Group B *Streptococcus* vaccine: state of the art

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Abstract: Group B *Streptococcus* (GBS) is cause of neonatal invasive diseases as well as of severe infections in the elderly and immune-compromised patients. Despite significant advances in the prevention and treatment of neonatal disease, sepsis and meningitis caused by GBS still represent a significant public health care concern globally and additional prevention and therapeutic strategies against infection are highly desirable. The introduction of national recommended guidelines in several countries to screen pregnant women for GBS carriage and the use of antibiotics during delivery significantly reduced disease occurring within the first hours of life (early-onset disease), but it has had no effect on the late-onset diseases occurring after the first week and is not feasible in most countries. Availability of an effective vaccine against GBS would provide an effective means of controlling GBS disease. This review provides an overview of the burden of invasive disease caused by GBS in infants and adults, and highlights the strategies for the development of an effective vaccine against GBS infections.

Keywords: CPS-based conjugate vaccine, early-onset disease, group B *Streptococcus*, late-onset disease, protein-based vaccine

Introduction

Lancefield group B *Streptococcus* (GBS), also referred as *Streptococcus agalactiae*, is a Gram-positive pathogen representing one of the most common causes of life-threatening bacterial infections in neonates and infants. Neonatal infections can result in pneumonia, sepsis, meningitis and in some cases death [Gibbs *et al.* 2004; Heath and Schuchat, 2007]. GBS has also been associated with high rates of invasive diseases in adults, especially in patients >65 years of age, black race, and with underlying medical conditions such as diabetes, cancer, cirrhosis and HIV infection [Blancas *et al.* 2004; Edwards and Baker, 2005; Skoff *et al.* 2009]. However, GBS is also a commensal organism able to primarily colonize the urogenital and gastrointestinal tracts of more than 30% of the healthy population and, in particular, 25–40% of healthy women are asymptotically colonized. It was estimated that, if 20–30% of pregnant women are colonized with GBS, approximately 50% of their infants become colonized and approximately 1% of these infants develop disease [Hansen *et al.* 2004; Jones *et al.* 2006; Heath and Schuchat, 2007].

Streptococcus agalactiae was first described as a cause of bovine mastitis in 1887 [Nocard and Mollereau, 1887] and in 1935 human strains were isolated for the first time as colonizing bacteria in pregnant women [Lancefield and Hare, 1935]. The first report describing GBS as a human pathogen associated with three cases of fatal puerperal sepsis was published in 1938 in United Kingdom [Fry, 1938]. Up to 1960s, infections in vaginal cultures from asymptomatic postpartum women were sporadically reported [Hood *et al.* 1961; Eickhoff *et al.* 1964; Butter and de Moor, 1967]. But, by the 1970s, GBS had emerged as the predominant pathogen causing septicaemia and meningitis in neonates and infants younger than 3 months in the United States. By the 1980s it was estimated that neonatal infections caused by GBS had an incidence of 0.5–2 per 1000 live births, a mortality rate of 20–25% and permanent neurologic sequelae in the majority of survivors [Schuchat, 1999].

Group B *Streptococci* appear in pairs or short chains and share a common antigen, the

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Lancefield group B polysaccharide antigen, and are distinguished on the basis of their type-specific capsular polysaccharides (CPS), which the microorganism expresses at high levels on its surface, into 10 structurally and antigenically unique types (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX) [Kong *et al.* 2002, 2005; Slotved *et al.* 2007]. The capsule surely represents the major virulence factor, which helps GBS evade host defence mechanisms by interfering with phagocytic clearance [Avci and Kasper, 2010]. Although it is known that all GBS serotypes are capable of causing invasive infections, five serotypes (Ia, Ib, II, III and V) account for the majority of disease both in neonates and adults. Epidemiological studies in different geographic areas (Africa, Western Pacific, Europe, the US and the Americas) reported similar serotype distribution data and little change has occurred over the past 30 years [Phares *et al.* 2008; Le Doare and Heath, 2013]. However, around 8–14% of the clinical isolates are classified as nontypeable (NT) strains because they cannot be distinguished on the basis of CPS antigenicity [Skoff *et al.* 2009]. Nevertheless, the association between NT GBS strains and invasive disease remains to be better documented.

The burden of GBS invasive disease in infants and adults

GBS diseases in neonates have been traditionally classified as early-onset disease (EOD) and late-onset disease (LOD) depending on the infants' age and disease manifestation.

EOD manifests in the first 6 days of life and accounts for between 60 and 70% of all GBS neonatal illness. The neonate is usually infected by exposure to GBS during birth and the transmission occurs vertically from mother to newborn, usually when the neonate aspirates contaminated amniotic and vaginal fluids. The bacteria can spread rapidly into the bloodstream and the disease can progress rapidly with clinical signs, typically pneumonia or sepsis, appearing within the first 12 hours in 98% of cases [Gibbs *et al.* 2004; Puopolo *et al.* 2005; Simonsen *et al.* 2014]. GBS serotypes Ia and III, following by Ib, II and V are responsible for the majority of EOD [Phares *et al.* 2008; Edmond *et al.* 2012].

