease is probably not among the highest [302]. Indeed, the conjugate vaccine for serogroup C has resulted in dramatic reductions of cases of the disease [303] and created herd immunity that seems to have had a significant effect on the carrier status of adolescents and young adults. Thus, the prospect of dominating this very serious disease lies decidedly in the medium term.

However, it must be borne in mind that we are immersed in a constellation of *Neisseriae*, whose only survival niche is man. Although Neisseriae such as *N. lactamica*, *N. sicca*, *N. elongata*, *N. cinerea*, and *N. flavescens* are usually able to establish silent infection in normal humans, it is not inconceivable that, given the microorganism's great capacity

Table 3: An overview of the currently available *Neisseria meningitidis* vaccines.

| Vaccine | Manufacturer | Serogroups | Licensed age group | Administration schedule | Components details |
|---|---|---|---|---|---|
| AC Vax | GlaxoSmithKline, UK | A, C | 2 y+ | Single dose | 50 $\mu$g each of meningococcal polysaccharides |
| ACWY Vax | GlaxoSmithKline, UK | A/C/Y/W-135 | 2 y+; can be given also at 2 mo+, even though less protective against C, Y, and W-135 | Single dose | 50 $\mu$g each of meningococcal polysaccharides |
| Bexsero (4CMenB) | Novartis Vaccines and Diagnostics | B | 2 mo–17 y | Complex dose schedule depending on age: 3 doses + booster for 2–5 mo; 2 doses + booster at 6–23 mo; 2 doses at 2+ y | 50 $\mu$g of each recombinant NHBA, NadA, fHbp fusion proteins, OMVs from strain NZ98/254 containing the PorA P1.4 (25 $\mu$g), adsorbed on 0.5 mg $Al^{3+}$ |
| HexaMen and HexaMix | National Institute for Public Health and the Environment, Bilthoven, Netherlands | B | — | 2, 3, and 4 mo, a booster dose at 12–18 mo | OMV from two recombinant engineered strains, each of which expressed three different PorA subtypes (P1.5-2, 10; P1.12-1, 13; P1.7-2, 4; P1.19, 15-1; P1.7, 16; and P1.5-1, 2-2) |
| Menactra (MenACWY-DT) | Sanofi Pasteur | A/C/Y/W-135 | 9 mo–55 y | Single dose | 4 $\mu$g each of meningococcal polysaccharides conjugated to 48 $\mu$g of a diphtheria toxoid protein carrier |
| MenAfriVac (MenA-TT) | Serum Institute of India | A | 1–29 y | Single dose | 10 $\mu$g of meningococcal group A polysaccharides conjugated to 10 to 33 $\mu$g tetanus toxoid |
| MenBvac | National Institute for Public Health, Norway, and Novartis | B | — | 3 doses (interval 5–12 w) | OMVs from the strain 44/76 adsorbed on $Al^{3+}$ |
| MencevaxA | GlaxoSmithKline and RIT, Belgium | A | 2 y+ | Single dose | 50 $\mu$g of meningococcal group A polysaccharides<br>No conjugation |
| MencevaxAC | GlaxoSmithKline | A, C | 2 y+ | Single dose | 50 $\mu$g each of meningococcal group polysaccharides<br>No conjugation |
| MencevaxACY | GlaxoSmithKline | A, C, Y | 2 y+ | Single dose | 50 $\mu$g each of meningococcal group polysaccharides<br>No conjugation |
| MencevaxACYW | GlaxoSmithKline | A/C/Y/W-135 | 2 y+ | Single dose | 50 $\mu$g each of meningococcal group polysaccharides<br>No conjugation |
| Mengivac A + C (MenPS) | Sanofi Pasteur | A, C | — | — | 50 $\mu$g of meningococcal group C polysaccharides |
| MenHibrix (HibMenCY-TT) | GlaxoSmithKline | C, Y | 6 w–18 mo | 2, 4, 6, and 12 to 15 mo | Meningococcal groups C and Y polysaccharides conjugated to tetanus toxoid |
| Meningitec (MenC-CRM) | Wyeth Vaccines, Canada, UK, and Australia | C | 2 mo+ | 3 doses at 2–12 mo, 1 dose at 12 mo+ | 10 $\mu$g of meningococcal group C polysaccharides conjugated to 15 $\mu$g $CRM_{197}$ |
| Meninvact | Sanofi Pasteur | C | 2 mo+ | 2 doses at 2–12 mo, 1 dose at 12 mo+ | Meningococcal group C polysaccharides conjugated to $CRM_{197}$ |

Table 3: Continued.

| Vaccine | Manufacturer | Serogroups | Licensed age group | Administration schedule | Components details |
|---|---|---|---|---|---|
| Menitorix (Hib-MenC-TT) | GlaxoSmithKline | C | 6 w–12 mo | Booster at 1-2 y | Meningococcal group C polysaccharides conjugated to tetanus toxoid |
| Menjugate (MenC-CRM) | Novartis Vaccines and Diagnostics | C | 2 mo+ | 3 doses at 2–12 mo; 1 dose at 12 mo+ | 10 $\mu$g of meningococcal group C polysaccharides conjugated to 12.5 to 25 $\mu$g $CRM_{197}$ |
| Menomune | Sanofi Pasteur | A, C | 2 y+ | Single dose | 50 $\mu$g each of meningococcal group polysaccharides No conjugation |
| Menomune | Sanofi Pasteur | A/C/Y/W-135 | 2 y+ | Single dose | 50 $\mu$g each of meningococcal group polysaccharides No conjugation |
| Menovac | Finlay Institute | A/C/Y/W-135 | 2–55 y | Single dose | Meningococcal group polysaccharides |
| Menveo (MenACWY-CRM197) | Novartis Vaccines and Diagnostics | A/C/Y/W-135 | 2–55 y | Single dose | 10 $\mu$g of meningococcal group A polysaccharides and 5 $\mu$g of meningococcal groups C, Y, and W-135 polysaccharides conjugated to $CRM_{197}$ |
| MeNZB | Institute for Public Health, New Zealand, Chiron, Novartis | B | — | — | OMVs from strain P1.7b, 4 |
| NeisVac-C (MenC-TT) | Baxter BioScience | C | 2 mo–65 y | 2 doses at 2–12 mo (with an interval of at least 2 mo), 1 dose at 12 mo+ | 10 $\mu$g of meningococcal group C polysaccharides conjugated to tetanus toxoid |
| Nimenrix | GlaxoSmithKline | A/C/Y/W-135 | 1 y+ | Single dose | 5 $\mu$g each of meningococcal group polysaccharides conjugated to 44 $\mu$g tetanus toxoid |
| NmVac4 | JN-International Medical Corporation | A/C/Y/W-135 | 2–55 y | Single dose | 50 $\mu$g each of meningococcal group polysaccharides |
| Trumenba | Pfizer | B | 10–25 y | 3 doses (0–2–6 mo) | 120 $\mu$g of recombinant fHbp adsorbed on 0.25 mg $Al^{3+}$ |
| Zamevax | Imunoloski Zavod, Croatia | A, C | — | — | No conjugation |

$CRM_{197}$: cross-reacting material 197; fHbp: factor H binding protein; mo: month; NadA: *Neisseria* adhesion A; NHBA: Neisseria heparin binding antigen, also named GNA2132; OMV: outer membrane vesicle; PorA: porin A; w: week; y: year; $Al^{3+}$: Aluminum.

for genetic variation, nonpathogenic *Neisseria* might become hazardous to humans [304].

The challenge is still open.

## Abbreviations

Abs: Antibodies
ACP: Adhesin complex protein
AIFA: Agenzia Italiana del Farmaco (Italian National Agency for Drugs)
AMPs: Antimicrobial peptides
AP: Alternative Pathway
*app*: Adhesion and penetration protein
ARF6: ADP-ribosylation factor 6
BBB: Blood-brain barrier
C3: Human complement 3 component
C3b: Human complement 3b component
C4: Human complement 4
C4bp: Human complement 4b binding protein
CAMPs: Cationic antimicrobial peptides
*cas*: CRISPR-associated
*cbp*: Calprotectin binding protein
CCL: Chemokine (C-C motif) ligand
CD: Cluster of Differentiation (CD4, CD25, CD45R, CD86, CD105, CD147, CDw29)
CDS: Coding sequence
CP: Capsule
CEACAMs: Carcinoembryonic antigen related cell adhesion molecules

CMP-NANA: Cytidine monophosphoacetyl *N*-Acetyl-neuraminic acid
CRs: Correia repeats
CREEs: Correia repeat-enclosed elements
CRISPR: Clustered regularly interspaced short palindromic repeats
CRM197: Cross-reacting material 197
CRP: C-reactive protein
CXCL: Chemokine (C-X-C motif) ligand
DCs: Dendritic cells
*dam*: DNA adenine methyltransferase
DNA: Deoxyribonucleic acid
*drg*: Dam replacing gene
dRS3: Duplicated repeat sequence 3
*E. coli*: *Escherichia coli*
EMA: European Medicines Agency
FDA: Food and Drug Administration
fH: Factor H
fHbp: Factor H binding protein
*fur*: Ferric uptake regulator
GEI: Genomic island
GGI: Gonococcal genetic island
*ggt*: Gamma-glutamyl aminopeptidase
GNA: Genome-derived neisserial antigen (GNA33, GNA1030, GNA1870, GNA2091, GNA2132)
GWAS: Genome-wide association studies
HAMPs: Host-associated molecular patterns
HAV: Hepatitis A virus
HGT: Horizontal Gene Transfer
HLA: Human leukocyte antigens
*hpu*: Haemoglobin-haptoglobin utilization
h-SBA: Human serum bactericidal activity
HSP: Heat-Shock Protein
HSPG: Heparan sulphate proteoglycan
HTTs: High-throughput technologies
IFN: Interferon
IgA: Immunoglobulin A
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IGR: Intergenic region
I$\kappa$B: Inhibitors of NF-$\kappa$B
IL: Interleukin (IL6, IL8, IL10, IL12)
IM: Inner membrane
IS: Insertion sequence (IS1016, IS1106-like, IS1301, IS1655)
*kat*A: Catalase A
LAMP1: Lysosomal-associated membrane protein 1
*lbp*: Lactoferrin-binding protein
*lct*: Lactate permease
LGT: Lateral Gene Transfer
*lgt*: Prolipoprotein diacylglyceryl transferase (*lgt*A, *lgt*B, *lgt*C, *lgt*D, *lgt*E, *lgt*G)
L-NNT: Lacto-N-neotetraose
LOS: Lipooligosaccharides
LPS: Lipopolysaccharides
MAC: Membrane attack complex
MAPK: Mitogen-activated protein kinase
MAPKK: Mitogen-activated protein kinase Kinase
MATS: Meningococcal Antigen Typing System
MDA: Meningococcal disease-associated island
MHC: Major histocompatibility complex
MIP: Macrophage infectivity potentiator
MMEs: Minimal mobile elements
*mlt*A: Membrane-boundlytic transglycosylase A
mo: Month
mRNA: messenger RNA
*msf*: Meningococcal surface fibril
*msr*: Methionine sulfoxide reductase
MS: Mass spectrometry
*msp*A: Meningococcal serine protease A
*mtr*: Multiple transferable resistance
MWNTs: Multiwalled carbon nanotubes
*N.*: Neisseria (*N. lactamica*, *N. sicca*, *N. elongata*, *N. cinerea*, and *N. flavescens*)
*nad*A: Neisserial adhesin A
NCAM-1: Neural cell adhesion molecule 1
NEMIS: *Neisseria* miniature insertion sequences
NETs: Neutrophil extracellular traps
NF-$\kappa$B: Nuclear factor kappa-light-chain-enhancer of activated B cells
*N. gonorrheae*: *Neisseria gonorrheae*
NHBA: Neisserial heparin binding antigen
*nhhA*: *Neisseria* hia/hsf homologue
NIMEs: Neisserial intergenic mosaic elements
NKs: Natural killer cells
NLRs: NOD-like receptors
*N. meningitidis*: *Neisseria meningitidis*
NO: Nitric oxide
NOD: Nucleotide-binding Oligomerization Domain
NTPase: Nucleoside triphosphatases
*oat*: O-acetyltransferase
OM: Outer membrane
OMPs: Outer membrane proteins
OMVs: Outer membrane vesicles
OMPLA: Outermembrane phospholipase A
Opa: Opacity-associated protein a
Opc: Opacity-associated protein c
ORF: Open reading frame
*pac*: Peptidoglycan O-acyltransferase
PAI: Pathogenic island
PAMPs: Pathogen-associated molecular patterns
*pil*: Pilin (*pil*C: OM/cell surface pilin; *pil*D: prepilin-processing leader peptidase; *pil*F: traffic NTPase; *pil*G: pilus assembly protein; *pil*M: biogenesis protein; *pil*P: pilot protein; *pil*Q: secretin; *pil*T: traffic NTPase; *pil*W: pilus stabilization protein)
PN: Peroxynitrite
*por*: Porin
*ppt*A: Pilin phosphorylcholine transferase A
PRRs: Pattern recognition receptors
PTK: Protein tyrosine kinase
RANTES: Regulated on activation, normal T cell expressed and secreted
RecA: Recombinase A

REP2: Repetitive extragenic palindromic sequence
RIG-I: Retinoic acid-inducible gene 1
rLP2086: Recombinant lipoprotein 2086
RLRs: RIG-1-like receptors
RNA: Ribonucleic acid
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
*sia*: Polysialic acid capsule biosynthesis protein (*sia*A, *sia*B, *sia*C, *sia*D)
SMAD: Small Mothers Against Decapentaplegic
*sod*: Superoxide dismutase (sodB, sodC)
SOMVs: Spontaneously released OMVs
SSM: Slipped strand mispairing
SSR: Simple Sequence Repeat
*tbp*: Transferrin-binding protein
TGF: Transforming Growth Factor
Th: T-cell helper
TI: T-cell Independent
TLR: Toll-like receptor (TLR2, TLR4, TLR5, TLR9)
TNF: Tumor Necrosis Factor
*tsp*A: T cell stimulating protein A
UK: United Kingdom
USA: United States of America
UTR: Untranslated Region
w: Week
WGS: Whole-genome sequencing
WNT: Wingless-related integration site
y: Year
*znu*D: Zinc uptake component D.

## Conflict of Interests

R. Gasparini, D. Panatto, N. L. Bragazzi, P. L. Lai, A. Bechini, M. Levi, P. Durando, and D. Amicizia declare that they have no conflict of interests regarding the publication of this paper.

## Acknowledgments

Although efforts to make a systematic overview have been made as much as possible, quoting the relevant scholarly literature, due to the interdisciplinary nature of the topic and its vastity, the authors apologize if they have missed some important contributions. The authors thank Dr. Bernard Patrick for revising the paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 710656, 9 pages
http://dx.doi.org/10.1155/2015/710656

*Research Article*

# Estimation of the Impact of Meningococcal Serogroup C Universal Vaccination in Italy and Suggestions for the Multicomponent Serogroup B Vaccine Introduction

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Received 9 January 2015; Revised 31 March 2015; Accepted 15 April 2015

Academic Editor: Nejat K. Egilmez



In Italy, the meningococcal C conjugate vaccine (MenC) has been offered in most regions since 2009-2010. The incidence of Invasive Meningococcal Disease (IMD) was 0.25 confirmed cases per 100,000 in 2011, but this may be considerably underestimated due to underdetection and underreporting. This study estimates the impact of the MenC universal vaccination (URV) in the Puglia region by assessing the completeness of three registration sources (notifications, hospitalizations, and laboratory surveillance). Capture-recapture analysis was performed on meningococcal meningitis collected within 2001–2013. The impact of URV among ≤18-year-olds was assessed by attributable benefit, preventable fraction, and prevented fraction. Missed opportunities for vaccination were evaluated from surveillance of IMD. The proportion of detected serogroups was applied to the number of IMD in the postvaccination period to compute the cases still preventable. The sensitivity of the three sources was 36.7% (95% CI: 17.5%–57.9%) and registrations lost nearly 28 cases/year in the period. Attributable benefit of URV was −0.5 cases per 100,000, preventable fraction 19.6%, and prevented fraction 31.3%. Three adolescent cases missed the opportunity to be vaccinated. The multicomponent serogroup B meningococcal vaccine has the potential to further prevent at least three other cases/year. Vaccination strategy against serogroup B together with existing programmes makes IMD a 100% vaccine-preventable disease.

## 1. Background

*Neisseria meningitidis* is one of the leading causes of bacterial meningitis and sepsis and can also cause pneumonia and other localized infections. Invasive Meningococcal Disease (IMD) is associated with substantial mortality and long-term morbidity worldwide. There are 12 serogroups, but the majority of invasive meningococcal infections are caused by organisms from the A, B, C, X, Y, or W-135 serogroups [1].

Despite significant gaps in data limit description of IMD epidemiology in some parts of the world, it is generally recognized that mass campaigns using conjugated meningococcal vaccines in the last decade have led to the control of serogroup C disease in many developed countries [2].

In Canada, the decline in IMD incidence was at least partly attributable to the universal infant serogroup C conjugate immunization programmes and adolescent catch-up programmes that started as early as 2001-2002 [3, 4]. In the USA, where in 2005 the Advisory Committee on Immunization Practices recommended routine vaccination with the quadrivalent meningococcal conjugate vaccine (MenACWY) for adolescents aged 11–18 years, the incidence of *Neisseria meningitidis* infections mostly decreased from 2006 to 2010 in the targeted population [5, 6].

