



**Panacea Biotec**

*Innovation in support of life*

**PRE-GRANT OPPOSITION**

Our Ref.: 6666/CHENP/2012

May 02, 2014

**BY HAND**

The Controller of Patents,  
The Patent Office,  
Intellectual Property Office Building,  
G.S.T. Road, Guindy, Chennai-600032

*EDP/SC*

*PGO*

*21/5/14*

*E-105/11/2014*

**Re. : PANACEA BIOTEC LIMITED**  
**Pre-grant Opposition against grant of Patent Application No**  
**6666/CHENP/2012 dated 27.07.2012, entitled 15-VALENT PNEUMOCOCCAL**  
**POLYSACCHARIDE PROTEIN CONJUGATE VACCINE COMPOSITION"**

Sir,

We respectfully submit herewith a pre-grant representation by way of opposition under Section 25(1) of the Patents Act, 1970 and rule 55(1) of the Patent Rules, 2005, against the grant of patent application, No. 6666/CHENP/2012, dated 27.07.2012 (the "Application")

The statement and evidence in support of the representation are submitted herewith.

We request the learned Controller to take the above on record and grant a hearing under Rule 55(1) of the Patent Rules, 2005.

Yours faithfully,

For **Panacea Biotec Ltd.**,

**Arindam Purkayastha**

**IN/PA-1344**

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**Enclosures:**

1. Statement of representation (in duplicate)
2. Extract of Patent Journal No 02/2014 dtd. 10/C1/2014 (page no 1134)
3. Prior art disclosures – D1, D2, D3, D4, D5, D6, D7 and D8 (all in duplicate)

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CIN: L33117PB1984PLC022350

**BEFORE THE CONTROLLER OF PATENTS**

**CHENNAI**

In the matter of Patents Act, 1970 *as amended by* Patents (Amendment) Act 2005,

And

In the matter of Patents Rules, 2006

And

IN THE MATTER of Patents Application  
No. 6666/CHENP/2012 filed on 27/07/2012  
(International Application filed on 03/02/2011)  
made by **MERCK SHARP & DOHME CORP**

126, East Lincoln Avenue, Rahway  
New Jersey 07065-0907, USA

.....**Applicant**

IN THE MATTER of representation by way of  
opposition of the grant of a patent thereto by

1. Panacea Biotec Ltd. GRAND  
Centre, 72/3 GEN Block, TTC Industrial  
Area, Malhape, Navi Mumbai – 400710

2. Panacea Biotec Ltd. B-1 Ext. /A-27,  
Mohan Co-op. Indl. Estate, Mathura  
Road, New Delhi, 110 044, INDIA

.....**Opponent**

## **REPRESENTATION UNDER SECTION 25(1)**

We, Panacea Biotec Ltd, (hereinafter called 'opponent') make the following representation under Section 25(1) of the Patents Act, 1970 (hereinafter called 'Act') in opposing the grant of patent on the application indicated in the cause title.

### **IMPUGNED APPLICATION**

The impugned application no. 6666/CHENP/2012 entitled "15-VALENT PNEUMOCOCCAL POLYSACCHARIDE PROTEIN CONJUGATE VACCINE COMPOSITION", entered national phase on 27 July 2012, and arises out of International application no. PCT/US2011/023526 filed on 03/02/2011 and published on January 10, 2014. For the purpose of priority date, the date considered is 09/02/2010.

The impugned application to the best of the information of the opponent is not granted and therefore the present opposition is within time and ought to be taken on record.

### **1. THE OPPONENT'S BUSINESS AND ACTIVITIES**

The opponent, Panacea Biotec Ltd, is a Company incorporated under laws of India and having its principal office at B-1 Ext. /A-27, Mohan Co-op Indl. Estate, Mathura Road, New Delhi, 110 044, India. The opponent is a leading manufacturer of medicines and vaccines in this country and the opponent's products are sold under different brands and enjoy considerable goodwill and reputation. The opponent is very well known and has been operating in this country for several decades. The opponent is also engaged in the research and development of vaccines, biopharmaceuticals, medicines and pharmaceutical products and preparations.

### **2. LOCUS STANDI**

Locus standi is not a condition precedent for an opposition under Section 25(1). In any event, it is stated that the application under opposition relates to an alleged invention in the field of medicinal products. The opponent being engaged in the research and development as well as in the manufacture of drugs / medicinal compositions for many years and is thus a person interested.

### **3. GROUNDS OF OPPOSITION**

The application is opposed on the following grounds of Section 25 (1) (Pre-grant opposition to the patents) which reads as under:

25(1): Where an application for a Patent has been published but a patent has not been granted, any person may in writing represent by way of opposition to the Controller against the grant of patents on the ground-

b. that the invention so far as claimed in any claim of the complete specification has been published before the priority date of the claim

(i) In any specification filed in pursuance of an application for a Patent made in India on or after the 1st day of January, 1912; or(ii) In India or elsewhere, in any other document.

Provided that the ground specified in sub-clause (ii) shall not be available where such publication does not constitute an anticipation of the invention by virtue of sub-section (2) or sub-section (3) of section 29.

c. that the invention so far as claimed in any claim of the complete specification is claimed in a claim of a complete specification published on or after the priority date of the applicant's claim and filed in pursuance of an application for a Patent in India, being a claim of which the priority date is earlier than that of the applicant's claim;

d. that the invention so far as claimed in any claim of the complete specification was publicly known or publicly used in India before the priority date of that claim;

e. that the invention so far as claimed in any claim of the complete specification is obvious and clearly does not involve any inventive step, having regard to the matter published as mentioned in clause (a) or having regard to what was used in India before the priority date of the applicant's claim;

f. that the subject of any claim of the complete specification is not an invention within the meaning of this Act, or is not Patentable under this Act;

g. that the complete specification does not sufficiently and clearly describe the invention or the method by which it is to be performed;

h. the applicant has failed to disclose to the Controller the information required by Section 8 or has furnished the information which in any material particular was false to his knowledge.

#### **4. CLAIMS**

All the claims of the instant application have been reproduced below.

1. An immunogenic composition comprising:
  - (1) a multivalent polysaccharide-protein conjugate mixture consisting of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *Streptococcus pneumoniae* conjugated to a carrier protein; and
  - (2) a pharmaceutically acceptable carrier.
2. The immunogenic composition of claim 1, wherein the carrier protein is CRM<sub>197</sub>.
3. The immunogenic composition of claim 1, further comprising an adjuvant.
4. The immunogenic composition claim 3, wherein the adjuvant is an aluminum-based adjuvant.
5. The immunogenic composition of claim 4, wherein the adjuvant is selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide.
6. The immunogenic composition of claim 5, wherein the adjuvant is aluminum phosphate.
7. A method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide, comprising administering to a human an immunologically effective amount of the immunogenic composition of claim 1.
8. The method of claim 7, wherein the immunogenic composition administered is a single 0.5 mL dose formulated to contain: 2 µg of each saccharide, except for 6B at 4 µg; about 32 µg CRM<sub>197</sub> carrier protein; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; 150 mM sodium chloride and 20 mM L-histidine buffer.

In an amendment dated 22/08/2012 the following amendments were made:

**1. An immunogenic composition comprising:**

(1) a multivalent polysaccharide-protein conjugate mixture consisting of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *Streptococcus pneumoniae* conjugated to a carrier protein; and

(2) a pharmaceutically acceptable carrier.

2. The immunogenic composition of claim 1, wherein the carrier protein is CRM<sub>197</sub>.

3. The immunogenic composition of claim 1, further comprising an adjuvant.

4. The immunogenic composition claim 3, wherein the adjuvant is an aluminum-based adjuvant.

5. The immunogenic composition of claim 4, wherein the adjuvant is selected from the group consisting of aluminum phosphate, aluminum sulfate and aluminum hydroxide.

6. The immunogenic composition of claim 5, wherein the adjuvant is aluminum phosphate.

7. The immunogenic composition of claim 1 formulated as single 0.5 mL dose containing 2 µg of each saccharide, except for 6B at 4 µg; about 32 µg CRM<sub>197</sub> carrier protein; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; 150 mM sodium chloride and 20 mM L-histidine buffer.

7. 8. A method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide, comprising administering to a human an immunologically effective amount of the immunogenic composition of claim 1.

8. 9. The method of claim 7 8, wherein the immunogenic composition administered is a single 0.5 mL dose formulated to contain: 2 µg of each saccharide, except for 6B at 4 µg; about 32 µg CRM<sub>197</sub> carrier protein; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; 150 mM sodium chloride and 20 mM L-histidine buffer.

## 5. DOCUMENTS RELIED UPON BY THE OPPONENT

D1-WO2007000342( Glaxo)

D2- US2009010959/WO2007/071707(Biemens et al)

D3- Gonzalez, BE et al, Journal of pediatric infectious disease, Vol 25, Pages 301-305, *Streptococcus pneumoniae* serogroups 15 and 33.

D4- WO2006110381, Wyeth

D5- WO2000056359, SKB

D6- CN101590224, Guangzhou Jingda Medical Science

D7-WO2009000826 (GSK)

D8- Pilishvli Tamara et al “ Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine” The Journal of infectious diseases 1 Jan 2010, Vol 201, no.1, 1<sup>st</sup> January 2010, pages 32-41

D1 discloses an immunogenic *S. pneumoniae* capsular polysaccharide –protein conjugate composition that comprises the instantly claimed 15 serotypes ( see page 27, lines 30-36, page 28, lines 10-15, also see claims 84 and 80 to 83). D1 discloses a kit which could contain *S. pneumoniae* capsular polysaccharide – protein conjugates. “Typically the *Streptococcus pneumoniae* vaccine in the vaccine kit of the present invention (or in any of the immunogenic compositions of the invention described above) will comprise saccharide antigens (optionally conjugated), wherein the saccharides are derived from at least four serotypes of pneumococcus chosen from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. Optionally, the four serotypes include 6B, 14, 19F and 23F. Optionally, at least 7 serotypes are included in the composition, for example those derived from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Optionally more than 7 serotypes are included in the composition, for instance at least 10, 11, 12, 13 or 14 serotypes. For example the composition in one embodiment includes 10 or 11 capsular saccharides derived from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F, and

optionally 3 (all optionally conjugated). In an embodiment of the invention at least 13 saccharide antigens (optionally conjugated) are included, although further saccharide antigens, for example 23 valent (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention”

D2 provides that “Typically the *Streptococcus pneumoniae* vaccine of the present invention will comprise capsular saccharide antigens (Preferably conjugated), wherein the saccharides are derived from at least ten serotypes of *S. pneumoniae*. The number of *S. pneumoniae* capsular saccharides can range from 10 different serotypes (or “V”, valences) to 23 different serotypes (23V). In one embodiment there are 10, 11, 12, 13, 14 or 15 different serotypes. In another embodiment of the invention, the vaccine may comprise conjugated *S. pneumoniae* saccharides and unconjugated *S. pneumoniae* saccharides. Preferably, the total number of saccharide serotypes is less than or equal to 23. For example, the invention may comprise 10 conjugated serotypes and 13 unconjugated saccharides. In a similar manner, the vaccine may comprise 11, 12, 13, 14 or 16 conjugated saccharides and 12, 11, 10, 9 or 7 respectively, unconjugated saccharides”..... “In one embodiment the multivalent pneumococcal vaccine of the invention will be selected from the following serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, although it is appreciated that one or two other serotypes could be substituted depending on the age of the recipient receiving the vaccine and the geographical location where the vaccine will be administered. For example, a 10-valent vaccine may comprise polysaccharides from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. An 11-valent vaccine may also include saccharides from serotype 3 A 12 or 13-valent paediatric (infant) vaccine may also include the 11 valent formulation supplemented with serotypes 6A and 19A, or 6A and 22F, or 19A and 22F, or 6A and 15B, or 19A and 15B, or 22F and 15B, whereas a 13-valent elderly vaccine may include the 10 or 11 valent formulation supplemented with serotypes 19A and 22F, 8 and 12F, or 8 and 15B, or 8 and 19A, or 8 and 22F, or 12F and 15B, or 12F and 19A, or 12F and 22F, or 15B and 19A, or 15B and 22F. A 14 valent paediatric vaccine may include the 10 valent formulation described above supplemented with serotypes 3, 6A, 19A and 22F, serotypes 6A, 8, 19A and 22F; serotypes 6A, 12F, 19A and 22F; serotypes 6A, 15B, 19A and 22F; serotypes 3, 8, 19A and 22F; serotypes 3, 12F, 19A and 22F; serotypes 3, 15B, 19A and 22F, serotypes 3, 6A, 8 and 22F; serotypes 3, 6A, 12F and 22F; or serotypes 3, 6A, 15B and 22F”. Beimens et al teaches preferred serotypes 1,3,4,5,6A,6B, 7F,9V,14, 18C, 19A,19F, 22F, 23F and 33F ( see page 5 lines 5-6 , page 35 lines 13-16) and also teach the importance of combining pneumococcal capsular serotypes that effect children into an



immunogenic composition, teach a pediatric /child specific immunogenic composition that is 14-valent capsular polysaccharide –protein conjugate composition that comprises 14 of the 15 identical serotypes( 1,3,4,5,6A, 6B, 7F,9V,14,18C,19A,19F,22F and 23F see page 5, lines 17-18). This reference also suggests a 15 valent immunogenic composition.

D3 specifically suggests that *S. pneumoniae* serotype 33F causes invasive disease in children (see Table 2, page 303, col.1; page 304, col.2 page 3) for the purpose of showing the need for inclusion of serotype 33F in pneumococcal immunogenic compositions due to increased incidence of invasive pneumococcal disease in vaccinated children that have not been vaccinated against serotype 33F.

D4 provides generally a multivalent immunogenic composition comprising 13 distinct polysaccharide-protein conjugates, wherein each of the conjugates contains a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae* conjugated to a carrier protein, together with a physiologically acceptable vehicle. Claim 1 is directed to “A multivalent immunogenic composition, comprising: 13 distinct polysaccharide-protein conjugates, together with a physiologically acceptable vehicle, wherein each of the conjugates comprises a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae* conjugated to a carrier protein, and the capsular polysaccharides are prepared from serotypes 1,3,4,5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F”. Claim 2 states that carrier protein is CRM197. Claims 3 and 4 recite aluminum-based adjuvant.

D5 provides “*Streptococcus pneumoniae* Polysaccharide Antigens of the Invention”

*Typically the Streptococcus pneumoniae vaccine of the present invention will comprise polysaccharide antigens (preferably conjugated), wherein the polysaccharides are derived from at least four serotypes of pneumococcus. Preferably the four serotypes include 6B, 14, 19F and 23F. More preferably, at least 7 serotypes are included in the composition, for example those derived from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. More preferably still, at least 11 serotypes are included in the composition, for example the composition in one embodiment includes capsular polysaccharides derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F (preferably conjugated). In a preferred embodiment of the invention at least 1" polysaccharide antigens (preferably conjugated) are included, although further polysaccharide*

*antigens, for example 23 valent (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention. For elderly vaccination (for instance for the prevention of pneumonia) it is advantageous to include serotypes 8 and 12F (and most preferably 15 and 22 as well) to the 11 valent antigenic composition described above to form a 15 valent vaccine, whereas for infants or toddlers (where otitis media is of more concern) serotypes 6A and 19A are advantageously included to form a 13 valent vaccine” ( see page numbers 11-12)*

*D7 teaches that “typically the Streptococcus pneumoniae vaccine of the present invention will comprise capsular saccharide antigens (preferably conjugated), wherein the saccharides are derived from at least ten serotypes of S. pneumoniae. The number of S. pneumoniae capsular saccharides can range from 10 different serotypes (or "v", valences) to 23 different serotypes (23v). In one embodiment there are 10, 11, 12, 13, 14 or 15 different serotypes. In another embodiment of the invention, the vaccine may comprise conjugated S. pneumoniae saccharides and unconjugated S. pneumoniae saccharides. Preferably, the total number of saccharide serotypes is less than or equal to 23. For example, the invention may comprise 10 conjugated serotypes and 13 unconjugated saccharides. In a similar manner, the vaccine may comprise 11, 12, 13, 14, 15 or 16 conjugated saccharides and 12, 11, 10, 9, 8 or 7, respectively, unconjugated saccharides”.*

According to D8, in the United States the two most relevant emerging serotypes causing invasive pneumococcal disease to children under 5 years of age are 22F and 33F ( See page 34, right hand column).

## **6. GROUNDS OF OPPOSITION**

### **6.1 Lack of novelty**

The following is a quotation of Section 25(1) (b) which forms the basis for all oppositions based on lack of novelty-

#### **Section 25(1) (b)**

1. Where an application for a patent has been published but a patent has not been granted, any person may, in writing, represent by way of opposition to the controller against the grant of patent on the ground:

b. that the invention so far as claimed in any claim of the complete specification has been published before the priority date of the claim

(i) In any specification filed in pursuance of an application for a Patent made in India on or after the 1st day of January, 1912; or

(ii) In India or elsewhere, in any other document.

Provided that the ground specified in sub-clause (ii) shall not be available where such publication does not constitute an anticipation of the invention by virtue of sub-section (2) or sub-section (3) of section 29.

**6.1.1) Lack of novelty of claims 1- 9 in view of D1 under section 25(1) (b) (ii) of the Indian Patents Act**

**The sole independent claim 1 read as**

1. An immunogenic composition comprising:  
 (1) a multivalent polysaccharide-protein conjugate mixture consisting of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *Streptococcus pneumoniae* conjugated to a carrier protein; and  
 (2) a pharmaceutically acceptable carrier

**Comparison of claim 1 of the instant invention vis- a- vis disclosure of D1**

<b><u>Essential elements of claim 1</u></b>	<b><u>D1 -WO2007000342</u></b>
1. multivalent polysaccharide-protein conjugate mixture consisting of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of <i>Streptococcus pneumoniae</i> conjugated to a carrier protein.	D1 on page 27, lines 30-36, page 28, lines 10-15, also see claims 84 and 80 to 83, discloses a multivalent pneumococcal vaccine, polysaccharides of which are selected from serotypes : 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.  <i>“Typically the Streptococcus pneumoniae vaccine in the vaccine kit of the present invention (or in any of the immunogenic compositions of the invention described above) will comprise saccharide antigens (optionally conjugated), wherein the</i>

<p>2. a pharmaceutically acceptable carrier</p>	<p><i>saccharides are derived from at least four serotypes of pneumococcus chosen from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. Optionally, the four serotypes include 6B, 14, 19F and 23F. Optionally, at least 7 serotypes are included in the composition, for example those derived from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Optionally more than 7 serotypes are included in the composition, for instance at least 10, 11, 12, 13 or 14 serotypes. For example the composition in one embodiment includes 10 or 11 capsular saccharides derived from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F, and optionally 3 (all optionally conjugated). In an embodiment of the invention at least 13 saccharide antigens (optionally conjugated) are included, although further saccharide antigens, for example 23 valent (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention”</i></p> <p>Further on page 27, last three lines D1 discloses the following :</p> <p><i>“The pneumococcal saccharides are independently conjugated to any known carrier protein, for example CRM197, tetanus toxoid, diphtheria toxoid, protein D or any other carrier proteins as mentioned above.”</i></p> <p>Page 24- lines 29-30 of D1 disclose :</p> <p><i>“A further aspect of the invention is a vaccine comprising the immunogenic composition of the invention and a pharmaceutically acceptable excipient”</i></p>
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The claim 1 is directed towards a multivalent pneumococcal immunogenic composition comprising polysaccharides from 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of

Optionally, the immunogenic composition or vaccine of the invention contains an amount of an adjuvant sufficient to enhance the immune response to the immunogen. Suitable adjuvants include, but are not limited to, aluminium salts (aluminium phosphate or aluminium hydroxide), squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, non-ionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) Nature 344:873-875.

D1 on page 22 lines 34-36 states

In general, the immunogenic composition of the invention may comprise a dose of each saccharide conjugate between 0.1 and 20µg, 2 and 10µg, 2 and 6µg or 4 and 7µg of saccharide.

Thus, the subject matter claimed in the claims 1-9 are not novel over the disclosure and the teachings of the prior art D1.

**6.1.2) Lack of novelty of claims 1 to 9 in view of D2 under section 25(1) (b) (ii) of the Indian Patents Act**

**The sole independent claim 1 read as**

1. An immunogenic composition comprising:
- (1) a multivalent polysaccharide-protein conjugate mixture consisting of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *Streptococcus pneumoniae* conjugated to a carrier protein; and
  - (2) a pharmaceutically acceptable carrier

**Comparison of claim 1 of the instant invention vis -a - vis prior art D2 disclosure**

<b><u>Essential elements of the instant claim 1</u></b>	<b><u>D2 WO2007/071707</u></b>
1. multivalent polysaccharide-protein	D2 on pages 4 and 5 discloses the following:

conjugate mixture consisting of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *Streptococcus pneumoniae* conjugated to a carrier protein.