LOD occurs after the first week of life within the first 90 days. It is characterized by meningitis in up to 50% of cases [Heath and Schuchat, 2007]. Unlike EOD pathogenesis, LOD may be acquired

perinatally from breast milk or from nosocomial and community sources. Prematurity is considered the main risk factor for developing LOD with a proportional increase of the risk for every week of prematurity [Lin *et al.* 2003]. LOD is caused mainly by GBS serotype III. The mortality rate is lower than EOD but morbidity is high, as around 50% of neonates that survive GBS infection suffer complications, including mental retardation, hearing loss, and speech and language delay [Schuchat, 1999; Heath and Schuchat, 2007]. Cases occurring 90 days after birth are also described, but rare and account for 0.37–0.73 per 100,000 in the USA [Phares *et al.* 2008]. The introduction in the US of national consensus guidelines for GBS disease prevention by the Centers for Disease Control and Prevention (CDC), first issued in 1996 and updated in 2002 [Schrag *et al.* 2002a], recommending universal screening of pregnant women for rectovaginal GBS colonization at 35–37 weeks' gestation and administering intrapartum antimicrobial prophylaxis (IAP) to carriers, was associated with a decline in the incidence of EOD from 0.47 per 1000 live births in 1999–2001 to 0.34 per 1000 live births in 2003–2005 [Phares *et al.* 2008]. Not surprisingly, the incidence of late-onset GBS infections among infants aged 7–89 days remained stable despite the implementation of prophylactic measures and occurred with an average of 0.34 per 1000 live births in the USA from 1999 to 2005 [Phares *et al.* 2008].

Since 2000, national population-based surveillance studies have been undertaken in different countries. Edmonds and colleagues have carried out a systematic review and meta-analysis to estimate the global burden of GBS and to define the current global incidence of group B streptococcal invasive disease in infants aged 0–89 days in the era of intrapartum prophylaxis [Edmond *et al.* 2012]. Although substantial heterogeneity was observed between studies and geographic areas, the analysis estimated an overall incidence of 0.53 per 1000 live births. Incidence was highest in Africa (1.21/1000) followed by the Americas (0.67/1000) and Europe (0.57/1000). Southeast Asia had the lowest incidence (0.02/1000), but it should be noted that few data were available from this region and the incidence value was the result of five studies only from Bangladesh, India and Thailand [Edmond *et al.* 2012]. However, the low incidence in low and middle income Asian countries could be also due to other factors, such as the widespread antibiotic usage prior to culturing,

high case fatality before sample collection, or just the lack of appropriate laboratory facility and culture methods. Moreover, the disease may remain unrecognized in countries where most newborns are delivered outside of health centres and access to hospitals is hampered [Dagneu *et al.* 2012; Johri *et al.* 2013]. Recent carriage studies carried out in the healthy population in India and Bangladesh reported a prevalence of GBS carriage between 7.7% and 12%, suggesting that the neonatal incidence in these countries could be underestimated [Chan *et al.* 2013; Johri *et al.* 2013]. It is evident that to obtain an accurate estimate of the true burden of GBS disease in these geographic areas, as well as in other developing countries, more studies of prospective, population-based carriage and/or disease incidence are needed. A low incidence of neonatal invasive diseases was reported in Japan. A nationwide surveillance study between 2004 and 2010 showed that the incidence of EOD and LOD was 0.08 and 0.10 per 1000 live births, respectively, although the mortality and morbidity rates remained substantial over time. Moreover, the issue of national guidelines did not affect the incidence rate [Matsubara *et al.* 2013]. A study by Edmonds and colleagues also confirmed that serotype III was the most common (48.9%) among different geographic areas, followed by serotypes Ia, Ib, II, and V. Altogether these five serotypes account for more than 94% of neonatal diseases caused by GBS [Edmond *et al.* 2012].

GBS has also emerged as an important cause of invasive infections in nonpregnant adults, particularly among the elderly (≥ 65 years), black persons, and adults with underlying medical conditions. Common clinical syndromes include mainly bacteremia with an unknown source (approximately 40%), skin and/or soft-tissue infection and pneumonia [Skoff *et al.* 2009]. More severe clinical syndromes were also described such as meningitis, streptococcal toxic shock syndrome and endocarditis, which are rare but often associated with severe morbidity and mortality. In patients >65 years of age, the mortality rate for invasive GBS disease is markedly higher than in neonates and is approximately 10% [Phares *et al.* 2008]. The underlying medical conditions associated with invasive GBS disease in nonpregnant adults include diabetes mellitus (41%), heart disease (36%) and malignancy (17%), with a significant increase of the proportion related to diabetes as reported in US studies during recent years. A population-based

surveillance involving 10 US states confirmed that the incidence of adult GBS disease increased significantly from 3.6 cases per 100,000 persons in 1990 to 7.3 cases per 100,000 persons in 2007 [Skoff *et al.* 2009]. Amongst adults aged 65 years or older, the incidence is much higher at approximately 30 cases per 100,000 population [Phares *et al.* 2008; Skoff *et al.* 2009; Le Doare and Heath, 2013]. Different to serotype distribution in neonatal infections, in adult diseases serotypes V and Ia are the most prevalent in US and Europe ranging from 20% to 30% of cases, followed by serotypes II, III and Ib [Edwards and Baker, 2005; Bergseng *et al.* 2008; Phares *et al.* 2008; Kothari *et al.* 2009; Skoff *et al.* 2009; Lambertsen *et al.* 2010; Tazi *et al.* 2011].