In Europe, the United Kingdom was the first country to introduce meningococcal serogroup C conjugate vaccine (MenC) in 1999, incorporating it into the routine childhood immunization schedule. In 2000, a catch-up campaign was

Table 1: Vaccination coverage (VC) with meningococcal conjugate vaccines in children ≤24 months and adolescents in Italy and in the Puglia region, within 2006–2013 (postvaccination period), by birth cohort.

| | Italy [10] | | Puglia region | |
|---|---|---|---|---|
| Birth cohort | Number of regions providing VC | MenC VC* | MenC VC | MenACWY VC |
| 1995§ | | | 45.60% | 1.80% |
| 1996§ | | | 51.20% | 1.90% |
| 1997§ | | | 56.20% | 2.10% |
| 1998§ | | | 62.00% | 8.50% |
| 1999§ | | | 46.80% | 4.70% |
| 2000§ | | | 37.30% | 21.70% |
| 2001§ | | | 27.00% | 32.30% |
| 2002§ | | | 24.70% | 22.90% |
| 2004† | | | 47.00% | |
| 2005† | 13 | 48.10% | 65.00% | |
| 2006† | 13 | 58.50% | 74.50% | |
| 2007† | 16 | 64.40% | 77.90% | |
| 2008† | 16 | 68.30% | 79.00% | |
| 2009† | 18 | 77.80% | 81.90% | |
| 2010† | 18 | 81.10% | 82.40% | |
| 2011† | | | 81.10% | |

*Average VC in regions which provided data. †One dose of MenC conjugate vaccine at 15 months of age. §One dose of MenC and, since 2012, of MenACWY conjugate vaccine at 11/12 years of age.

implemented for adolescents ≤18 years, later extended to young adults up to 24 years of age. As a consequence, in England, hospital admissions decreased from 34.54 per 100,000 children <15 years old in 1999 to 12.40 per 100,000 in 2011 [7].

At a broader level, in 2011, 3,814 confirmed cases of IMD were reported by 29 European Union/European Economic Area (EU/EEA) countries (0.75 per 100,000), mostly in children younger than five years of age (5.73 per 100,000), followed by adolescents and young adults aged 15–24 years (1.29 per 100,000). The majority of cases were due to serogroups B and C, with serogroup B being dominant, mainly attributable to the introduction of the MenC universal routine vaccination (URV) in some EU/EEA countries [8].

In Italy, although only recommended at a national level for at risk groups under the National Immunization Plan 2005–2007, MenC has also been offered to other targets based on individual regional vaccination policies [9]. Since 2009-2010, most regions have established a universal infant free-of-charge programme, most commonly based on active call, reaching an average vaccination coverage of 68% in the 2008 birth cohort [10]. The Puglia region (Southern Italy, with approximately 4,000,000 inhabitants) introduced 1-dose MenC URV for children aged 15 months in 2006 [11], achieving a vaccination coverage higher than 80% (Table 1).

In the same period, 12 regions recommended the immunization of adolescents aged between 11 and 16 years, with one dose [12]. Puglia also introduced the active, free-of-charge offer of 1-dose MenC at 11-12 years of age in 2006 [11] and replaced it with MenACWY in 2012 [13], reaching a vaccination coverage against meningococcus C of nearly 60% (Table 1).

The National Vaccination Plan 2012–2014 included MenC URV in the list of "Essential Health Interventions" for toddlers between 13 and 15 months of age and 11–18-year-old adolescents [14].

IMD is rare in Italy where 0.25 confirmed cases per 100,000 population were observed in 2011, based on surveillance data submitted to The European Surveillance System [8]. Reported incidence, however, may be considerably underestimated due to underdiagnosis (underascertainment) and underreporting affecting IMD surveillance, particularly in some regions [12].

Monitoring the incidence of meningococcal disease is important to evaluate the impact of the implemented vaccination strategies, and to advise on the use of the new multicomponent serogroup B meningococcal (4CMenB) vaccine. This has recently been introduced in Puglia [15] and in other three Italian regions and is under discussion for the introduction on a national scale. This study aims to estimate the impact of MenC URV on the burden of IMD in Puglia by assessing the completeness (sensitivity) of registration systems on meningococcal disease.

## 2. Methods

*2.1. Sensitivity Analysis of Data Sources.* In Italy, three surveillance sources are available for monitoring meningococcal disease:

(i) *Mandatory Notification System* (referred to as the Sistema Informativo delle Malattie Infettive (SIMI)), implemented in 1990 by the Ministry of Health, according to which physicians have to report every case of meningococcal meningitis (International Classification of Diseases, Ninth Revision (ICD9) code: 036.0). The notification database contains: a unique patient number, date of birth, gender, first and last name, postal code, municipality, date of notification, date of first symptoms, and date of diagnosis.

(ii) *National Surveillance of Invasive Bacterial Diseases* (referred to as MIB), implemented in 1994 by the Istituto Superiore di Sanità (ISS), with the aim of collecting data on the type of *Neisseria* causing invasive disease from blood and/or cerebrospinal fluid (CSF) isolates. This source contains: a unique patient number, date of birth, gender, first and last name, municipality, laboratory that submitted the strain, date of collection and receipt of the sample, and typing results.

(iii) *Hospital Discharge Registry* (HDR), where IMD is identified by the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD9-CM) codes 036.xx as main or secondary diagnosis. Data in this source are: a personal ID

number, first and last name, date of birth, gender, postal code, municipality, date of hospital admission and discharge, ICD9-CM codes, and outcome (deceased or not).

To evaluate the completeness of these three sources in describing IMD incidence, a capture-recapture analysis, similar to that proposed by de Greeff et al. in 2006 [16], was performed on data collected between 2001 and 2013 in Puglia, assuming that the region had a closed population in the considered years (0.6% increase, data from Italy's National Census Bureau (ISTAT) estimate of native- and foreign-born [17]; first assumption). To ensure that each individual had the same chance of being included in all three sources [18], the evaluation was restricted to meningococcal meningitis (ICD9-CM 036.0), as meningococcal sepsis or other clinical pictures of IMD are not notifiable by law in Italy [19] (second assumption). For the capture-recapture analysis involving three or more sources, the independence assumption (third assumption) was not crucial because interaction terms can be incorporated into regression models to adjust for source dependencies [20]. The homogeneity of capture (fourth assumption) was directly fulfilled by the linkage of records between the three sources by three patient variables: first name, last name, and date of birth. The three-source analysis was performed by fitting eight log-linear models using STATA's user-written program "recap" module, providing standard three-source capture-recapture analyses without covariates [21]. The population size (that is the total number of cases, including the number of cases not registered in any of the three sources) and the Confidence Interval estimates were computed according to a goodness-of-fit based method proposed by Regal and Hook [22]. The choice of the final model was based on the likelihood ratio test statistic ($G^2$), the Akaike Information Criterion (AIC), and the Bayesian Information Criterion (BIC) [23, 24]. The best-fitting model was the one with the lowest $G^2$, AIC, and BIC [25].

Sensitivity of each source was estimated by dividing the observed number of cases in each source by the capture-recapture estimate of the total population [16]; overall sensitivity of the three surveillance registries was computed by dividing the number of cases registered in at least one of the three sources by the capture-recapture estimate of the total population.

*2.2. Impact of Meningococcal URV on the Burden of IMD.* Record linkage in accordance with the above-mentioned design was performed to obtain the number of IMD registered in at least one of the three sources (pool of cases). Crude and age-adjusted annual incidence rates were calculated by dividing the pool of cases by the number of residents in Puglia and applying the region's age-specific rates to the Italian 2001–2013 standard population, respectively (data from Italy's National Census Bureau estimate) [17]. Data were compared to the national hospitalization rates [10]. Crude and age-specific incidence rates before the introduction of URV (calculated as the average annual rates between 2001 and 2005, prevaccination period) were compared to the average annual rates within 2006–2013 (postvaccination period) by calculating the Incidence Rate Ratios (IRRs) with 95% Confidence Interval (95% CI), by using Poisson regression models.

The impact of the vaccination programme was also assessed in the target population, considering subjects ≤18 years of age resident in Puglia, by the following measures:

(i) The *attributable benefit*, that is the reduction in incidence of the disease among vaccinated individuals attributable to the introduction of URV in 2006 [26], calculated as

$$A_{\text{le}}B = I_{v+} - I_{v-} = I_{2006-2013} - I_{2001-2005}. \quad (1)$$

(ii) The *preventable fraction*, that is the proportion of the disease that would be prevented if the whole population was vaccinated, calculated as

$$P_{\text{le}}F = \frac{I_p - I_v}{I_p}, \quad (2)$$

where $I_p$ was the incidence rate in the population and $I_v$ was the incidence rate in the vaccinated people [26]. Considering the introduction of URV in 2006, the formula was computed as

$$P_{\text{le}}F = \frac{I_{2001-2013} - I_{2006-2013}}{I_{2001-2013}}. \quad (3)$$

(iii) The *prevented fraction*, that is the proportion of hypothetical total cases that were prevented by the introduction of URV, calculated as

$$P_{\text{ed}}F = \frac{I_u - I_p}{I_u}, \quad (4)$$

where $I_p$ was the incidence of the disease in the population and $I_u$ was the rate among unvaccinated people [26]. The formula was computed as

$$P_{\text{ed}}F = \frac{I_{2001-2005} - I_{2001-2013}}{I_{2001-2005}}. \quad (5)$$

The prevented fraction could also be calculated as

$$P_{\text{ed}}F = P_p \times (1 - \text{RR}), \quad (6)$$

where $P_p$ was the prevalence of subjects protected by the vaccination [26]. In this study, the prevalence of protected subjects was computed as

$$P_p = \text{VC} \times \text{VE}, \quad (7)$$

where VC was the vaccination coverage reached in the target population and VE was the vaccine efficacy reported for the marketed vaccines [27]. The Relative Risk was

$$\text{RR} = \frac{I_{2006-2013}}{I_{2001-2005}}. \quad (8)$$

*2.3. Missed Opportunities in the Meningococcal Vaccination Programme.* To review the missed opportunities for vaccination occurring in the meningococcal URV programme, data from an *ad hoc* surveillance system on IMD cases was evaluated.

A prospective population-based, laboratory-confirmed surveillance of possible IMD cases started in Puglia in January 2013 with the aim of describing the epidemiology of IMD in the most affected age groups (0–30 years, residents) over a two-year period. The surveillance network included Infectious Disease and Intensive Care Divisions of all hospitals in the region and the Reference Laboratory for Invasive Bacterial Diseases. Subjects admitted to the participating wards and meeting the clinical case definition for IMD set out by the EU Commission Decision 28/IV/2008 were enrolled as possible cases. This included any person with at least one of the following five clinical signs: fever, meningeal signs, petechial rash, septic shock, and septic arthritis. A confirmed case was any person meeting at least one of the following four laboratory criteria: (i) isolation of *Neisseria meningitidis* from a normally sterile site, including purpuric skin lesions; (ii) detection of *Neisseria meningitidis* nucleic acid from a normally sterile site, including purpuric skin lesions; (iii) detection of *Neisseria meningitidis* antigen in CSF; (iv) detection of gram negative stained diplococcus in CSF [28].

From all specimens taken for routine diagnostic ascertainment within 24 h of enrolment of a possible case, an aliquot was stored at −20°C and sent to the Regional Reference Laboratory for standardized testing of *N. meningitidis* by RT-PCR and multiplex sequential PCR.

For each enrolled subject, physicians involved in the surveillance network collected the following information in an electronic case report form:

(i) clinical symptoms, date and time of presentation,

(ii) demographics and immunization history for meningococcal vaccines (number of doses, date of vaccination, etc., later validated by the regional immunization registry),

(iii) risk factors and comorbidities,

(iv) data on clinical outcome (recovery, worsening condition, other complications, and death) and hospital resources utilization (length of hospital stay, days in intensive care, daily dosages (DDDs) of antibiotics, and diagnostic tests).

Patients were followed up to 30 days after the beginning of the disease.

The surveillance was conducted in accordance with The Guidelines for Good Clinical Practice and the ethical principles that originate in the Declaration of Helsinki. The protocol was approved by the Institutional Review Board at the Regional Observatory for Epidemiology. For each enrolled subject, written informed consent was obtained from the legal guardians, according to the Italian law. No incentive was provided to encourage study participation.

A missed opportunity was defined as an IMD case occurring in the study period, who was supposed to be vaccinated according to the meningococcal conjugate regional immunization schedule. For each case, the month of disease onset, gender, age, serogroup, *exitus* or sequelae, administered vaccine, and scheduled time of vaccination were reported.

*2.4. Estimation of Meningococcal URV Further Potential Impact.* To estimate the further potential impact of the meningococcal vaccination programme, now including the new 4CMenB vaccine, the distribution of *N. meningitidis* serogroups detected by the MIB surveillance was applied to the total number of cases reported among subjects ≤18 years old in the postvaccination period, in order to compute the annual number of cases that could still be preventable.

For the 4CMenB vaccine, a predicted vaccine strain coverage of 87% was considered [29].

## 3. Results

*3.1. Sensitivity Analysis of Data Sources.* At a regional level, in the period within 2001–2013,

(i) 118 cases of meningococcal meningitis were notified to the SIMI,

(ii) 102 cases of meningococcal meningitis were reported to the MIB surveillance,

(iii) 144 hospitalizations for meningococcal meningitis were recorded in the HDR.

Moreover, 873 hospitalizations were coded as meningitis due to unspecified bacterium (ICD9-CM 320.9).

In the study period, 213 cases of meningococcal meningitis were recorded in at least one of the three sources. Of these, 49 were identified in the three sources, a further 13 were matches between HDR and MIB sources, 34 were matches between MIB and SIMI, and 6 were matches between HDR and SIMI (Figure 1). The log-linear model with the lowest $G^2$, AIC, and BIC included two interactions between sources (MIB, SIMI and MIB, HDR) and provided an estimate of 580 (95% CI: 368–1,216) total cases (Table 2).

The overall sensitivity was estimated at 36.7% (95% CI: 17.5%–57.9%). The completeness of HDR was 24.8% (95% CI: 11.8%–39.1%), higher than that of SIMI (20.3%, 95% CI: 9.7%–32.1%) and of MIB (17.6%, 95% CI: 8.4%–27.7%).

*3.2. Impact of Meningococcal URV on the Burden of IMD.* The overall annual incidence trend of IMD showed a sharp reduction immediately after the introduction of the URV in 2006, both in Italy and in the Puglia region (Figure 2).

In the Puglia region, the IRR before and after the introduction of the vaccination programme was 0.7 (95% CI: 0.4–1.4, Table 3). The annual incidence decreased from 1.29 per 100,000 in the prevaccination period to 0.79 per 100,000 in the postvaccination period among subjects ≤18 years of age. The attributable benefit of URV was −0.5 cases per 100,000, while the preventable fraction was 19.6% and the prevented fraction was 31.3%.

Vaccination coverage against meningococcus C among subjects ≤18 years old was 57.3%. Thus, the prevented fraction

Table 2: Log-linear models fitted to three sources of data on meningococcal meningitis and the estimated number of cases in the Puglia region, within 2001–2013.

| Models | df* | $G^{2\dagger}$ | $p^{\ddagger}$ | AIC** | BIC*** | $x^{\S}$ | $N^{\P}$ | 95% CI for $N^{\P}$ |
|---|---|---|---|---|---|---|---|---|
| Independent (no interaction) | 3 | 114.63 | 0 | 108.63 | 108.79 | 29 | 242 | 229–260 |
| Interaction (MIB#, SIMI##) | 2 | 21.81 | 0 | 17.81 | 17.92 | 77 | 290 | 260–333 |
| Interaction (MIB, HDR###) | 2 | 114.46 | 0 | 110.46 | 110.57 | 30 | 243 | 228–266 |
| Interaction (SIMI, HDR) | 2 | 87.01 | 0 | 83.01 | 83.12 | 6 | 219 | 214–231 |
| **Interaction (MIB, SIMI) and (MIB, HDR)** | **1** | **.58** | **.44** | **−1.42** | **−1.36** | **367** | **580** | **368–1,216** |
| Interaction (MIB, SIMI) and (SIMI, HDR) | 1 | 18.63 | 0 | 16.63 | 16.69 | 35 | 248 | 223–307 |
| Interaction (MIB, HDR) and (SIMI, HDR) | 1 | 84.92 | 0 | 82.92 | 82.97 | 5 | 218 | 213–228 |
| Interaction (MIB, SIMI) and (MIB, HDR) and (SIMI, HDR) | 0 | 0 | 1 | 0 | 0 | 244 | 457 | 271–1,225 |

*df, degrees of freedom. †$G^2$, likelihood ratio statistic. ‡$p$ value for the likelihood ratio statistic. **AIC, Akaike Information Criterion. ***BIC, Bayesian Information Criterion. §Estimate of the number of cases not reported to any source. ¶Estimate of the total number of cases.
#MIB, Invasive Bacterial Diseases Surveillance. ##SIMI, Infectious Diseases Routine Notification System. ###HDR, Hospital Discharge Registry.

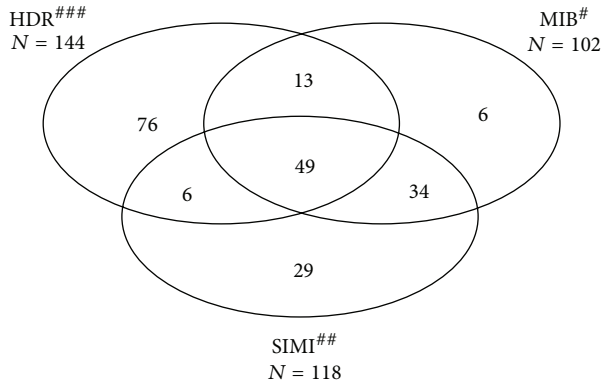


Figure 1: Venn diagram of the number of meningococcal meningitis cases identified by the three sources MIB#, SIMI##, and HDR### ($N$ = 213) in the Puglia region, within 2001–2013. #MIB, Invasive Bacterial Diseases Surveillance; ##SIMI, Infectious Diseases Routine Notification System; ###HDR, Hospital Discharge Registry.