2. a pharmaceutically acceptable carrier

*“Typically the Streptococcus pneumoniae vaccine of the present invention will comprise capsular saccharide antigens (optionally conjugated), wherein the saccharides are derived from at least ten serotypes of S. pneumoniae. The number of S. pneumoniae capsular saccharides can range from 10 different serotypes (or “V”, valences) to 23 different serotypes (23V). In one embodiment there are 10, 11, 12, 13, 14 or **15 different serotypes**. In another embodiment of the invention, the vaccine may comprise conjugated S. pneumoniae saccharides and unconjugated S. pneumoniae saccharides. Optionally, the total number of saccharide serotypes is less than or equal to 23. For example, the invention may comprise 10 conjugated serotypes and 13 unconjugated saccharides. In a similar manner, the vaccine may comprise 11, 12, 13, 14 or 16 conjugated saccharides and 12, 11, 10, 9 or 7 respectively, unconjugated saccharides”..... “In one embodiment the multivalent pneumococcal vaccine of the invention will be selected from the following serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, although it is appreciated that one or two other serotypes could be substituted depending on the age of the recipient receiving the vaccine and the geographical location where the vaccine will be administered. For example, a 10-valent vaccine may comprise polysaccharides from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. An 11-valent vaccine may also include saccharides from serotype 3 A 12 or 13-valent paediatric (infant) vaccine may also include the 11 valent formulation supplemented with serotypes 6A and 19A, or 6A and 22F, or 19A and 22F, or 6A and 15B, or 19A and 15B, or 22F and 15B, whereas a 13-valent elderly vaccine may include the 10 or 11 valent formulation supplemented with serotypes 19A and 22F, 8 and 12F, or 8 and 15B, or 8 and 19A, or 8 and 22F, or 12F and 15B, or 12F and 19A, or 12F and 22F, or 15B and 19A, or 15B and 22F. A 14 valent paediatric vaccine may*

	<p><i>include the 10 valent formulation described above supplemented with serotypes 3, 6A, 19A and 22F, serotypes 6A, 8, 19A and 22F; serotypes 6A, 12F, 19A and 22F; serotypes 6A, 15B, 19A and 22F; serotypes 3, 8, 19A and 22F; serotypes 3, 12F, 19A and 22F; serotypes 3, 15B, 19A and 22F, serotypes 3, 6A, 8 and 22F; serotypes 3, 6A, 12F and 22F; or serotypes 3, 6A, 15B and 22F”.</i></p>
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The claim 1 is directed towards a multivalent pneumococcal immunogenic composition comprising polysaccharides from 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *Streptococcus pneumoniae* conjugated to a carrier protein. The prior art D2 teaches the inclusion of these serotypes in a vaccine. Further it also teaches that each of the polysaccharide is conjugated to a carrier protein. The claim 1 of the alleged application further states the inclusion of a physiologically acceptable vehicle as an essential component of the vaccine. The prior art D2 also discloses the formulation of the vaccine, which is either in solution form (which would include a carrier) or in a lyophilized state, preferably in the presence of a sugar such as sucrose or lactose. Thus, the use of the carrier is taught in the prior art D2.

Thus, all the essential elements of claim 1, has been disclosed in the prior art D2. Hence, claim 1 lacks novelty in the light of the disclosure of D2.

Dependent claims 2- 9 define the carrier protein used in the immunogenic composition (claim 2 claims CRM-197) and inclusion of an adjuvant (claim 3) which can be a aluminum-based adjuvant (claim 4) selected from aluminum phosphate, aluminum sulfate and aluminum hydroxide (claims 5 and 6). Claim 7 relates to dose of saccharides and excipients, claim 8 relates to a method of inducing immune response and claim 9 to a method using the composition with specific dose.

On page 6 lines 21-28 D2 discloses

Each *Streptococcus pneumoniae* capsular saccharide may be conjugated to a carrier protein independently selected from the group consisting of TT, DT, CRM197, fragment C of TT, PhtD, PhtDE fusions (particularly those described in WO 01/98334 and WO 03/54007), detoxified pneumolysin and protein D, other than saccharide from serotype 19F which is always conjugated to DT or CRM 197, preferably DT. A more complete list of protein carriers that may be used in the conjugates of the invention is presented below

On page 17 lines 20-33 D2 discloses

In general, the immunogenic composition of the invention may comprise a dose of each saccharide conjugate between 0.1 and 20 $\mu$ g, 1 and 10 $\mu$ g or 1 and 3 $\mu$ g of saccharide.

In an embodiment, the immunogenic composition of the invention contains each *S. pneumoniae* capsular saccharide at a dose of between 0.1-20 $\mu$ g; 0.5-10 $\mu$ g; 0.5- 5 $\mu$ g or 1-3 $\mu$ g of saccharide. In an embodiment, capsular saccharides may be present at different dosages, for example some capsular saccharides may be present at a dose of exactly 1 $\mu$ g or some capsular saccharides may be present at a dose of exactly 3 $\mu$ g. In an embodiment, saccharides from serotypes 3, 18C and 19F (or 4, 18C and 19F) are present at a higher dose than other saccharides. In one aspect of this embodiment, serotypes 3, 18C and 19F (or 4, 18C and 19F) are present at a dose of around or exactly 3  $\mu$ g whilst other saccharides in the immunogenic composition are present at a dose of around or exactly 1 $\mu$ g.



On page 23 lines 19-27 D2 discloses -

The present invention further provides a vaccine containing the immunogenic compositions of the invention and a pharmaceutically acceptable excipient.

The vaccines of the present invention may be adjuvanted, particularly when intended for use in an elderly population but also for use in infant populations. Suitable adjuvants include an aluminum salt such as aluminum hydroxide gel or aluminum phosphate or alum, but may also be a salt of calcium, magnesium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatized saccharides, or polyphosphazenes.

Hence claims 1-9 lack novelty over the disclosure of D2.

**6.1.3) Lack of novelty of claim 1 to 9 in view of D7 under section 25(1) (b) (ii) of the Indian Patents Act**

**The sole independent claim 1 read as**

1. An immunogenic composition comprising:
- (1) a multivalent polysaccharide-protein conjugate mixture consisting of capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *Streptococcus pneumoniae* conjugated to a carrier protein; and
- (2) a pharmaceutically acceptable carrier

**Comparison of claim 1 of the instant invention vis –a - vis prior art D7 disclosure**

<b><u>Essential elements of the instant claim 1</u></b>	<b><u>D7- WO2009000826</u></b>
1. multivalent polysaccharide-protein conjugate mixture consisting of capsular polysaccharides	D7 on page 5 lines 11 to 21 states “typically the <i>Streptococcus pneumoniae</i> vaccine of the present invention will comprise capsular saccharide antigens (preferably conjugated), wherein the saccharides are derived from at least ten serotypes of <i>S.</i>

<p>from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of <i>Streptococcus pneumoniae</i> conjugated to a carrier protein.</p> <p>2. a pharmaceutically acceptable carrier</p>	<p><i>pneumoniae. The number of S. pneumoniae capsular saccharides can range from 10 different serotypes (or "v", valences) to 23 different serotypes (23v). In one embodiment there are 10, 11, 12, 13, 14 or 15 different serotypes. In another embodiment of the invention, the vaccine may comprise conjugated S. pneumoniae saccharides and unconjugated S. pneumoniae saccharides. Preferably, the total number of saccharide serotypes is less than or equal to 23. For example, the invention may comprise 10 conjugated serotypes and 13 unconjugated saccharides. In a similar manner, the vaccine may comprise 11, 12, 13, 14, 15 or 16 conjugated saccharides and 12, 11, 10, 9, 8 or 7, respectively, unconjugated saccharides".</i></p>
--	---

The claim 1 is directed towards a multivalent pneumococcal immunogenic composition comprising polysaccharides from 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F of *Streptococcus pneumoniae* conjugated to a carrier protein. The prior art D7 teaches the inclusion of these serotypes in a vaccine. Further it also teaches that each of the polysaccharide is conjugated to a carrier protein. The claim 1 of the alleged application further states the inclusion of a physiologically acceptable vehicle as an essential component of the vaccine. The prior art D7 also discloses the formulation of the vaccine, which is either in solution form (which would include a carrier) or in a lyophilized state, preferably in the presence of a sugar such as sucrose or lactose. Thus, the use of the carrier is taught in the prior art D7.

On page 6 last paragraph to page 7 first and second paragraph D7 states

In one embodiment the multivalent pneumococcal vaccine of the invention will be selected from the following serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, although it is appreciated that one or two other serotypes could be substituted depending on the age of the recipient receiving the vaccine and the geographical location where the vaccine will be administered, e.g. serotype 6A may be included on the list. For example, an 10-valent vaccine may comprise polysaccharides from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. An 11-valent vaccine may also include saccharides from serotype 3. A 12 or 13-valent

paediatric (infant) vaccine may also include the 10 or 11 valent formulation supplemented with serotypes 6A and 19A, or 6A and 22F, or 19A and 22F, or 6A and 15, or 19A and 15, or 22F and 15, whereas a 13-valent elderly vaccine may include the 11 valent formulation supplemented with serotypes 19A and 22F, 8 and 12F, or 8 and 15, or 8 and 19A, or 8 and 22F, or 12F and 15, or 12F and 19A, or 12F and 22F, or 15 and 19A, or 15 and 22F. A 14 valent paediatric vaccine may include the 10 valent formulation described above supplemented with serotypes 3, 6A, 19A and 22F; serotypes 6A, 8, 19A and 22F; serotypes 6A, 12F, 19A and 22F; serotypes 6A, 15, 19A and 22F; serotypes 3, 8, 19A and 22F; serotypes 3, 12F, 19A and 22F; serotypes 3, 15, 19A and 22F; serotypes 3, 6A, 8 and 22F; serotypes 3, 6A, 12F and 22F; or serotypes 3, 6A, 15 and 22F.

The composition in one embodiment includes capsular saccharides derived from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F (preferably conjugated). In a further embodiment of the invention at least 11 saccharide antigens (preferably conjugated) are included, for example capsular saccharides derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. In a further embodiment of the invention, at least 12 or 13 saccharide antigens are included, for example a vaccine may comprise capsular saccharides derived from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F or capsular saccharides derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F, although further saccharide antigens, for example 23 valent (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention.

Thus, all the essential elements of claim 1, has been disclosed in the prior art D7. Hence, claim 1 lacks novelty in the light of the disclosure of D7.

Dependent claims 2- 9 define the carrier protein used in the immunogenic composition (claim 2 claims CRM-197) and inclusion of an adjuvant (claim 3) which can be a aluminum-based adjuvant (claim 4) selected from aluminum phosphate, aluminum sulfate and aluminum hydroxide (claims 5 and 6). Claim 7 relates to dose of saccharides and excipients, claim 8 relates to a method of inducing immune response and claim 9 to a method using the composition with specific dose.

Regarding protein carriers D7 states on page 10 lines 3-9

Examples of carrier proteins which may be used in the present invention are DT (Diphtheria toxoid), TT (tetanus toxoid) or fragment C of TT, DT CRM197 (a DT mutant) other DT point mutants, such as CRM176, CRM228, CRM 45 (Uchida et al J. Biol. Chem. 218; 3838-3844, 1973); CRM 9, CRM 45, CRM102, CRM 103 and CRM107 and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala

On page 19 lines 4-16 D7 states

In general, the immunogenic composition of the invention may comprise a dose of each saccharide conjugate between 0.1 and 20 $\mu$ g, 1 and 10 $\mu$ g or 1 and 3 $\mu$ g of saccharide.

In an embodiment, the immunogenic composition of the invention contains each *S. pneumoniae* capsular saccharide at a dose of between 0.1-20 $\mu$ g; 0.5-10 $\mu$ g; 0.5- 5 $\mu$ g or 1-3 $\mu$ g of saccharide. In an embodiment, capsular saccharides may be present at different dosages, for example some capsular saccharides may be present at a dose of around or exactly 1 $\mu$ g or some capsular saccharides may be present at a dose of around or exactly 3 $\mu$ g. In an embodiment, saccharides from serotypes 3, 18C and 19F (or 4, 18C and 19F) are present at a higher dose than other saccharides. In one aspect of this embodiment, serotypes 3, 18C and 19F (or 4, 18C and 19F) are present at a dose of around or exactly 3  $\mu$ g whilst other saccharides in the immunogenic composition are present at a dose of around or exactly 1 $\mu$ g.

On page 25 lines 4-13 D7 discloses

The present invention further provides a vaccine containing the immunogenic compositions of the invention and a pharmaceutically acceptable excipient.

The vaccines of the present invention may be adjuvanted, particularly when intended for use in an elderly population but also for use in infant populations. Suitable adjuvants include an aluminum salt such as aluminum hydroxide gel or aluminum phosphate or alum, but may also be a salt of calcium, magnesium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatized saccharides, or polyphosphazenes.

Hence claims 1-9 lack novelty over the disclosure of D7.

#### **6.2) Lack of inventive step**

The following is the quotation of Section 25(1) (e) which forms the basis for all oppositions based on obviousness and lack of inventive step.

#### **Section 25(1) (e):**

“that the invention so far as claimed in any claim of the complete specification is obvious and clearly does not involve any inventive step, having regard to the matter published as mentioned in clause (b) or having regard to what was used in India before the priority date of the applicant’s claim”.

The subject matter of claims 1-9 of the opposed application lacks inventive step in view of the matter published in prior art as elaborated below.

#### **6.2.1) Claims 1 – 9 are obvious and lack inventive step over disclosure of D4 and D8 under section 25(1) (e) of the Indian Patents Act**

D4 provides generally a multivalent immunogenic composition comprising 13 distinct polysaccharide-protein conjugates, wherein each of the conjugates contains a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae* conjugated to a carrier protein, together with a physiologically acceptable vehicle. Claim 1 is directed to “A multivalent immunogenic composition, comprising: 13 distinct polysaccharide-protein conjugates, together

with a physiologically acceptable vehicle, wherein each of the conjugates comprises a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae* conjugated to a carrier protein, and the capsular polysaccharides are prepared from serotypes 1,3,4,5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F". Claim 2 states that carrier protein is CRM197. Claims 3 and 4 recite aluminum-based adjuvant. D4 further discloses all the limitations of dependent claims 2-9. The only difference between D4 and alleged invention is addition of 2 serotypes 22F and 33F. The 13 valent composition disclosed in D4 includes serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F.

The 15 valent vaccines according to instant application contains serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F.

According to D8, in the United States the two most relevant emerging serotypes causing invasive pneumococcal disease to children under 5 years of age are 22F and 33F ( See page 34, right hand column).

Thus, the inclusion of all the claimed serotypes in the alleged application, in a pneumococcal vaccine has been already taught in the prior arts D4 and D8.

The claims of the alleged application are mere reproduction of what is already been given in the prior art D4 and D8. There has been no due experimentation carried out for arriving to the invention in the alleged application. Also the so-called 'invention' in the alleged application does not provide any technological advancement over the known prior art. Thus, it is stated that the claims 1-9 are obvious and not inventive over the prior art D4 and D8.

### **6.3 Not an Invention under Section 25(1) (f) of Indian Patents Act**

**6.3.1 ) The subject matter of claims 8 and 9 of the alleged application, is not an invention as per section 3(i) of Indian Patents Act 1970 as amended by Patents (Amendment) Act 2005, so is opposed under Section 25(1) (f) of Indian Patents Act.**

**Section 25 (1) (f): states**

'that the subject of any claim of the complete specification is not an invention within the meaning of this Act, or is not patentable under this Act'

**Section 3 (i):**

“any process for the medicinal, surgical, curative, prophylactic, diagnostic, therapeutic or other treatment of human beings or any process for a similar treatment of animals to render them free of disease or to increase their economic value or that of their products”.

Claims 8 and 9 relate to a method of inducing immune response by administering an immunogenic composition. As per section 3(i) of the Indian Patent Act, a method of treatment is not considered an invention and thus, does not qualify as a patentable subject matter. The instant claims of the alleged application are not patentable and hence are subject matter of opposition as per section 25(1) (f) of Indian Patents Act.

Thus, given the above, the claims 8 and 9 of the alleged application relate to a method of treatment which is not an invention as per section 3(i) of Indian Patents Act 1970 as amended by Patents (Amendment) Act 2005. Also as mentioned above, given the section 25(1) (f) of Indian Patents Act as amended by Patents (Amendment) Act 2005, ‘any claim of the complete specification is not an invention within the meaning of this Act, or is not patentable under this Act’, is a ground for pre-grant opposition. Thus, claims 8 and 9 are opposed under section 25(1) (f) of Indian Patents Act.

**CONCLUSION**

Given the foregoing, the Opponent humbly requests the Patent Office to reject the application on all or any of the following grounds:

- The alleged invention lacks novelty and inventive step over the prior art disclosures and hence it is not new;
- The subject matter of the alleged application is not an invention under the provisions of the Act.

All these grounds relate to material flaws that go to the heart of the Application and each is sufficient for it to be rejected in its entirety, rather than requiring a claim-by-claim assessment.

8.0) The Opponent further requests that the Patent Office grant a hearing as per Rule 55(1) of the Patent Rules.

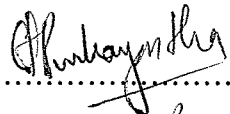
9.0) Relief Sought

In the circumstances aforesaid the opponent prays for the following relief:

- i) Revocation of the patent in entirety.
- ii) Award of costs in favor of the opponents
- iii) Such other relief or relief as the controller may deem appropriate.

**Respectfully submitted,**

On behalf of Panacea Biotec Ltd,



ARINDAM PURKAYASTHA

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(57) Abstract :

The present invention provides a multivalent immunogenic composition having 15 distinct polysaccharide protein conjugates. Each conjugate consists of a capsular polysaccharide prepared from a different serotype of (1 3 4 5 6A 6B 7F 9V 14 18C 19A 19F 22F 23F or 33F) conjugated to a carrier protein preferably CRM197. The immunogenic composition preferably formulated as a vaccine on an aluminum based adjuvant provides broad coverage against pneumococcal disease particularly in infants and young children.

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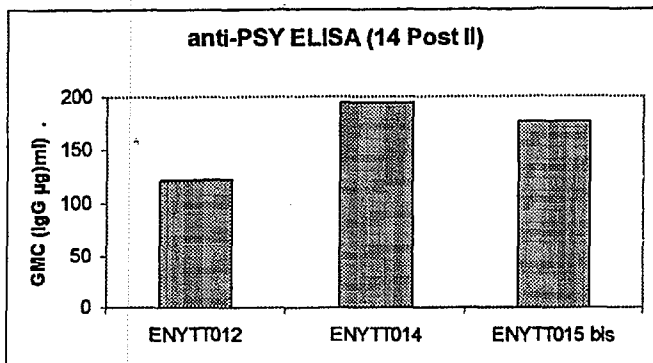
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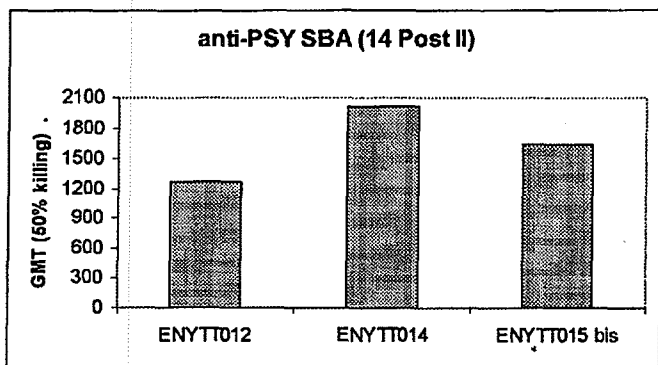
[Continued on next page]

(54) Title: IMMUNOGENIC COMPOSITION

A



B



(57) Abstract: The present application discloses an immunogenic composition comprising at least 2 different *N. meningitidis* capsular saccharides, wherein one or more is/are selected from a first group consisting of MenA, MenC, MenY and MenW which is/are conjugated through a linker to a carrier protein(s), and one or more different saccharides is/are selected from a second group consisting of MenA, MenC, MenY and MenW which is/are directly conjugated to a carrier protein(s).

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## Immunogenic composition

The present invention relates to immunogenic compositions comprising bacterial capsular saccharides conjugated to a carrier protein, in particular those saccharides of *N. meningitidis*. It additionally relates to vaccines and vaccine kits comprising such saccharide conjugates, processes for making the immunogenic compositions and vaccines and the use of the vaccines and immunogenic compositions of the invention in therapy. It also relates to methods of immunising against infection using the saccharide conjugates and the use of the saccharide conjugates in the manufacture of a medicament.

10

*Neisseria meningitidis* is a Gram-negative human pathogen which causes bacterial meningitis. Based on the organism's capsular polysaccharide, twelve serogroups of *N. meningitidis* have been identified (A, B, C, H, I, K, L, 29E, W135, X, Y and Z). Serogroup A (MenA) is the most common cause of epidemic disease in sub-Saharan Africa. Serogroups B and C are responsible for the majority of cases in developing countries, with the remaining cases being caused by W135 and Y).