GBS prevention strategies: chemoprophylaxis and vaccination

By the late 1980s, a number of efforts had been made to prevent and treat GBS disease in infants and pregnant women. The intravenous antimicrobial prophylaxis, administered to women who are GBS colonized during labour and delivery, was found to be the most practical and effective mode to prevent vertical transmission of GBS from the mother to the infant and reduce the risk of perinatal sepsis [Boyer and Gotoff, 1986]. Since the first report at the end of the 1970s by Baker and Kasper related to the correlation of maternal antibody deficiency to increased susceptibility to neonatal GBS infection [Baker and Kasper, 1976], maternal active vaccination has been considered an attractive alternative to protect neonates against both EOD and LOD.

IAP

IAP is the most widely used strategy for GBS prevention and is applied after the identification of GBS carriers through a screening-based approach or by identification of clinical risk factors [Melin, 2008, 2011]. The screening approach recommends that all pregnant women should be screened between 35 and 37 weeks' gestation for GBS vaginal and rectal colonization. The risk-based approach does not require routine screening but instead evaluates potential risk factors for perinatal GBS diseases. The risk factors include prematurity (<37 weeks' gestation), prolonged rupture of membranes (PROM) ≥ 18 hours, intra-amniotic infection (IAI), temperature of $\geq 38^\circ\text{C}$, previous baby with invasive GBS disease or GBS isolation at any time during the pregnancy. Both

approaches recommend antibiotic administration during labour to women who were GBS positive at the screening time to GBS infection and/or presenting one of the risk factors mentioned above. For women with a negative GBS result or any risk factor, no prophylactic antibiotic is recommended.

National consensus guidelines for the prevention of GBS perinatal disease recommending use of IAP have been issued by public health authorities in a number of developed countries. The screening-based approach is recommended in the US, Japan and a number of European countries (Belgium, France, Germany, Italy, Poland, Spain and Switzerland). A risk-based approach is recommended in guidelines from Australia, Denmark, the Netherlands, New Zealand and the United Kingdom [Melin and Efstratiou, 2013].

A retrospective cohort study in a population of over 600,000 liveborn infants compared the effectiveness of the screening and risk-based approaches in preventing EOD. Data showed that the screening approach was more effective than the risk-based approach at preventing perinatal group B streptococcal disease [Schrage *et al.* 2002]. In the US, after the widespread implementation of preventive strategies, the incidence decreased dramatically from 1.7 cases per 1000 live births in 1990 to <0.4 cases per 1000 live births in 2010 [Schuchat, 1999; Schrage *et al.* 2000, 2002a, 2002b]. However, no prevention strategy is currently totally effective in the eradication of EOD and, most important, IAP has had no impact on late-onset infection where the burden is still substantial. Globally, over the same 20-year period, the incidence of GBS LOD has remained relatively steady with 0.3–0.4 cases per 1000 live births and GBS remains the most important cause for neonatal meningitis in children aged less than 5 years [Schrage *et al.* 2000, 2002; Phares *et al.* 2008; Verani and Schrage, 2010; Verani *et al.* 2010].

Furthermore, an important drawback to wider implementation of the screening-based method for early identification of GBS carriers is its complexity in terms of speed and/or accuracy of prenatal screening culture; even with optimized and sensitive methods it will not possibly identify all GBS carriers at time of delivery due to intermittent colonization. Missed opportunities for prevention were also identified among women delivering preterm and among those showing

adverse antibiotic events, such as anaphylaxis. Moreover, the widespread use of β -lactam antimicrobials for the intrapartum prophylaxis raises concerns about the selection of antibiotic resistance among GBS isolates or other newborn pathogens. In recent years a small number of clinical isolates have been characterized as having decreased penicillin susceptibility [Kimura *et al.* 2008]. However, resistance to β -lactams remains rare and does not appear to be increasing, whereas resistance to alternative agents such as erythromycin and clindamycin is an increasing concern [Castor *et al.* 2008]. GBS resistance to clindamycin and erythromycin has already affected IAP options for penicillin allergic women. Very recently a comparative genome and phylogenetic analysis of a large collection of clinical isolates showed that the emergence of GBS diseases in neonates is associated with the selection and worldwide dissemination of only a limited number of tetracycline resistant (TcR) clones selected through the extensive use of tetracycline from 1948 [Da Cunha *et al.* 2014].

Vaccination

Vaccination surely represents the most attractive strategy for GBS disease prevention. Effective vaccines would stimulate the production of functionally active antibodies that could cross the placenta and provide protection against neonatal GBS infection. The most promising vaccine candidates able to confer protection and induce functionally active antibodies are represented by CPS and surface proteins, although to date clinical trials in humans have been conducted only by immunizing subjects with polysaccharide-based vaccines.