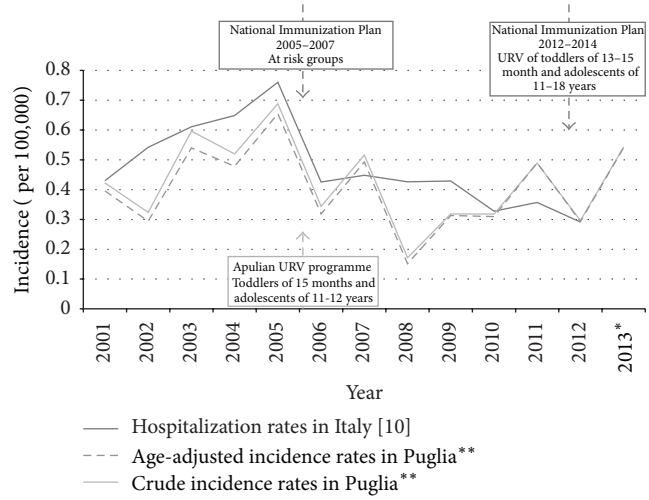


Figure 2: Annual incidence trend of IMD and MenC recommendations in Italy and in the Puglia region, within 2001–2013. *National data not available [10]. **Pool of cases from the three sources linkage.

could be estimated to vary between 18.4% and 22.2% for a VE of 83–100%. (Figure 3(a)). On the other hand, an observed prevented fraction of 31.3% implied that vaccination coverage could range from 80.8% to 97.4% (Figure 3(b)).

*3.3. Missed Opportunities in the Meningococcal Vaccination Programme.* Of 11 confirmed IMD cases among those enrolled ≤30 years of age, three adolescents missed the opportunity to be protected by the vaccination (Table 4).

*3.4. Estimation of Meningococcal URV Further Potential Impact.* Between 2006 and 2013, in the MIB surveillance, serogroup B accounted for 53.8% of isolates, serogroup C for 15.4%, serogroup W for 23.1%, and serogroup Y for 7.7% among subjects ≤18 years of age. Considering an average annual IMD incidence of 0.79 per 100,000 (≈6 cases/year), three cases/year could be attributable to serogroup B, one to group C, and two cases to the other serogroups. Estimating a vaccine strain coverage of 87%, the 4cMenB vaccine has the potential to further prevent at least three other cases/year.

## 4. Discussion

To the best of our knowledge, this is the first study in Italy to provide an assessment of the sensitivity of data sources available for monitoring the incidence of meningococcal meningitis. In some areas, all three registries have the disadvantage of incompleteness due to underdiagnosis (underascertainment), misclassification, and underreporting of IMD [12]. Capture-recapture analysis represents a unique tool to estimate the sensitivity of surveillance registrations and hence the total number of cases [16].

In this study, the completeness of each source alone is no guarantee of an adequate description of disease incidence. Concerning the three linked registries, they are not sufficiently comprehensive in terms of the cases they contain (37%). The number of cases not registered in any of the three sources amounted to 367, meaning that our surveillance systems lost nearly 28 cases/year in the study period. In a similar study by de Greeff et al. in Netherlands, the sensitivity

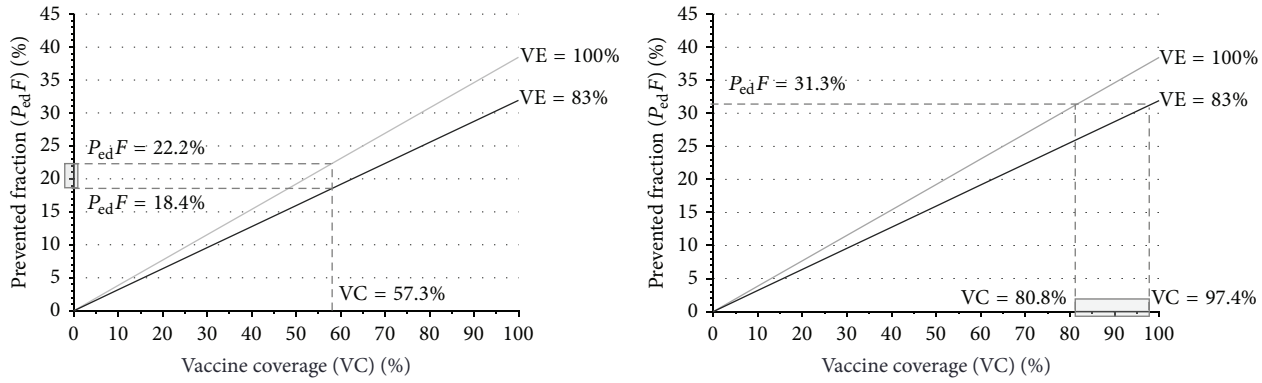


Figure 3: Prevented fraction of IMD cases ≤18 years of age and vaccination coverage against meningococcus C in the Puglia region, within 2001–2013, by vaccine efficacy (VE [27]). (a) Estimated $P_{ed}F$ for VC of 57.3% among subjects ≤18 years of age, assuming VE of 83–100%. (b) Estimated VC among subjects ≤18 years of age for $P_{ed}F$ of 31.3%, assuming VE of 83–100%.

Table 3: Annual incidence rates per 100,000 Rate Ratios (RRs) and 95% CIs of IMD* between the prevaccination and the postvaccination period in the Puglia region, within 2001–2013, by class of age.

| Class of age | 2001–2005 | | 2006–2013 | | |
|---|---|---|---|---|---|
| | *N* | Rate per 100,000 | *N* | Rate per 100,000 | RR (95% CI) |
| <1 year | 1 | 2.48 | 1 | 2.7 | 1.1 (0.1–17.1) |
| 1–4 years | 3 | 1.82 | 1.7 | 1.1 | 0.6 (0.1–3.9) |
| 5–9 years | 2.2 | 1.01 | 1.3 | 0.6 | 0.6 (0.1–5.5) |
| 10–14 years | 1.8 | 0.75 | 1 | 0.5 | 0.6 (0.1–7) |
| 15–19 years | 2.2 | 0.86 | 1.3 | 0.6 | 0.6 (0.1–5.5) |
| 20–24 years | 2.4 | 0.84 | 2.7 | 1.1 | 1.2 (0.2–7.2) |
| 25–49 years | 3.4 | 0.23 | 1.2 | 0.1 | 0.3 (0.1–2.8) |
| ≥50 years | 4.6 | 0.34 | 4.5 | 0.3 | 0.9 (0.2–3.3) |
| Total | **20.6** | **0.51** | **14.7** | **0.4** | **0.7 (0.4–1.4)** |

*Pool of cases from the three sources' linkage.

was estimated at 49% for mandatory notifications, 67% for hospital registrations, and 58% for laboratory surveillance [16]. As in our findings, surveillance of meningococcal disease based on hospital admissions seems to capture the most cases, though the data lack serotyping information and are not as timely available as notification data and laboratory surveillance. This seems reasonable as meningococcal disease is so severe that all patients are expected to show up in the hospital [10, 16]. In addition, changes in completeness of registration/reporting by any of the three sources could have affected the results of the capture-recapture analysis, making the interpretation of the chronological trend a challenge.

A significant underreporting affects the Infectious Diseases Routine Notification System in several Italian regions, complicating efforts to understand their occurrence and burden, particularly when the planning and evaluation of vaccination programmes need timely, reliable incidence data. The pattern of this underreporting is a complex mix of factors, including availability and use of appropriate diagnostic services, reporting practices by physicians, and the operation of the surveillance system itself [30]. As regards Invasive Bacterial Diseases, underascertainment remains considerable for the scarce attitude to investigate cases using adequate laboratory tests, as a large number of the discharge records coded as "meningitis due to unspecified bacterium" in this study shows. Real time-PCR is significantly more sensitive than culture, which, so far, has been the most commonly used technique for meningococcal surveillance, leading to an important underestimation of disease burden. Furthermore, it is well known that culture-based methods have an even lower sensitivity compared to molecular methods when the patient has been treated with antibiotics [31]. Real time-PCR also has the advantage of providing diagnosis in the presence of culture-negative samples [32, 33] and timely results.

According to other studies conducted in Italy [10, 34], the incidence rate of IMD decreased after the introduction of the meningococcal URV in 2006, leading to a reduction in the attributable risk among vaccinated individuals. A study by Pascucci et al. in the Emilia Romagna region indicated a 70% decrease in the incidence of meningococcus C-related invasive disease and no cases attributable to serogroup C in children aged 1–4 years after the introduction of the MenC universal vaccination in 2006 [35].

From a public health perspective, it is important to determine the proportion of cases associated with a disease that could be prevented if the target population had received the vaccine for the entire period instead of only a part [36]. In this study, the preventable fraction was 19.6%, meaning that almost one case in five could have theoretically been prevented if URV had been introduced in 2001. The proportion of total cases presumably prevented by the introduction of URV amounted to almost one in three cases (prevented fraction of 31.3%), higher than what could be estimated considering the current coverage against meningococcus C of 57.3% in the target population. Thus, the proportion of subjects protected by the vaccination programme could be higher, up to 80.8–97.4%, due to the indirect herd effect in the unvaccinated population. A nationwide study by Bijlsma et al.

Table 4: Missed opportunities in the meningococcal vaccination programme. Active surveillance of IMD cases ≤30-year-olds, in Puglia region, January 2013–September 2014.

| Enrolment date | Gender | Age | Serogroup | Sequelae | Exitus | Vaccine to receive | Active call to vaccination |
|---|---|---|---|---|---|---|---|
| June 2013 | M | 11 | Y | Partial deafness | No | MenACWY | February 2013 |
| October 2013 | F | 13 | C | None | No | MenACWY | January 2011 |
| May 2014 | F | 18 | C | None | Yes | MenC | February 2007 |

in Netherlands has provided further evidence that herd protection, resulting from the reduced carriage of virulent meningococci, was responsible for >36% of MenC vaccine impact [37].

Some missed opportunities occurred in the adolescent meningococcal vaccination programme in Puglia, leading to one death and one case developing long-term sequelae. This highlights the importance of strengthening the ongoing vaccination programme against all preventable serogroups and increasing adequately vaccine timeliness and coverage.

Despite the URV-attributable reduction in the proportion of meningococcal infection due to serogroup C, nearly one case from group C and two cases from groups W and Y could still be preventable every year; cases from serogroup B remain dominant.

Vaccination strategy against serogroup B in infants, now implemented in Puglia, and the existing programmes against serogroups A, C, W, and Y targeting children aged 15 months and 11-12 years old are a full spectrum armoury against all five serogroups, making invasive meningococcal infection a 100% vaccine-preventable disease.

## Disclosure

Portions of the study data shown in this paper were previously presented as an oral presentation at the European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE), held on 5–7 November 2014 in Stockholm, Sweden, and as a lecture at the 47th National Congress of the Italian Society of Hygiene, Preventive Medicine and Public Health (SItI) in Riccione, Italy, 1–4 October 2014.

## Conflict of Interests

The *ad hoc* laboratory-confirmed surveillance of IMD was supported by an unrestricted grant from Novartis Vaccines and Diagnostics S.r.l. Rosa Prato has served in advisory committees and as a speaker in conferences related to meningococcal vaccines for both Novartis and GSK. She has received a research grant from Novartis to act as the Principal Investigator in the IMD surveillance. Domenico Martinelli has received travel expenses from Novartis to take part in expert meetings and conferences. All other coauthors have no conflicts relevant to this paper to disclose, with the exception of funding needed to conduct the surveillance. Rosa Prato's institution has taken charge of conceiving and designing the study, analysing and interpreting data, and drafting the paper.

## Acknowledgments

The authors would like to thank the reporting physicians and staff at Apulian hospital Infectious Disease and Intensive Care Divisions and Local Health Units involved in the surveillance network (Giovanni Caputi, Giuseppina Moffa, Annalisa Saracino, Maurizio Del Vecchio, Domenico La Rovere, Giovanni Buccoliero, Piergiorgio Chiriacò, and Vera Laforgia). The authors express their appreciation to Anna Sallustio and Daniela Loconsole, at the Reference Laboratory for Invasive Bacterial Diseases, for their technical support.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 353461, 9 pages
http://dx.doi.org/10.1155/2015/353461

*Review Article*

# Meningococcal Antigen Typing System Development and Application to the Evaluation of Effectiveness of Meningococcal B Vaccine and Possible Use for Other Purposes

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Received 24 November 2014; Revised 16 January 2015; Accepted 17 February 2015

Academic Editor: Saul N. Faust



Development of the 4-component meningococcal serogroup B vaccine (4CMenB) has required new assays for the reliable evaluation of the expression and cross-reactivity of those specific antigen variants that are predicted to be targeted by bactericidal antibodies elicited by the vaccine in different isolates. Existing laboratory techniques, such as multilocus sequence typing, are poorly suited to this purpose, since they do not provide information on the contribution of single vaccine components and therefore cannot be applied to estimate the potential coverage of the multicomponent vaccine. The hSBA, the only correlate of protection against invasive meningococcal disease accepted thus far, cannot conveniently be used to test large number of strains. To overcome these issues, the meningococcal antigen typing system (MATS) has been specifically developed in order to predict 4CMenB coverage of individual meningococcus serogroup B strains. To date, MATS has proved advantageous for several reasons, including its ability to assess both qualitative and quantitative aspects of surface antigens of single strains in a highly reproducible, rapid, and resource-saving manner, while its shortcomings include a possible underestimation of 4CMenB coverage and the use of pooled sera to calculate the positive bactericidal threshold. This paper provides an overview of MATS development and its field application.

## 1. Introduction

*Neisseria meningitidis* is a major causative agent of invasive bacterial diseases that affect mostly children between 3 and 12 months of age, followed by adolescents. Of thirteen known serogroups of *N. meningitidis*, only six (A, B, C, W-135, X, and Y) cause invasive disease [1, 2]. Active immunization is the most effective way to prevent invasive meningococcal disease; vaccines against serogroups A, C, W-135, and Y and a recently approved universal vaccine against serogroup B (MenB) are available [3]. This latter, a 4-component meningococcal serogroup B vaccine (4CMenB, commercially available as Bexsero), is the first vaccine to be developed by means of reverse vaccinology [4, 5]. 4CMenB consists of three recombinant proteins, namely, factor H binding protein (fHbp), Neisserial heparin-binding antigen (NHBA), and *Neisseria* adhesin A (NadA), combined with OMV from MenB strain NZ98/254, which contains porin A (PorA) serosubtype P1.4 (see Figure 1) [6].

Evaluating the protective efficacy of a vaccine without measuring clinical outcomes is of great practical importance [7, 8]. This is particularly true for vaccines against *N. meningitidis*, the incidence of which is relatively low [9, 10]. A correlate or surrogate of protection can be defined as an immunological measurement that correlates statistically with the level of a trial endpoint used to measure vaccine efficacy [11]. The serum bactericidal assay with human complement (hSBA) is a universally accepted correlate of protection against meningococcal disease that quantifies the complement-mediated killing of bacteria by functional antibodies in sera from vaccinees [12]; in general, an hSBA titer $\geq 1:4$ is considered to be a correlate of protection [12, 13].

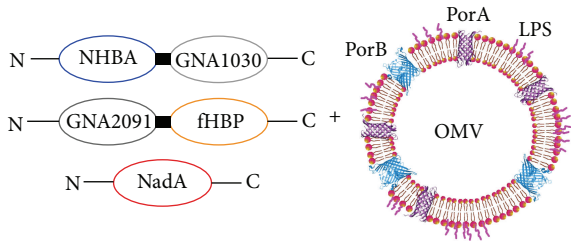


FIGURE 1: 4CMenB vaccine composition. Antigens NHBA and fHbp are fused with two accessory proteins, GNA1030 and GNA2091, respectively. Adapted with permission from [15].

Despite its strengths, hSBA has some shortcomings. First, hSBA is a labor-intensive technique and testing a large number of single circulating strains would produce logistical difficulties. Second, it requires collecting considerable amounts of sera from immunized individuals, which would be ethically debatable, especially in pediatric studies. Third, the standardization of hSBA across numerous strains and complement sources is also burdensome [5, 14, 15]. Fourth, while hSBA is able to assess the effectiveness of a vaccine by measuring bactericidal antibody titers, it does not provide information on the contribution of each vaccine component [14]. Indeed, the surface-exposed proteins fHbp, NHBA, and NadA of MenB display considerable sequence variation and expression, as well as different degrees of cross-reactivity among variants of a protein antigen to the antibodies induced by the vaccine [16–20]. We therefore need new assays that can reliably assess the expression of those specific antigen variants that are predicted to be targeted by bactericidal antibodies elicited by the vaccine on different bacterial isolates.

Today, the most widely used approach for characterizing single meningococci is multilocus sequence typing (MLST), which defines strains from the sequences of seven housekeeping genes, including *arcC* (carbamate kinase), *aroE* (shikimate dehydrogenase), *glpF* (glycerol kinase), *gmk* (guanylate kinase), pta (phosphate acetyltransferase), *tpi* (triosephosphate isomerase), and *yqiL* (acetyl coenzyme A acetyltransferase) [21, 22]. It should, however, be noted that the classification of strains based on MLST does not give direct indication on the 4CMenB antigenic repertoire. A study by Bambini et al. [18] demonstrated that each MLST clonal complex has an almost specific antigen variant repertoire resulting in a weak correlation between MLST and antigenic variability. It has been confirmed that the clonal complex alone generally has no discriminatory power to predict which strain will be killed on hSBA. These considerations make MLST only partially suitable for determining the phenotype profile, predicting vaccine antigen diversity, and, thus, assessing potential strain coverage.

Another potentially useful method to predict strain coverage is flow cytometry, which uses arrays of mono- and polyclonal antibodies and enables a considerable number of strains to be analyzed; currently, however, the method is implemented in few laboratories and may have standardization difficulties and, by using monoclonal antibodies only, it gives indication on the amount of antigens on the surface but not on their sequence diversity [5, 23].