15

Immunogenic compositions comprising *N. meningitidis* saccharides conjugated to carrier proteins are known in the art; the carrier protein having the known effect of turning the T-independent polysaccharide antigen into a T-dependent antigen capable of triggering an immune memory response. For instance WO 02/58737 discloses a vaccine comprising purified capsular polysaccharides from *N. meningitidis* serogroups A, C, W135 and Y conjugated to a carrier protein. However, this application teaches that all polysaccharides should essentially be conjugated in the same way (through the same linker to the same protein carrier).

20

25

There remains a need to develop improved conjugate vaccines against neisserial meningitis. The present invention concerns the provision of a meningococcal polysaccharide conjugate vaccine where conjugation of each polysaccharide is tailored (rather than being uniform) to achieve an efficacious combination vaccine. In particular it is advantageous to use linker molecules to conjugate certain meningococcal saccharides to their protein carriers in combination with others that are directly conjugated. In this way polysaccharides that are less good immunogens may be presented to the immune system via a linker, and those that are very good immunogens may be directly conjugated so that they do not dominate the immune response to the combination.

30

35

Accordingly, in one aspect of the present invention there is provided an immunogenic composition comprising at least 2 different *N. meningitidis* capsular saccharides, wherein one or more is/are selected from a first group consisting of MenA, MenC, MenY and MenW which is/are conjugated through a linker to a carrier protein(s), and one or more  
5 different saccharides is/are selected from a second group consisting of MenA, MenC, MenY and MenW which is/are directly conjugated to a carrier protein(s).

In a MenAC vaccine, for example, MenA may be conjugated through a linker and MenC directly. In a MenCY vaccine, MenC may be conjugated through a linker and MenY  
10 directly. In a MenACWY vaccine Men A may be conjugated through a linker and MenCWY directly, or MenAC may be conjugated through a linker and MenWY directly.

A further consideration in a combination vaccine comprising various saccharides conjugated to the same carrier is the issue of carrier immune suppression: too much  
15 carrier may be used and the immune response may be dampened. With a uniform approach to conjugation the carrier will present a similar blend of B- and T- cell epitopes to the immune system. However if conjugation takes place at different chemical groups within the carrier protein for one saccharide versus another, the protein carriers are likely to be different to some extent in how they present themselves to the immune system.

20

Accordingly, in a separate embodiment of the invention there is provided an immunogenic composition comprising at least 2 different saccharides conjugated separately to the same type of carrier protein (for instance tetanus toxoid), wherein one or more saccharide(s) is/are conjugated to the carrier protein via a first type of chemical group on the protein  
25 carrier, and one or more saccharide(s) is/are conjugated to the carrier protein via a second (different) type of chemical group on the protein carrier.

The first and second types of chemical group may be present in the protein carrier on a mutually exclusive first and second set of amino acids of the protein carrier (for instance  
30 certain aspartic acid / glutamic acid residues in one set and certain lysine residues in the second). One saccharide may be conjugated to a carboxyl group on the carrier, and another on an amino group for instance. Such conjugation may involve conjugation on separate B- and/or T-cell epitopes for each different conjugate.

35 For instance in a MenAC vaccine, MenA may be linked to a first type of chemical group (such as carboxyl) on the carrier protein and MenC linked to a second (such as amino). In

a MenCY vaccine MenC may be linked to a first type of chemical group (such as carboxyl) on the carrier protein and MenY linked to a second (such as amino). In a MenACWY vaccine, MenAC may be linked to a first type of chemical group (such as carboxyl) on the carrier protein and MenWY linked to a second (such as amino), or MenA may be linked to a first type of chemical group (such as carboxyl) on the carrier protein and MenCWY linked to a second (such as amino).

According to a further aspect of the invention there is provided a method of immunising a human host against disease caused by *Neisseria meningitidis* comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine of the invention.

According to a further aspect of the invention there is provided an immunogenic composition of the invention for use in the treatment or prevention of disease caused by *Neisseria meningitidis*.

According to a further aspect of the invention there is provided a use of the immunogenic composition or vaccine of the invention in the manufacture of a medicament for the treatment or prevention of diseases caused by *Neisseria meningitidis*.

## Description of figures

**Figure 1 – A –** Bar chart showing GMC responses in an anti-MenY ELISA. ENYTT012 is a MenY-TT conjugate prepared from native MenY polysaccharide. ENYTT014 is a MenY-TT conjugate prepared from microfluidised MenY polysaccharide which had undergone 40 cycles of microfluidisation. ENYTT015bis is a MenY-TT conjugate prepared from microfluidised MenY polysaccharide which had undergone 20 cycles of microfluidisation.

**- B –** Bar chart showing GMT responses in an anti-MenY SBA assay. ENYTT012 is a MenY-TT conjugate prepared from native MenY polysaccharide. ENYTT014 is a MenY-TT conjugate prepared from microfluidised MenY polysaccharide which had undergone 40 cycles of microfluidisation. ENYTT015bis is a MenY-TT conjugate prepared from microfluidised MenY polysaccharide which had undergone 20 cycles of microfluidisation.

## Detailed description

In one aspect of the present invention there is provided an immunogenic composition comprising at least 2 different *N. meningitidis* capsular saccharides, wherein one or more is/are selected from a first group consisting of MenA, MenC, MenY and MenW which is/are conjugated through a linker to a carrier protein(s), and one or more different  
5 saccharides is/are selected from a second group consisting of MenA, MenC, MenY and MenW which is/are directly conjugated to a carrier protein(s).

More specifically, the first group may consist of MenA and MenC, and the second group consist of MenC, MenY and MenW. Particular embodiments of the invention are  
10 immunogenic compositions comprising: MenA capsular saccharide conjugated through a linker to a carrier protein and MenC capsular saccharide directly conjugated to a carrier protein; MenC capsular saccharide conjugated through a linker to a carrier protein and MenY capsular saccharide directly conjugated to a carrier protein; MenA and MenC  
15 capsular saccharides conjugated through a linker to a carrier protein(s) and MenY and Men W capsular saccharides directly conjugated to a carrier protein(s); MenA capsular saccharide conjugated through a linker to a carrier protein and MenC, MenY and Men W capsular saccharides directly conjugated to a carrier protein(s). In any of these  
embodiments a Hib conjugate may also be included, which is linked to a carrier protein (see list of carriers above and below, for example TT) directly or through a linker.

20

The term "saccharide" throughout this specification may indicate polysaccharide or oligosaccharide and includes both. Polysaccharides are isolated from bacteria or isolated from bacteria and sized to some degree by known methods (see for example EP497524 and EP497525) and optionally by microfluidisation. Polysaccharides can be sized in order  
25 to reduce viscosity in polysaccharide samples and/or to improve filterability for conjugated products. Oligosaccharides have a low number of repeat units (typically 5-30 repeat units) and are typically hydrolysed polysaccharides.

Each *N. meningitidis* (and/or Hib) capsular saccharide may be conjugated to a carrier  
30 protein independently selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D. A more complete list of protein carriers that may be used in the conjugates of the invention is presented below. Although one or more *N. meningitidis* (and/or Hib) capsular saccharide may be conjugated to different carrier proteins from the others, in one embodiment they are all conjugated to the same carrier protein. For  
35 instance they may all be conjugated to the same carrier protein selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D. In this context CRM197

and DT may be considered to be the same carrier protein as they differ by only one amino acid. In an embodiment all the *N. meningitidis* (and/or Hib) capsular saccharides present are conjugated to TT.

5 If the protein carrier is the same for 2 or more saccharides in the composition, the saccharide could be conjugated to the same molecule of the protein carrier (carrier molecules having 2 more different saccharides conjugated to it) [see for instance WO 04/083251; for example, a single carrier protein might be conjugated to MenA and MenC; MenA and MenW; MenA and MenY; MenC and MenW; MenC and MenY; Men W and  
10 MenY; MenA, MenC and MenW; MenA, MenC and MenY; MenA, MenW and MenY; MenC, MenW and MenY; MenA, MenC, MenW and MenY; Hib and MenA; Hib and MenC; Hib and MenW; or Hib and MenY]. Alternatively the saccharides may each be separately conjugated to different molecules of the protein carrier (each molecule of protein carrier only having one type of saccharide conjugated to it).

15

Immunogenic compositions of the first aspect of the invention may also have any or all the additional characteristics of the second aspect of the invention and vice versa.

In a second aspect of the invention there is presented an immunogenic composition  
20 comprising at least 2 different saccharide conjugates conjugated separately to the same type of carrier protein, wherein one or more saccharide(s) is/are conjugated to the carrier protein via a first type of chemical group on the protein carrier, and one or more saccharide(s) is/are conjugated to the carrier protein via a second (different) type of chemical group on the protein carrier.

25

In one embodiment the 2 conjugates involve the same saccharide linked to the same carrier, but by different conjugation chemistries. In an alternative embodiment 2 different saccharides are conjugated to different groups on the protein carrier.

30 By "conjugated separately to the same type of carrier protein" it is meant that the saccharides are conjugated to the same carrier individually (for example, MenA is conjugated to tetanus toxoid through an amine group on the tetanus toxoid and MenC is conjugated to tetanus toxoid through a carboxylic acid group on a different molecule of tetanus toxoid.)

35



The capsular saccharide(s) may be conjugated to the same carrier protein independently selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D. A more complete list of protein carriers that may be used in the conjugates of the invention is presented below. In this context CRM197 and DT may be considered to be the same carrier protein as they differ by only one amino acid. In an embodiment all the capsular saccharides present are conjugated to TT.

In one embodiment the first and second type of chemical group on the protein carrier are present on separate B- and/or T-cell epitopes on the carrier protein. That is, they are present on a different set of B- and/or T-cell epitopes from each other. To predict B-cell epitopes for a carrier known methods may be used such as either or both of the following two methods: 2D-structure prediction and/or antigenic index prediction. 2D-structure prediction can be made using the PSIPRED program (from David Jones, Brunel Bioinformatics Group, Dept. Biological Sciences, Brunel University, Uxbridge UB8 3PH, UK). The antigenic index can be calculated on the basis of the method described by Jameson and Wolf (CABIOS 4:181-186 [1988]). The parameters used in this program are the antigenic index and the minimal length for an antigenic peptide. An antigenic index of 0.9 for a minimum of 5 consecutive amino acids can be used as the thresholds in the program. T-helper cell epitopes are peptides bound to HLA class II molecules and recognized by T-helper cells. The prediction of useful T-helper cell epitopes can be based on known techniques, such as the TEPITOPE method describe by Sturniolo at al. (Nature Biotech. 17: 555-561 [1999]).

The saccharides may be selected from a group consisting of: *N. meningitidis* serogroup A capsular saccharide (MenA), *N. meningitidis* serogroup C capsular saccharide (MenC), *N. meningitidis* serogroup Y capsular saccharide (MenY), *N. meningitidis* serogroup W capsular saccharide (MenW), *H. influenzae* type b capsular saccharide (Hib), Group B Streptococcus group I capsular saccharide, Group B Streptococcus group II capsular saccharide, Group B Streptococcus group III capsular saccharide, Group B Streptococcus group IV capsular saccharide, Group B Streptococcus group V capsular saccharide, *Staphylococcus aureus* type 5 capsular saccharide, *Staphylococcus aureus* type 8 capsular saccharide, Vi saccharide from *Salmonella typhi*, *N. meningitidis* LPS (such as L3 and/or L2), *M. catarrhalis* LPS, *H. influenzae* LPS, and from any of the capsular pneumococcal saccharides such as from serotype: 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F or 33F. In one embodiment the immunogenic composition of the invention consists of or comprises two or more different

saccharides from the same genus of bacteria (e.g. *Neisseria*, *Streptococcus*, *Staphylococcus*, or *Haemophilus*).

5 The first and second chemical groups present on the protein carrier are different from each other and are ideally natural chemical groups that may be readily used for conjugation purposes. They may be selected independently from the group consisting of: carboxyl groups, amino groups, sulphhydryl groups, Hydroxyl groups, Imidazolyl groups, Guanidyl groups, and Indolyl groups. In one embodiment the first chemical group is carboxyl and the second is amino, or vice versa. These groups are explained in greater  
10 detail below.

In a specific embodiment the immunogenic composition comprises at least 2 different *N. meningitidis* capsular saccharides, wherein one or more is/are selected from a first group consisting of MenA and MenC which is/are conjugated to the carrier protein via the first  
15 type of chemical group on the protein carrier (for instance carboxyl), and one or more different saccharides is/are selected from a second group consisting of MenC, MenY and MenW which is/are conjugated to the carrier protein via the second type of chemical group on the protein carrier (for instance amino).

20 In a further embodiment the immunogenic composition of the invention comprises MenA conjugated via the first type of chemical group (for instance carboxyl), and MenC conjugated via the second type of chemical group (for instance amino).

In another embodiment the immunogenic composition comprises MenC conjugated via  
25 the first type of chemical group (for instance carboxyl), and MenY conjugated via the second type of chemical group (for instance amino).

In another embodiment the immunogenic composition comprises MenA conjugated via  
30 the first type of chemical group (for instance carboxyl), and MenC, MenY and MenW conjugated via the second type of chemical group (for instance amino).

In another embodiment the immunogenic composition comprises MenA and MenC  
35 conjugated via the first type of chemical group (for instance carboxyl), and MenY and MenW conjugated via the second type of chemical group (for instance amino).

In any of the above embodiments Hib may also be present also conjugated to the same type of protein carrier. Hib may be conjugated to the carrier by the first or second type of chemical group. In one embodiment it is conjugated via a carboxyl group.

## 5 General considerations in the aspects of the invention

The saccharides of the invention (in particular the *N. meningitidis* saccharides and/or the Hib capsular saccharide) included in pharmaceutical (immunogenic) compositions of the invention are conjugated to a carrier protein such as tetanus toxoid (TT), tetanus toxoid  
10 fragment C, non-toxic mutants of tetanus toxin [note all such variants of TT are considered to be the same type of carrier protein for the purposes of this invention], diphtheria toxoid (DT), CRM197, other non-toxic mutants of diphtheria toxin [such as CRM176, CRM 197, CRM228, CRM 45 (Uchida et al J. Biol. Chem. 218; 3838-3844, 1973); CRM 9, CRM 45, CRM102, CRM 103 and CRM107 and other mutations described  
15 by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in US 4709017 or US 4950740; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in US 5917017 or US 6455673; or fragment disclosed in US 5843711] (note all such variants of  
20 DT are considered to be the same type of carrier protein for the purposes of this invention), pneumococcal pneumolysin (Kuo et al (1995) Infect Immun 63; 2706-13), OMPC (meningococcal outer membrane protein – usually extracted from *N. meningitidis* serogroup B – EP0372501), synthetic peptides (EP0378881, EP0427347), heat shock proteins (WO 93/17712, WO 94/03208), pertussis proteins (WO 98/58668, EP0471177),  
25 cytokines, lymphokines, growth factors or hormones (WO 91/01146), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen derived antigens (Falugi et al (2001) Eur J Immunol 31; 3816-3824) such as N19 protein (Baraldoi et al (2004) Infect Immun 72; 4884-7) pneumococcal surface protein PspA (WO 02/091998), iron uptake proteins (WO 01/72337), toxin A or B of *C. difficile* (WO 00/61761) or Protein  
30 D (EP594610 and WO 00/56360).

In an embodiment, the immunogenic composition of the invention uses the same type of carrier protein (independently) in at least two, three, four or each of the saccharides (e.g. *N. meningitidis* capsular saccharides and/or Hib) contained therein. In an embodiment  
35 where Hib and *N. meningitidis* capsular saccharides are present, Hib may be conjugated to the same type of carrier protein as the at least two, three, four or each of the *N.*

*meningitidis* saccharides. For example, 2, 3 or 4 of the *N. meningitidis* saccharides (MenA,C,Y,W) are independently conjugated to tetanus toxoid to make 2, 3 or 4 conjugates, and optionally Hib is also conjugated to TT.

5 In an embodiment, the immunogenic composition of the invention comprises a *N. meningitidis* saccharide conjugated to a carrier protein selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D. In an embodiment, the immunogenic composition of the invention comprises a Hib saccharide conjugated to a carrier protein selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D.

10

The immunogenic composition of the invention optionally comprises at least one meningococcal saccharide (for example MenA; MenC; MenW; MenY; MenA and MenC; MenA and MenW; MenA and MenY; MenC and Men W; Men C and MenY; Men W and MenY; MenA, MenC and MenW; MenA, MenC and MenY; MenA, MenW and MenY; 15 MenC, MenW and MenY or MenA, MenC, MenW and MenY) conjugate having a ratio of Men saccharide to carrier protein of between 1:5 and 5:1, between 1:2 and 5:1, between 1:0.5 and 1:2.5 or between 1:1.25 and 1:2.5(w/w).

20

The immunogenic composition of the invention optionally comprises a Hib saccharide conjugate having a ratio of Hib to carrier protein of between 1:5 and 5:1; 1:2 and 2:1; 1:1 and 1:4; 1:2 and 1:3.5; or around or exactly 1:2.5 or 1:3 (w/w).

25

The ratio of saccharide to carrier protein (w/w) in a conjugate may be determined using the sterilized conjugate. The amount of protein is determined using a Lowry assay (for example Lowry et al (1951) J. Biol. Chem. 193, 265-275 or Peterson et al Analytical Biochemistry 100, 201-220 (1979)) and the amount of saccharide is determined using ICP-OES (inductively coupled plasma-optical emission spectroscopy) for MenA, DMAP assay for MenC and Resorcinol assay for MenW and MenY (Monsigny et al (1988) Anal. Biochem. 175, 525-530).

30

In an embodiment, the immunogenic composition of the invention comprises *N. meningitidis* saccharide conjugate(s) and/or the Hib saccharide conjugate wherein the *N. meningitidis* saccharide(s) and/or the Hib saccharide is conjugated to the carrier protein via a linker, for instance a bifunctional linker. The linker is optionally heterobifunctional or homobifunctional, having for example a reactive amino group and a reactive carboxylic acid group, 2 reactive amino groups or two reactive carboxylic acid groups. The linker has 35

for example between 4 and 20, 4 and 12, 5 and 10 carbon atoms. A possible linker is ADH. Other linkers include B-propionamido (WO 00/10599), nitrophenyl-ethylamine (Gever et al (1979) Med. Microbiol. Immunol. 165; 171-288), haloalkyl halides (US4057685), glycosidic linkages (US4673574, US4808700), hexane diamine and 6-aminocaproic acid (US4459286).

The saccharide conjugates present in the immunogenic compositions of the invention may be prepared by any known coupling technique. The conjugation method may rely on activation of the saccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated saccharide may thus be coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using GMBS) or a holoacetylated carrier protein (for example using iodoacetimide or N-succinimidyl bromoacetatebromoacetate). Optionally, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or ADH and the amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g. EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described in PCT published application WO 93/15760 Uniformed Services University and WO 95/08348 and WO 96/29094.

Other suitable techniques use carbimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU. Many are described in WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with CDI (Bethell et al J. Biol. Chem. 1979, 254; 2572-4, Hearn et al J. Chromatogr. 1981. 218; 509-18) followed by reaction of with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group' reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

The conjugates can also be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide (or CDAP) activated saccharide derivatised with adipic acid hydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256), for example using EDAC.

5

In an embodiment, a hydroxyl group (optionally an activated hydroxyl group for example a hydroxyl group activated by a cyanate ester) on a saccharide is linked to an amino or carboxylic group on a protein either directly or indirectly (through a linker). Where a linker is present, a hydroxyl group on a saccharide is optionally linked to an amino group on a linker, for example by using CDAP conjugation. A further amino group in the linker for example ADH) may be conjugated to a carboxylic acid group on a protein, for example by using carbodiimide chemistry, for example by using EDAC. In an embodiment, the Hib or *N. meningitidis* capsular saccharide(s) (or saccharide in general) is conjugated to the linker first before the linker is conjugated to the carrier protein. Alternatively the linker may be conjugated to the carrier before conjugation to the saccharide.

10  
15

In general the following types of chemical groups on a protein carrier can be used for coupling / conjugation:

20 A) Carboxyl (for instance via aspartic acid or glutamic acid). In one embodiment this group is linked to amino groups on saccharides directly or to an amino group on a linker with carbodiimide chemistry e.g. with EDAC.

25 B) Amino group (for instance via lysine). In one embodiment this group is linked to carboxyl groups on saccharides directly or to a carboxyl group on a linker with carbodiimide chemistry e.g. with EDAC. In another embodiment this group is linked to hydroxyl groups activated with CDAP or CNBr on saccharides directly or to such groups on a linker; to saccharides or linkers having an aldehyde group; to saccharides or linkers having a succinimide ester group.

30

C) Sulphydryl (for instance via cysteine). In one embodiment this group is linked to a bromo or chloro acetylated saccharide or linker with maleimide chemistry. In one embodiment this group is activated/modified with bis diazobenzidine.

35 D) Hydroxyl group (for instance via tyrosine). In one embodiment this group is activated/modified with bis diazobenzidine.

E) Imidazolyl group (for instance via histidine). In one embodiment this group is activated/modified with bis diazobenzidine.