Polysaccharide-based vaccines

Native polysaccharide vaccines

The first evidence of the protective nature of CPS-specific antibodies was in the 1930s when Rebecca Lancefield demonstrated that, using CPS-specific polyclonal rabbit serum, mice could be protected against GBS infections [Lancefield and Hare, 1935; Lancefield, 1938].

In 1976 Baker and Kasper provided the proof of concept that maternal vaccination could be a suitable effective strategy to prevent GBS infection in newborns, supporting the rationale for the development of a vaccine against GBS using CPS

as antigen [Baker and Kasper, 1976]. This study demonstrated that low levels of maternal antibodies against type III CPS were strongly correlated with neonatal susceptibility to GBS EOD or LOD. Maternal CPS-specific antibodies, transferred from the mother to the newborn by transplacental transmission of immunoglobulin (IgG), were able to confer protection to babies against GBS infections [Baker and Kasper, 1976]. The association between maternal antibody levels against serotype Ia and III and infant susceptibility was later confirmed by subsequent multicentre seroepidemiology studies [Lin *et al.* 2001, 2004; Baker *et al.* 2014].

Unmodified type-specific polysaccharides were used in the first generation of GBS vaccines. The first human clinical trials were conducted in the 1980s with purified native type Ia, II or III polysaccharides injected in healthy adult volunteers, including pregnant women. Although phase I trials demonstrated that these vaccines were safe and well tolerated, it was evident that the immunogenicity needed to be improved. Type II CPS was found to be the most immunogenic, while types Ia and III showed an immune response in about half of the recipients [Baker and Kasper, 1985; Baker *et al.* 1988]. Moreover, unconjugated polysaccharide vaccines induced T cell independent B-cell activation without B-cell memory response, booster vaccine doses did not result in an enhanced immune response to the vaccine antigen, and the variability among the responders was quite high, especially in GBS-naïve individuals [Baker and Kasper, 1985; Avci and Kasper, 2010].

Polysaccharide-protein conjugate vaccines

The immunogenicity of polysaccharides as human vaccines is enhanced by conjugation to protein carriers. Following the development of the *Haemophilus influenzae* type b (Hib) conjugate vaccine in the early 1990s [Eskola *et al.* 1990], glycoconjugate vaccines to *Neisseria meningitidis* and *Streptococcus pneumoniae* have been introduced and incorporated in the childhood immunization schedule [Finn and Heath, 2005]. To improve the immunogenicity of CPS-based vaccines, the second generation of GBS vaccines was based on the generation of glycoconjugates. The conjugation of a highly immunogenic protein to CPS antigens potentially induces a strong and long-lasting immune response against the polysaccharide. The carbohydrate epitopes are presented to T cells, which stimulate B cells to undergo class switching and proliferation [Avci and Kasper, 2010].

Indeed, conjugate vaccines prepared with GBS type-specific CPS (Ia, Ib, II, III, IV, V, VI, VII and VIII) coupled to tetanus toxoid (TT) showed higher immunogenicity than uncoupled CPS when tested preclinically in animal models [Wessels *et al.* 1990; Paoletti *et al.* 1992, 1994, 1999; Paoletti and Kasper, 2002; Paoletti and Madoff, 2002]. The success of the preclinical tests in animals constituted the rationale to proceed with the clinical studies of CPS-TT conjugated in humans.

The first GBS conjugate vaccine for phase I clinical trials was obtained from purified type III CPS coupled by reductive amination to monomeric TT [Kasper *et al.* 1996]. The trial was conducted in healthy nonpregnant women and compared three dosages of III-TT, unconjugated type III CPS, and saline placebo. The vaccine was safe, the immunogenicity of III-TT was dose-dependent and women who received the unconjugated type III CPS had significantly inferior immune responses. Moreover, the proportion of recipients who responded to vaccination with III-TT was substantially greater than that responding to type III CPS alone [Kasper *et al.* 1996].

Despite the positive results collected during this trial, some aspects of the effect in pregnant women and their babies still needed to be investigated. To this purpose, a subsequent trial comparing III-TT with unconjugated type III CPS in pregnant women showed that, after glycoconjugate vaccination, titres of protective IgG to type III CPS were elevated in cord blood, persisted for at least 2 months in the neonates, and correlated with levels of type III CPS-specific antibody in maternal serum [Baker *et al.* 2003b; Baker and Edwards, 2003]. Monovalent conjugate vaccines representing the most frequent disease-causing serotypes (Ia, Ib, II, III and V) have been prepared coupled to TT and tested in phase I and II clinical trials in healthy women. For each vaccine, an improved immunogenicity was demonstrated over the isotypic unconjugated polysaccharide, which was dose-dependent and more consistent with a memory response. Moreover, glycoconjugate vaccines were able to induce functionally active serotype-specific IgG [except for type V CPS-TT conjugate, which primarily elicited immunoglobulin M (IgM) antibodies] which, in the presence of complement, were able to opsonize and induce killing of GBS by human peripheral blood leukocytes in *in vitro* assays [Baker *et al.* 1999, 2000, 2004, 2007]. Altogether these studies have demonstrated that

CPS–TT vaccines were technically feasible and could be administered safely to women early in the third trimester of pregnancy, providing important health benefits to both mother and infant.