To overcome the aforementioned limitations, a novel approach, termed the meningococcal antigen typing system (MATS), has been developed [14], its main aim being to predict the coverage of individual MenB strains provided by vaccination with 4CMenB by measuring the amount of antigen and its cross-reactivity. At the same time, since most capsular strains of *N. meningitidis* may express the same protein antigens [24, 25], the application of MATS could be extended to other serogroups. Moreover, this technique could be potentially adapted to other bacterial pathogens [26]. For these reasons recent advances and applications of MATS in the field of epidemiologic surveillance of bacteria should now be reviewed.

## 2. MATS Development and Interpretation

*2.1. MATS as a Qualitative and Quantitative Assay.* MATS was designed as a rapid and robust binding assay able to predict the susceptibility of individual MenB strains to be killed by bactericidal antibodies elicited by 4CMenB; this method enables both qualitative (level of sequence relatedness) and quantitative (level of expression) evaluation of the antigens expressed on the surface of single strains [14, 15]. Both quantitative and qualitative aspects are highly important and should be assessed for the reasons described below.

The density and spatial orientation of an antigen on the bacterial surface are critical in the process of classical pathway of complement activation, which is initiated when a sufficient density of antigen-antibody complexes allows proximate fragment crystallizable (Fc) regions of the antibody to bind C1q. An increase in surface antigen density results in a reduced distance between bound antibodies, thus leading to a higher probability of engagement and activation of the complement system [27, 28]. On the other hand, the level of surface antigen expression is not the only factor that influences killing; the quality of the fit antigen-antibody is also crucial [29].

Basically, MATS is a modified sandwich enzyme-linked immunosorbent assay (ELISA) that quantifies expression and the level of matching with the corresponding antigen in the vaccine (fHbp, NHBA, and NadA) on bacterial lysates. Moreover, the PorA serosubtype is identified by means of the traditional PCR genotypic approach by assessing its variable region 2, and an individual strain matching for PorA (PorA 1.4) is considered to be covered by 4CMenB. This subsequently enables antigenic cross-reactivity with the main corresponding 4CMenB components to be measured [14, 15].

Methodologically, the MATS ELISA procedure comprises several steps, which can be schematized as follows (see Figure 2). At the first step, cultures are grown overnight on chocolate agar plates at 37°C, 95% relative humidity, and $CO_2$ concentration of 5%. Subsequently, bacteria are suspended in Mueller-Hinton broth to achieve an optical density (OD) at 600 nm of 0.4 and then lysed with the detergent Empigen BB 5% added to a final volume of 1 : 11 (0.45%) and inactivated at 45°C for 1 hour in a water bath. Twofold serial dilutions of the bacterial lysates are incubated in duplicate in three different ELISA microwell plates coated

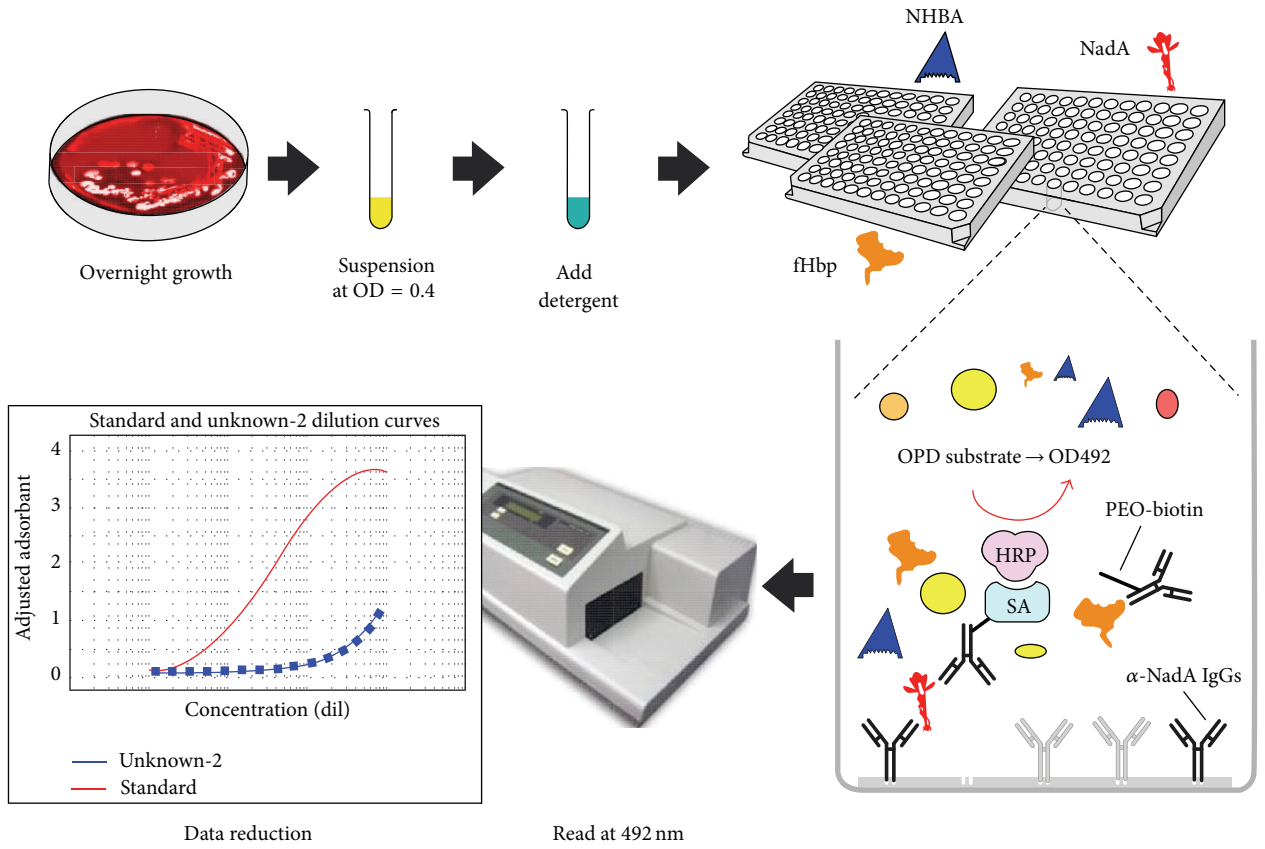


Figure 2: Schematic representation of the MATS ELISA method [15]. Bacteria from overnight cultures on agar chocolate plates are suspended in Mueller-Hinton broth and lysed with a detergent (Empigen BB 5%) added to a final volume of 1/11 and inactivated for 1 hour at 45°C in a water bath; bacterial lysates are added to three different ELISA microwell plates coated with polyclonal rabbit antibodies raised against the single vaccine components fHbp, NHBA, and NadA; the antigens are captured from the suspension to the plate. Plates are then incubated for 1 hour at 37°C with biotinylated rabbit polyclonal antibodies against each of the antigens, washed, incubated with streptavidin-HRP, and developed with the OPD substrate. Relative potency is calculated by interpolating the regression curve of the unknown sample versus that of a reference strain added to each plate. Adapted with permission from [15].

with rabbit polyclonal antibodies against fHbp, NHBA, and NadA, respectively. The plates are incubated for 1 hour at 37°C and washed with a solution of phosphate buffered saline (PBS1x) plus 0.05% Tween. The amount of antigen bound to the antibodies is detected by incubating for 1 hour at 37°C with purified rabbit immunoglobulin G (IgG) raised against each of the three recombinant proteins, labeled with biotin. The plates are then washed and incubated for 30 min at 37°C with streptavidin-horseradish peroxidase and for 20 min at room temperature with the *ortho*-phenylene diamine substrate; the reaction is stopped by adding 50 $\mu$L of sulfuric acid solution (4 N). Immediately afterwards, the plates are read at 492 nm by means of an ELISA reader [14, 15].

The MATS ELISA readout expresses the relative potency (RP) for fHbp, NHBA, and NadA of single strains; RPs of tested strains are calculated by means of a variance-weighted regression method by comparing the serial dilution curves of tested strains with those of the appropriate reference strains for each of the three antigens. The reference strains are as follows: H44/76 for fHbp, NGH38 for NHBA, and 5/99 for NadA; the RPs are calculated by assigning the arbitrary value of 1 (or 100%) to each reference strain. Subsequently, in order to determine cut-off values of RPs that would predict susceptibility on hSBA, MATS RPs have been related to hSBA of pooled serum from 13-month-old children immunized with 4CMenB at 2, 4, 6, and 12 months of age. The positive bactericidal threshold (PBT) has been defined as the MATS RP point estimate, above which the majority of strains are killed in hSBA. These RP values are 0.021 (2.1%) for fHbp, 0.294 (29.4%) for NHBA, and 0.009 (0.9%) for NadA [14]. In sum, the MATS phenotype is defined as follows: each of the four antigens can be either positive (when RP > PBT or PorA is P1.4) or negative (when RP ≤ PBT and PorA is not P1.4) so that the number of possible MATS phenotypes is $2^4 = 16$ [14, 30].

*2.2. Impact of Antigen Sequence Variation on the Relative Potency Values (Qualitative Aspects of MATS).* As described above, the level of sequence relatedness of an antigen in a given MenB strain to the corresponding antigen included in the vaccine is a determining factor. The main 4CMenB antigens, fHbp, NHBA, and NadA, have a substantial level

of sequence variability [16–18]. For instance, fHbp has been divided into three variants, namely, 1, 2, and 3, each of which may be further classified into subvariants. Sequence conservation within variants is high (91.6–100%), while that between variants is approximately 63% [17, 18]. Other fHbp classifications based on two subfamilies [31] and nine modular groups [20] have also been described. Similarly, several peptides of NHBA and at least five NadA variants have been established [16]. 4CMenB recombinant proteins include fHbp subvariant 1.1, NHBA subvariant 2, and NadA variant 3 [24]. MATS RPs of single antigens in MenB strains are highly influenced by such complex genetic variations. In a panel of 124 MenB isolates, selected to represent a broad range of MLST and PorA types from different geographic areas, the highest RPs of fHbp (46–140%) have been found among strains classified within fHbp subvariant 1.1. Strains expressing other subvariants of the fHbp variant 1 have shown significantly lower RPs of 1.6–38%, while those expressing fHbp variants 2 and 3 have displayed RPs below the lower limit of quantitation. Although all tested strains harbor the *nhba* gene, only 70% of them have RPs above the lower limit of quantitation and a 6.5-fold range in RPs has been observed (from 20% to 130%). The *nadA* gene has been found in only one-third of isolates and less than 20% showed RPs above the lower limit of quantitation, with a more than 1000-fold range in RP values [14].

Analogous results have been reported from Canada, where all isolates expressing fHbp subvariant 1.1 showed RPs > PBT, while the proportions of strains predicted to be covered by 4CMenB were 33%, 85%, and 95% for those expressing the more distant subvariants 1.13, 1.15, and 1.4, respectively. None of the fHbp variant 2 or 3 strains had RPs above the PBT for fHbp and would require expression of a different vaccine antigen (i.e., PorA, NHBA, and NadA) in order to be covered [32]. Among European MenB isolates, strains with different subvariants of fHbp-1 have generally shown RPs > PBT, while almost all strains with fHbp-2 or fHbp-3 have had RPs close to 0% or, in any case, below the PBT. A high variation in RPs for different variants of NHBA has also been documented in three studies [5, 32, 33].

*2.3. Association between MATS and hSBA.* To investigate the relationship between MATS RPs and bactericidal titers, 57 strains from the 124 MenB panel were tested by means of hSBA using pooled infant serum from 13-month-old children immunized with 4CMenB at 2, 4, 6, and 12 months of age. To assess the contribution of each antigen to the bactericidal activity, a subset of 5 strains for fHbp, 11 for NHBA, and 7 for NadA that were mismatched to the vaccine for PorA and had only one vaccine antigen RP above the MATS lower limit of quantitation was selected. In this subset of strains the correlations between hSBA titers and relative potencies were statistically significant. Spearman's correlation coefficients were as follows: $\rho = 0.97$ ($P = 0.005$) for fHbp; $\rho = 0.75$ ($P = 0.008$) for NHBA; $\rho = 0.81$ ($P = 0.027$) for NadA. Eighty-nine percent (39/44) of strains with RPs above the PBT for at least one antigen have been seen to be killed on hSBA (titer of $\geq 1:8$ or 4-fold rise) with pooled sera from 13-month-old children immunized with four 4CMenB doses. When considering RPs above the PBT for the single antigens, the highest positive predictive value was for fHbp (100%, 7/7), followed by NadA (83%, 5/6) and NHBA (82%, 9/11). The negative predictive value (proportion of strains with all four RPs ≤ PBT that are not killed on hSBA) was also high (77%, $n = 13$) [14]. Similar findings have been observed among adults who received three 4CMenB doses: within the 124-strain panel, 83 out of 91 (91%) strains showing RPs above the PBT for one or more antigens were killed on hSBA with pooled sera. Positive predictive values increased as the number of antigens with RP > PBT increased (1 antigen: 85%, $n = 41$; 2 antigens: 94%, $n = 34$; 3 antigens: 100%, $n = 16$) [14].

Table 1: Positive bacterial threshold (PBT) values with 95% CIs for three antigens [26].

| Antigen | Estimate % | 95% CI |
|---|---|---|
| fHbp | 2.1 | 1.4–3.1 |
| NHBA | 29.4 | 16.9–51.1 |
| NadA | 0.9 | 0.4–1.9 |

The use of pooled immune sera, however, may not accurately predict how each of the sera that compose the pool would react against a tested strain. Indeed, it is possible that a few subjects with unusually high or low hSBA titers may impact on the overall pooled response or that the potential synergy between antibodies from different subjects might generate a bactericidal response which would not be achieved individually. However, a recent study [34] aiming at investigating the relationship between pooled and individual sera has shown that individual responses to 4CMenB are homogeneously distributed and that pooled hSBA titers reflect the arithmetic mean of the individual titers with good approximation.

*2.4. Reliability and Reproducibility of MATS.* Interlaboratory standardization of MATS has been also achieved by using 17 shared MenB strains [26]. The log-transformed RPs obtained from five laboratories showed excellent robustness at different temperatures of sample inactivation (37°C and 45°C), with Lin's coefficient of accuracy being 0.999 (95% CI: 0.997–1) and Pearson's correlation coefficient being 0.993 (95% CI: 0.988–0.995). Log-transformed RPs from seven laboratories displayed almost perfect accuracy and concordance, with the corresponding coefficients exceeding 0.99. Within-laboratory variations in RPs for individual antigens differed slightly, being highest for NadA and lowest for fHbp; this variance, however, was not affected by systematic bias. Similarly, between-laboratory coefficients of variation were 7.85% for fHbp, 12.60% for NHBA, and 16.51% for NadA. Moreover, in this study empirical estimates of 95% CI of PBTs (Table 1) for each individual antigen were calculated as $10^{\lg(\mathrm{PBT})\pm 1.96\bullet\sigma}$. The authors proposed using 95% confidence limits to predict 4CMenB coverage, as these would account for between- and within-laboratory variances. Laboratory qualification criteria were also clearly described.

Table 2: Relative contribution (%) of all possible antigen combinations to MATS-predicted coverage in three countries [5, 30, 32, 33].

| Antigen (MATS phenotype) | England and Wales ($n = 535$) | Canada ($n = 157$) | Greece ($n = 148$) |
|---|---|---|---|
| fHbp+/NHBA−/NadA−/PorA− | 14.6 | 12.7 | 9.5 |
| fHbp−/NHBA+/NadA−/PorA− | 7.9 | 13.4 | 33.8 |
| fHbp−/NHBA−/NadA+/PorA− | — | — | 0.7 |
| fHbp−/NHBA−/NadA−/PorA+ | 0.4 | — | 0.7 |
| fHbp+/NHBA+/NadA−/PorA− | 29.9 | 25.5 | 37.1 |
| fHbp+/NHBA−/NadA+/PorA− | — | 0.6 | — |
| fHbp+/NHBA−/NadA−/PorA+ | 3.2 | 1.9 | — |
| fHbp−/NHBA+/NadA−/PorA+ | 0.9 | — | 1.3 |
| fHbp−/NHBA+/NadA+/PorA− | 0.4 | 0.6 | — |
| fHbp−/NHBA−/NadA+/PorA+ | — | — | — |
| fHbp+/NHBA+/NadA+/PorA− | 0.2 | — | — |
| fHbp+/NHBA+/NadA−/PorA+ | 15.7 | 11.5 | 6.1 |
| fHbp+/NHBA−/NadA+/PorA+ | — | — | — |
| fHbp−/NHBA+/NadA+/PorA+ | — | — | — |
| fHbp+/NHBA+/NadA+/PorA+ | — | — | — |
| fHbp−/NHBA−/NadA−/PorA− | 26.9 | 33.8 | 10.8 |

## 3. Field Applications of MATS

*3.1. Application of MATS to Estimate Country-Specific Serogroup B Strain Coverage of 4CMenB.* In a large study [5] (1,052 MenB isolates from five countries) carried out in Europe, the results of MATS predicted an overall strain coverage of 78% (95% CI: 63–90%), and half of the tested strains would have been covered by at least two vaccine antigens. The most common MATS phenotypes were fHbp plus NHBA, no antigen, fHbp plus NHBA plus PorA, fHbp, and NHBA; expression of NadA was above the positive bactericidal thresholds in only a small proportion [7% (16/235)] of isolates that harboured the NadA gene. The robustness of estimates was further established by MATS testing of additional MenB isolates from another two countries (Spain and the Czech Republic). Some level of between-country variation in coverage estimates was observed: 87% (95% CI: 70–93%) in Italy, 85% (95% CI: 76–98%) in Norway, 85% (95% CI: 69–93%) in France, 82% (95% CI: 69–92%) in Germany, 74% (95% CI: 58–87%) in the Czech Republic, 73% (95% CI: 57–85%) in England and Wales, and 69% (95% CI: 48–85%) in Spain. However, this difference was not statistically significant at an $\alpha$ level of <0.05. A somewhat lower predicted strain coverage of 66% (95% CI: 46–78%) has been reported in Canada. As in the European study, most tested strains could have been targeted by bactericidal antibodies against >1 vaccine antigen. Thus, 29% and 11.5% of isolates were covered by two and three antigens, respectively. The highest relative contribution to coverage was made by fHbp and NHBA [32]. A more recent Greek study has revealed an MATS-predicted coverage of 89.2% (95% CI: 63.5–98.6%) by at least one antigen and 44.5% by at least two antigens. Again, NHBA and fHbp were the greatest contributors to coverage, while PorA and NadA contributed significantly less [33]. Table 2 reports the contribution of each 4CMenB antigen and their combinations to MATS-predicted coverage in three countries.