5 F) Guanidyl group (for instance via arginine).

G) Indolyl group (for instance via tryptophan).

10 On a saccharide, in general the following groups can be used for a coupling: OH, COOH or NH<sub>2</sub>. Aldehyde groups can be generated after different treatments known in the art such as: periodate, acid hydrolysis, hydrogen peroxide, etc.

*Direct coupling approaches:*

15 Saccharide-OH + CNBr or CDAP ----> cyanate ester + NH<sub>2</sub>-Prot ----> conjugate  
 Saccharide-aldehyde + NH<sub>2</sub>-Prot ----> Schiff base + NaCNBH<sub>3</sub> ----> conjugate  
 Saccharide-COOH + NH<sub>2</sub>-Prot + EDAC ----> conjugate  
 Saccharide-NH<sub>2</sub> + COOH-Prot + EDAC ----> conjugate

20 *Indirect coupling via spacer (linker) approaches:*

Saccharide-OH + CNBr or CDAP --> cyanate ester + NH<sub>2</sub>---NH<sub>2</sub> ----> saccharide---NH<sub>2</sub>  
 + COOH-Prot + EDAC ----> conjugate

25 Saccharide-OH + CNBr or CDAP ----> cyanate ester + NH<sub>2</sub>---SH ----> saccharide---SH  
 + SH-Prot (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance) ----> saccharide-S-S-Prot

30 Saccharide-OH + CNBr or CDAP --> cyanate ester + NH<sub>2</sub>---SH ----> saccharide---SH  
 + maleimide-Prot (modification of amino groups) ----> conjugate

Saccharide-COOH + EDAC + NH<sub>2</sub>---NH<sub>2</sub> --> saccharide---NH<sub>2</sub> + EDAC + COOH-Prot ----> conjugate

Saccharide-COOH + EDAC+ NH<sub>2</sub>---SH -----> saccharide---SH + SH-Prot (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance) -----> saccharide-S-S-Prot

- 5 Saccharide-COOH + EDAC+ NH<sub>2</sub>---SH -----> saccharide---SH + maleimide-Prot (modification of amino groups) -----> conjugate

Saccharide-Aldehyde + NH<sub>2</sub>---NH<sub>2</sub> -----> saccharide---NH<sub>2</sub> + EDAC + COOH-Prot -----> conjugate

10

Note: instead of EDAC above, any suitable carbodiimide may be used.

In summary, the types of protein carrier chemical group that may be generally used for coupling with a saccharide are amino groups (for instance on lysine residues), COOH groups (for instance on aspartic and glutamic acid residues) and SH groups (if accessible) (for instance on cysteine residues).

15

In an embodiment, the Hib saccharide, where present, is conjugated to the carrier protein using CNBr, or CDAP, or a combination of CDAP and carbodiimide chemistry (such as EDAC), or a combination of CNBr and carbodiimide chemistry (such as EDAC). Optionally Hib is conjugated using CNBr and carbodiimide chemistry, optionally EDAC. For example, CNBr is used to join the saccharide and linker and then carbodiimide chemistry is used to join linker to the protein carrier.

20

25 In an embodiment, at least one of the *N. meningitidis* capsular saccharides (or saccharide in general) is directly conjugated to a carrier protein; optionally Men W and/or MenY and/or MenC saccharide(s) is directly conjugated to a carrier protein. For example MenW; MenY; MenC; MenW and MenY; MenW and MenC; MenY and MenC; or MenW, MenY and MenC are directly linked to the carrier protein. Optionally, at least one of the *N. meningitidis* capsular saccharides is directly conjugated by CDAP. For example MenW; MenY; MenC; MenW and MenY; MenW and MenC; MenY and MenC; or MenW, MenY and MenC are directly linked to the carrier protein by CDAP (see WO 95/08348 and WO 96/29094). In an embodiment, all *N. meningitidis* capsular saccharides are conjugated to tetanus toxoid.

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35



In an embodiment, the ratio of Men W and/or Y saccharide to carrier protein is between 1:0.5 and 1:2 (w/w) and/or the ratio of MenC saccharide to carrier protein is between 1:0.5 and 1:4 or 1:0.5 and 1:1.5 (w/w), especially where these saccharides are directly linked to the protein, optionally using CDAP.

5

In an embodiment, at least one of the *N. meningitidis* capsular saccharide(s) (or saccharide in general) is conjugated to the carrier protein via a linker, for instance a bifunctional linker. The linker is optionally heterobifunctional or homobifunctional, having for example a reactive amine group and a reactive carboxylic acid group, 2 reactive amine groups or 2 reactive carboxylic acid groups. The linker has for example between 4 and 20, 4 and 12, 5 and 10 carbon atoms. A possible linker is ADH.

10

In an embodiment, MenA; MenC; or MenA and MenC is conjugated to a carrier protein (for example tetanus toxoid) via a linker.

15

In an embodiment, at least one *N. meningitidis* saccharide is conjugated to a carrier protein via a linker using CDAP and EDAC. For example, MenA; MenC; or MenA and MenC are conjugated to a protein via a linker (for example those with two hydrazino groups at its ends such as ADH) using CDAP and EDAC as described above. For example, CDAP is used to conjugate the saccharide to a linker and EDAC is used to conjugate the linker to a protein. Optionally the conjugation via a linker results in a ratio of saccharide to carrier protein of between 1:0.5 and 1:6; 1:1 and 1:5 or 1:2 and 1:4, for MenA; MenC; or MenA and MenC.

20

In an embodiment, the MenA capsular saccharide, where present is at least partially O-acetylated such that at least 50%, 60%, 70%, 80%, 90%, 95% or 98% of the repeat units are O-acetylated at at least one position. O-acetylation is for example present at least at the O-3 position of at least 50%, 60%, 70%, 80%, 90%, 95% or 98% of the repeat units.

25

In an embodiment, the MenC capsular saccharide, where present is at least partially O-acetylated such that at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 98% of ( $\alpha$ 2  $\rightarrow$ 9)-linked NeuNAc repeat units are O-acetylated at at least one or two positions. O-acetylation is for example present at the O-7 and/or O-8 position of at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 98% of the repeat units.

30

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In an embodiment, the MenW capsular saccharide, where present is at least partially O-acetylated such that at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 98% of the repeat units are O-acetylated at at least one or two positions. O-acetylation is for example present at the O-7 and/or O-9 position of at least 30%. 40%, 50%, 60%, 70%, 80%, 90%,  
5 95% or 98% of the repeat units.

In an embodiment, the MenY capsular saccharide, where present is at least partially O-acetylated such that at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 98% of the repeat units are O-acetylated at at least one or two positions. O-acetylation is present  
10 at the 7 and/or 9 position of at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 98% of the repeat units.

The percentage of O-acetylation refers to the percentage of the repeat units containing O-acetylation. This may be measured in the saccharide prior to conjugate and/or after  
15 conjugation.

In one embodiment of the invention the immunogenic composition, saccharide present, or each *N. meningitidis* capsular saccharide present, is conjugated to TT. In a further embodiment each *N. meningitidis* capsular saccharide is separately conjugated to a  
20 separate carrier protein. In a further embodiment each *N. meningitidis* capsular saccharide conjugate has a saccharide:carrier ratio of 1:5-5:1 or 1:1-1:4(w/w). In a further embodiment at least one, two or three *N. meningitidis* capsular saccharide conjugate(s) is directly conjugated to a carrier protein. In a further embodiment Men W and/or MenY, MenW and/or MenC, MenY and/or MenC, or MenW and MenC and MenY are directly  
25 conjugated to a carrier protein. In a further embodiment at least one, two or three *N. meningitidis* saccharide conjugate(s) is directly conjugated by CDAP chemistry. In a further embodiment the ratio of Men W and/or Y saccharide to carrier protein is between 1:0.5 and 1:2 (w/w). In a further embodiment the ratio of MenC saccharide to carrier protein is between 1:0.5 and 1:2 (w/w). In a further embodiment at least one, two or three  
30 *N. meningitidis* capsular saccharide(s) are conjugated to the carrier protein via a linker (which may be bifunctional such as having two reactive amino groups (such as ADH) or two reactive carboxyl groups, or a reactive amino group at one end and a reactive carboxyl group at the other). The linker can have between 4 and 12 carbon atoms. In a further embodiment the or each *N. meningitidis* capsular saccharide(s) conjugated via a  
35 linker are conjugated to the linker with CDAP chemistry. In a further embodiment the carrier protein is conjugated to the linker using carbodiimide chemistry, for example using

EDAC. In a further embodiment the or each *N. meningitidis* capsular saccharide is conjugated to the linker before the carrier protein is conjugated to the linker. In a further embodiment MenA is conjugated to a carrier protein via a linker (the ratio of MenA saccharide to carrier protein may be between 1:2 and 1:5 (w/w)). In a further embodiment  
5 MenC is conjugated to a carrier protein via a linker (the ratio of MenC saccharide to carrier protein may be between 1:2 and 1:5 (w/w)).

The inventors have also noted that the focus of the art has been to use oligosaccharides for ease of conjugate production. The inventors have found that by using native or slightly  
10 sized polysaccharide conjugates, one or more of the following advantages may be realised: 1) a conjugate having high immunogenicity which is filterable through a 0.2 micron filter; 2) immune memory may be enhanced (as in example three); 3) the alteration of the ratio of polysaccharide to protein in the conjugate such that the ratio of polysaccharide to protein (w/w) in the conjugate may be increased (this can result in a reduction of the  
15 carrier suppression effect); 4) immunogenic conjugates prone to hydrolysis (such as MenA conjugates) may be stabilised by the use of larger polysaccharides for conjugation. The use of larger polysaccharides can result in more cross-linking with the conjugate carrier and may lessen the liberation of free saccharide from the conjugate. The conjugate vaccines described in the prior art tend to depolymerise the polysaccharides  
20 prior to conjugation in order to improve conjugation. The present inventors have found that meningococcal (or saccharide) conjugate vaccines retaining a larger size of saccharide can provide a good immune response against meningococcal disease.

The immunogenic composition of the invention may thus comprise one or more  
25 saccharide conjugates wherein the average size of each saccharide before conjugation is above 50kDa, 75kDa, 100kDa, 110kDa, 120kDa or 130kDa. In one embodiment the conjugate post conjugation should be readily filterable through a 0.2 micron filter such that a yield of more than 50, 60, 70, 80, 90 or 95% is obtained post filtration compared with the pre filtration sample.

30 In particular, the immunogenic composition of the invention comprises *N. meningitidis* capsular saccharides from at least one, two, three or four of serogroups A, C, W and Y conjugated to a carrier protein, wherein the average size (weight-average molecular weight; Mw) of at least one, two, three or four or each *N. meningitidis* saccharide is above  
35 50kDa, 60kDa, 75kDa, 100kDa, 110kDa, 120kDa or 130kDa.

The immunogenic composition may comprise *N. meningitidis* capsular saccharides from at least one, two, three or four of serogroups A, C, W and Y conjugated to a carrier protein, wherein at least one, two, three or four or each *N. meningitidis* saccharide is either a native saccharide or is sized by a factor up to x2, x3, x4, x5, x6, x7, x8, x9 or x10  
5 relative to the weight average molecular weight of the native polysaccharide.

For the purposes of the invention, "native polysaccharide" refers to a saccharide that has not been subjected to a process, the purpose of which is to reduce the size of the saccharide. A polysaccharide can become slightly reduced in size during normal  
10 purification procedures. Such a saccharide is still native. Only if the polysaccharide has been subjected to sizing techniques would the polysaccharide not be considered native.

For the purposes of the invention, "sized by a factor up to x2" means that the saccharide is subject to a process intended to reduce the size of the saccharide but to retain a size  
15 more than half the size of the native polysaccharide. X3, x4 etc. are to be interpreted in the same way i.e. the saccharide is subject to a process intended to reduce the size of the polysaccharide but to retain a size more than a third, a quarter etc. the size of the native polysaccharide.

20 In an aspect of the invention, the immunogenic composition comprises *N. meningitidis* capsular saccharides from at least one, two, three or four of serogroups A, C, W and Y conjugated to a carrier protein, wherein at least one, two, three or four or each *N. meningitidis* saccharide is native polysaccharide.

25 In an aspect of the invention, the immunogenic composition comprises *N. meningitidis* capsular saccharides from at least one, two, three or four of serogroups A, C, W and Y conjugated to a carrier protein, wherein at least one, two, three or four or each *N. meningitidis* saccharide is sized by a factor up to x1.5, x2, x3, x4, x5, x6, x7, x8, x9 or x10.

30 The immunogenic compositions of the invention optionally comprise conjugates of : *N. meningitidis* serogroup C capsular saccharide (MenC), serogroup A capsular saccharide (MenA), serogroup W135 capsular saccharide (MenW), serogroup Y capsular saccharide (MenY), serogroup C and Y capsular saccharides (MenCY), serogroup C and A capsular  
35 saccharides (MenAC), serogroup C and W capsular saccharides (MenCW), serogroup A and Y capsular saccharide (MenAY), serogroup A and W capsular saccharides (MenAW), serogroup W and Y capsular saccharides (Men WY), serogroup A, C and W capsular

saccharide (MenACW), serogroup A, C and Y capsular saccharides (MenACY); serogroup A, W135 and Y capsular saccharides (MenAWY), serogroup C, W135 and Y capsular saccharides (MenCWY); or serogroup A, C, W135 and Y capsular saccharides (MenACWY). This is the definition of "one, two, three or four", or "at least one of" of serogroups A, C, W and Y, or of each *N. meningitidis* saccharide where mentioned herein.

In an embodiment, the average size of at least one, two, three, four or each *N. meningitidis* saccharide is between 50kDa and 1500kDa, 50kDa and 500kDa, 50 kDa and 300 kDa, 101kDa and 1500kDa, 101kDa and 500kDa, 101kDa and 300kDa as determined by MALLS.

In an embodiment, the MenA saccharide, where present, has a molecular weight of 50-500kDa, 50-100kDa, 100-500kDa, 55-90kDa, 60-70kDa or 70-80kDa or 60-80kDa.

In an embodiment, the MenC saccharide, where present, has a molecular weight of 100-200kDa, 50-100kDa, 100-150kDa, 101-130kDa, 150-210kDa or 180-210kDa.

In an embodiment the MenY saccharide, where present, has a molecular weight of 60-190kDa, 70-180kDa, 80-170kDa, 90-160kDa, 100-150kDa or 110-140kDa, 50-100kDa, 100-140kDa, 140-170kDa or 150-160kDa.

In an embodiment the MenW saccharide, where present, has a molecular weight of 60-190kDa, 70-180kDa, 80-170kDa, 90-160kDa, 100-150kDa, 110-140kDa, 50-100kDa or 120-140kDa.

The molecular weight or average molecular weight of a saccharide herein refers to the weight-average molecular weight (Mw) of the saccharide measured prior to conjugation and is measured by MALLS.

The MALLS technique is well known in the art and is typically carried out as described in example 2. For MALLS analysis of meningococcal saccharides, two columns (TSKG6000 and 5000PWxl) may be used in combination and the saccharides are eluted in water. Saccharides are detected using a light scattering detector (for instance Wyatt Dawn DSP equipped with a 10mW argon laser at 488nm) and an interferometric refractometer (for instance Wyatt Otilab DSP equipped with a P100 cell and a red filter at 498nm).

In an embodiment the *N. meningitidis* saccharides are native polysaccharides or native polysaccharides which have reduced in size during a normal extraction process.

5 In an embodiment, the *N. meningitidis* saccharides are sized by mechanical cleavage, for instance by microfluidisation or sonication. Microfluidisation and sonication have the advantage of decreasing the size of the larger native polysaccharides sufficiently to provide a filterable conjugate (for example through a 0.2 micron filter). Sizing is by a factor of no more than x20, x10, x8, x6, x5, x4, x3, x2 or x1.5.

10 In an embodiment, the immunogenic composition comprises *N. meningitidis* conjugates that are made from a mixture of native polysaccharides and saccharides that are sized by a factor of no more than x20. For example, saccharides from MenC and/or MenA are native. For example, saccharides from MenY and/or MenW are sized by a factor of no more than x20, x10, x8, x6, x5, x4, x3 or x2. For example, an immunogenic composition  
15 contains a conjugate made from MenY and/or MenW and/or MenC and/or MenA which is sized by a factor of no more than x10 and/or is microfluidised. For example, an immunogenic composition contains a conjugate made from native MenA and/or MenC and/or MenW and/or MenY. For example, an immunogenic composition comprises a conjugate made from native MenC. For example, an immunogenic composition comprises  
20 a conjugate made from native MenC and MenA which is sized by a factor of no more than x10 and/or is microfluidised. For example, an immunogenic composition comprises a conjugate made from native MenC and MenY which is sized by a factor of no more than x10 and/or is microfluidised.

25 In an embodiment, the polydispersity of the saccharide is 1-1.5, 1-1.3, 1-1.2, 1-1.1 or 1-1.05 and after conjugation to a carrier protein, the polydispersity of the conjugate is 1.0-2.5, 1.0-2.0, 1.0-1.5, 1.0-1.2, 1.5-2.5, 1.7-2.2 or 1.5-2.0. All polydispersity measurements are by MALLS.

30 Saccharides are optionally sized up to 1.5, 2, 4, 6, 8, 10, 12, 14, 16, 18 or 20 times from the size of the polysaccharide isolated from bacteria.

In one embodiment each *N. meningitidis* saccharide is either a native polysaccharide or is sized by a factor of no more than x10. In a further embodiment each *N. meningitidis*  
35 capsular saccharide is a native polysaccharide. In a further embodiment at least one, two, three or four *N. meningitidis* capsular saccharide(s) is sized by microfluidization. In a

further embodiment each *N. meningitidis* capsular saccharide is sized by a factor of no more than x10. In a further embodiment the *N. meningitidis* conjugates are made from a mixture of native polysaccharides and saccharides that are sized by a factor of no more than x10. In a further embodiment the capsular saccharide from serogroup Y is sized by a factor of no more than x10. In a further embodiment capsular saccharides from serogroups A and C are native polysaccharides and saccharides from serogroups W135 and Y are sized by a factor of no more than x10. In a further embodiment the average size of each *N. meningitidis* capsular saccharide is between 50 kDa and 300 kDa or 50kDa and 200kDa. In a further embodiment the immunogenic composition comprises a MenA capsular saccharide having an average size of above 50kDa, 75kDa, 100kDa or an average size of between 50-100kDa or 55-90kDa or 60-80kDa. In a further embodiment the immunogenic composition comprises a MenC capsular saccharide having an average size of above 50kDa, 75kDa, 100kDa or between 100-200kDa, 100-150kDa, 80-120kDa, 90-110kDa, 150-200kDa, 120-240kDa, 140-220kDa, 160-200kDa or 190-200kDa. In a further embodiment the immunogenic composition comprises a MenY capsular saccharide, having an average size of above 50kDa, 75kDa, 100kDa or between 60-190kDa or 70-180kDa or 80-170kDa or 90-160kDa or 100-150kDa, 110-145kDa or 120-140kDa. In a further embodiment the immunogenic composition comprises a MenW capsular saccharide having an average size of above 50kDa, 75kDa, 100kDa or between 60-190kDa or 70-180kDa or 80-170kDa or 90-160kDa or 100-150kDa, 140-180kDa, 150-170kDa or 110-140kDa.

The immunogenic composition of the invention may comprise a *H. influenzae* b capsular saccharide (Hib) conjugated to a carrier protein. This may be conjugated to a carrier protein selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D, for instance TT. The Hib saccharide may be conjugated to the same carrier protein as for at least one, two, three or all of the *N. meningitidis* capsular saccharide conjugates, for instance TT. The ratio of Hib to carrier protein in the Hib capsular saccharide conjugate may be between 1:5 and 5:1 (w/w), for instance between 1:1 and 1:4, 1:2 and 1:3.5 or around 1:3 (w/w). The Hib capsular saccharide may be conjugated to the carrier protein via a linker (see above). The linker may be bifunctional (with two reactive amino groups, such as ADH, or two reactive carboxylic acid groups, or a reactive amino group at one end and a reactive carboxylic acid group at the other end). It may have between 4 and 12 carbon atoms. Hib saccharide may be conjugated to the carrier protein or linker using CNBr or CDAP. The carrier protein may be conjugated to the Hib saccharide via the linker using a method comprising carbodiimide chemistry, for example

EDAC chemistry (thus using the carboxyl chemical group on the carrier). The dose of the Hib saccharide conjugate may be between 0.1 and 9 $\mu$ g, 1 and 5 $\mu$ g or 2 and 3 $\mu$ g of saccharide.

- 5 In a further embodiment, the immunogenic composition of the invention comprises a Hib saccharide conjugate and at least two *N. meningitidis* saccharide conjugates wherein the Hib conjugate is present in a lower saccharide dose than the mean saccharide dose of the at least two *N. meningitidis* saccharide conjugates. Alternatively, the Hib conjugate is present in a lower saccharide dose than the saccharide dose of each of the at least two *N.*
- 10 *meningitidis* saccharide conjugates. For example, the dose of the Hib conjugate may be at least 10%, 20%, 30%, 40%, 50%, 60%, 70% or 80% lower than the mean or lowest saccharide dose of the at least two further *N. meningitidis* saccharide conjugates.