Multivalent conjugate vaccines

Although the immunogenicity in humans of GBS type-specific CPS antigens was successfully increased through conjugation to TT, the immune responses were serotype-specific. Thus, to obtain a broadly effective vaccine against the most common disease-causing strains circulating worldwide, conjugate formulations need to be multivalent [Phares *et al.* 2008; Barcaite *et al.* 2008; Le Doare and Heath, 2013]. A combination of four TT-conjugated serotypes (Ia, Ib, II and III) was successfully tested in a mouse infection model [Paoletti *et al.* 1994], and subsequently the safety and immunogenicity of this tetravalent vaccine formulation was evaluated in healthy adults [Kotloff *et al.* 1996]. Vaccination was generally well tolerated and higher IgG titres were observed in those subjects with pre-existing anti-capsular antibody to GBS. In the following years a bivalent vaccine containing II–TT and III–TT glycoconjugates was tested in humans in terms of safety and immunogenicity [Baker *et al.* 2003a]. The immune response in the subjects who had received the bivalent vaccine did not differ statistically from the antibody responses to monovalent vaccine; moreover, there was no significant interference between the two single vaccines.

The majority of the clinical trials have involved only one dosage of conjugate vaccine. In one clinical trial, however, 36 healthy adults vaccinated previously with GBS type III–TT conjugate were given a second dose 21 months later. This second dose restored specific antibody levels to those obtained after the primary vaccination. However, 22% of the participants who had undetectable III CPS-specific IgG before the first dose of III–TT vaccine exhibited a booster response to the second dose, with a fourfold increase of type III CPS-specific IgG than after the initial immunization. [Paoletti *et al.* 2001].

Although the data are not yet available, the safety and immunogenicity of a trivalent GBS vaccine based on type Ia, Ib and III CPS, conjugated to CRM₁₉₇ (see next section) has been evaluated in a phase I study in healthy nonpregnant women sponsored by Novartis Vaccines [ClinicalTrials.gov identifier: NCT01193920; NCT01150123].

Carrier proteins and adjuvants

Most of the clinical studies performed so far administrated GBS conjugate vaccines employing TT and without adjuvants. An important concern raised from the use of TT in humans is based on the pre-existing immunity to the carrier supposedly causing significant adverse effects. Although no serious adverse effects have been reported in the clinical trials conducted so far, tetanus immunization in the 12 months before vaccination was an exclusion criterion for subjects recruited in the trials. Another protein carrier tested in human GBS vaccine trial was CRM₁₉₇, which is a natural nontoxic mutant of diphtheria toxin (DT) isolated from *Corynebacterium diphtheriae* C7 (β197) cultures. It is a nontoxic variant of the DT that differs from wildtype toxin in a point mutation, which eliminates enzymatic activity and toxicity. CRM₁₉₇ is antigenically indistinguishable from DT, but has advantages as a conjugate protein: it is nontoxic, and has more lysyl side chains available for conjugation, showing the greatest versatility for conjugates to multiple polysaccharides in the same product. Indeed, CRM₁₉₇ is the elective carrier used in some licensed conjugate vaccines against *Neisseria meningitidis* and *Streptococcus pneumoniae* [Pichichero, 2013]. CRM₁₉₇ was used to prepare a glycoconjugate GBS vaccine consisting of type V–CPS. CRM–V vaccine was administered to healthy women to compare immune response with that obtained by vaccination with TT–V conjugates. In the two groups receiving or CRM–V or TT–V, no statistically significant difference was observed [Baker *et al.* 2004].

An interesting attractiveness is surely to use native GBS proteins as carriers for CPS–conjugate vaccine formulations. Two surface proteins (C5a-peptidase and alpha C protein) selected for their ability to confer protection in animal models against GBS infections have been successfully used as carriers for type-III CPS [Gravekamp *et al.* 1999; Cheng *et al.* 2002]. These studies conducted in animals represent the proof of the concept for a dual potential use of native GBS proteins both as carrier for the generation of CPS conjugates and as vaccine antigens able to themselves induce protective immune responses.

To date only one study have been performed by administering GBS type III CPS–TT conjugate vaccine adsorbed with aluminium hydroxide in healthy adult recipients. The vaccine did not show enhanced immunogenicity to either the CPS or the tetanus components [Paoletti *et al.* 2001].

This result was unexpected based on previous experiments demonstrating increasing immune responses in mice and baboons vaccinated with GBS type III–TT conjugate with alum [Paoletti *et al.* 1996; Guttormsen *et al.* 1998].

Protein-based vaccines

Although multivalent capsular conjugate vaccines are in advanced stage of development, to overcome serotype-specific immunity, the lack of coverage against NT isolates and the potential problems of serotype replacement/switching, efforts have been also directed to the identification of highly protective antigens for the development of a broad coverage GBS protein-based vaccine. Promising vaccine proteins should be surface-exposed (to be more accessible to antibodies), conserved and expressed in a wide panel of strains, strongly immunogenic, and able to confer protection from disease. Different to the CPS antigens, the proteins are able to induce protective T-cell dependent antibody responses and long-lasting immunity.