*3.2. Application of MATS to Estimate Serogroup X Strain Coverage of 4CMenB.* Today, the *N. meningitidis* group X (MenX), which has caused several outbreaks in Africa, remains the only serogroup for which no vaccine exists. The cross serogroup immunogenicity elicited by 4CMenB is biologically plausible, since the majority of capsular strains may express the vaccine antigens [24]. In a paper by Hong et al. [35], nine African MenX isolates and two French MenX isolates were tested by means of MATS in order to determine the presence, diversity, and expression level of 4CMenB antigens. In this study, the PBT values estimated for MenB isolates were applied to MenX strains; the strains were also analyzed by hSBA by using preimmunization and postimmunization sera from different age groups. All African isolates displayed MATS RP values for fHbp above PBT (2.5–5%); conversely, the two French strains showed RP values below the lower limit of quantitation for fHbp, as a result of mismatching for fHbp. Among the African isolates, RPs for NHBA ranged from 11.3% to 31.5%; only three isolates displayed RP values above the PBT. Moreover, one African and one French isolate had the *nadA* gene, but its expression was very low. Although a correlation between MATS RP and hSBA has not yet been established for non-B strains, good agreement was observed between MATS RPs and killing in hSBA in all the age groups for the MenX strains tested in this study.

*3.3. Application of MATS to Estimate the Potential Impact of 4CMenB on Carriage.* The impact of meningococcal vaccination on asymptomatic nasopharyngeal carriage is of primary importance, since healthy carriers are the main reservoir of *N.*

*meningitidis* [36]. Again, MATS may provide useful insights into carriage status, as has been documented in two recent studies [37, 38]. Claus et al. [37] applied MATS to investigate whether 41 capsule-null locus meningococci isolated from Germany, the Czech Republic, and Burkina Faso harbored the genes and expressed the main 4CMenB antigens. However, as PBT had not yet been assessed for capsule-null locus meningococci, PBT values were not applied in their study. Six strains belonging to the clonal complex ST-198 expressed fHbp subvariant 1.4, with RPs ranging from 7.9% to 11.2%. NHBA was expressed in all 41 strains, with RP values of 2.3–58.2%; the highest RPs were seen in isolates belonging to clonal complexes ST-198 and ST-845. The *nadA* gene was found in only one isolate; the expression of the NadA protein was below the limit of detection. No strain matched for the PorA included in 4CMenB. Interestingly, the authors documented that the presence of a capsule has almost no impact on antigen detection levels. In particular, MenB strains MC58 and H44/76 and their corresponding capsule-null isogenic mutants, as well as the capsule-null isolate $\alpha$30 and its encapsulated derivative, showed very similar RPs for fHbp and NHBA [37].

In an Italian longitudinal carrier study [38] 32 out of 173 students tested positive for *N. meningitidis* of different serogroups in at least 1 of 4 examinations. As shown by MATS, strains expressing subvariants of fHbp-1 isolated from 5 students showed detectable expression levels of the antigen, with RPs of 1.2–122.1%. Conversely, isolates carrying fHbp-2 and fHbp-3 were negative on MATS. NHBA was detectable in all isolates (RPs of 2.5–131.2%), while, as expected, NadA expression was detected in only one subject, being NadA very poorly expressed in carrier strains. This study also revealed that RPs for individual antigens remained comparable in subjects on consecutive positive swabs, with the exception of NadA, for which the only positive subject showed a more than 10-fold higher RP on the second swab (43%) than on the first, third, and fourth (3.7%, 3.4%, and 3.1%, resp.).

These two studies [37, 38] have indicated that 4CMenB will very probably affect the carriage and acquisition of *N. meningitidis*; the impact on the unencapsulated strains will probably be less pronounced. It must, however, be borne in mind that, in the case of capsule-null locus strains, the association between hSBA and MATS is quite difficult to establish, owing to their intrinsic susceptibility to complement-mediated killing [38]. In any case, vaccine-induced changes in circulating meningococcal strains and detailed typing of both invasive and carrier isolates should be subject to strict monitoring in the near future.

## 4. MATS May Underestimate 4CMenB Coverage

Intrinsically, MATS is a conservative predictor of MenB strain coverage; indeed, in comparison with hSBA, it underestimates coverage. Frosi et al. [30] applied a stratified proportional random sampling procedure to select a representative panel of 40 MenB isolates from the overall panel of 535 isolates collected in England and Wales over 2007-2008. The selected isolates were tested in the hSBA assay with pooled sera from infant and adolescent vaccinees, and the results compared by means of MATS. In this study, MATS-based predictions of coverage of 70% (95% CI: 55–85%) were largely confirmed by 88% killing in hSBA (95% CI: 72–95%); 27 true positives and 4 true negatives were found, yielding overall accuracy of 78%; positive and negative predictive values were 96% and 33%, respectively [30]. Thus, coverage predicted by MATS has been largely confirmed by subsequent hSBA on pooled infant and adolescent postvaccination sera. This discrepancy between MATS and hSBA assessments of coverage could be explained by various factors.

*4.1. Non-PorA Components of OMV.* One of the four main 4CMenB components is OMV from MenB strain NZ98/254 [39]. OMVs are released by several bacterial species and contain various outer membrane proteins, lipopolysaccharide, and a lumen with periplasmic constituents [40]. Apart from PorA, other proteins identified in the outer membrane have been shown to be immunogenic, including, for example, opacity-associated proteins (Opc and Opa) [41] and *Neisseria* surface protein A (NspA) [42]. MATS ELISA does not detect antibodies elicited against such OMV components.

*4.2. Synergistic or Additive Effects of Antibodies against Multiple Antigens.* MATS does not consider the possible effects of bactericidal antibodies against more than one antigen present in 4CMenB, including different outer membrane proteins. Giuliani et al. [43] have documented that antibodies against non-PorA antigens present in OMV, which may be either bactericidal or not, are able to induce a synergistic bactericidal activity with antibodies against fHbp. Similarly, anti-NHBA antibodies may exert a cooperative activity with antibodies against other antigens [44]. Sera from six subjects immunized with a meningococcal vaccine containing recombinant GNA2091-fHbp, NHBA-GNA1030, and NadA were tested in hSBA both before and after depletion of anti-fHbp and/or anti-NHBA antibodies. All vaccinees showed at least a 4-fold increase in hSBA titers in comparison with their preimmune serum against the test strain H44/76, which matched the fHbp present in the vaccine. When anti-fHbp antibodies were depleted, hSBA titers decreased by at least 88% in all subjects. Four of six vaccinees showed at least a 4-fold increase in hSBA titers against the strain M4407, which expresses a heterologous to the vaccine fHbp-2 and a homologous to the vaccine NHBA amino acid sequence. In one vaccinee, hSBA was directed mostly against NHBA, in another vaccinee against fHbp, while in the remaining two subjects depletion of antibodies against either fHbp or NHBA more than halved the hSBA titer. In all four subjects, depletion of both anti-fHbp and anti-NHBA antibodies suppressed bactericidal activity to a greater degree than depletion of these antibodies individually, indicating a cooperative bactericidal activity between antibodies against fHbp and NHBA. The same study showed that, in mice immunized with only fHbp variant 1 antigen or NHBA vaccine antigen, the percentage survival of strain M4407 incubated with a 1 : 1 mixture of pooled sera from mice immunized with NHBA and pooled

sera from mice immunized with fHbp was lower than that when the strain was incubated with a 1 : 1 mixture of pooled sera from mice immunized with NHBA and pooled sera from negative control mice immunized with aluminum hydroxide [44].

*4.3. NadA In Vitro Downregulation.* In comparison with both NHBA and fHbp, the relative contribution of NadA to 4CMenB coverage has proved to be very low in all studies conducted so far [5, 14, 32, 33], despite the fact that this antigen is carried by approximately one-third of pathogenic isolates and by three of four hypervirulent lineages. The most probable reason for this observation is the very complex mechanisms of *nadA* gene regulation. The phase-variable expression of nadA is mostly mediated by NadR, which represses *nadA* [45]. Indeed, under *in vitro* conditions in both hSBA and MATS, *nadA* repression by NadR results in inefficient killing of various MenB strains by anti-NadA antibodies. However, it has been suggested that *nadA* expression *in vitro* may differ from that *in vivo*. In this regard, sera from children infected with strains that do not express NadA (RP = 0%) or displaying low NadA expression (RP < PBT) have been seen to recognize NadA recombinant proteins, also confirming the hypothesis that NadA is less expressed *in vitro*; this degree of recognition is much higher than that observed in sera from subjects infected by *nadA* negative strains [46]. Knocking out *nadR* results in a higher level of NadA expression and efficient killing by sera from subjects vaccinated with 4CMenB. Moreover, the MenB strain NGP165, which is mismatched for fHbp, NHBA, and PorA and has NadA RP < PBT, is not killed by sera from infants vaccinated with 4CMenB when it is grown *in vitro*. However, the same infant sera provide passive *in vivo* protection against NGP165 bacteremia, as has been shown in an infant rat model [47].

## 5. Conclusion

This paper attempts to critically review available studies that have used MATS. Implementation of MATS is expected to grow steadily, as 4CMenB has recently been approved for human use in several countries [48–50]. MATS could be useful in tracking spatial and temporal changes in MenB epidemiology and their repercussions on 4CMenB coverage. As anticipated by Vogel et al. [23], there are two main reasons for the increased use of MATS as follows: (i) implementation of the vaccine will potentially affect the population structure of *N. meningitidis*, in that the proportion of strains not covered by 4CMenB will probably increase, thus requiring additional surveillance efforts and (ii) careful assessment of vaccination failures.

A possible limitation of this method could be the lack of information on the relationship between pooled sera, which are used to calculate PBT, and the response rate of individual subjects; this aspect has not yet been fully investigated and is currently under evaluation.

Although MATS has been established for MenB strains, the method is flexible enough to be modified for application to other *N. meningitidis* serogroups and bacteria species. To date, PBT values have been established only for serogroup B, thereby limiting MATS application to this serogroup. However, as the genetic diversity of other serogroups is substantially lower, the establishment of PBTs for these should require a lower number of strains. Moreover, reverse vaccinology is being applied to a number of other bacteria [51], and MATS ELISA could be adapted in order to assess these pathogens [26].

Across the studies conducted so far [5, 32, 33], the MATS-predicted MenB strain coverage provided by 4CMenB has shown some differences among areas or countries, with minimum values observed in Canada and maximum values in Greece. Apart from the diversity in circulating strains, a possible explanation for this variability is that MenB strain panels may not be fully representative, since they generally come from passive surveillance systems [5]. Likewise, it must be taken into account that MATS underestimates the vaccine coverage [30, 41, 46, 47].

Accurate data on the potential strain coverage provided by the new multicomponent vaccine are of crucial interest to policy-makers in order to decide whether to introduce the vaccine into national immunization programs. MenB coverage estimates based on MATS have already been used in various economic evaluations of 4CMenB [52, 53].

In conclusion, MATS is an innovative technique that has several advantages, including (but not limited to) the following:

(i) assessment of both the level of expression and antigenic relatedness of fHbp, NHBA, and NadA vaccine antigens in meningococci isolates;

(ii) from the technical standpoint, MATS is more rapid and resource-saving than other approaches;

(iii) despite some level of underestimation, MATS strongly correlates with the universally accepted correlate of protection and provides satisfactorily accurate MenB coverage predictions;

(iv) it is highly reproducible and can be successfully transferred to new laboratories;

(v) it enables near real-time estimation of the postimplementation effectiveness of 4CMenB;

(vi) PBT values may be used to efficiently predict 4CMenB strain coverage on a representative panel of invasive isolates from any geographical setting; such estimates are crucial to economic and health technology assessment (HTA) studies.

## Conflict of Interests

Giuseppe Boccadifuoco and Marzia Monica Giuliani are employees of Novartis Vaccines. Alexander Domnich, Roberto Gasparini, Daniela Amicizia, and Donatella Panatto declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 402381, 6 pages
http://dx.doi.org/10.1155/2015/402381

*Review Article*

# Meningococcal B Vaccination (4CMenB) in Infants and Toddlers

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Received 9 October 2014; Revised 27 January 2015; Accepted 3 March 2015

Academic Editor: Saul N. Faust



*Neisseria meningitidis* is a Gram-negative pathogen that actively invades its human host and leads to the development of life-threatening pathologies. One of the leading causes of death in the world, *N. meningitidis* can be responsible for nearly 1,000 new infections per 100,000 subjects during an epidemic period. The bacterial species are classified into 12 serogroups, five of which (A, B, C, W, and Y) cause the majority of meningitides. The three purified protein conjugate vaccines currently available target serogroups A, C, W, and Y. Serogroup B has long been a challenge but the discovery of the complete genome sequence of an MenB strain has allowed the development of a specific four-component vaccine (4CMenB). This review describes the pathogenetic role of *N. meningitidis* and the recent literature concerning the new meningococcal vaccine.

## 1. Introduction

*Neisseria meningitidis* is one of the most frequent causes of meningitis and septicemia worldwide [1, 2], being not only responsible for 10–20% of specific meningococcal-related mortality but also the cause of (particularly pediatric) long-term morbidity [1] as it leads to permanent neurological *sequelae* and disabilities in an additional 20% [3–5]. Furthermore, the pediatric mortality rate among children with sepsis is over 20% [6, 7]. The incidence of meningococcal meningitis is greatest amongst children, adolescents, and adults aged up to 29 years, but young children are the most susceptible. The risk of *N. meningitidis* infection is particularly high in some regions of the world but, despite the introduction of innovations in health care, morbidity and mortality rates are high in both developed and undeveloped countries, and prevention is therefore a priority.

The bacteria colonise the nasopharyngeal tract of human hosts and are spread from subject to subject via air droplets. Transmission rates vary and are also related to individual risk factors such as age and/or underlying medical and social conditions (e.g., primary or secondary immunodeficiencies, a history of travel, and overcrowded living condition). Twelve different serogroups are known, but most invasive meningococcal diseases are caused by one of the six capsular groups A, B, C, W, X, and Y.

A number of excellent conjugate vaccines against serogroups A, C, W, and Y have been licensed, and the introduction of conjugate meningococcal C vaccine (MenC) has led to a rapid and sustained reduction in the incidence of invasive MenC disease across all age groups in Italy [8, 9]. However, a vaccine against capsular group B (MenB), which has now become responsible for most cases in Italy and the rest of the world [7, 9], has long eluded vaccinologists, particularly because of the problems associated with the B polysaccharide [10–12]. Unlike the highly immunogenic polysaccharides of serogroups A, C, W, and Y, the serogroup B polysaccharidic capsule contains a polysialic acid whose antigenic structure resembles the cell surface glycoproteins of human neurological tissue, and this has proved to be a formidable challenge [13].

The new protein-based vaccine against MenB (4CmenB; Bexsero, Novartis Vaccines and Diagnostics, Siena, Italy) has now overcome this barrier by using a cocktail of four main immunogenic components: two recombinant fusion proteins (*Neisseria* heparin-binding antigen [NHBA-GNA1030] and

factor H binding protein [fHbp-GNA2091]), recombinant *Neisseria* adhesion A (NadA), and detergent-treated outer membrane vesicles (OMVs) derived from the NZ98/254 New Zealand meningococcal outbreak strain in which porin A (PorA 1.4) is the major immunodominant antigen. These components were identified using reverse vaccinology [14], a technique that analyses the whole bacterial genome in order to predict meningococcal antigens (exposed on the pathogen's surface or secreted) that can act as vaccine targets. NHBA is a surface b-barrel lipoprotein that binds to the anticoagulant heparin and induces protective immunity in human hosts [15–17], and fHbp is a surface-exposed protein that allows binding exclusively to human fH [18], mediates host serum resistance, and induces bactericidal antibodies upon host detection [15, 19]. NadA is a surface adhesin and invasin whose interactions with abundantly expressed human heat shock protein 90 (Hsp90) [20] also induce bactericidal antibodies [21, 22]. NadA expression can be regulated by the Nad repressor (NadR) [23]. Finally, porA (one of the two b-barrel porin proteins produced by *N. meningitides*) [24] assists in opsonophagocytic activity and is also involved in host actin reorganisation during infection, which depends on its ability to nucleate actin filaments.

This four-component meningococcal serogroup B vaccine (4CMenB), the first successful vaccine against the endemic form of this cause of serious bacterial meningitis and septicemia, has been in development for almost 20 years and has recently been approved for the active immunisation of subjects aged ≥2 months [9, 10] by licensing authorities in Europe, Canada, and Australia.