The mean dose is determined by adding the doses of all the further saccharides and

15 dividing by the number of further saccharides. Further saccharides are all the saccharides within the immunogenic composition apart from Hib and can include *N. meningitidis* capsular saccharides. The "dose" is in the amount of immunogenic composition or vaccine that is administered to a human.

- 20 A Hib saccharide is the polyribosyl phosphate (PRP) capsular polysaccharide of *Haemophilus influenzae* type b or an oligosaccharide derived therefrom.

At least two further bacterial saccharide conjugates is to be taken to mean two further bacterial saccharide conjugates in addition to a Hib conjugate. The two further bacterial

25 conjugates may include *N. meningitidis* capsular saccharide conjugates.

The immunogenic compositions of the invention may comprise further saccharide conjugates derived from one or more of *Neisseria meningitidis*, *Streptococcus pneumoniae*, Group A Streptococci, Group B Streptococci, *S. typhi*, *Staphylococcus aureus* or *Staphylococcus epidermidis*. In an embodiment, the immunogenic composition comprises capsular saccharides derived from one or more of serogroups A, C, W135 and Y of *Neisseria meningitidis*. A further embodiment comprises capsular saccharides derived from *Streptococcus pneumoniae*. The pneumococcal capsular saccharide antigens are optionally selected from serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A,

30 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (optionally from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F). A further embodiment comprises the Type



5, Type 8 or 336 capsular saccharides of *Staphylococcus aureus*. A further embodiment comprises the Type I, Type II or Type III capsular saccharides of *Staphylococcus epidermidis*. A further embodiment comprises the Vi saccharide from *S. typhi*. A further embodiment comprises the Type Ia, Type Ic, Type II, Type III or Type V capsular saccharides of Group B streptococcus. A further embodiment comprises the capsular saccharides of Group A streptococcus, optionally further comprising at least one M protein and optionally multiple types of M protein.

10 The immunogenic compositions of the invention may also comprise a DTPa or DTPw vaccine (for instance one containing DT, TT, and either a whole cell pertussis (Pw) vaccine or an acellular pertussis (Pa) vaccine (comprising for instance pertussis toxoid, FHA, pertactin, and, optionally agglutinogens 2 and 3). Such combinations may also comprise a vaccine against hepatitis B (for instance it may comprise hepatitis B surface antigen [HepB], optionally adsorbed onto aluminium phosphate). In one embodiment the immunogenic composition of the invention comprises a DTPwHepBHibMenAC vaccine where the HibMenAC component is as described above.

20 Immunogenic compositions of the invention optionally comprise additional viral antigens conferring protection against disease caused by measles and/or mumps and/or rubella and/or varicella. For example, immunogenic composition of the invention contains antigens from measles, mumps and rubella (MMR) or measles, mumps, rubella and varicella (MMRV). In an embodiment, these viral antigens are optionally present in the same container as the meningococcal and/or Hib saccharide conjugate(s). In an embodiment, these viral antigens are lyophilised.

25 In an embodiment, the immunogenic composition of the invention further comprises an antigen from *N. meningitidis* serogroup B. The antigen is optionally a capsular polysaccharide from *N. meningitidis* serogroup B (MenB) or a sized polysaccharide or oligosaccharide derived therefrom, which may be conjugated to a protein carrier. The antigen is optionally an outer membrane vesicle preparation from *N. meningitidis* serogroup B as described in EP301992, WO 01/09350, WO 04/14417, WO 04/14418 and WO 04/14419.

35 In general, the immunogenic composition of the invention may comprise a dose of each saccharide conjugate between 0.1 and 20 $\mu$ g, 2 and 10 $\mu$ g, 2 and 6 $\mu$ g or 4 and 7 $\mu$ g of saccharide.

In an embodiment, the immunogenic composition of the invention contains each *N. meningitidis* capsular saccharide at a dose of between 0.1-20 $\mu$ g; 1-10 $\mu$ g; 2-10 $\mu$ g, 2.5-5 $\mu$ g, around or exactly 5 $\mu$ g; or around or exactly 2.5 $\mu$ g. In an embodiment, the immunogenic  
5 composition of the invention comprises MenA, MenC, MenW and MenY (optionally conjugated to tetanus toxoid) in doses of 2.5, 2.5, 2.5 and 2.5 $\mu$ g respectively, 5, 5, 5 and 5 $\mu$ g respectively or 5, 5, 2.5 and 2.5 $\mu$ g respectively.

In an embodiment, the immunogenic composition of the invention for example contains  
10 the Hib saccharide conjugate at a saccharide dose between 0.1 and 9 $\mu$ g; 1 and 5 $\mu$ g or 2 and 3 $\mu$ g or around or exactly 2.5 $\mu$ g. In a further embodiment the immunogenic composition of the invention for example contains the Hib saccharide conjugate at a saccharide dose between 0.1 and 9 $\mu$ g; 1 and 5 $\mu$ g or 2 and 3 $\mu$ g or around or exactly 2.5 $\mu$ g and each of the *N. meningitidis* polysaccharide conjugates at a saccharide dose of  
15 between 2 and 20 $\mu$ g, 3 and 10 $\mu$ g, or between 4 and 7 $\mu$ g or around or exactly 5 $\mu$ g.

"Around" or "approximately" are defined as within 10% more or less of the given figure for the purposes of the invention.

20 In an embodiment, the immunogenic composition of the invention may contain a saccharide dose of the Hib saccharide conjugate which is for example less than 90%, 80%, 75%, 70%, 60%, 50%, 40%, 30%, 20% or 10% of the mean saccharide dose of at least two, three, four or each of the *N. meningitidis* saccharide conjugates. The saccharide dose of the Hib saccharide is for example between 20% and 60%, 30% and  
25 60%, 40% and 60% or around or exactly 50% of the mean saccharide dose of at least two, three, four or each of the *N. meningitidis* saccharide conjugates.

In an embodiment, the immunogenic composition of the invention contains a saccharide  
30 dose of the Hib saccharide conjugate which is for example less than 90%, 80%, 75%, 70%, 60%, 50%, 40%, 30%, 20% or 10% of the lowest saccharide dose of the at least two, three, four or each of the *N. meningitidis* saccharide conjugates. The saccharide dose of the Hib saccharide is for example between 20% and 60%, 30% and 60%, 40% and 60% or around or exactly 50% of the lowest saccharide dose of the at least two,  
35 three, four or each of the *N. meningitidis* saccharide conjugates.

In an embodiment of the invention, the saccharide dose of each of the at least two, three, four or each of the *N. meningitidis* saccharide conjugates is optionally the same, or approximately the same.

5 Examples of immunogenic compositions of the invention are compositions consisting of or comprising:

Hib conjugate and MenA conjugate and MenC conjugate, optionally at saccharide dose ratios of 1:2:2, 1:2:1, 1:4:2, 1:6:3, 1:3:3, 1:4:4, 1:5:5, 1:6:6 (w/w). Optionally, the saccharide dose of MenA is greater than the saccharide dose of MenC.

10 Hib conjugate and MenC conjugate and MenY conjugate, optionally at saccharide dose ratios of 1:2:2, 1:2:1, 1:4:2, 1:4:1, 1:8:4, 1:6:3, 1:3:3, 1:4:4, 1:5:5, 1:6:6 (w/w). Optionally, the saccharide dose of MenC is greater than the saccharide dose of MenY.

Hib conjugate and MenC conjugate and MenW conjugate, optionally at saccharide dose ratios of 1:2:2, 1:2:1, 1:4:2, 1:4:1, 1:8:4, 1:6:3, 1:3:3, 1:4:4, 1:5:5, 1:6:6 (w/w). Optionally  
15 the saccharide dose of MenC is greater than the saccharide dose of MenW.

Hib conjugate and MenA conjugate and MenW conjugate, optionally at saccharide dose ratios of 1:2:2, 1:2:1, 1:4:2, 1:4:1, 1:8:4, 1:6:3, 1:3:3, 1:4:4, 1:5:5, 1:6:6 (w/w). Optionally,  
20 the saccharide dose of MenA is greater than the saccharide dose of MenW.

Hib conjugate and MenA conjugate and MenY conjugate, optionally at saccharide dose ratios of 1:2:2, 1:2:1, 1:4:2, 1:4:1, 1:8:4, 1:6:3, 1:3:3, 1:4:4, 1:5:5, 1:6:6 (w/w). Optionally  
25 the saccharide dose of MenA is greater than the saccharide dose of MenY.

Hib conjugate and MenW conjugate and MenY conjugate, optionally at saccharide dose ratios of 1:2:2, 1:2:1, 1:1:2, 1:4:2, 1:2:4, 1:4:1, 1:1:4, 1:3:6, 1:1:3, 1:6:3, 1:3:3, 1:4:4,  
1:5:5, 1:6:6 (w/w). Optionally the saccharide dose of MenY is greater than the saccharide  
30 dose of MenW.

MenA, MenC, MenW and MenY at saccharide dose ratios of 1:1:1:1 or 2:1:1:1 or 1:2:1:1 or 2:2:1:1 or 1:3:1:1 or 1:4:1:1 (w/w).

A further aspect of the invention is a vaccine comprising the immunogenic composition of  
35 the invention and a pharmaceutically acceptable excipient.

In an embodiment, the immunogenic composition of the invention is adjusted to or buffered at, or adjusted to between pH 7.0 and 8.0, pH 7.2 and 7.6 or around or exactly  
35 pH 7.4.

The immunogenic composition or vaccines of the invention are optionally lyophilised in the presence of a stabilising agent for example a polyol such as sucrose or trehalose.

Optionally, the immunogenic composition or vaccine of the invention contains an amount of an adjuvant sufficient to enhance the immune response to the immunogen. Suitable adjuvants include, but are not limited to, aluminium salts (aluminium phosphate or aluminium hydroxide), squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, non-ionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) Nature 344:873-875.

- 10 For the *N. meningitidis* or HibMen combinations discussed above, it may be advantageous not to use any aluminium salt adjuvant or any adjuvant at all.

As with all immunogenic compositions or vaccines, the immunologically effective amounts of the immunogens must be determined empirically. Factors to be considered include the immunogenicity, whether or not the immunogen will be complexed with or covalently attached to an adjuvant or carrier protein or other carrier, route of administrations and the number of immunising dosages to be administered.

The active agent can be present in varying concentrations in the pharmaceutical composition or vaccine of the invention. Typically, the minimum concentration of the substance is an amount necessary to achieve its intended use, while the maximum concentration is the maximum amount that will remain in solution or homogeneously suspended within the initial mixture. For instance, the minimum amount of a therapeutic agent is optionally one which will provide a single therapeutically effective dosage. For bioactive substances, the minimum concentration is an amount necessary for bioactivity upon reconstitution and the maximum concentration is at the point at which a homogeneous suspension cannot be maintained. In the case of single-dosed units, the amount is that of a single therapeutic application. Generally, it is expected that each dose will comprise 1-100 $\mu$ g of protein antigen, optionally 5-50 $\mu$ g or 5-25 $\mu$ g. For example, doses of bacterial saccharides are 10-20 $\mu$ g, 5-10 $\mu$ g, 2.5-5 $\mu$ g or 1-2.5 $\mu$ g of saccharide in the conjugate.

The vaccine preparations of the present invention may be used to protect or treat a mammal (for example a human patient) susceptible to infection, by means of administering said vaccine via systemic or mucosal route. A human patient is optionally an infant (under 12 months), a toddler (12-24, 12-16 or 12-14 months), a child (2-10, 3-8

50

or 3-5 years) an adolescent (12-21, 14-20 or 15-19 years) or an adult. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Intranasal administration of vaccines for the treatment of pneumonia or otitis media is preferred (as nasopharyngeal carriage of pneumococci can be more effectively prevented, thus attenuating infection at its earliest stage). Although the vaccine of the invention may be administered as a single dose, components thereof may also be co-administered together at the same time or at different times (for instance if saccharides are present in a vaccine these could be administered separately at the same time or 1-2 weeks after the administration of a bacterial protein vaccine for optimal coordination of the immune responses with respect to each other). In addition to a single route of administration, 2 different routes of administration may be used. For example, viral antigens may be administered ID (intradermal), whilst bacterial proteins may be administered IM (intramuscular) or IN (intranasal). If saccharides are present, they may be administered IM (or ID) and bacterial proteins may be administered IN (or ID). In addition, the vaccines of the invention may be administered IM for priming doses and IN for booster doses.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

A further aspect of the invention is a vaccine kit for concomitant or sequential administration comprising two multi-valent immunogenic compositions for conferring protection in a host against disease caused by *Bordetella pertussis*, *Clostridium tetani*, *Corynebacterium diphtheriae* and *Neisseria meningitidis* and optionally *Haemophilus influenzae*. For example, the kit optionally comprises a first container comprising one or more of:

tetanus toxoid (TT),  
diphtheria toxoid (DT), and  
whole cell or acellular pertussis components

and a second container comprising:  
an immunogenic composition of the invention as described above (for instance those comprising Men or HibMen saccharide conjugate combinations).

A further aspect of the invention is a vaccine kit for concomitant or sequential administration comprising two multi-valent immunogenic compositions for conferring protection in a host against disease caused by *Streptococcus pneumoniae* and *Neisseria meningitidis* and optionally *Haemophilus influenzae*. For example, the kit optionally  
5 comprises a first container comprising:

one or more conjugates of a carrier protein and a capsular saccharide from *Streptococcus pneumoniae* [where the capsular saccharide is optionally from a pneumococcal serotype selected from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F,  
10 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F].

and a second container comprising:

an immunogenic composition of the invention as described above (for instance those comprising Men or HibMen saccharide conjugate combinations).

15 Examples of the Hib conjugate and the *N. meningitidis* polysaccharide conjugates are as described above.

Typically the *Streptococcus pneumoniae* vaccine in the vaccine kit of the present invention (or in any of the immunogenic compositions of the invention described above)  
20 will comprise saccharide antigens (optionally conjugated), wherein the saccharides are derived from at least four serotypes of pneumococcus chosen from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. Optionally, the four serotypes include 6B, 14, 19F and 23F. Optionally, at least 7 serotypes are included in the composition, for example those derived  
25 from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Optionally more than 7 serotypes are included in the composition, for instance at least 10, 11, 12, 13 or 14 serotypes. For example the composition in one embodiment includes 10 or 11 capsular saccharides derived from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F, and optionally 3 (all optionally conjugated). In an embodiment of the invention at least 13 saccharide antigens  
30 (optionally conjugated) are included, although further saccharide antigens, for example 23 valent (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention.

The pneumococcal saccharides are independently conjugated to any known carrier  
35 protein, for example CRM197, tetanus toxoid, diphtheria toxoid, protein D or any other carrier proteins as mentioned above.

Optionally, the vaccine kits of the invention comprise a third component. For example, the kit optionally comprises a first container comprising one or more of:

- 5 tetanus toxoid (TT),
- diphtheria toxoid (DT), and
- whole cell or acellular pertussis components

and a second container comprising :

- 10 one or more conjugates of a carrier protein and a capsular saccharide from *Streptococcus pneumoniae* [where the capsular saccharide is optionally from a pneumococcal serotype selected from the group consisting of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F].

- 15 and a third container comprising:  
an immunogenic composition of the invention as described above (for instance those comprising Men or HibMen saccharide conjugate combinations).

20 A further aspect of the invention is a process for making the immunogenic composition or vaccine of the invention, comprising the step of mixing the saccharides of the invention, for instance mixing *N. meningitidis* capsular saccharides from at least one, two, three or all four of serogroups A, C, W and Y conjugated to a carrier protein with a pharmaceutically acceptable excipient.

25 A further aspect of the invention is a method of immunising a human host against disease caused by bacteria, for example *N. meningitidis* and optionally *Haemophilus influenzae* infection comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine or kit of the invention, optionally using a single dose.

30 An independent aspect of the invention is a method of immunising a human host with an immunogenic composition comprising at least 2 different *N. meningitidis* capsular saccharide conjugates selected from the group consisting of serogroup A, C, W and Y (optionally MenA, C, W and Y) wherein a single dose administration (optionally to teenagers, adults or children) results in a blood test taken one month after administration  
35 giving over 50%, 60%, 70%, 80%, 90% or 95% responders in an SBA assay measuring

levels of response against MenA, MenC, MenW and/or MenY. Optionally the SBA assay is as described in Example 9 with responder assessed as described in Example 9.

5 A further independent aspect of the invention is an immunogenic composition comprising MenA , MenC, MenW and/or MenY conjugates which is capable of eliciting an immune response after a single dose such that over 50%, 60%, 70%, 80%, 90% or 95% of human subjects (children, teenagers or adults) inoculated are classified as responders in an SBA assay on blood extracted a month after inoculation (optionally using the criteria described in example 9).

10

Such an immunogenic composition optionally has the further structural characteristics described herein.

15

A further aspect of the invention is an immunogenic composition of the invention for use in the treatment or prevention of disease caused by bacteria, for example *N. meningitidis* and optionally *Haemophilus influenzae* infection.

20

A further aspect of the invention is use of the immunogenic composition or vaccine or kit of the invention in the manufacture of a medicament for the treatment or prevention of diseases caused by bacteria for example *N. meningitidis* and optionally *Haemophilus influenzae* infection.

25

The terms "comprising", "comprise" and "comprises" herein are intended by the inventors to be optionally substitutable with the terms "consisting of", "consist of" and "consists of", respectively, in every instance.

All references or patent applications cited within this patent specification are incorporated by reference herein.

30

The invention is illustrated in the accompanying examples. The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative , but do not limit the invention.



## Examples

### Example 1 – preparation of polysaccharide conjugates

5 The covalent binding of *Haemophilus influenzae* (Hib) PRP polysaccharide to TT was carried out by a coupling chemistry developed by Chu et al (Infection and Immunity 1983, 40 (1); 245-256). Hib PRP polysaccharide was activated by adding CNBr and incubating at pH10.5 for 6 minutes. The pH was lowered to pH8.75 and adipic acid dihydrazide (ADH) was added and incubation continued for a further 90 minutes. The activated PRP  
10 was coupled to purified tetanus toxoid via carbodiimide condensation using 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDAC). EDAC was added to the activated PRP to reach a final ratio of 0.6mg EDAC/mg activated PRP. The pH was adjusted to 5.0 and purified tetanus toxoid was added to reach 2mg TT/mg activated PRP. The resulting solution was left for three days with mild stirring. After filtration through a 0.45µm  
15 membrane, the conjugate was purified on a sephacryl S500HR (Pharmacia, Sweden) column equilibrated in 0.2M NaCl.

MenC –TT conjugates were produced using native polysaccharides ( of over 150kDa as measured by MALLS) or were slightly microfluidised. MenA-TT conjugates were produced  
20 using either native polysaccharide or slightly microfluidised polysaccharide of over 60kDa as measured by the MALLS method of example 2. MenW and MenY-TT conjugates were produced using sized polysaccharides of around 100-200kDa as measured by MALLS (see example 2). Sizing was by microfluidisation using a homogenizer Emulsiflex C-50 apparatus. The polysaccharides were then filtered through a 0.2µm filter.  
25

Activation and coupling were performed as described in WO96/29094 and WO 00/56360. Briefly, the polysaccharide at a concentration of 10-20mg/ml in 2M NaCl pH 5.5-6.0 was mixed with CDAPsolution (100mg/ml freshly prepared in acetonitrile/WFI, 50/50) to a final CDAP/polysaccharide ratio of 0.75/1 or 1.5/1. After 1.5 minutes, the pH was raised with  
30 sodium hydroxide to pH10.0. After three minutes tetanus toxoid was added to reach a protein/polysaccharide ratio of 1.5/1 for MenW, 1.2/1 for MenY, 1.5/1 for MenA or 1.5/1 for MenC. The reaction continued for one to two hours.

After the coupling step, glycine was added to a final ratio of glycine/PS (w/w) of 7.5/1 and  
35 the pH was adjusted to pH9.0. The mixture was left for 30 minutes. The conjugate was clarified using a 10µm Kleenpak filter and was then loaded onto a Sephacryl S400HR column using an elution buffer of 150mM NaCl, 10mM or 5mM Tris pH7.5. Clinical lots were filtered on an Opticap 4 sterilizing membrane. The resultant conjugates had an average polysaccharide:protein ratio of 1:1-1:5 (w/w).  
40

Example 1a – preparation of MenA and MenC polysaccharide conjugates of the invention

5 MenC –TT conjugates were produced using native polysaccharides (of over 150kDa as measured by MALLS) or were slightly microfluidised. MenA-TT conjugates were produced using either native polysaccharide or slightly microfluidised polysaccharide of over 60kDa as measured by the MALLS method of example 2. Sizing was by microfluidisation using a homogenizer Emulsiflex C-50 apparatus. The polysaccharides were then filtered through a 0.2µm filter.