During the past decades, the application of recombinant DNA techniques and the availability of complete bacterial genomes have favoured a shift from conventional culture-based approaches to genome-based vaccinology with the identification of new protein vaccine candidates [Rinaudo *et al.* 2009; Rappuoli *et al.* 2011]. Conventional vaccine development relied on the cultivation of microorganisms under laboratory conditions by which potential antigens were isolated through biochemical and microbiological methods. Several limitations are associated with this traditional approach. It has often been proven to be inadequate in the development of vaccines against microbes that do not grow *in vitro*, those that lack suitable animal models of infection, and for those pathogens with antigenic hypervariability. Until the publication of the first complete genome sequences of two GBS clinical isolates in 2002 [Glaser *et al.* 2002; Tettelin *et al.* 2002], only a limited number of proteins involved in GBS pathogenesis were identified also as potential vaccine candidates for their ability to elicit protective immunity in animal models and induce complement dependent opsonophagocytosis killing of bacteria *in vitro* [Lindahl *et al.* 2005]. Among these proteins there are the tandem repeat-containing α and β components of the C protein complex [Michel *et al.* 1991], Rib protein [Stalhammar-Carlemalm *et al.* 1993], the

surface-immunogenic protein (Sip) [Brodeur *et al.* 2000], and the serine-protease C5a peptidase that interferes with host defences by inactivating human complement component C5a [Cheng *et al.* 2001].

The genome sequence of a microorganism provides unprecedented access to the complete repertoire of its antigens, from which vaccine candidates can be selected through rapid high-throughput screening processes. The application of genome and bioinformatics analysis and molecular biology tools to vaccine development, a concept termed reverse vaccinology, was first applied for the development of *Neisseria meningitidis* serogroup B (MenB) vaccine [Pizza *et al.* 2000]. This is the first vaccine based on reverse vaccinology that has recently received a positive opinion from the European Medicines Agency (EMA) and has been approved with the commercial name of Bexsero. The success of reverse vaccinology in the design of MenB vaccine served as a milestone for this approach, which has been further applied to other pathogens [Serruto and Rappuoli, 2006], including *Streptococcus agalactiae* [Maione *et al.* 2005], with significant improvements over the original approach [Donati and Rappuoli, 2013].

In 2005, the identification of novel protein vaccine candidates suitable for an effective vaccine against GBS infections represented the first important example of how reverse vaccinology has been refined, shifting from a classical approach based on one genome sequence to a pan-genome concept describing better a bacterial species [Maione *et al.* 2005; Medini *et al.* 2005; Tettelin *et al.* 2005]. In effect, the first GBS genome sequences, generated in 2002 and used as references in comparative genome hybridization (CGH) studies [Glaser *et al.* 2002; Tettelin *et al.* 2002], had revealed substantial genetic heterogeneity, even among strains with the same serotype, and in particular between genes that were expected to play a role in disease, such as transcriptional regulators and surface proteins [Tettelin *et al.* 2002]. This analysis suggested that a single genome would not be representative in describing the global genetic variability within a single species and that to develop a broad coverage vaccine formulation a deep understanding of the population structure of the pathogen was needed.

A few year later, comparative analyses of eight GBS complete genomes belonging to different serotypes confirmed the high degree of intraspecies diversity in GBS, introducing the concept of ‘pan-genome’

to define the global gene repertoire of a species [Tettelin *et al.* 2005]. In this comparative genomic study, Tettelin and colleagues suggested that each strain contained a 'core genome' that was present in all strains (an average of 1806 genes typically involved in housekeeping functions), a 'dispensable genome' comprising a set of genes present in 2 or more strains (an average of 439 genes typically with hypothetical or unknown functions) and strain-specific genes found only in a single isolate [Tettelin *et al.* 2005]. Moreover, mathematical extrapolations indicated that the GBS pan-genome was open and theoretically unlimited, meaning that for every new GBS genome sequenced, new strain-specific genes could be added to the complete genetic repertoire of the GBS species.

From a vaccine discovery perspective, the distinction between core genes and dispensable genes determined significant implications. Search for promising vaccine candidates only in the core genome (in order to identify antigens able to protect all the strains) contains the inherent risk of losing interesting proteins that are not essential for bacterial growth, but can be important virulence factors involved into the pathogenesis of bacterium. To avoid this risk, Maione and co-workers, by applying the pan-genomic reverse vaccinology approach through multiple genomes screening (Figure 1a), have identified 4 potential vaccine antigens among 312 putative surface/secreted proteins that were produced as recombinant proteins in *Escherichia coli* and tested for their capacity to elicit protection in infant mice in an active maternal/neonatal challenge model. Interestingly, only one of these antigens, the Sip protein, belonged to the core genome; indeed, it had been previously identified as a potential vaccine candidate [Brodeur *et al.* 2000]. The other three proteins were never described and were part of the dispensable portion of GBS pan-genome, not present in all analysed strains. Nevertheless, the authors demonstrated that the combination of these proteins, each effective against overlapping populations of isolates, in a multivalent vaccine formulation can confer broad serotype-independent protection [Maione *et al.* 2005].