A bivalent fHbp recombinant vaccine (also known as LP2086; Trumenba, Pfizer Inc., Philadelphia, PA, USA) has been developed since 2006 and has now been approved by the US Food and Drug Administration for use in 10-to-25-year olds [25]. This vaccine appeared safe in a phase 3 study in approximately 5,600 healthy individuals 10 to 25 years of age and immunogenic and safe when coadministered with routine meningococcal A, C, Y, and W and tetanus, diphtheria, and pertussis (Tdap) vaccines in a phase 2 study in more than 2,600 healthy individuals 10 to 12 years of age [26]. Studies on this vaccine are ongoing in Europe and approval from European Medicines Agency is expected in 2017.

The aim of this review is to discuss the immunogenicity, safety, and tolerability of 4CMenB vaccine in infants and toddlers, and the efficacy of different vaccination strategies.

## 2. Immunogenicity of 4CMenB

An important challenge for the licensing of 4CMenB vaccine was the difficulty in showing its activity against epidemiologically relevant strains of *N. meningitidis* [27, 28]. After its development and phases I and II trials, some large-scale randomised phase III studies were planned in order to assess its efficacy and describe adverse reactions, but due to the rarity of the diseases caused by *N. meningitides* serotype B (which have annual rates of 0.5–5 per 100,000 people) laboratory-based methods were developed with the aim of predicting the vaccine's effectiveness and coverage [29]. In Europe, the vaccine was licensed on the basis of a correlate of protection calculated using a titre of human serum bactericidal activity (hSBA) that is present in convalescent patients which was shown to be protective in US Army recruits. HSBA assay is a recognised *in vitro* surrogate for evaluating protective immunity against *N. meningitidis*, and an adequate response is a crucial criterion for licensing vaccines against serogroup B meningococci. In order to justify the inclusion of each antigen in the formulation, it is necessary to run four hSBA assays on each serum sample, each using a *N. meningitidis* strain expressing the target antigen independently from the others in order to evaluate immunogenicity of each component of the vaccine, for example, a meningococcal strain that uniquely expresses NadA but not factor-H-binding protein or *Neisseria* heparin-binding antigen. Typically, an hSBA titre of 4 is initially used to assess immunogenicity. Subsequent phase III studies of 4CMenB in children used a more conservative titre of ≥5, which ensures that the level is >4 with 95% confidence taking into account within-assay variability.

The safety and immunogenicity of 4CMenB vaccine has been studied when administered at the same time as other routine infant vaccines (diphtheria, tetanus, acellular pertussis, inactivated poliovirus, hepatitis B, *Haemophilus influenzae* type b [DTaP-IPV-HepB/Hib], and 7-valent pneumococcal conjugate vaccine [PCV7]), and it has been found that the antibody responses to the routine vaccines are equivalent to those observed when the routine vaccines are given alone in the case of all of the antigens except for the pertactin component of acellular pertussis and pneumococcal serotype 6B [10]. However, this laboratory observation seems to be of no clinical significance, and published data also suggest that the incidence of pneumococcal disease due to serotype 6B is low in the countries in which PCV7 vaccination is used.

Other studies have investigated the persistence of bactericidal antibodies in young children after primary immunisation and the level of immunogenicity after a preschool booster [29, 30]. The levels of bactericidal antibodies after primary 4CMenB vaccination at the ages of two, four, six, and 12 months had waned when measured at 40–44 months, but an anamnestic response was observed following a booster dose given at the age of 40–44 months [14]. Similarly, bactericidal antibody levels in infants who originally received 4CMenB during late infancy (6, 8, and 12 months) had also waned when measured at the age of 40 months but, once again, there was an anamnestic response to a booster dose given at 40 months [30].

## 3. Safety and Tolerability of 4CMenB

The studies assessing the immunogenicity of 4CMenB also evaluated its safety and tolerability. The participating infants were observed for 30 minutes after each vaccine administration, and their parents were given a diary card on which to record the occurrence and severity of solicited local (i.e., injection site tenderness, erythema, induration, and swelling) and systemic reactions (i.e., changes in eating habits, sleepiness, vomiting, diarrhea, irritability, unusual

crying, rash, and increased/decreased body temperature) and any other adverse events, during the following seven days. The rates of local and systemic reactions were similar to those seen following other routine infant and early childhood vaccinations, but injection site pain was consistently reported more frequently, especially by older children.

Fever was more frequent in the children who received 4CMenB together with other routine infant vaccines. It mainly occurred during the first 24 hours after administration but, as in case of other vaccines, it has been found that the prophylactic administration of paracetamol before and 4–6 hours after vaccination significantly reduces postvaccination fever without affecting immunological responses [31, 32].

The pivotal and phase IIb studies found that the most frequently reported local reaction of tenderness affected 87% of the 4CMenB injection sites, 80% of the DTaP- IPV-HepB/Hib sites, and 79% of the PCV7 sites when all three vaccines were administered together [33]. The frequency of reported tenderness after DTaP-IPV-HepB/Hib and PCV7 injections when they were administered without 4CmenB was respectively 59% and 53%, whereas when DTaP-IPV-HepB/Hib and PCV7 injections were administered with 4CmenB it was respectively 68% and 62%.

The reported rates of local reactions to 4CMenB were slightly higher than those related to routine vaccines [10], but the majority were transient, most intense on the day after vaccination, and resolved within a week.

Although the systemic reactions that occurred when 4CMenB was administered concomitantly with routine vaccines cannot be specifically attributed to one or other of the vaccines, it is possible to assess the overall profile. The occurrence of 4CmenB-related seizures is rare: the combined data of infant studies including >20,000 vaccinations in the primary 4CMenB study arm indicate an overall rate 0.1 febrile seizures/1000 vaccinations on the day of vaccination or the day after and no events in the control arm. They are similarly rare in toddlers: 0.4 events/1000 vaccinations (95% confidence interval (CI): 0.05–1.46) after a total of 11,000 4CMenB vaccinations administered with or without routine vaccines, as against 0.3 event/1000 visits (95% CI: 0.04–1.05) in the case of those receiving routine vaccines alone [10, 33].

Six suspected cases of Kawasaki disease reported during the course of two infant studies (four in the pivotal trial and two in the phase IIb study) were evaluated by an independent external expert panel in order to assess whether they were true Kawasaki cases and whether they were vaccine related [33]. Analysis of the Kawasaki cases indicates an annual incidence of 72/100,000 person-years (95% CI: 23–169) after 4CmenB vaccinations, as against 56/100,000 person-years (95% CI: 1–311) after routine vaccinations alone.

The overall data from different studies indicate that the frequency of febrile seizures, the incidence of Kawasaki disease, and the proportion of infants using antipyretics are similar to those observed during clinical licensure programmes. However, as the number of exposed infants is still too small to exclude any relationship with rare adverse events, further postmarketing surveillance is necessary.

## 4. Coverage and Efficacy of 4CMenB

The new 4CMenB vaccine may not protect against all invasive meningococcal B strains because the antigens included in the vaccine are expressed by only some of the strains in circulation. However, it is not known what protection 4CMenB vaccine provides against invasive meningococcal disease (IMD) because it depends on the vaccine antigens expressed by the meningococcal strains in any given geographical area and their cross-reactivity with the antigens included in the vaccine. Epidemiological and microbiological data regarding the circulating meningococcal strains are important in order to predict the theoretical coverage provided by 4CMenB vaccine and assess its impact on disease burden.

Further postmarketing surveillance will allow a more precise estimate of the effectiveness of 4CMenB. However, although an hSBA can be used to demonstrate whether the vaccine induces antibodies capable of killing meningococcal strains, the presence of four antigens means that it is more complex than in the case of other meningococcal vaccines (i.e., MenC). Moreover, the genetic diversity of serogroup B strains means that not all of them have the genes coding for each of the antigens, and their expression may vary over time or from place to place.

For these reasons, the Meningococcal Antigen Typing System (MATS) is used to measure bacterial antigen expression in order to predict whether bactericidal serum is capable of killing particular strains [34–36]. This method is characterised by both phenotypic and genotypic analyses: the expression of the individual antigens that cross-react with the corresponding vaccine antigen is quantified using polyclonal antibodies against NHBA, NadA, and fHbp in an enzyme-linked immunosorbent assay (ELISA), and DNA sequence homology to the variable region sequence of the vaccine strain PorA gene is assessed, in order to estimate coverage in a specific region [32, 33]. It has not yet been proved that there is a correlation between the MATS results and real vaccination coverage, but the predicted protection based on the expression of at least one matched antigen ranges from 73% to 87% [36]. The MATS has been applied to isolates of 1,052 MenB strains causing IMD in Europe submitted to reference laboratories in France, Germany, Italy, Norway, and the UK between 2007 and 2008 [37], and the analysis demonstrated estimated efficacy values ranging from 73% in the UK to 87% in Italy. Furthermore, a study conducted in Canada between 2006 and 2009 analysed 157 MenB isolates collected from children and adults with IMD and found that the potential coverage of 4CMenB vaccine was 75–90%. On the basis of the MATS ELISA findings, the authors predicted that 66% of the circulating strains were covered by at least one vaccine antigen although none were covered by all four [38].

A new meningococcal serotype X has recently been isolated in Africa, against which no vaccine is currently available. However, some authors have recently used the MATS and bactericidal assays of 11 serogroup X isolates taken from nine African and two French patients and found that 4CMenB vaccine could have a good coverage against the strains from Africa but not those from France [39]. In regions where meningococcal strains are appropriately

monitored, the MATS can evaluate the real effectiveness of 4CmenB vaccine, and the development of changes in the MenB serogroup over time. It is also a very useful means of monitoring the emergence of new MenB mutants due to selective vaccination pressure [39].

## 5. Vaccination Strategies and Future Perspectives

Although the development of a meningococcal B serogroup vaccine was slow and difficult, the new Bexsero 4CMenB vaccine has recently been licensed in the EU, Australia, and Canada. The introduction of any new vaccine is never easy, but it is especially complex in this case.

The success of a vaccination programme is based on both cost-effectiveness and public acceptance and although meningococcal B infection is a cause for concern in the general population, the acceptability of the vaccine by parents is influenced by worries concerning its potential side effects, its real effectiveness, and the consequences of its coadministration with other routine vaccines in terms of the number of injections and possible immunological interference [40]. For example, its acceptance may be reduced by the fact that it has been associated with increased rates of fever when coadministered with the routine vaccinations provided during infancy on the basis of the national immunisation schedules.

One recent study of parental attitudes to 4CMenB showed that 82.5% of the interviewees wanted their children vaccinated. The most frequent concerns were side effects including fever (41.3%) and adequate vaccine testing (11.7%), but 26% of the parents said that they had no concerns. Moreover, as in the case of other vaccinations (e.g., HPV), the authors found that most parents (81.7%) were more likely to accept the vaccination if their immunisation providers recommended it [41].

The vaccination schedule should take into account the age of the subjects most frequently affected by meningococcal disease and the epidemiology of meningococcal infections. One recent study aimed at defining the optimal age for administering 4CMenB to children has shown that the incidence is highest in those aged <5 years (particularly those in their in the first year of life, when deaths are more frequent) and, on the basis of this finding, the authors suggested that vaccination should be started in the first year of life, with a catch-up dose being given at the age of five years [42].

Other recent studies have evaluated the cost-effectiveness of a potential MenB vaccination programme [43, 44]. One study carried out in The Netherlands estimated that an infant MenB vaccination programme would prevent 14% of cases over the lifetime of a birth cohort and concluded that this was not cost-effective [44] and although another study carried out in the UK estimated that routine vaccination would prevent 27–56% of cases over the lifetime of a birth cohort, the authors also considered it not cost-effective [41]. Finally, a recent study of the economic impact of MenB vaccination in Canada found that the MenB vaccination programme exceeds the generally cost-effectiveness thresholds and therefore should not be considered economically advantageous [45].

Nevertheless, 4CMenB is now being used in Canada and, upon parents' request, also in all of the countries and it has been licensed and its use in at-risk populations has been implemented. It has also been announced that it may be introduced into the UK's routine infant immunisation schedule using a 2 + 1 regimen although only a 3 + 1 regimen with the concomitant use of paracetamol is currently licensed [46], and some other countries are reconsidering their cost-effectiveness calculations by also considering its possible impact on carrier status [47].

With the availability of the bivalent rLP2086 vaccine for the adolescent age, it will be important to compare vaccination strategies that cover different age groups as well as to understand the impact of the two vaccines against IMD overall, meningococcal disease due to serogroups different from MenB and meningococcal carriage in the nasopharynx.

## 6. Conclusions

*N. meningitidis* is still one of the major causes of sepsis and meningitis among children worldwide and is associated with a high mortality rate. Considerable efforts have therefore been made to prevent meningococcal disease by means of vaccination, and two effective conjugate vaccines have recently been licensed and led to good results (Men C and MenACYW). However, it proved to be very difficult to develop a vaccine against serogroup B because of the poor immunogenicity of its capsular polysaccharide, even when conjugated with a carrier that could also induce an autoimmune response, until experts used reverse vaccinology to identify appropriate antigenic recombinant proteins. The overall findings of various studies have shown that the administration of three doses of 4CmenB to young children (alone or with routine vaccines) does not interfere with immune responses, and most have found that its safety and tolerability are acceptable. However, its coadministration with other vaccines does lead to increased reactogenicity (particularly fever) and so such coadministration should be combined with paracetamol given both before and after vaccination.

The new 4CMenB vaccine represents an important opportunity to fight pediatric IMDs, but its introduction should take into account the need to maintain the appropriate use of meningococcal conjugate vaccines that cover serogroups other than B, community opinion, and cost-effectiveness data. Moreover, it will be important to compare 4CMenB potential efficacy with that of bivalent rLP2086 vaccine. Finally, it is very important to continue surveillance in order to monitor the emergence of new meningococcal B strains in order to identify any that are not susceptible to 4CMenB.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgment

This review was supported by a grant from the Italian Ministry of Health (Bando Giovani Ricercatori 2009).

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 394368, 8 pages
http://dx.doi.org/10.1155/2015/394368

*Review Article*

# Nasopharyngeal Bacterial Carriage in the Conjugate Vaccine Era with a Focus on Pneumococci

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Received 6 February 2015; Revised 29 May 2015; Accepted 10 June 2015

Academic Editor: Jacek Tabarkiewicz



Seven-valent pneumococcal conjugate vaccine (PCV7) was included in the UK national immunisation program in 2006, and this was replaced by thirteen-valent PCV in 2010. During this time, the carriage of vaccine-type *Streptococcus pneumoniae* decreased but pneumococcal carriage remained stable due to increases in non-vaccine-type *S. pneumoniae*. Carriage studies have been undertaken in various countries to monitor vaccine-type replacement and to help predict the serotypes, which may cause invasive disease. There has been less focus on how conjugate vaccines indirectly affect colonization of other nasopharyngeal bacteria. If the nasopharynx is treated as a niche, then bacterial dynamics are accepted to occur. Alterations in these dynamics have been shown due to seasonal changes, antibiotic use, and sibling/day care interaction. It has been shown that, following PCV7 introduction, an eradication of pneumococcal vaccine types has resulted in increases in the abundance of other respiratory pathogens including *Haemophilus influenzae* and *Staphylococcus aureus*. These changes are difficult to attribute to PCV7 introduction alone and these studies do not account for further changes due to PCV13 implementation. This review aims to describe nasopharyngeal cocarriage of respiratory pathogens in the PCV era.

## 1. Introduction

Invasive pneumococcal disease (IPD) is a cause of substantial morbidity and mortality worldwide, with over 5,000 cases reported in the UK per year [1]. For patients with IPD in the United States, around 10% will die from the illness [2]. In 2008, globally there were an estimated 476,000 deaths attributed to pneumococcal infection among children less than five years of age. As of 2014, globally 59% of infants still live in countries where a PCV has yet to be added to the national immunisation program [3]. The seven-valent pneumococcal conjugate vaccine (PCV7) (Prevenar, Pfizer, previously Wyeth) was added to the US immunisation schedule in 2000 and to the UK immunisation schedule in 2006. The effect of PCV7 on pneumococcal carriage in children has been investigated, with vaccine serotypes decreasing since PCV introduction [4–6]. In the UK and elsewhere, the incidence of IPD has decreased after the implementation of PCVs [7–10]. As PCV13 was introduced, the rates of vaccine-type carriage and IPD have similarly declined [11–13], and as additional higher valency PCVs are introduced it is expected that IPD incidence will continue to decrease [14].

The effect of pneumococcal vaccination on other bacterial species known to occupy the same niche as *S. pneumoniae* has not been fully investigated, in particular determination of how changes in the human microbiome can be attributed to external pressures or vaccine introductions [15, 16]. This paper reviews what is known about cocarriage of nasopharyngeal bacteria.

## 2. Pneumococcal Disease

*S. pneumoniae* are Gram-positive diplococci often found to occupy the nasopharynx. Pneumococci are typed according to the serological response to their external polysaccharide capsule. Strains of *S. pneumoniae* that do not react with type-specific antisera are deemed nontypeable (NT) *S. pneumoniae*. Currently 94 pneumococcal serotypes have been characterised [17–22]. Individuals become colonised with *S. pneumoniae* and other nasopharyngeal flora during their first few months of life [23], although the age of pneumococcal colonisation varies and may be attributed to environmental factors such as having siblings, attending day care, or geographical location [23, 24]. Colonisation with a pneumococcal isolate is a prerequisite for pneumococcal infection; the capsule type of *S. pneumoniae* rather than genotype is thought to modulate the degree of infection [25]. The capsular type of the pneumococcus dictates the duration of colonization in children with common serotypes retained longer in carriage. As the age of a child increases, so does their immune mediated clearance of pneumococcal serotypes [26]. Disease caused by pneumococcal infections can be divided into two groups: invasive pneumococcal disease (IPD) and noninvasive disease.

## 3. Immunisation to Reduce Disease Burden

The 23-valent pneumococcal polysaccharide vaccine (PPV23) (Pneumovax II, Aventis Pasteur) is a plain polysaccharide vaccine that induces an immune response to the polysaccharide capsule of an infectious organism to induce short-term memory B-cells and antibody production. As the immune response produced is classed as "slow, no immune memory," this type of vaccine is not effective in young children and infants (IPD risk groups). T-cell independent vaccines also appear not to prevent carriage of the bacterial species [27] after the short-lived immune response has finished.