10

In order to conjugate MenA capsular polysaccharide to tetanus toxoid via a spacer, the following method was used. The covalent binding of the polysaccharide and the spacer (ADH) is carried out by a coupling chemistry by which the polysaccharide is activated under controlled conditions by a cyanylating agent, 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP). The spacer reacts with the cyanylated PS through its hydrazino groups, to form a stable isourea link between the spacer and the polysaccharide.

15

20 A 10mg/ml solution of MenA (pH 6.0) [3.5 g] was treated with a freshly prepared 100mg/ml solution of CDAP in acetonitrile/water (50/50 (v/v)) to obtain a CDAP/MenA ratio of 0.75 (w/w). After 1.5 minutes, the pH was raised to pH 10.0. Three minutes later, ADH was added to obtain an ADH/MenA ratio of 8.9. The pH of the solution was decreased to 8.75 and the reaction proceeded for 2 hours maintaining this pH (with temperature kept at 25 °C).

20

25 The PSAAH solution was concentrated to a quarter of its initial volume and then diafiltered with 30 volumes of 0.2M NaCl using a Filtron Omega membrane with a cut-off of 10kDa, and the retentate was filtered.

25

Prior to the conjugation (carbodiimide condensation) reaction, the purified TT solution and the PSAAH solution were diluted to reach a concentration of 10 mg/ml for PSAAH and 10mg/ml for TT.

30

EDAC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide) was added to the PSAH solution (2g saccharide) in order to reach a final ratio of 0.9 mg EDAC/mg PSAAH. The pH was adjusted to 5.0. The purified tetanus toxoid was added with a peristaltic pump (in 60 minutes) to reach 2 mg TT/mg PSAAH. The resulting solution was left 60 min at +25°C under stirring to obtain a final coupling time of 120 min. The solution was neutralised by addition of 1M Tris-HCl pH 7.5 (1/10 of the final volume) and left 30 minutes at +25°C then overnight at +2°C to +8°C.

35

40 The conjugate was clarified using a 10µm filter and was purified using a Sephacryl S400HR column (Pharmacia, Sweden). The column was equilibrated in 10 mM Tris-HCl (pH 7.0), 0.075 M NaCl and the conjugate (approx. 660mL) was loaded on the column (+2°C to +8°C). The elution pool was selected as a function of optical density at 280 nm. Collection started when absorbance increased to 0.05. Harvest continued until the Kd reached 0.30. The conjugate was filter sterilised at +20°C, then stored at +2°C to +8°C. The resultant conjugate had a polysaccharide:protein ratio of 1:2-1:4 (w/w).

40

45 In order to conjugate MenC capsular polysaccharide to tetanus toxoid via a spacer, the following method was used. The covalent binding of the polysaccharide and the spacer (ADH) is carried out by a coupling chemistry by which the polysaccharide is activated under controlled conditions by a cyanylating agent, 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP). The spacer reacts with the cyanylated PS through its hydrazino groups, to form a stable isourea link between the spacer and the polysaccharide.

50

5 A 20mg/ml solution of MenC (pH6.0) (3.5 g) was treated with a freshly prepared 100mg/ml solution of CDAP in acetonitrile/water (50/50 (v/v)) to obtain a CDAP/MenC ratio of 1.5 (w/w). After 1.5 minutes, the pH was raised to pH 10.0. At activation pH 5M NaCl was added to achieve a final concentration of 2M NaCl. Three minutes later, ADH was added to obtain an ADH/MenC ratio of 8.9. The pH of the solution was decreased to 8.75 and the reaction proceeded for 2 hours (retained at 25 °C).

The PSCAH solution was concentrated to a minimum of 150 mL and then diafiltered with 30 volumes of 0.2M NaCl using a Filtron Omega membrane with a cut-off of 10kDa, and the retentate was filtered.

10 Prior to the conjugation reaction, the purified TT solution and the PSCAH solution (2g scale) were diluted in 0.2M NaCl to reach a concentration of 15 mg/ml for PSCAH and 20mg/ml for TT.

15 The purified tetanus toxoid was added to the PSCAH solution in order to reach 2 mg TT/mg PSCAH. The pH was adjusted to 5.0. EDAC (16.7 mg/ml in Tris 0.1M pH 7.5) was added with a peristaltic pump (in 10 minutes) to reach a final ratio of 0.5 mg EDAC/mg PSCAH. The resulting solution was left 110 min at +25°C under stirring and pH regulation to obtain a final coupling time of 120 min. The solution was then neutralized by addition of 1M Tris-Hcl pH 9.0 (1/10 of final volume) and left 30 minutes at +25°C then overnight at +2°C to +8°C.

20 The conjugate was clarified using a 10µm filter and was purified using a Sephacryl S400HR column (Pharmacia, Sweden). The column was equilibrated in 10 mM Tris-HCl (pH 7.0), 0.075 M NaCl and the conjugate (approx. 460mL) was loaded on the column (+2°C to +8°C). The elution pool was selected as a function of optical density at 280 nm. Collection started when absorbance increased to 0.05. Harvest continued until the Kd reached 0.20. The conjugate was filter sterilised at +20°C, then stored at +2°C to +8°C. The resultant conjugate had a polysaccharide:protein ratio of 1:2-1:4 (w/w).

#### Example 2 – determination of molecular weight using MALLS

30 Detectors were coupled to a HPLC size exclusion column from which the samples were eluted. On one hand, the laser light scattering detector measured the light intensities scattered at 16 angles by the macromolecular solution and on the other hand, an interferometric refractometer placed on-line allowed the determination of the quantity of sample eluted. From these intensities, the size and shape of the macromolecules in solution can be determined.

40 The mean molecular weight in weight ( $M_w$ ) is defined as the sum of the weights of all the species multiplied by their respective molecular weight and divided by the sum of weights of all the species.

a) Weight-average molecular weight: - $M_w$ -

$$M_w = \frac{\sum W_i \cdot M_i}{\sum W_i} = \frac{m_2}{m_1}$$

45

b) Number-average molecular weight: - $M_n$ -

$$M_n = \frac{\sum N_i \cdot M_i}{\sum N_i} = \frac{m_1}{m_0}$$

c) Root mean square radius:  $-R_w-$  and  $R^2_w$  is the square radius defined by:

5

$$R^2_w \text{ or } (r^2)_w = \frac{\sum m_i \cdot r_i^2}{\sum m_i}$$

( $-m_i-$  is the mass of a scattering centre  $i$  and  $-r_i-$  is the distance between the scattering centre  $i$  and the center of gravity of the macromolecule).

10

d) The polydispersity is defined as the ratio  $-M_w / M_n-$ .

15 Meningococcal polysaccharides were analysed by MALLS by loading onto two HPLC columns (TSKG6000 and 5000PWxl) used in combination. 25 $\mu$ l of the polysaccharide were loaded onto the column and was eluted with 0.75ml of filtered water. The polysaccharides are detected using a light scattering detector (Wyatt Dawn DSP equipped with a 10mW argon laser at 488nm) and an interferometric refractometer (Wyatt Otilab DSP  
20 equipped with a P100 cell and a red filter at 498nm).

The molecular weight polydispersities and recoveries of all samples were calculated by the Debye method using a polynomial fit order of 1 in the Astra 4.72 software.

25

### Example 3 – clinical trial comparing immunisation with Meningitec or a larger sized MenC-TT conjugate

30 A phase II , open, controlled study was carried out to compare GSK Biologicals meningococcal serogroup C conjugate vaccine (MenC) with GSK Biological's *Haemophilus influenzae* b-meningococcal serogroup C conjugate vaccine (Hib-MenC) or Meningitec®. Each dose of Meningitec® contains 10 $\mu$ g of meningococcal serogroup C oligosaccharide conjugated to 15 $\mu$ g of CRM197 and is produced by Wyeth. The GSK  
35 MenC conjugates contained native polysaccharides of about 200kDa conjugated to tetanus toxoid (TT).

The study consisted of five groups, each planned to contain 100 subjects, allocated to two parallel arms as follows:

40

In this present study, all subjects in both arms received one-fifth (1/5) of a dose of Mencevax™ ACWY and a concomitant dose of Infanrix™ hexa at 12-15 months of age

(Study Month 0). Two blood samples were collected from all subjects (Study Month 0 and Study Month 1). Arm 1 consisted of four groups from a primary vaccination study who were primed at their age of 3, 4 and 5 months with the following vaccines:

- 5 • Group K: MenC (10 µg), non-adsorbed (non-ads), tetanus toxoid (TT) conjugate and Infanrix™ hexa (MenC10-TT + Infanrix™ hexa)
- Group L: Hib (10 µg)-MenC (10 µg), non-ads TT conjugate and Infanrix™ penta (Hib10-MenC10-TT + Infanrix™ penta)
- Group M: Hib (5 µg)-MenC (5 µg), non-ads, TT conjugate and Infanrix™ penta (Hib5-MenC5-TT + Infanrix™ penta)
- 10 • Group N: Meningitec™ and Infanrix™ hexa (Meningitec™ + Infanrix™ hexa)

The two Hib-MenC-TT vaccine groups (Groups L and M) were kept blinded in the booster study as to the exact formulation of the candidate vaccine.

15 Arm 2-(Group O) consisted of age-matched subjects not previously vaccinated with a meningococcal serogroup C vaccine (naïve) but who had received routine pediatric vaccines according to the German Permanent Commission on Immunization.

#### Criteria for evaluation:

20 *Immunogenicity:* Determination of bactericidal antibody titers against meningococcal C (SBA-MenC) by a bactericidal test (cut-off: a dilution of 1:8) and ELISA measurement of antibodies against meningococcal serogroup C (assay cut-off : 0.3 µg/ml), the Hib polysaccharide PRP (assay cut-off: 0.15 µg/ml) and tetanus toxoid (assay cut-off: 0.1 IU/ml) in blood samples obtained prior to vaccination and approximately one month after vaccination in all subjects.

25

#### Statistical methods:

30 *Demographics:* Determination of mean age in months (with median, range and standard deviation [SD]), and racial and gender composition of the ATP and Total vaccinated cohorts.

#### *Immunogenicity:*

Two analyses of immunogenicity were performed based on the ATP cohort for immunogenicity (for analyses of immune memory and booster response) or the ATP cohort for safety (for analysis of persistence). These included:

35 *Evaluation of immune memory for MenC and booster response for Hib and Tetanus* (before and one month after administration of 1/5 dose of the plain polysaccharide vaccine):

- Determination of geometric mean titers and concentrations (GMTs and GMCs) with 95% confidence intervals (95% CI)
- 40 • Determination of the percentage of subjects with antibody titer/concentration above the proposed cutoffs with exact 95% CI (seropositivity/seroprotection rates)
- Investigation of antibody titers/concentration after vaccination using reverse cumulative curves
- Computation of standardized asymptotic 95% CI for the difference in
- 45 seropositivity/seroprotection rate
- between the primed group (Groups K, L, M and N) and the unprimed group (Group O)
- Determination of the geometric mean of individual ratio of SBA-MenC titer over anti-PSC concentration, with 95% CI
- 50 • Determination of the 95% CI for the post-vaccination GMT/C ratio between the groups K, L, M and the control group N for anti-PRP and anti-tetanus and between each primed group (Groups K, L, M and N) and the unprimed group (Group O) for SBA-MenC and anti-PSC using an ANOVA model

ResultsTable 1. SBA-MenC titres and anti-PSC antibody concentration after booster vaccination

5

Antibody	Group	N	GMT/C	95% CL LL	95% CL UL
SBA-MenC	K -MenC-TT	71	3508.9	2580.1	4772.2
	L - HibMenC	79	2530.1	1831.7	3494.7
	M-HibMenC	81	5385.4	4425.0	6554.2
	N -Meningitec	85	1552.6	1044.4	2307.9
	O - Control	91	9.3	6.3	13.6
Anti-PSC	K -MenC-TT	70	28.10	22.59	34.95
	L - HibMenC	71	30.01	24.09	37.38
	M-HibMenC	76	34.58	29.10	41.09
	N -Meningitec	78	16.59	12.98	21.21
	O - Control	94	3.05	2.36	3.93

10 Group K: subjects primed with MenC10-TT + Infanrix. hexa; Group L: subjects primed with Hib10-MenC10-TT + Infanrix. penta; Group M: subjects primed with Hib5-MenC5-TT + Infanrix. penta; Group N: subjects primed with Meningitec. + Infanrix. hexa; Group O: control subjects (i.e. subjects not primed with MenC conjugate vaccine)  
N: number of subjects with available results

15 Higher titres of antibodies against MenC and higher SBA titres were achieved by priming with the larger sized MenC polysaccharide conjugate vaccines (groups K, L and M) compared with the Meningitec oligosaccharide conjugate vaccine.

Table 2: Geometric mean ratio for SBA MenC titre/anti-PSC concentration

Group	Timing	N	GMR	LL	UL
K	Pre	70	49.470	34.939	70.044
	Post	66	126.138	101.419	156.882
L	Pre	76	36.528	25.849	51.621
	Post	70	90.200	70.153	115.975
M	Pre	77	51.298	36.478	72.139
	Post	74	164.950	139.304	195.318
N	Pre	84	22.571	16.521	30.837
	Post	76	90.168	67.757	119.991
O	Pre	3	91.634	0.651	12889.8
	Post	87	2.708	1.767	4.149

20 In all four primed groups (Groups K, L, M and N), the GMR increased significantly from pre to post booster vaccination indicating the presence of antibody maturation and functionality. GMR in the Group M (primed with Hib5-MenC5-TT) was higher than in the Group N (primed with Meningitec™).

25 Table 3: Persistence at 12-15 months of age just prior to administration of the booster vaccines

Endpoints	Group	N	%	Group	N	%	Difference	Value%
SBAMenC ≥ 1:8	K	79	88.6	N	91	80.2	N-K	-8.4
	L	84	93.3	N	91	80.2	N-L	-3.1
	M	85	87.1	N	91	80.2	N-M	-6.8

SBAMenC ≥ 1:128	K	79	65.8	N	91	51.6	N-K	-14.2
	L	84	56.0	N	91	51.6	N-L	-4.3
	M	85	64.7	N	91	51.6	N-M	-13.1
Anti-PSC ≥0.3µg/ml	K	79	100.0	N	91	100.0	N-K	0.0
	L	84	100.0	N	91	100.0	N-L	0.0
	M	88	98.9	N	91	100.0	N-M	1.1
Anti-PSC ≥2µg/ml	K	79	72.2	N	91	81.3	N-K	9.2
	L	84	64.3	N	91	81.3	N-L	17.0
	M	88	64.3	N	91	81.3	N-M	8.6
Anti-PRP ≥0.15µg/ml	K	81	88.9	N	91	85.7	N-K	-3.2
	L	86	96.5	N	91	85.7	N-L	-10.8
	M	90	98.9	N	91	85.7	N-M	-13.2
Anti-PRP ≥1µg/ml	K	81	33.3	N	91	28.6	N-K	-4.8
	L	86	55.8	N	91	28.6	N-L	-27.2
	M	90	74.4	N	91	28.6	N-M	-45.9
Anti-tetanus ≥0.1 IU/ml	K	81	100.0	N	91	96.7	N-K	-3.3
	L	86	100.0	N	91	96.7	N-L	-3.3
	M	90	100.0	N	91	96.7	N-M	-3.3

Group K: subjects primed with MenC10-TT + Infanrix™ hexa; Group L: subjects primed with Hib10-MenC10-TT + Infanrix™ penta; Group M: subjects primed with Hib5-MenC5-TT + Infanrix™ penta; Group N: subjects primed with Meningitec™ + Infanrix™ hexa;

5 N: number of subjects with available results

Higher SBA titres against MenC were achieved by priming with the larger size of MenC (groups K, L and M) compared to priming with the MenC-oligosaccharide conjugate Meningitec™.

10

#### Immune memory (ATP cohort for immunogenicity)

Administration of 1/5 dose of the plain polysaccharide ACWY vaccine elicited very high SBA-MenC titer in all four primed groups with 98.7-100% and 97.5-100% of subjects primed with a candidate vaccine regimen exhibiting titers ≥1:8 and ≥1:128, respectively. In the group primed with the Meningitec™ regimen, there was a trend for a lower percentage of subjects with titers ≥1:128 (91.8%). In comparison, 17.6% of unprimed subjects had SBA MenC titers ≥ 1:8 and ≥1:128.

15

#### Example 4 Phase II clinical trial on HibMenAC –TT conjugate vaccine mixed with DTPw-HepB

20

**Study design:** Open, randomized (1:1:1:1:1), single centre study with five groups. The five groups received the following vaccination regimen respectively, at 6, 10 and 14 weeks of age.

25

- Tritanrix™-HepB/Hib-MenAC 2.5/2.5/2.5: henceforth referred to as 2.5/2.5/2.5

- Tritanrix™-HepB/Hib-MenAC 2.5/5/5: henceforth referred to as 2.5/5/5
- Tritanrix™-HepB/Hib-MenAC 5/5/5: henceforth referred to as 5/5/5
- Tritanrix™-HepB + Hiberix™: henceforth referred to as Hiberix
- Tritanrix.-HepB/Hiberix™ + Meningitec™: henceforth referred to as Meningitec

5 Blood samples were taken at the time of the first vaccine dose (Pre) and one month after the third vaccine dose (Post-dose 3).

Tritanrix is a DTPw vaccine marketed by GlaxoSmithKline Biologicals S.A.

10 105 subjects were used in each of the five groups giving a total of 525 subjects in the study.

Table 4 Content of GSK vaccine formulations

Components per dose (0.5ml)	2.5/2.5/2.5*	2.5/5/5	5/5/5
Hib capsular polysaccharide PRP conjugated to tetanus toxoid (TT)	2.5µg	2.5µg	5µg
<i>Neisseria meningitidis</i> A capsular polysaccharide (PSA) conjugated to TT	2.5µg	5µg	5µg
<i>Neisseria meningitidis</i> C capsular polysaccharide (PSC) conjugated to TT	2.5µg	5µg	5µg

15 \* The 2.5/2.5/2.5 vaccine was a dose dilution of GSK Biologicals' Hib-MenAC 5/5/5 vaccine containing 2.5µg of each of PRP-TT, MenA-TT and MenC-TT.

The Hib-MenAC vaccine formulations were mixed extemporaneously with Tritanrix-HepB.  
 20 GSK Biologicals' combined diphtheria-tetanus-whole cell *Bordetella pertussis* – hepatitis B (DTPw-HB) vaccine (Tritanrix-HepB) contains not less than 30 International Units (IU) of diphtheria toxoid, not less than 60 IU of tetanus toxoid, not less than 4IU of killed *Bordetella pertussis* and 10µg of recombinant hepatitis B surface antigen.

25 **Reference therapy, dose, mode of administration, lot No.:**

*Vaccination schedule/site:* One group received Tritanrix.-HepB vaccine intramuscularly in the left thigh and Hiberix. intramuscularly in the right thigh at 6, 10 and 14 weeks of age. Another group received Tritanrix.-HepB/Hiberix. vaccine intramuscularly in the left thigh and Meningitec. vaccine intramuscularly in the right thigh at 6, 10 and 14 weeks of age.



Vaccine/composition/dose/lot number: The Tritanrix.-HepB vaccine used was as described above.

One dose (0.5 ml) of GSK Biologicals' *Haemophilus influenzae* type b conjugate vaccine: Hiberix™ contained 10 µg of PRP conjugated to tetanus toxoid. In the Hiberix™ Group, it was mixed with sterile diluent and in the Meningitec™ Group it was mixed with Tritanrix™-HepB.

One dose (0.5 ml) of Wyeth Lederle's MENINGITEC™ vaccine contained: 10 µg of capsular oligosaccharide of meningococcal group C conjugated to 15 µg of *Corynebacterium diphtheria* CRM197 protein and aluminium as salts.