Further work performed on dissecting the role of the novel identified proteins revealed that the three dispensable genome antigens were structural components of the pilus-like structures, unknown previously [Lauer *et al.* 2005]. Pili are high molecular weight (HMW) polymers, visible by electron microscopy as long filamentous structures

extending out from the bacterial surface (Figure 1b). Pili have now been identified in many Gram-positive pathogens, even if they had been missed by conventional molecular microbiology for a century [Telford *et al.* 2006]. In GBS they are constituted by three structural components, corresponding to the major pilus subunit (known as the backbone protein, BP) forming the pilus shaft and the two ancillary proteins (APs), covalently assembled and linked to the cell wall by a series of sortase-mediated transpeptidase reactions. The genes coding for the pilus subunits and the specific sortases involved in their assembly are clustered in genomic pilus islands (PIs), inserted in specific loci of the genome (Figure 1b) [Telford *et al.* 2006].

Following the identification in GBS of three pilus variants whose genes are present in three different PIs (named PI-1, PI-2a and PI-2b) (Figure 1b) [Rosini *et al.* 2006] and the evidence that each pilus contains two structural subunits eliciting protective immunity in mice (Figure 1b) [Maione *et al.* 2005; Rosini *et al.* 2006; Margarit *et al.* 2009], Margarit and colleagues demonstrated, in an extensive study of pili distribution and conservation in a large collection of clinical isolates, that all GBS isolates carried at least one or a combination of two PIs and that a pilus-based vaccine exclusively constituted by three pilus components, one from each pilus type, can be effective in preventing GBS infections and capable of providing a broad protection [Margarit *et al.* 2009].

This evidence provides a rationale for the development of a universal pilus-based vaccine that is potentially capable of preventing disease by all GBS serotypes (Figure 1c). However, not all the protective pilin subunits could be included in the vaccine due to their antigenic variation. For instance, although the backbone subunit of type 2a pilus (BP-2a) was capable of eliciting high opsonophagocytic titres, it was excluded from that vaccine formulation due to its high gene variability and variant-specific protection *in vivo* [Margarit *et al.* 2009].

Recent advances in vaccine design have demonstrated that it is possible to overcome antigenic variation and to provide a broadened coverage. Structural vaccinology technology was successfully applied for the rational design of an optimized BP-2a protein. By solving the crystallographic structure of BP-2a, discerning four IgG-like domains (D1, D2, D3, D4), individual domains

Figure 1. Genome-based and structural approaches applied to Group B Streptococcus (GBS) for antigen discovery and development. (a) Pan-genomic reverse vaccinology through multiple genome analysis has identified 4 potential new vaccine antigens among 312 putative surface/secreted proteins screened in an active maternal immunization/neonatal pup challenge model. Bioinformatic tools were used to analyse and compare the genome sequences of eight GBS strains in order to identify all potential vaccine candidates according to their topological features; selected open reading frames (ORFs) were amplified, cloned in expression vectors, purified and used to immunize mice; mice immune sera were analysed by fluorescence-activated cell sorting (FACS) to verify whether the antigens were expressed and surface-exposed; and finally, a mouse model was used to assess protective immunity of the antigens [Maione *et al.* 2005]. (b) Schematic representation of the three genomic pilus islands (PIs) identified in GBS. Each pilus contains two protective subunits (indicated by red circles) identified as novel vaccine candidates by genome-based approaches. Pili are high molecular weight polymers, visible by electron microscopy as long filamentous structures extending out from the bacterial surface [Rosini *et al.* 2006; Margarit *et al.* 2009]. (c) Antigen variability studies in large collection of clinical isolates representative of circulating strains causing diseases worldwide (selected through molecular epidemiology studies) allowed the design of a trivalent pilus-based vaccine potentially capable to confer broad serotype-independent protection [Margarit *et al.* 2009]. (d) Structural vaccinology, based on the atomic resolution of the structures of potential antigens, allowed the rational engineering of target epitopes to use as vaccine candidates to achieve a more comprehensive coverage against the major circulating species [Nuccitelli *et al.* 2011].

were tested as subunit vaccines; only the D3 domain elicited protection similar to the full-length BP-2a (Figure 1d). Since the other BP-2a variants showed a similar structural organization, the protective D3 domains of six variant BP-2a molecules were cobbled together to generate in *E. coli* stable hybrid product (named 6xD3) (Figure 1d) which provided protection against all six prototypic pilus variant GBS strains examined [Nuccitelli *et al.* 2011].