Conjugate vaccines contain bacterial polysaccharides from the outer capsule of an organism, for example, PCV7 (seven-valent pneumococcal conjugate vaccine, Prevenar, Pfizer); 10-valent PCV (Synflorix, GSK) and 13-valent PCV (Prevenar 13, Pfizer) are currently licensed pneumococcal conjugate vaccines. The polysaccharide is converted into a T-cell dependent antigen through the presence of the carrier protein. Long-term memory B-cells mature so that the immune system has both a short-term and long-term response invoked when those polysaccharides are encountered again. This reduces colonisation of the serotypes included within the vaccine, helping to prevent infection even in the very young. PCVs are given to children rather than a PPV to produce a stronger and more long-lasting immune response [28] and PCVs have also been found to be more effective against vaccine serotypes than PPV in older adults [29]. The 10-valent pneumococcal vaccine, PHiD-CV10 (GSK), also includes conjugation of nontypeable *Haemophilus influenzae* protein D. PCV10 is comparable to PCV7 at preventing invasive pneumococcal disease [30] and has been found to have a higher immunologic coverage for acute otitis media than PCV13 [31].

## 4. Pneumococcal Conjugate Vaccination

Prior to implementation of 7-valent pneumococcal conjugate vaccination, the majority of invasive disease globally was caused by seven of the pneumococcal serotypes [32]. PCV13 introduction has further addressed IPD caused by the 6 serotypes included in the new vaccine in Europe and North America [33]. PCV effectiveness is subject to strains undergoing capsular changes including (a) serotype replacement/shifting [34], where prevalence of a nonvaccine serotype increases as prevalence of a vaccine serotype decreases and the non-vaccine-type bacteria overcome vaccine challenges in a community [35], and (b) capsular switching, where an individual bacterium can undergo changes in the capsular genes, causing the bacteria to change serotype [36]. Through alteration of capsular expression and the increase in prevalence of serotypes not included in vaccine formulations, serotypes in carriage may be replaced with more virulent serotypes [37]. However it has been reported that capsular switching resulting from vaccine pressures will only contribute to an increase of a maximum of three extra cases of IPD per 100,000 vaccinated children cumulated over a ten-year period [38]. The additional maximum of three cases per year was deduced using a mathematical model of pneumococcal transmission based on IPD data presented in previous European publications [38]. However until there is evidence from more studies of capsular switching after PCV, IPD from non-vaccine-type pneumococci may be a more pressing issue [39]. Antibiotic resistance in pneumococcal isolates has also been shown to be present globally in both carriage [40–42] and disease [43–45] cases. Reasons for pneumococcal antibiotic resistance include vaccine pressures as well as overprescribing and overuse of antibiotics acting as a selective pressure for current strains to undergo clonal expansion [46, 47].

## 5. Interactions of Nasopharyngeal Microbiota

The microbiota of the human nasopharynx contains both commensal and potentially pathogenic species with external environmental factors and the presence of antibiotic resistant species contributing to disease states [48].

Bacteria found to reside in the nasopharynx other than *S. pneumoniae* include *H. influenzae*, *Moraxella catarrhalis*, alpha-haemolytic streptococci ($\alpha$-HS), *Staphylococcus aureus*, and *Neisseria meningitidis* which are included in this review as respiratory bacteria capable of causing significant infections. *M. catarrhalis* is a nonmotile Gram-negative human commensal and opportunistic pathogen responsible for a range of infections, including causing an estimated 10% of adult chronic obstructive airways disease (COPD) exacerbations [49]. It has been shown that even the colonisation of *M. catarrhalis* in a COPD patient can contribute to the progression of airway disease [50]. *S. pneumoniae*, *Streptococcus mutans*, and *Streptococcus sanguis* are all species of $\alpha$-haemolytic streptococci ($\alpha$-HS) that are both commensal and pathogenic in people; *Streptococcus viridans* are a group of streptococcal organisms including *Streptococcus mitis*, *Streptococcus salivarius*, and *S. mutans*. *S. viridians* have

been shown to be involved in causing empyema thoracis and lung abscesses [51]. *S. aureus* is a commensally carried Gram-positive bacterium that can act as an opportunistic respiratory pathogen in susceptible individuals. Nasal carriage of *S. aureus* is positively associated with noninvasive and invasive infections, compared to those who do not carry *S. aureus* [52]. It is important to assess the carriage of *S. aureus* and to monitor if carriage of methicillin-resistant (MRSA) bacteria is increasing in the community. Carriage of the Gram-negative diplococcus *N. meningitidis* is higher in young children under 1 year of age and then, after 15 years of age [53], *N. meningitidis* can result in a wide range of infections and bacterial load is associated with mortality, particularly for serogroup C that produces a higher bacterial load in patients [54]. *H. influenzae* are Gram-negative coccobacilli, serologically typed a–f, as well as a large, distinct population [55] that are unencapsulated, termed nontypeable *H. influenzae* (NTHi). Certain bacterial genes of NTHi have been found to be associated to aid bacterial persistence within the lower airways of patients with COPD [56] as well as now being known to cause invasive disease in risk groups [57].

## 6. Monitoring Bacterial Carriage

Bacterial colonization is thought to be a prerequisite for an individual to become infected, but bacterial colonisation does not normally result in infection [58]. There are many relevant studies, both completed and ongoing, that monitor bacterial carriage in individuals [59–63]. Bacterial carriage may be monitored to detect before and after changes following the implementation of a preventative vaccination strategy [4, 59, 64–68]. Carriage can be monitored for changes attributed to age, health status, geographical location, ethnicity, and many other environmental factors [69, 70]. Carriage of a number of bacteria or carriage of a single bacterial species can be monitored. Respiratory bacteria can be detected using relatively noninvasive means such as a nasopharyngeal [71, 72] or nose swab, meaning that larger numbers of patients can be recruited to strengthen the results gained from the study.

Pneumococcal carriage studies are highly informative and beneficial for a number of reasons. Through surveillance of invasive disease studies, we have seen how PCVs are effective against invasive vaccine-type (VT) pneumococcal disease [73], and through carriage studies we can see the reduction of VT pneumococcal colonisation and VT pneumococcal transmission [74]. It is also possible to monitor indirect effects of PCVs (Table 1), such as changes in dynamics of the nasopharynx to detect microbial shifts [75], where the bacterial species change in content or numbers due to an external pressure.

## 7. A Niche of Carriage and Infection

Bacterial cocolonisation of the nasopharynx niche can be classed as dynamic as it comprises both synergistic and competitive associations. These associations can change depending on whether or not the niche is in a healthy or a disease state [76]. A lower diversity of nasopharyngeal flora has been positively associated with higher carriage rates of nasopharyngeal pathogens including *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* [77]. Individuals with spontaneous otorrhea that have multiple pneumococcal serotypes colonising at any one time were more likely to present with other species cocolonising [78]. Viral infection has been to leave the middle ear vulnerable to infection by bacteria that normally reside in the nasopharynx [79].

External factors such as sibling interaction and interaction with other children can play a part in polymicrobial carriage, and such relationships are associated with more frequent nasopharyngeal carriage of potential pathogens [80]. Adult associations with more frequent carriage of potential pathogenic species include, but are not limited to, the presence of children either at home or at work, preexisting allergic conditions, and respiratory conditions including COPD and asthma [80]. Such data imply that children are reservoirs for bacterial pathogens. With increased contact between children and other children or children and adults, there is a greater chance for bacterial transmission between the individuals.

*S. pneumoniae* and *H. influenzae* are frequently found to cocolonise the nasopharynx, and competition may exist between the two organisms for nutritional resources and for dominance of the niche. It has been shown that *H. influenzae* when colonising with *S. pneumoniae* may outcompete them for survival through signaling of nucleotide-binding oligomerisation domain-1 (Nod1) to facilitate clearance of *S. pneumoniae* [81], but virulent *S. pneumoniae* serotypes show resistance to host cell-mediated clearance as a mechanism to overcome these attacks [82]. Both organisms cause immune responses in colonised individuals and cocolonisation by these two pathogens can result in exaggerated immune responses with prolonged hospitalization particularly for young asthmatics experiencing their first count of wheezes [83].

Before the inclusion of the *H. influenzae* type b (Hib) conjugate vaccine in the UK routine paediatric immunisation schedule in 1992 [84], around 95% of invasive *H. influenzae* disease was attributed to serotype b alone [85]. After Hib vaccination there was a dramatic 98% decrease in invasive disease by 1998 [86]. However a small amount of Hib disease has still been reported in some vaccinated populations, ranging from invasive disease due to vaccine failure in the UK and elsewhere [86, 87]. Increased disease incidence has been reported for non-Hib serotypes [88–90]. NTHi has also been reported as a cause for invasive disease [90, 91]. The surveillance following Hib vaccination indicates that vaccine-type replacement is seen with other strains not included in vaccine formulation; however studies have not yet fully elucidated the effects on cocolonising niche species.

Polymicrobial cocarriage can consist of more than one bacterial species; this can also include viruses and fungi [92]. A polymicrobial infection combining both bacteria and fungi can mount a greater immune response within a host than infection by either bacteria or fungi [93]. Preinfection with a virus can destroy epithelial cells and allow better adhesion for bacteria, thus priming the middle ear for further infection that can result in otitis media [94]. Bacterial

Table 1: Examples of studies identifying respiratory cocarriage of bacterial species in the PCV era.

| Study | Study detail: vaccination status, species selected, and origin of samples | Methodology |
|---|---|---|
| Wiertsema et al. [104] | Post-PCV7, Spn, and Hflu (Australia) | Nasopharyngeal swab (NP) and conventional culture |
| Xu et al. [101] | Post-PCV7, Spn, Hflu, and Mcat (USA) | Nasal swab (NS), oropharyngeal sample (OP), and conventional culture |
| Spijkerman et al. [105] | Post-PCV7, Spn, SA, Hflu, and Mcat (Netherlands) | NP swab and conventional culture |
| Principi et al. [24] | Pre-PCV7, Spn, Hflu, and Mcat (Italy) | NP swab and conventional culture |
| Biesbroek et al. [75] | Post-PCV7 and pre-PCV7, Spn, Mcat, SA, and Spn (Netherlands) | NP swab and 454 pyrosequencing |
| Xu et al. [76] | Post-PCV7, Spn, Hflu, Mcat, and SA (USA) | NP, OP, and conventional culture |
| Pettigrew et al. [77] | Post-PCV7, Spn, Hflu, and Mcat (USA) | NP and 454 pyrosequencing |
| Laufer et al. [102] | Post-PCV7, Spn (USA) | NS swab and 454 pyrosequencing |
| Bogaert et al. [103] | Post-PCV7 and reduced dose PCV7, Spn, Hflu, Mcat, and SA (Netherlands) | NP swab and 454 pyrosequencing |

This table includes vaccination status, species chosen for monitoring, and the origin of the samples as well as the methodology used for a comparison. Spn: *S. pneumoniae*; Hflu: *H. influenzae*; SA: *S. aureus*; Mcat: *M. catarrhalis*. 454 pyrosequencing is nonculture based identification.

communities that aggregate together on a surface are known as biofilms. Biofilms have a number of mechanisms to increase persistence and survive, which protect from both therapeutic attack and host immune responses [95, 96]. Bacterial biofilms contribute to a range of chronic respiratory and otolaryngeal diseases. Bacterial biofilms are commonly detected and implicated in pathogenicity in children with recurrent acute otitis media [97, 98], chronic middle ear effusion [99], and other chronic respiratory conditions.

## 8. Indirect Effects of PCV Implementation

Reports are emerging of the effect of PCV implementation on cocarriage and disease caused by bacteria other than pneumococci. Where vaccine serotypes of *S. pneumoniae* have been eradicated, there has been an increase in nontypeable *H. influenzae* isolated in cases of otitis media [100]. A large study of healthy children and children with recurrent otitis media, all less than 36 months of age in Western Australia, has shown that with a decrease in *S. pneumoniae* and *S. pneumoniae* PCV7 VT serotypes there is a corresponding increase in *H. influenzae,* particularly NTHi. Another study has shown that colonisation with of *S. pneumoniae* invasive serotype 19A is associated with a decrease in colonisation of *H. influenzae* [101]. To study the indirect effects of PCV implementation without introducing bias through standard microbiology culture, 16S-sequencing was used to sequence nasopharyngeal swabs of children with or without otitis media, which demonstrated that an infection is associated with increased *S. pneumoniae* and *H. influenzae* being present with a lack of protective flora present [102]. Another study set out to characterize the nasopharyngeal niche to deduce which, if any, external factors (such as viral carriage and day care level) have an effect on the microbiota of the nasopharynx of young children. Results showed that seasonal changes were occurring but that these were unrelated to viral or antibiotic causes and that seasonal variations corresponded to "healthy" probiotic species being more abundant in summer rather than autumn [103].

## 9. Summary

Pneumococcal conjugate vaccines (PCVs) affect the carriage of *S. pneumoniae* and the carriage of vaccine-type (VT) serotypes. With a decrease in *S. pneumoniae* PCV7 VT serotypes, there was a corresponding increase in *H. influenzae,* particularly NTHi [100], exemplifying the dynamic, potentially competitive relationship between these two organisms [106–108]. The eradication of PCV7 VT in the nasopharynx has also been associated with higher rates of *H. influenzae* and *S. aureus* carriage in young children and infants, highlighting those virulent serotypes of *S. pneumoniae* also having a competitive relationship with *S. aureus* as well as *H. influenzae* [75, 105]. Incidences of bacterial cocarriage are important to report to inform future vaccine developments. This is important as the effect of vaccines targeting nasopharyngeal pathogens may produce indirect effects, as the ultimate balance between cocolonising organisms is unknown. The future of vaccination is under scrutiny with each vaccination implemented against specific serotypes or serogroups, as vaccine-type replacement is detected in the years following vaccination. When it is so difficult to predict the effects after vaccination of the target species, it is even more difficult to account for the indirect effects on cocolonising species.

(i) IPD remains an important disease both in the UK and worldwide responsible for morbidity and mortality.

(ii) PCVs are currently the most effective pneumococcal vaccinations available at both reducing colonization of invasive serotypes and invasive disease.

(iii) The role of conjugate vaccines in the nasopharyngeal niche remains unclear as studies initially focused on serotype/serogroup replacement of the target species.

(iv) Serotype replacement occurs after PCV implementation, driving the need to develop new vaccination strategies independent of pneumococcal serotype inclusion.

(v) Bacterial carriage studies in the conjugate vaccine era have primarily focused on carriage of the target species; there have not been as many studies looking at nasopharyngeal cocarriage.

(vi) Studies that have looked at nasopharyngeal cocarriage in the PCV era have shown changes in carriage of *H. influenzae* and *S. aureus* following PCV implementation, implying that a reduction in vaccine-type pneumococci will result in an increase of carried *H. influenzae* and *S. aureus*.

(vii) It is currently difficult to define all potential indirect effects of PCV on the carriage of nonpneumococcal organisms.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

S. C. Clarke and S. N. Faust have contributed equally to this work.

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Hindawi Publishing Corporation
Journal of Immunology Research
Volume 2015, Article ID 206757, 6 pages
http://dx.doi.org/10.1155/2015/206757

*Research Article*

# Impact of Pneumococcal Conjugate Universal Routine Vaccination on Pneumococcal Disease in Italian Children

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Received 11 January 2015; Revised 6 June 2015; Accepted 10 June 2015

Academic Editor: Jiri Mestecky



In Italy, the effectiveness of pneumococcal universal vaccination in preventing vaccine-type invasive pneumococcal disease (IPD) in the PCV7/PCV13 shifting period was estimated to be 84.3% (95% CI: 84.0–84.6%) in children <5 years. This study aims at corroborating the estimation of both the effectiveness (VE) of PCVs and its impact in reducing pneumococcal diseases. A 1 : 3 matched-case-control study was conducted among children <5 years old hospitalized for IPD or pneumococcal pneumonia (PP) between 2006 and 2012 in the Puglia region. Moreover, hospitalizations for pneumococcal outcomes in the pre- and postvaccination period and the hospitalization risk ratios (HRRs) with 95% CIs were computed in Italy and in the first eight regions that introduced PCVs in 2006. The overall effectiveness of PCVs was 75% (95% CI: 61%–84%); it was 69% (95% CI: 30%–88%) against IPD and 77% (95% CI: 61%–87%) against PP. PCVs showed a significant impact on IPD and acute otitis media either at a national level or in those regions with a longer vaccination history, with a nearly 40% reduction of hospitalizations for both outcomes. Our findings provide further evidence of the effectiveness of PCVs against pneumococcal diseases and its impact on nasopharyngeal carriage in children <5 years, indicating the importance of maintaining high immunization coverage.

## 1. Introduction

Pneumococcal infection is a major cause of morbidity and mortality worldwide [1]. Among young children and the elderly population, *Streptococcus pneumoniae* causes invasive disease (IPD), such as severe blood infection, meningitis, and pneumonia [2].

Since the introduction of the 7-valent conjugate vaccine (PCV7, containing serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F) in 2000, there has been an overall reduction in the incidence of all-serotype IPD, ranging from 80% in the USA to 30–40% in Europe, in children under 5, despite the reported increase in the incidence of pneumococcal diseases due to non-PCV7 serotypes (NVT) in all age groups [3, 4].