10

Results – immune responses generated against Hib, MenA and MenC

Table 5a Anti – PRP (µg/ml)

Group	2.5/2.5/2.5			2.5/5/5			5/5/5			Hiberix™			Meningitec™		
	%	95%CL		%	95%CL		%	95%CL		%	95%CL		%	95%CL	
	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL
%≥0.15	100	96.5	100	99.0	94.8	100	100	96.5	100	100	96.5	100	100	96.5	100
GMC	20.80	15.96	27.10	22.62	17.72	28.88	19.36	15.33	24.46	38.55	29.93	49.64	10.94	8.62	13.88

15

Table 5b SBA –MenC

Group	2.5/2.5/2.5			2.5/5/5			5/5/5			Hiberix™			Meningitec™		
	%	95%CL		%	95%CL		%	95%CL		%	95%CL		%	95%CL	
	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL
%≥1:8	99	94.7	100	100	96.5	100	100	96.5	100	2.9	0.6	8.4	100	96.5	100
GMT	3132	2497	3930	4206	3409	5189	3697	3118	4384	4.7	3.9	5.6	4501	3904	5180

20

Table 5c SBA MenA

Group	2.5/2.5/2.5			2.5/5/5			5/5/5			Hiberix™			Meningitec™		
	%	95%CL		%	95%CL		%	95%CL		%	95%CL		%	95%CL	
	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL
%≥1:8	99.7	91.9	99.7	100	95.8	100	100	96.2	100	6.8	2.5	14.3	9.1	4.0	17.1
GMT	316.7	251.4	398.9	418.5	358.6	488.5	363	310.5	424.4	5.6	4.3	7.4	5.6	4.4	7.2

Table 5d Anti-PSC (µg/ml)

Group	2.5/2.5/2.5			2.5/5/5			5/5/5			Hiberix™			Meningitec™		
	%	95%CL		%	95%CL		%	95%CL		%	95%CL		%	95%CL	
	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL	GMC/T	LL	UL

	GMC/T LL UL	GMC/T LL UL	GMC/T LL UL	GMC/T LL UL	
% $\geq$ 0.3	100 96.5 100	100 96.4 100	100 96.5 100	8.2 3.6 15.6	100 96.5 100
GMC	49.03 43.24 55.59	71.11 62.49 80.92	61.62 54.88 69.20	0.17 0.15 0.19	58.02 51.42 65.46

Table 5e Anti – PSA ( $\mu$ g/ml)

Group	2.5/2.5/2.5	2.5/5/5	5/5/5	Hiberix™	Meningitec™
	% 95%CL GMC/T LL UL	% 95%CL GMC/T LL UL	% 95%CL GMC/T LL UL	% 95%CL GMC/T LL UL	% 95%CL GMC/T LL UL
% $\geq$ 0.3	100 96.4 100	100 96.5 100	99.0 94.8 100	1.0 0.0 5.4	5.9 2.2 12.5
GMC	18.10 15.34 21.35	26.51 22.93 30.79	23.40 20.05 27.30	0.15 0.15 0.15	0.17 0.15 0.18

5 Conclusion

A comparison of the immunogenicity results achieved using the oligosaccharide MenC-CRM197 conjugate vaccine and the three GSK formulations which contain polysaccharide MenA-TT and MenC –TT conjugates showed that the polysaccharide Men conjugates were able to elicit a good immunogenic response similar to that achieved using the oligosaccharide conjugate vaccine Meningitec. All formulations tested gave a response to MenC in 100% of patients.

15 Example 5 – Phase II clinical trial administering Hib MenCY concomitantly with Infanrix penta according to a 2, 3 and 4 month schedule

**Study design:** A Phase II, open (partially double-blind\*) randomized controlled multi-center study with 5 groups receiving a three-dose primary schedule with vaccines as follows:

20 Group Hib-MenCY 2.5/5/5: Hib-MenCY (2.5/5/5) + Infanrix™ penta

Group Hib-MenCY 5/10/10: Hib-MenCY (5/10/10) + Infanrix™ penta

Group Hib-MenCY 5/5/5: Hib-MenCY (5/5/5) + Infanrix™ penta

Group Hib-MenC: Hib-MenC (5/5) + Infanrix™ penta

Group Menjugate: Menjugate™\*\* + Infanrix™ hexa (control).

25 \*Hib-MenCY 2.5/5/5, Hib-MenCY 5/10/10 and Hib-MenC were administered in a double-blind manner while the Hib-MenCY 5/5/5 group and the Menjugate group were open. The 2.5/5/5, 5/10/10 and 5/5/5 formulations of Hib-MenCY contain MenC native polysaccharides and MenY polysaccharides which are microfluidized.

\*\*Menjugate™ contains 10 $\mu$ g of MenC oligosaccharides conjugated to 12.5-25 $\mu$ g of

30 CRM197 per dose and is produced by Chiron.

Vaccination at +/- 2, 3, 4 months of age (Study Month 0, Month 1 and Month 2), and blood samples (3.5ml) from all subjects prior to and one month post primary vaccination (Study Month 0 and Month 3).

- 5 **Study vaccine, dose, mode of administration, lot number:** Three doses injected intramuscularly at one month intervals, at approximately 2, 3 and 4 months of age as follows:

10 **Table 6: Vaccines administered (study and control), group, schedule/site and dose**

Group	Schedule (months of age)	Vaccine dose administered Site- Left upper thigh	Concomitant vaccine administered Site Right upper thigh
Hib-MenCY 2.5/5/5	2, 3, and 4	Hib (2.5µg)- MenC-TT (5µg)-MenY-TT (5 µg)	DTPa-HBV-IPV (Infanrix™ penta)
Hib-MenCY 5/10/10	2, 3, and 4	Hib (5µg)-MenC-TT (10µg)-MenY-TT (10µg)	DTPa-HBV-IPV (Infanrix™ penta)
Hib-MenCY 5/5/5	2, 3, and 4	Hib (5µg)-MenC-TT (5µg)-MenY-TT (5µg)	DTPa-HBV-IPV (Infanrix™ penta)
Hib-MenC	2, 3, and 4	Hib (5µg)-Men C (5µg)	DTPa-HBV-IPV (Infanrix™ penta)
Menjugate™	2, 3, and 4	Menjugate™	DTPa-HBV-IPV/Hib (Infanrix™hexa)

15 **Immunogenicity:** Measurement of antibody titres/concentrations against each vaccine antigen:

Prior to the first dose (Month 0) and approximately one month after the third dose (Month 3) in all subjects for: SBA-MenC and SBA-MenY, anti-PSC and anti-PSY, anti-PRP, anti-T, anti-FHA, anti-PRN and anti-PT. Using serum bactericidal activity against *N.*

*meningitidis* serogroups C and Y (SBA-MenC and SBA-MenY cut-off: 1:8 and 1:128);

- 20 ELISA assays with cut-offs:  $\geq 0.3$  µg/ml and  $\geq 2$ µg/ml for anti- *N. meningitidis* serogroups C and Y polysaccharides (anti-PSC IgG and anti-PSY IgG);  $\geq 0.15$  µg/ml and  $\geq 1.0$ µg/ml for Hib polysaccharide polyribosil-ribitol-phosphate (anti-PRP IgG); 5EL.U/ml for anti-FHA, anti-PRN, anti-PT;  $\geq 0.1$  IU/ml anti-tetanus toxoid (anti-TT). Only at one month after the third dose (Month 3) in all subjects for: anti-D, anti-HBs and anti-polio 1, 2 and 3. Using
- 25 ELISA assays with cut-offs: 0.1 IU/ml for anti-diphtheria (anti-D);  $\geq 10$  mIU/ml for

antihepatitis B (anti-HBs); and microneutralization test cut-off: 1:8 for anti-polio type 1, 2 and 3 (anti-polio 1, 2 and 3).

**Statistical methods:**

5 The seroprotection/seropositivity rates and geometric mean concentrations/titres (GMCs/GMTs) with 95% confidence intervals (95% CI) were computed per group, for SBA-MenC, anti-PSC, SBA-MenY, anti-PSY, anti-PRP, anti-Tetanus, anti-PT, anti-FHA and anti-PRN prior to and one month after vaccination; for anti-Diphtheria, anti-HBs, anti-Polio 1, anti-Polio 2 and anti-Polio 3 one month after vaccination. Vaccine response  
 10 (appearance of antibodies in subjects initially seronegative or at least maintenance of antibody concentrations in subjects initially seropositive) with 95% CI for anti-PT, anti-PRN and anti-FHA were also computed one month after vaccination. Reverse cumulative curves for each antibody at Month 3 are also presented. The differences between the Hib-MenCY and the Hib- MenC groups, compared with the Menjugate™ control group were  
 15 evaluated in an exploratory manner for each antibody, except for SBA-MenY and anti-PSY, in terms of (1) the difference between the Menjugate™ group (minus) the Hib-MenCY and Hib-MenC groups for the percentage of subjects above the specified cut-offs or with a vaccine response with their standardized asymptotic 95% CI, (2) the GMC or  
 20 GMC or GMT ratios of the Menjugate™ group over the Hib-MenCY and Hib-MenC groups with their 95% CI. The same comparisons were done to evaluate the difference between each pair of Hib-MenCY formulations for anti-PRP, SBA-MenC, anti-PSC, SBA-MenY, anti-PSY and anti-TT antibodies.

The overall incidences of local and general solicited symptoms were computed by group according to the type of symptom, their intensity and relationship to vaccination (as  
 25 percentages of subjects reporting general, local, and any solicited symptoms within the 8 days following vaccination and their exact 95% CI). Incidences of unsolicited symptoms were computed per group. For Grade 3 symptoms, onset ≤48 hours, medical attention, duration, relationship to vaccination and outcomes were provided. Serious Adverse Events were fully described.

30 **Seroprotection/seropositivity rates & GMC/Ts (ATP cohort for immunogenicity)**

35 Table 7a Anti – PRP (µg/ml)

Group	N	% ≥ 0.15	LL	UL	≥1	LL	UL	GMC	LL	UL
Hib MenCY 2.5/5/5	67	100.0	94.6	100.0	98.5	92.0	100.0	9.01	7.25	11.21

Hib MenCY 5/10/10	67	100.0	94.6	100.0	98.5	92.0	100.0	9.49	7.72	11.65
Hib MenCY 5/5/5	70	100.0	94.9	100.0	98.6	92.3	100.0	8.08	6.53	9.98
Hib MenC	74	100.0	95.1	100.0	98.6	92.7	100.0	10.44	8.49	12.83
Menjugate™	71	100.0	94.9	100.0	80.3	69.1	88.8	2.60	1.97	3.43

Table 7b SBA –MenC (Titre)

5

Group	N	% $\geq$ 1:8	LL	UL	$\geq$ 1:128	LL	UL	GMT	LL	UL
Hib MenCY 2.5/5/5	70	100.0	94.9	100.0	95.7	88.0	99.1	1005.8	773.5	1308.0
Hib MenCY 5/10/10	67	100.0	94.6	100.0	94.0	85.4	98.3	1029.8	799.7	1326.0
Hib MenCY 5/5/5	71	100.0	94.9	100.0	94.4	86.2	98.4	906.9	691.3	1189.8
Hib MenC	74	100.0	95.1	100.0	95.9	88.6	99.2	871.0	677.3	1120.0
Menjugate™	71	100.0	94.9	100.0	100.0	94.9	100.0	3557.6	2978.8	4248.8

Table 7c Anti-PSC ( $\mu$ g/ml)

Group	N	% $\geq$ 0.3	LL	UL	$\geq$ 2	LL	UL	GMC	LL	UL
Hib MenCY 2.5/5/5	69	100.0	94.8	100.0	100.0	94.8	100.0	21.70	18.36	25.65
Hib MenCY 5/10/10	66	100.0	94.6	100.0	100.0	94.6	100.0	27.26	23.26	31.95
Hib MenCY 5/5/5	70	100.0	94.9	100.0	100.0	94.9	100.0	19.02	16.49	21.93
Hib MenC	74	100.0	95.1	100.0	100.0	95.1	100.0	21.08	18.24	24.35
Menjugate™	71	100.0	94.9	100.0	100.0	94.9	100.0	38.49	33.64	44.05

10 Table 7d SBA-MenY (Titre)

Group	N	% $\geq$ 1:8	LL	UL	$\geq$ 1:128	LL	UL	GMT	LL	UL
Hib MenCY 2.5/5/5	69	97.1	89.9	99.6	92.8	83.9	97.6	470.7	351.1	631.2
Hib MenCY 5/10/10	66	97.0	89.5	99.6	86.4	75.7	93.6	437.1	322.0	593.4.8
Hib MenCY 5/5/5	71	98.6	92.4	100.0	95.8	88.1	99.1	635.3	501.5	804.8
Hib MenC	74	21.6	12.9	32.7	13.5	6.7	23.5	9.3	6.3	13.7
Menjugate™	71	19.7	11.2	30.9	9.9	4.1	19.3	7.5	5.4	10.4

Table 7e Anti – PSY ( $\mu$ g/ml)

Group	N	% $\geq$ 0.3	LL	UL	$\geq$ 2	LL	UL	GMC	LL	UL
Hib MenCY 2.5/5/5	69	100.0	94.8	100.0	100.0	94.8	100.0	26.86	22.86	31.56
Hib MenCY 5/10/10	66	100.0	94.6	100.0	100.0	94.6	100.0	37.02	31.84	43.04
Hib MenCY 5/5/5	70	100.0	94.9	100.0	100.0	94.9	100.0	23.57	19.94	27.86

Hib MenC	74	8.1	3.0	16.8	4.1	0.8	11.4	0.19	0.15	0.25
Menjugate™	71	5.6	1.6	13.8	1.4	0.0	7.6	0.17	0.15	0.19

Table 7f Anti-tetanus (IU/ml)

Group	N	% ≥ 0.1	LL	UL	GMC	LL	UL
Hib MenCY 2.5/5/5	68	100.0	94.7	100.0	3.06	2.63	3.55
Hib MenCY 5/10/10	67	100.0	94.6	100.0	3.25	2.88	3.68
Hib MenCY 5/5/5	70	100.0	94.9	100.0	2.97	2.59	3.41
Hib MenC	74	100.0	95.1	100.0	3.15	2.73	3.64
Menjugate™	71	100.0	94.9	100.0	1.66	1.39	1.97

Group Hib-MenCY 2.5/5/5: Hib-MenCY (2.5/5/5) + Infanrix™ penta

5 Group Hib-MenCY 5/10/10: Hib-MenCY (5/10/10) + Infanrix™ penta

Group Hib-MenCY 5/5/5: Hib-MenCY (5/5/5) + Infanrix™ penta

Group Hib-MenC: Hib-Men (5/5) + Infanrix™ hexa

Group Menjugate: Menjugate™ + Infanrix™ penta

10 N = number of subjects with available results. % = percentage of subjects with concentration/titre within the specified range

GMC/T: geometric mean concentration/titre 95% CI = 95% confidence interval; LL = Lower Limit; UL = Upper Limit

### Conclusion

15

The MenC and Y polysaccharide conjugates produced a good immune response in all subjects with 100% of subjects producing above 0.3 µg/ml responses against MenC and MenY.

20 Example 6 – Phase II clinical trial comparing three formulations of MenACWY-TT with Meningitec MenC-CRM197 oligosaccharide-conjugate vaccine.

25 This example reports a phase II, open (partially-blind), randomized, controlled dose-range study to evaluate the immunogenicity of three different formulations of GlaxoSmithKline Biological's meningococcal serogroups A, C, W-135, Y tetanus toxoid conjugate (MenACWY-TT) vaccine in comparison to a MenC oligosaccharide-CRM197 conjugate vaccine (Meningitec™) when given as one dose to children aged 12-14 months.

30 The clinical trial was an open (partially double-blind\*), controlled, multicentric study in which eligible subjects of 12-14 months were randomized (1:1:1:1) to one of four parallel groups of 50 subjects to receive a single primary dose at Visit 1 as follows:

35 Form 1T: MenACWY-TT at a dose of 2.5µg of MenA polysaccharide conjugated to tetanus toxoid (TT), 2.5µg of MenC polysaccharide conjugated to TT, 2.5µg of MenW polysaccharide conjugated to TT and 2.5µg of MenY polysaccharide conjugated to TT.

Form 2T: MenACWY-TT at a dose of 5µg of MenA polysaccharide conjugated to TT, 5µg of MenC polysaccharide conjugated to TT, 5µg of MenW polysaccharide conjugated to TT and 5µg of MenY polysaccharide conjugated to TT.

Form 3T: MenACWY-TT at a dose of 2.5µg of MenA polysaccharide conjugated to TT, 10µg of MenC polysaccharide conjugated to TT, 2.5µg of MenW polysaccharide conjugated to TT and 2.5µg of MenY polysaccharide conjugated to TT.

Ctrl T: 10µg MenC oligosaccharide conjugated to 12.5-25µg CRM197 (Meningitec).

\*The three different MenACWY-TT formulations were administered in a double-blind manner.

*Vaccination schedule/site:* A single vaccine dose was administered intramuscularly in the left deltoid at Visit 1 (Study Month 0) according to randomized assignment. All candidate vaccines were supplied as a lyophilized pellet in a monodose vial (0.5 ml after reconstitution with the supplied saline diluent).

*Immunogenicity:* Measurement of titers/concentrations of antibodies against meningococcal vaccine antigen components in blood samples obtained prior to the study vaccine dose (Month 0) and approximately one month after the study vaccine dose (Month 1) in all subjects. Determination of bactericidal antibody titers against *N. meningitidis* serogroups A, C, W-135 and Y (SBA-MenA, SBA-MenC, SBA-MenW and SBA-MenY) by a bactericidal test (assay cut-offs: a dilution of 1:8 and 1:128) and ELISA measurement of antibodies against *N. meningitidis* serogroups A, C, W-135 and Y (anti-PSA, anti-PSC, anti-PSW and anti-PSY, assay cut-offs  $\geq 0.3\mu\text{g/ml}$  and  $\geq 2\mu\text{g/ml}$ ), and tetanus toxoid (anti-tetanus, assay cut-off 0.1 IU/ml).

## Results

Antibody response in terms of the percentage of SBA-MenA, SBA-MenC, SBA-MenW and SBA-MenY responders one month after vaccination (the primary endpoint) is shown in Table 8. A response is defined as greater than or equal to a 4-fold increase for seropositive subjects or seroconversion for seronegative subjects before vaccination.

Table 8: Vaccine responses for SBA antibody one month after vaccination

Antibody	Group	N	%	LL	UL
SBA-MenA	Form 1T	42	61.9	45.6	76.4
	Form 2T	39	82.1	66.5	92.5
	Form 3T	40	62.5	45.8	77.3
	Meningitec™	36	11.1	3.1	26.1
SBA-MenC	Form 1T	46	97.8	88.5	99.9
	Form 2T	43	100.0	91.8	100.0

	Form 3T	44	95.5	84.5	99.4
	Meningitec™	49	91.8	80.4	97.7
SBA-MenW	Form 1T	45	100.0	92.1	100.0
	Form 2T	43	97.7	87.7	99.9
	Form 3T	45	100.0	92.1	100.0
	Meningitec™	46	15.2	6.3	28.9
SBA-MenY	Form 1T	47	97.9	88.7	99.9
	Form 2T	44	88.6	75.4	96.2
	Form 3T	45	93.3	81.7	98.6
	Meningitec™	49	4.1	0.5	14.0

Table 9 shows the numbers of subjects achieving SBA titres over cutoff points of 1:8 and 1:128 as well as GMTs.

5

Table 9: Seropositivity rates and GMTs for SBA antibodies one month after vaccination

	Group	N	%	≥1:8 LL	UL	%	≥1:128 LL	UL	GMT
SBA-MenA	Form 1T	46	100	92.3	100	100	92.3	100	1457.3
	Form2T	45	100	92.1	100	97.8	88.2	99.9	1776.9
	Form3T	48	97.9	88.9	99.9	97.9	88.9	99.9	1339.5
	Meningitec™	41	51.2	35.1	67.1	43.9	28.5	60.3	42.8
SBA-MenC	Form 1T	47	97.9	88.7	99.9	78.7	64.3	89.3	281.3
	Form2T	45	100	92.1	100	84.4	70.5	93.5	428.6
	Form3T	47	95.7	85.5	99.5	85.1	71.7	93.8	478.4
	Meningitec™	50	94.0	83.5	98.7	62.0	47.2	75.3	200.1
SBA-MenW	Form 1T	47	100	92.5	100	100	92.5	100	2529.1
	Form2T	45	100	92.1	100	100	92.1	100	2501.6
	Form3T	48	100	92.6	100	97.9	88.9	99.9	2300.2
	Meningitec™	48	27.1	15.3	41.8	6.3	1.3	17.2	9.4
SBA-MenY	Form 1T	47	100	92.5	100	100	92.5	100	1987.4
	Form2T	45	100	92.1	100	100	92.1	100	2464.8
	Form3T	48	100	92.6	100	97.9	88.9	99.9	2033.7
	Meningitec™	49	49.0	34.4	63.7	28.6	16.6	43.3	25.0

10

Vaccination with all three formulations of the ACWY-TT polysaccharide conjugate led to good SBA responses against MenA, MenC, MenW and MenY with 95-100% of subjects with titres greater than 1:8. In particular, the 5/5/5/5 and 2.5/10/2.5/2.5 formulations of the polysaccharide conjugates produced a higher response against MenC than the oligosaccharide Meningitec™ vaccine as seen by a higher proportion of subjects having a titre greater than 1:128 and the GMT readings.