Vaccine target population: pregnant women, elderly and immunocompromised patients

Vaccination in pregnancy

The concept of vaccination during pregnancy is not new. Effective antibodies raised in pregnant women would be able to protect the mothers, cross the placenta and confer protection to the infants during the delivery and in the following months. Vaccination in pregnancy was

introduced in the late 19th century, when maternal immunization with vaccinia virus has been described to confer protection in young infants. Later, maternal immunization with whole cell pertussis vaccines was shown to be responsible for protecting young infants before their own active vaccination [Mooi and de Greeff, 2007; Jones and Heath, 2014]. In the beginning of the 1960s, TT vaccine administered to pregnant women prevented millions of maternal and neonatal deaths from tetanus infections [Baker, 2013]. The increased mortality and morbidity caused by influenza infection in pregnant women led to a recommendation for the vaccine during pregnancy. Currently, influenza and Tdap (TT, reduced diphtheria toxoid and acellular pertussis) vaccines in pregnant women to passively protect their babies are recommended by the US Advisory Committee on Immunization Practices [Rasmussen *et al.* 2013]. The main concerns regarding vaccination in pregnancy are often focused on the potential risks of vaccines to the foetus, but many data have been accumulated on the absence of increased risks for adverse events associated with vaccination during pregnancy and on their benefits to mothers and infants. Out of 2.4 million pregnant women who received influenza vaccine in the US alone during the winter season of 2009–2010, no pattern of adverse outcomes for mothers or children has been reported. A similar safety profile has been observed for Tdap maternal immunization, with no adverse event reported between 2005 and 2010 [Jones and Heath, 2014].

Only one clinical study has been published so far in which GBS type III–TT conjugate vaccine was administered to pregnant women [Baker *et al.* 2003b]. The immunization was well tolerated, transplacental transfer of elevated antibody titres was achieved and neutralizing IgG to type III CPS persisted up to 2 months of age in newborns, meaning it could prevent maternal, neonatal and young infant GBS disease [Baker *et al.* 2003b]. A phase II study sponsored by Novartis Vaccines is currently ongoing to evaluate safety, tolerability and immunogenicity of a trivalent CRM₁₉₇-conjugate vaccine in mothers and infants by immunizing healthy pregnant women (18–40 years of age, 24–35 weeks weeks' gestation). The study will also evaluate the levels of GBS serotype-specific (Ia, Ib and III) antibodies in infants, placental transfer from the pregnant women to the infant [ClinicalTrials.gov identifier: NCT01446289].

The increasing acceptance of vaccination (i.e. tetanus vaccine, pertussis, influenza) during pregnancy is providing a new opportunity to introduce a glycoconjugate GBS vaccine for use in pregnancy as the most suitable strategy to prevent neonatal GBS infection. Optimally, the administration of the vaccine should be early in the third trimester of pregnancy to eliminate the risk of not including the approximately 30% of GBS cases that are born prematurely. Another approach would be to incorporate the GBS vaccine within a routine female adolescent vaccine schedule, combined with other important vaccines such as papilloma virus. However, additional studies are required to understand the persistence of immunity, as pregnancy could occur 20 years after the vaccination, and a booster vaccine may be needed to restore protective antibody levels.

Vaccination in adults

Since GBS has been recently addressed as an important cause of invasive infections in the elderly and in those patients with underlying disorders, these subjects could represent two natural target populations for a GBS vaccine. However, to date only one clinical study targeting the elderly has been published [Palazzi *et al.* 2004]. Type V CPS–TT (V–TT) conjugate vaccine was administered to healthy adults (65–85 years old) to assess the safety and immunogenicity of the formulation. The glycoconjugate V–TT vaccine elicited specific antibodies against type V CPS, which were able to mediate opsonophagocytosis killing of type V GBS strain *in vitro*. Immune responses in elderly subjects did not show statistically significant differences to immune responses in adults 18–50 years old [Palazzi *et al.* 2004]. These data confirmed the possibility of eliciting an effective immune response *via* vaccination in the elderly. Additional studies would be required to understand if the same effect could be obtained in those elderly with underlying medical conditions.

Future perspectives for GBS vaccine development

During the past 20 years, many advances in the prevention and treatment of GBS disease have been achieved. Unfortunately, despite all the efforts, GBS remains one of the major health problems for infants. The glycoconjugate generation vaccine has been demonstrated to be able to prevent GBS disease. At present, the licencing of GBS vaccines is difficult because of the challenge

in conducting efficacy clinical trials in humans due to the low incidence of neonatal diseases. A possible solution came from earlier studies [Lin *et al.* 2001, 2004] and a recent study [Baker *et al.* 2014] establishing that maternal CPS-specific antibody levels at the time of delivery above a quantified threshold can be predicted to confer high level of protection against EOD. Baker and colleagues performed a prospective, multicentre, case-control study of 33 mothers delivering neonates with early onset GBS infection, and 99 age- and ethnicity-matched mothers colonized with the same CPS types delivering healthy neonates (controls). They measured the concentrations of type Ia, III or V CPS-specific antibody in maternal serum to calculate the relative and absolute risk associated with EOD. Using statistical analysis they found that maternal CPS-specific antibody serum concentrations of $\geq 1 \mu\text{g/ml}$ at the time of delivery appear to protect most neonates from early onset GBS type Ia and III disease. Interestingly, the concentrations of maternal CPS-specific IgG associated with neonatal protection correlated with opsonophagocytic activity against types Ia, III and V in human vaccine trials [Baker *et al.* 2014].

These data support a new strategy for the registration of a vaccine against GBS based on the quantification of specific antibodies induced by the vaccines, as a correlate of protection, instead of more complex efficacy clinical trials. Regulatory authorities may also take into consideration opsonophagocytosis killing of GBS induced by sera of immunized subjects as a serological correlate of efficacy.

Conflict of interest statement

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