Following the globally observed changes in the overall serotype distribution of *S. pneumoniae*, particularly a rise in serotype 19A, higher-valent pneumococcal conjugate vaccines (PCV10, comprising the additional serotypes 1, 5, and 7F, and PCV13, comprising the additional serotypes 1, 3, 5, 6A, 7F, and 19A) were developed and widely employed to provide improved serotype coverage against pneumococcal diseases. Second-generation PCVs, routinely implemented since 2010, have showed an early impact and effectiveness for reducing pneumococcal infections in both vaccinated and unvaccinated children and in reducing nasopharyngeal carriage of vaccine-serotypes [5–7]. In the first study from Miller et al. in England and Wales, PCV13 effectiveness in preventing IPD was estimated to be 78% (95% CI: −18% to 96%) and 73% (95% CI: 29%–90%) for two priming doses and for one dose over a year, respectively [8]. Moreover, PCV13 showed an early impact on overall pneumococcal nasopharyngeal carriage in young children <2 years with acute otitis media (AOM) [9].

In Italy, PCV7 was available on the market from 2002 to 2010. Between 2006 and 2010, all 21 regions had recommended or introduced the vaccine in their childhood immunization schedule [10, 11]. In May 2010, the Ministry of Health issued a recommendation to replace PCV7 with PCV13 [12].

Finally, the National Vaccination Plan 2012–2014 included the active offer of 3 PCV13 doses in the list of "Essential Health Interventions" for newborns at 3, 5-6, and 11–13 months of age. The plan set the objective to achieve and maintain PCV13 vaccination coverage (VC) of at least 95% in children <24 months of age [13]. Throughout the transition period between PCV7 and PCV13, children who had received one or two doses of PCV7 completed their immunization series with PCV13. One PCV13 catch-up dose was recommended for children fully vaccinated with PCV7 [12, 14].

In 2013, a survey conducted among 14 Italian regions showed that PCV vaccination coverage in children aged 24 months progressively increased from 2005 to 2009 birth cohort, with a considerable variability between regions, from 44.7% to 98.5% in 2011. Due to the reduction in circulation of the vaccine-serotypes, the incidence of IPD in children aged 0–4 years decreased from 7.1 per 100,000 in 2008 to 3.8 per 100,000 in 2012 [10].

The Puglia region (southeast Italy, ~4,000,000 habitants) was one of the first eight Italian regions to introduce PCVs in the childhood immunization schedule in 2006 [15, 16]. Vaccination coverage in children under 24 months of age increased from 75.3% in the 2006 birth cohort (PCV7 only) to 95.1% in the 2010 birth cohort (PCV7/PCV13) [17] and was 93% in the 2011 birth cohort (PCV13 only). In our previous study (Martinelli et al.), the overall effectiveness of the pneumococcal immunization programme in preventing vaccine-type IPD during and after the PCV7/PCV13 shifting in the immunization schedule was estimated at 84.3% (95% CI: 84.0%–84.6%). The impact of vaccination on hospitalizations for pneumococcal diseases in children under 5 years of age recorded the most important reduction for pneumococcal pneumonia (HRR: 0.43, 95% CI: 0.21–0.90), followed by IPD (HRR: 0.72, 95% CI: 0.21–2.43), acute otitis media (HRR: 0.75, 95% CI: 0.65–0.88), and all-cause pneumonia (HRR: 0.92, 95% CI: 0.86–0.99) [17].

This study aims at corroborating the estimation of both the effectiveness of PCVs and its impact in reducing severe pneumococcal diseases in children under 5 years of age in Italy.

## 2. Methods

*2.1. PCVs Programme Effectiveness.* To estimate the vaccination programme effectiveness (VE) in the PCV7/PCV13 period between 2006 and 2012, a 1 : 3 matched-case-control study was conducted among children <5 years of age and resident in Puglia.

A *case* was defined as a child born between January 2006 and June 2012, hospitalized (when aged at least 6 months) for IPD or pneumococcal pneumonia (PP) between June 2006 and December 2012. The study included 28 pediatric wards of all hospitals in the region (admitting about 60,000 children aged ≤60 months per year). Hospitalization records were extracted from the regional discharge registry. IPD was defined as ICD9-CM (International Classification of Diseases, Ninth Revision, Clinical Modification) code 320.1 (pneumococcal meningitis) or 038.2 (pneumococcal septicemia) or as each of the following codes: 320.8 (the other specified meningitis), 790.7 (bacteremia), or 038.9 (unspecified septicemia) if associated with 041.2 (bacterial infection in conditions classified elsewhere and of unspecified site—pneumococcus). Pneumonia with diagnosed *S. pneumoniae* infection was defined as ICD9-CM code 481 (*Streptococcus pneumoniae* pneumonia) [17]. Records were selected if these codes were either in the main or in the secondary discharge diagnosis.

A *control* was defined as a presumed healthy child, matched by gender, age (±1 month), and municipality, retrieved from the general population registry, and checked for previous history of hospital admission due to pneumococcal diseases (exclusion criteria).

Information on vaccination status against pneumococcus was retrieved from the regional immunization registry. According to the vaccine received, a case was defined as "fully vaccinated" if the child had been vaccinated with ≥3 doses of PCV7/PCV13 at least one month before the date of hospitalization; a fully vaccinated control was a child who had received ≥3 PCV7/PCV13 doses. A case was defined as "incompletely vaccinated" if the child had received <3 doses of PCV7/PCV13 at least one month before the hospitalization; an incompletely vaccinated control was a child who had received <3 doses of vaccine.

IPD and PP cases were described by age, sex, and vaccination status. Odds Ratios (ORs) with 95% confidence intervals (95% CIs) and the Exact McNemar Test were computed by using Stata MP 12 for Mac OS tool for matched studies. Post hoc power analysis was conducted after the study had been completed and used the obtained sample size and effect size to determine what the power was in the study, assuming the effect size in the sample is equal to the effect size in the population [18]. Overall and outcome specific VE was calculated as 1 − OR with 95% CIs.

The study was conducted in accordance with The Guidelines for Good Clinical Practice and the ethical principles that originate in the Declaration of Helsinki and within the Italian law. The protocol was approved by the Institutional Review Board at the Regional Observatory for Epidemiology.

*2.2. Impact of the Vaccination Programme.* In order to assess the impact of the vaccination programme in Italy, the disease burden on the population of children <5 years of age was estimated, choosing as indicators the hospitalization rates for pneumococcal diseases before (average annual hospital admission rates between 2001 and 2005) and after (annual rates between 2006 and 2011) the introduction of the pneumococcal vaccination.

Episodes of pneumococcal diseases were extracted from the national hospital discharge registry [19] and specific hospitalization rates were calculated for the following outcomes:

(i) *IPD*, ICD9-CM codes as defined above;

(ii) *PP*, ICD9-CM code as defined above;

(iii) *All-cause pneumonia*, defined as ICD9-CM codes 480.xx–486.xx without mention of a diagnosis of IPD as defined above;

Table 1: Cases of IPD and pneumococcal pneumonia recruited among children hospitalized between June 2006 and December 2012 in the Puglia region, by gender, age, and year of admission.

| | IPD ($N = 9$) | | Pneumococcal pneumonia ($N = 30$) | | Total cases ($N = 39$) | |
|---|---|---|---|---|---|---|
| | $N$ | % | $N$ | % | $N$ | % |
| Gender | | | | | | |
| Male | 4 | *44.44* | 15 | *50* | 19 | *48.72* |
| Female | 5 | *55.56* | 15 | *50* | 20 | *51.28* |
| Age | | | | | | |
| <24 months | 7 | *77.78* | 7 | *23.33* | 14 | *35.90* |
| 24–<60 months | 2 | *22.22* | 23 | *76.67* | 25 | *64.10* |
| Year of admission | | | | | | |
| 2006 | — | — | 1 | *3.32* | 1 | *2.56* |
| 2007 | 1 | *11.11* | — | — | 1 | *2.56* |
| 2008 | 1 | *11.11* | 2 | *6.67* | 3 | *7.69* |
| 2009 | 1 | *11.11* | 2 | *6.67* | 3 | *7.69* |
| 2010 | 1 | *11.11* | 11 | *36.67* | 12 | *30.78* |
| 2011 | 2 | *22.22* | 8 | *26.67* | 10 | *25.64* |
| 2012 | 3 | *33.34* | 6 | *20.00* | 9 | *23.08* |

(iv) *Unspecified AOM*, defined as ICD9-CM codes 382.xx—*suppurative and unspecified otitis media* [17].

These ICD9-CM codes were scanned across discharge diagnoses in each child record for any mention of these diseases. The hospitalization rates in the pre- and postvaccination period and the hospitalization risk ratios (HRRs) with 95% CIs were computed using outcome specific Poisson regression models [17]. National data and data from the first eight Italian regions that introduced PCVs in 2006 were computed. Analysis was performed by using STATA MP 12 for Mac OS.

## 3. Results

*3.1. PCVs Programme Effectiveness.* A total of 39 cases were recruited, nine of IPD (four males, seven aged <24 months) and 30 of PP (15 males, seven aged <24 months) (Table 1). Each case was matched to three controls. Both cases and controls were citizens of Italy.

Twenty-seven/39 (69.23%, eight IPD and 19 PP) cases had been fully vaccinated with ≥3 doses of PCV7/PCV13 compared to 107/117 (91.4%) vaccinated controls (26 among those matched to IPD and 81 among those matched to PP). The overall PCVs programme effectiveness was 75% (95% CI: 61%–84%). It was 69% (95% CI: 30%–88%) and 77% (95% CI: 61%–87%) against IPD and PP, respectively (Table 2).

*3.2. Impact of the Vaccination Programme.* Reduction in hospitalization rates for IPD was observed either at a national level or in the first eight regions that had introduced PCVs since 2006, with HRR of 0.66 (95% CI: 0.52–0.84) and 0.51 (95% CI: 0.32–0.81), respectively. A decrease was also recorded for AOM, with HRR of 0.61 (95% CI: 0.58–0.65) and 0.63 (95% CI: 0.57–0.70), respectively. In contrast, the hospitalization rates for PP increased either in Italy (HRR: 1.4, 95% CI: 1.25–1.57) or in the eight regions (HRR: 1.57, 95% CI: 1.26–1.967) (Table 3).

## 4. Discussion

Our findings provide further evidence of pneumococcal conjugate vaccination effectiveness against IPD in children aged <5 years, although data of 69% (95% CI: 30%–88%) was slightly lower than what had been estimated by the same authors in a previous study [17] and from other similar studies [9, 20]. In a postlicensure assessment of serotype-specific vaccine effectiveness from Andrews et al. in England, Wales, and Northern Ireland, PCV13 effectiveness after two doses before the age of 12 months or one dose from 12 months was 75% (95% CI: 58%–84%) [21]. It is important to notice that, compared to other studies estimating effectiveness against vaccine-type IPD, this case-control design evaluated hospital discharge records where serotype information is not reported.

To our knowledge this is the first study in Italy assessing the effectiveness of PCVs in preventing severe pneumococcal pneumonia in children. Our finding of 77% (95% CI: 61%–87%) corroborates the reduction in hospitalization rates for PP observed in Puglia after the introduction of the vaccine (HRR: 0.43, 95% CI: 0.21–0.90) [17]. A systematic review of PCV dosing from Whitney et al. summarized studies of pneumonia endpoints documenting drops in disease rates or reported cases following PCV introduction, on a range of schedules [22].

A limitation of this case-control study could be seen in the relatively small number of cases recruited. This was predictable given the high vaccination coverage reported in our territory; however the post hoc estimation of the power of the study was fairly high (84.6%) (Table 3).

The PCVs programme has showed a significant impact on invasive disease and AOM in children aged <5 years both at a national level and in those regions with a longer vaccination history, with nearly a 40% reduction of hospitalizations for both outcomes. A Danish nationwide population-based

Table 2: Cases of IPD and pneumococcal pneumonia and matched controls. PCVs effectiveness (95% CIs) in Puglia, 2006–2012.

| Vaccination status | IPD cases ($N = 9$) | | Matched controls ($N = 27$) | | OR (95% CI) | $p^*$ | VE (95% CI) |
|---|---|---|---|---|---|---|---|
| | $N$ | % | $N$ | % | | | |
| Fully vaccinated[#] | 8 | *88.89* | 26 | *96.30* | 0.31 (0.12–0.7) | 0.003 | 69% (30%–88%) |
| Incompletely vaccinated[##] | 1 | *11.11* | 0 | *0* | n.c.[**] | 0.31 | |
| Not vaccinated | 0 | *0* | 1 | *3.70* | Ref. | Ref. | |
| Vaccination status | Pneumococcal pneumonia cases ($N = 30$) | | Matched controls ($N = 90$) | | OR (95% CI) | $p^*$ | VE (95% CI) |
| | $N$ | % | $N$ | % | | | |
| Fully vaccinated[#] | 19 | *63.33* | 81 | *90.00* | 0.23 (0.13–0.39) | <0.001 | 77% (61%–87%) |
| Incompletely vaccinated[##] | 11 | *36.67* | 7 | *7.78* | 1.57 (0.56–4.78) | >0.05 | |
| Not vaccinated | 0 | *0* | 2 | *2.22* | Ref. | Ref. | |
| Vaccination status | Total cases ($N = 39$) | | Matched controls ($N = 117$) | | OR (95% CI) | $p^*$ | VE (95% CI) |
| | $N$ | % | $N$ | % | | | |
| Fully vaccinated[#] | 27 | *69.23* | 107 | *91.46* | 0.25 (0.16–0.39)[***] | <0.001 | 75% (61%–84%) |
| Incompletely vaccinated[##] | 12 | *30.77* | 7 | *5.98* | 1.71 (0.62–5.13) | >0.05 | |
| Not vaccinated | 0 | *0* | 3 | *2.56* | Ref. | Ref. | |

[#] *Fully vaccinated cases*: children vaccinated with ≥3 doses of PCV7/PCV13 at least one month before the date of hospitalization; *fully vaccinated control*: presumed healthy children vaccinated with ≥3 doses.
[##] *Incompletely vaccinated cases*: children vaccinated with <3 doses of PCV7/PCV13 at least one month before the date of hospitalization; *incompletely vaccinated control*: presumed healthy children vaccinated with <3 doses.
[*] Exact McNemar significance probability. [**] n.c.: not calculable. [***] *Power of estimation: 84.6%*.

Table 3: Hospitalization rates (per 100,000) and HRRs (95% CIs) for IPD, *Streptococcus pneumoniae* pneumonia, all-cause pneumonia, and AOM in the pre- and postvaccination period in Italy and in the first eight regions that introduced PCVs in 2006.

| Italy | 2001–2005 | | 2006–2011 | | HRR (95% CI) | |
|---|---|---|---|---|---|---|
| | $N$ | Rate per 100,000 | $N$ | Rate per 100,000 | | |
| IPD | 163 | 6.14 | 112 | 4.04 | 0.66 | (0.52–0.84) |
| Pneumococcal pneumonia | 488 | 18.37 | 715 | 25.74 | 1.4 | (1.25–1.57) |
| All-cause pneumonia | 21,406 | 805.76 | 22,354 | 804.90 | 0.99 | (0.98–1.02) |
| AOM | 2,658 | 100.07 | 1.703 | 61.33 | 0.61 | (0.58–0.65) |
| Eight regions which introduced PCV in 2006 | 2001–2005 | | 2006–2011 | | HRR (95% CI) | |
| | $N$ | Rate per 100,000 | $N$ | Rate per 100,000 | | |
| IPD | 53 | 5.49 | 28 | 2.90 | 0.51 | (0.32–0.81) |
| Pneumococcal pneumonia | 127 | 13.23 | 205 | 21.40 | 1.57 | (1.26–1.96) |
| All-cause pneumonia | 7,287 | 760.09 | 7,430 | 775.03 | 0.99 | (0.95–1.02) |
| AOM | 870 | 90.79 | 565 | 58.94 | 0.63 | (0.57–0.70) |

cohort study observed a 71% reduction (95% CI: 62%–79%) in IPD incidence in children aged <2 years [23]. In a study from Norway, a reduced risk of maternal report of AOM was identified among children who had received three or more PCV7 immunizations by 12 months of age (RR: 0.86; 95% CI: 0.81–0.91) and by 18 months (RR: 0.92, 95% CI: 0.90–0.94), respectively, when compared with nonimmunized children [24, 25].

In contrast, hospitalization rates for PP have increased in the most recent years. A main aspect influencing this data is that the number of hospitalizations for pneumonia has showed a peak in all age groups during and after the flu pandemic in 2009 [10].

Moreover, surveillance of pneumococcal diseases presents a number of challenges because of differences in surveillance systems and reporting practices among Italian regions, therefore producing false trends [11]. In addition, underascertainment remains considerable for the scarce attitude to investigate cases using adequate laboratory tests, as the large number of the discharge records coded as all-cause pneumonia in this study has showed. In general, the use of hospital discharge diagnoses might either underestimate or

overestimate the number of pneumococcal outcomes as it relies on the review of the discharge forms (where laboratory confirmation is not reported) and not on the review of the medical records of the children hospitalized for diseases caused by or related to *S. pneumoniae*.

In conclusion, the PCVs programme has confirmed its effectiveness against the most severe cases of *S. pneumoniae* diseases and its substantial impact on nasopharyngeal carriage in children aged <5 years. Our findings indicate the importance of maintaining high routine immunization coverage, especially in a mature programme, such as in Italy, in which indirect (herd) effects help enhance protection.

## Disclosure

Portions of the study data shown in this paper were previously presented as a poster at the 32nd Annual Meeting of the European Society for Paediatric Infectious Diseases, held in Dublin, Ireland, May 6–10, 2014.

## Conflict of Interests

Dr. Prato reports grants and nonfinancial support from Pfizer, GSK, Novartis, and Sanofi Pasteur MSD, outside this work. All other coauthors have no conflict relevant to this paper to disclose.

## Acknowledgments

The authors acknowledge The Italian Ministry of Health, Direzione Generale della Programmazione Sanitaria, Ufficio V, for providing national hospitalization records and Biagio Pedalino for his suggestions for the paper.

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