Table 10 Seropositivity rates and GMCs for anti polysaccharide antibodies one month after vaccination

5

	Group	N	%	≥0.3µg/ml LL	UL	%	≥2µg/ml LL	UL	GMC µg/ml
Anti-MenA	Form 1T	47	93.6	82.5	98.7	68.1	52.9	80.9	2.35
	Form2T	45	100	92.1	100	64.4	48.8	78.1	3.11
	Form3T	48	95.8	85.7	99.5	37.5	24.0	52.6	1.65
	Meningitec™	50	10.0	3.3	21.8	2.0	0.1	10.6	0.18
Anti-MenC	Form 1T	47	100	92.5	100	100	92.5	100	9.57
	Form2T	45	100	92.1	100	100	92.1	100	12.53
	Form3T	47	100	92.5	100	97.9	88.7	99.9	19.29
	Meningitec™	49	98.0	89.1	99.9	93.9	83.1	98.7	7.95
Anti-MenW	Form 1T	47	100	92.5	100	80.9	66.7	90.9	4.56
	Form2T	45	100	92.1	100	93.3	81.7	98.6	6.83
	Form3T	48	93.8	82.8	98.7	72.9	58.2	84.7	2.88
	Meningitec™	50	0.0	0.0	7.1	0.0	0.0	7.1	0.15
Anti-MenY	Form 1T	47	100	92.5	100	97.9	88.7	99.9	8.90
	Form2T	45	100	92.1	100	100	92.1	100	12.78
	Form3T	47	97.9	88.7	99.9	87.2	74.3	95.2	5.67
	Meningitec™	50	2.0	0.1	10.6	0.0	0.0	7.1	0.15

All three formulations of the ACWY-TT polysaccharide conjugate vaccine produced good immune responses against MenA, MenC, MenW and MenY with between 93% and 100% of subjects achieving titres greater than 0.3µg/ml. Higher GMC readings were achieved using the 5/5/5/5 and 2/5/10/2.5/2.5 formulations of the ACWY-TT polysaccharide conjugate vaccine in comparison with Meningitec™.

Example 7 – comparison of immunogenicity of native and sized MenY polysaccharide conjugates

Mice (female DBA/2 of 6-8 wk) received two injections, 2 weeks apart, of PSY-TT by the subcutaneous route. Blood samples were taken 14 days after the second injection in order to perform anti-PSY ELISA and SBA using S1975 menY strain. Per injection, mice received 1 µg of PSY-TT( lyo non-ads formulation).

The conjugates described in table 11 were used .

Table 11

Conjugates	ENYTT012	ENYTT014	ENYTT015 bis
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PSY microfluidisation	NO	Yes (40 cycles)	Yes (20 cycles)
TT/PS ratio	1/1	1/1	1/1

### Results

The results (Figure 1) show a trend towards higher immunogenicity for conjugates prepared using sized PSY. Figure 1A shows the GMC results obtained in an ELISA for antisera raised against conjugates prepared from native MenY (ENYTT012), microfluidised MenY – 40 cycles (ENYTT014) and microfluidised MenY – 20 cycles (ENYTT015 bis). Higher GMCs were obtained where the MenY-TT was prepared from microfluidised MenY.

Similar results were obtained when the antisera were assessed by SBA assay (Figure 1B). Again the higher GMT values were achieved using conjugates prepared from microfluidised MenY.

### Example 8 – Clinical trial assessing the effect of a linker in MenA in a MenACWY conjugate vaccine

A single dose of different formulations of MenACWY vaccine was administered to teenagers of 15-19 years in 5 groups of 25 subjects in a 1:1:1:1:1 randomised trial. The formulations tested were:

F1 – MenACWY conjugated to tetanus toxoid with the MenA conjugate containing an AH spacer – 5/5/5/5µg

F2 – MenACWY conjugated to tetanus toxoid with the MenA conjugate containing an AH spacer – 2.5/5/2.5/2.5µg

F3 – MenACWY conjugated to tetanus toxoid with the MenA conjugate containing an AH spacer – 5/5/2.5/2.5µg

F4 – MenACWY conjugated to tetanus toxoid with no spacer in any conjugate – 5/5/5/5µg

Control group – Mencevax™ ACWY

On day 30 after inoculation, a blood sample was taken from the patients.

The blood samples were used to assess the percentage of SBA-MenA, SBA-MenC, SBA-MenW135 and SBA-MenY responders one month after the vaccine dose. A vaccine response was defined as 1) for initially seronegative subjects – a post-vaccination antibody titre  $\geq 1/32$  at 1 month or 2) for initially seropositive subjects – antibody titre of  $\geq 4$  fold the pre-vaccination antibody titre.

### Results

As shown in Table 13, the use of a spacer in the MenA conjugate led to an increased immune response against MenA. The percentage of responders rose from 66% to 90-95% when the AH spacer was added. This was reflected in an increase in SBA GMT from 4335 to 10000 and an increase in GMC from 5 to 20-40. Surprisingly, the use of a AH spacer also led to an increased immune response against MenC as seen by an increase in the percentage of responders and an increase in the SBA GMT. An increase could also be seen in the SBA-GMT against MenY (6742-7122) and against MenW (4621-5418) when a spacer was introduced.

10 Table 12

Formulation	% SBA MenA responders	SBA-MenA GMT	Anti-PSA GMC $\mu\text{g/ml}$ ELISA
F 1 5AH/5/5/5	90.9	9805	20.38
F2 2.5AH/5/2.5/2.5	75	8517	29.5
F3 5AH/5/2.5/2.5	95.5	10290	47.83
F4 5/5/5/5	66.7	4335	5.46
Mencevax™	85.7	8022	27.39
Formulation	% SBA MenC responders	SBA-MenC GMT	Anti-PSC GMC $\mu\text{g/ml}$ ELISA
F 1 5AH/5/5/5	69.6	3989	12.11
F2 2.5AH/5/2.5/2.5	81.8	3524	12.78
F3 5AH/5/2.5/2.5	81.8	3608	8.4
F4 5/5/5/5	73.9	2391	8.84
Mencevax™	90.0	5447	38.71
Formulation	% SBA MenW responders	SBA-MenW GMT	Anti-PSW GMC $\mu\text{g/ml}$ ELISA
F 1 5AH/5/5/5	95	5418	9.65
F2 2.5AH/5/2.5/2.5	85	4469	14.55
F3 5AH/5/2.5/2.5	95.5	4257	6.39
F4 5/5/5/5	95.5	4621	10.7
Mencevax™	86.4	2714	13.57
Formulation	% SBY MenY responders	SBA-MenY GMT	Anti-PSY GMC $\mu\text{g/ml}$ ELISA
F 1 5AH/5/5/5	91.3	7122	16.3
F2 2.5AH/5/2.5/2.5	87.5	5755	12.52
F3 5AH/5/2.5/2.5	80	5928	8.88
F4 5/5/5/5	91.3	6742	13.88
Mencevax™	91.7	4854	21.02

Example 9 – Clinical trial assessing the effect of a linker in MenA and MenC conjugates in a MenACWY conjugate vaccine

5 A single dose of different formulations of MenACWY vaccine was administered to teenagers of 15-19 years in 5 groups of 25 subjects in a 1:1:1:1:1 randomised trial. The formulations tested were:

- 10 F1 – MenACWY conjugated to tetanus toxoid with the MenA and MenC conjugates containing an AH spacer – 2.5/2.5/2.5/2.5µg  
 F2 – MenACWY conjugated to tetanus toxoid with the MenA and MenC conjugates containing an AH spacer – 5/5/2.5/2.5µg  
 F3 – MenACWY conjugated to tetanus toxoid with the MenA and MenC conjugates containing an AH spacer – 5/5/5/5µg  
 15 F4 – MenACWY conjugated to tetanus toxoid with the MenA conjugate containing an AH spacer – 5/5/5/5µg  
 Control group – Mencevax™ ACWY

20 On day 30 after inoculation, a blood sample was taken from the patients.

The blood samples were used to assess the percentage of SBA-MenA, SBA-MenC, SBA-MenW135 and SBA-MenY responders one month after the vaccine dose. A vaccine response was defined as 1) for initially seronegative subjects – a post-vaccination antibody titre  $\geq 1/32$  at 1 month or 2) for initially seropositive subjects – antibody titre of  $\geq 4$  fold the pre-vaccination antibody titre.

25

Results

30 The introduction of an AH spacer into the MenC conjugate led to an increase in the immune response against MenC as shown in Table 14. This is demonstrated by an increase in SBA GMT from 1943 to 4329 and an increase in anti-PSC GMC from 7.65 to 13.13. Good immune responses against MenA, MenW and MenY were maintained.

35 Table 13

Formulation	% SBA MenA responders	SBA-MenA GMT	Anti-PSA GMC µg/ml ELISA
F 12.5AH/2.5AH/2.5/2.5	75	8417	20.23
F2 5AH/5AH/2.5/2.5	72	6299	16.07
F3 5AH/5AH/5/5	87	9264	27.26
F4 5AH/5/5/5	77.3	9632	20.39
Mencevax™	78.3	8284	12.93
Formulation	% SBA MenC	SBA-MenC GMT	Anti-PSC GMC

	responders		µg/ml ELISA
F 12.5AH/2.5AH/2.5/2.5	88	3619	12.82
F2 5AH/5AH/2.5/2.5	88	2833	13.32
F3 5AH/5AH/5/5	95.8	4329	13.13
F4 5AH/5/5/5	95.8	1943	7.65
Mencevax™	91.7	1567	16.55
Formulation	% SBA MenW responders	SBA-MenW GMT	Anti-PSW GMC µg/ml ELISA
F 12.5AH/2.5AH/2.5/2.5	100	5656	7
F2 5AH/5AH/2.5/2.5	96	4679	5.4
F3 5AH/5AH/5/5	91.3	4422	4.45
F4 5AH/5/5/5	88	4947	7.67
Mencevax™	96	3486	11.93
Formulation	% SBY MenY responders	SBA-MenY GMT	Anti-PSY GMC µg/ml ELISA
F 1 2.5AH/2.5AH/2.5/2.5	75	3891	17.81
F2 5AH/5AH/2.5/2.5	92	3968	11.96
F3 5AH/5AH/5/5	79.2	2756	9.51
F4 5AH/5/5/5	80	3914	16.76
Mencevax™	88	3056	21.41

## CLAIMS

- 5 1. An immunogenic composition comprising at least 2 different *N. meningitidis* capsular saccharides, wherein one or more is/are selected from a first group consisting of MenA, MenC, MenY and MenW which is/are conjugated through a linker to a carrier protein(s), and one or more different saccharides is/are selected from a second group consisting of MenA, MenC, MenY and MenW which is/are directly conjugated to a carrier protein(s).
- 10 2. The immunogenic composition of claim 1 comprising at least 2 different *N. meningitidis* capsular saccharides, wherein one or more is/are selected from a first group consisting of MenA and MenC which is/are conjugated through a linker to a carrier protein(s), and one or more different saccharides is/are selected from a second group consisting of MenC, MenY and MenW which is/are directly conjugated to a carrier protein(s).
- 15 3. The immunogenic composition of claim 2 comprising MenA capsular saccharide conjugated through a linker to a carrier protein, and MenC capsular saccharide directly conjugated to a carrier protein.
- 20 4. The immunogenic composition of claim 2 comprising MenC capsular saccharide conjugated through a linker to a carrier protein, and MenY capsular saccharide directly conjugated to a carrier protein.
- 25 5. The immunogenic composition of claim 2 comprising MenA and MenC capsular saccharides conjugated through a linker to a carrier protein(s), and MenY and Men W capsular saccharides directly conjugated to a carrier protein(s).
- 30 6. The immunogenic composition of claim 2 comprising MenA capsular saccharide conjugated through a linker to a carrier protein, and MenC, MenY and Men W capsular saccharides directly conjugated to a carrier protein(s).
- 35 7. The immunogenic composition of any preceding claim wherein each *N. meningitidis* capsular saccharide is conjugated to a carrier protein independently selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D.
- 40 8. The immunogenic composition of any preceding claim wherein each *N. meningitidis* capsular saccharide is conjugated to the same carrier protein selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D.

9. The immunogenic composition of any preceding claim wherein each *N. meningitidis* capsular saccharide is conjugated to TT.
- 5 10. The immunogenic composition of any preceding claim wherein each *N. meningitidis* capsular saccharide is separately conjugated to a separate carrier protein.
- 10 11. The immunogenic composition of any preceding claim wherein each *N. meningitidis* capsular saccharide conjugate has a saccharide:carrier ratio of 1:5-5:1 or 1:1-1:4(w/w).
- 15 12. The immunogenic composition of any preceding claim wherein at least one, two or three *N. meningitidis* capsular saccharide conjugate(s) is directly conjugated to a carrier protein.
- 20 13. The immunogenic composition of claim 12 wherein Men W and/or MenY, MenW and/or MenC, MenY and/or MenC, or MenW and MenC and MenY are directly conjugated to a carrier protein.
- 25 14. The immunogenic composition of claim 12 or 13 wherein at least one, two or three *N. meningitidis* saccharide conjugate(s) is directly conjugated by CDAP chemistry.
- 30 15. The immunogenic composition of any one of claims 12-14 wherein the ratio of Men W and/or Y saccharide to carrier protein is between 1:0.5 and 1:2 (w/w).
- 35 16. The immunogenic composition of any one of claims 12-15 wherein the ratio of MenC saccharide to carrier protein is between 1:0.5 and 1:2 (w/w).
- 40 17. The immunogenic composition of any one of claims 1-16, wherein at least one, two or three *N. meningitidis* capsular saccharide(s) are conjugated to the carrier protein via a linker.
18. The immunogenic composition of claim 17 wherein the linker is bifunctional.
19. The immunogenic composition of claim 17 or 18 wherein the linker has two reactive amino groups.
20. The immunogenic composition of claim 17 or 18 wherein the linker has two reactive carboxylic acid groups.

21. The immunogenic composition of claim 17 or 18 wherein the linker has a reactive amino group at one end and a reactive carboxylic acid group at the other end.
- 5 22. The immunogenic composition of claim 17-21 wherein the linker has between 4 and 12 carbon atoms.
23. The immunogenic composition of claim 17 or 18 wherein the linker is ADH.
- 10 24. The immunogenic composition of any one of claims 17-23 wherein the or each *N. meningitidis* capsular saccharide(s) conjugated via a linker are conjugated to the linker with CDAP chemistry.
- 15 25. The immunogenic composition of claim 17-24 wherein the carrier protein is conjugated to the linker using carbodiimide chemistry, optionally using EDAC.
- 20 26. The immunogenic composition of any one of claims 17-25 wherein the or each *N. meningitidis* capsular saccharide is conjugated to the linker before the carrier protein is conjugated to the linker.
- 25 27. The immunogenic composition of any one of claims 17-26 wherein MenA is conjugated to a carrier protein via a linker.
28. The immunogenic composition of claim 27 wherein the ratio of MenA saccharide to carrier protein is between 1:2 and 1:5 (w/w).
29. The immunogenic composition of any one of claims 17-28 wherein MenC is conjugated to a carrier protein via a linker.
- 30 30. The immunogenic composition of claim 34 wherein the ratio of MenC saccharide to carrier protein is between 1:2 and 1:5 (w/w).
- 35 31. The immunogenic composition of any preceding claim comprising *N. meningitidis* capsular saccharides from at least two of serogroups A, C, W135 and Y conjugated to a carrier protein to produce a *N. meningitidis* capsular saccharide conjugate, wherein the average size of each *N. meningitidis* saccharide is above 50kDa, 75kDa, 100kDa, 110kDa, 120kDa or 130kDa.
- 40 32. The immunogenic composition of claim 31, wherein each *N. meningitidis* saccharide is either a native polysaccharide or is sized by a factor of no more than x10.



33. The immunogenic composition of claim 31 or 32 wherein each *N. meningitidis* capsular saccharide is a native polysaccharide.
- 5 34. The immunogenic composition of claim 31 or 32, wherein at least one, two, three or four *N. meningitidis* capsular saccharide(s) is sized by microfluidization.
35. The immunogenic composition of claim 31 or 32 wherein each *N. meningitidis* capsular saccharide is sized by a factor of no more than x10.
- 10 36. The immunogenic composition of claim 31 or 32 wherein the *N. meningitidis* conjugates are made from a mixture of native polysaccharides and saccharides that are sized by a factor of no more than x10.
- 15 37. The immunogenic composition of claim 36 wherein capsular saccharide from serogroup Y is sized by a factor of no more than x10.
38. The immunogenic composition of claim 36 or 37 wherein capsular saccharides from serogroups A and C are native polysaccharides and saccharides from serogroups W135 and Y are sized by a factor of no more than x10.
- 20 39. The immunogenic composition of claims 31-38 wherein the average size of each *N. meningitidis* capsular saccharide is between 50 kDa and 300 kDa or 50kDa and 200kDa.
- 25 40. The immunogenic composition of claims 31-39 comprising a MenA capsular saccharide having an average size of above 50kDa, 75kDa, 100kDa or an average size of between 50-100kDa or 55-90kDa or 60-80kDa.
- 30 41. The immunogenic composition of claims 31-40 comprising a MenC capsular saccharide having an average size of above 50kDa, 75kDa, 100kDa or between 100-200kDa, 100-150kDa, 80-120kDa, 90-110kDa, 150-200kDa, 120-240kDa, 140-220kDa, 160-200kDa or 190-200kDa.
- 35 42. The immunogenic composition of claims 31-41 comprising a MenY capsular saccharide, having an average size of above 50kDa, 75kDa, 100kDa or between 60-190kDa or 70-180kDa or 80-170kDa or 90-160kDa or 100-150kDa, 110-145kDa or 120-140kDa.
- 40 43. The immunogenic composition of claims 31-42 comprising a MenW capsular saccharide having an average size of above 50kDa, 75kDa, 100kDa or between 60-190kDa or 70-180kDa or 80-170kDa or 90-160kDa or 100-150kDa, 140-180kDa, 150-170kDa or 110-140kDa.

- 5
44. The immunogenic composition of any preceding claim wherein the dose of each saccharide conjugate is between 2 and 20 $\mu$ g, 3 and 10 $\mu$ g or 4 and 7 $\mu$ g of saccharide.
- 10
45. The immunogenic composition of any preceding claim further comprising a *H. influenzae* b capsular saccharide (Hib) conjugated to a carrier protein.
46. The immunogenic composition of claim 45 wherein the *H. influenzae* b capsular saccharide is conjugated to a carrier protein selected from the group consisting of TT, DT, CRM197, fragment C of TT and protein D.
- 15
47. The immunogenic composition of claim 45 or 46, wherein the Hib saccharide is conjugated to the same carrier protein as for at least one, two, three or all of the *N. meningitidis* capsular saccharide conjugates.
- 20
48. The immunogenic composition of claims 45-47, wherein the Hib saccharide is conjugated to TT.
- 25
49. The immunogenic composition of claims 45-48 wherein the ratio of Hib to carrier protein in the Hib capsular saccharide conjugate is between 1:5 and 5:1 (w/w).
50. The immunogenic composition of claim 49 wherein the ratio of Hib to carrier protein in the Hib capsular saccharide conjugate is between 1:1 and 1:4, 1:2 and 1:3.5 or around 1:3 (w/w).
- 30
51. The immunogenic composition of claims 45-50 wherein the Hib capsular saccharide is conjugated to the carrier protein via a linker.
52. The immunogenic composition of claim 51 wherein the linker is bifunctional.
- 35
53. The immunogenic composition of claim 51 or 52 wherein the linker has two reactive amino groups.
54. The immunogenic composition of claim 51 or 52 wherein the linker has two reactive carboxylic acid groups.
- 40
55. The immunogenic composition of claim 51 or 52 wherein the linker has a reactive amino group at one end and a reactive carboxylic acid group at the other end.

56. The immunogenic composition of any one of claims 51-55 wherein the linker has between 4 and 12 carbon atoms.
57. The immunogenic composition of claim 51 or 52 wherein the linker is ADH.
58. The immunogenic composition of any one of claims 45-57 wherein the Hib saccharide is conjugated to the carrier protein or linker using CNBr or CDAP.
59. The immunogenic composition of claim 51-58 wherein the carrier protein is conjugated to the Hib saccharide via the linker using a method comprising carbodiimide chemistry, optionally EDAC chemistry.
60. The immunogenic composition of any one of claims 45-59 comprising a Hib saccharide conjugate and at least two further bacterial saccharide conjugates wherein the Hib conjugate is present in a lower dose than the mean dose of the at least two further bacterial saccharide conjugates.
61. The immunogenic composition of claim 60 wherein the Hib conjugate is present in a lower dose than the dose of each of the at least two further bacterial saccharide conjugates.
62. The immunogenic composition of claim 60 or 61 wherein the at least two further bacterial saccharide conjugates comprises *N. meningitidis* serogroup C capsular saccharide (MenC) conjugate.
63. The immunogenic composition of any one of claims 60-62 wherein the at least two further bacterial saccharide conjugates comprises *N. meningitidis* serogroup Y capsular saccharide (MenY) conjugate.
64. The immunogenic composition of any one of claims 60-63 wherein the at least two further bacterial saccharide conjugates comprises *N. meningitidis* serogroup A capsular saccharide (MenA) conjugate.
65. The immunogenic composition of any one of claims 60-64 wherein the at least two further bacterial saccharide conjugates comprises *N. meningitidis* serogroup W135 capsular saccharide (MenW) conjugate.
66. The immunogenic composition of any one of claims 60-65 wherein the at least two further bacterial saccharide conjugates comprise a *S. pneumoniae* capsular saccharide derived from a strain selected from the group consisting of serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

- 5
67. The immunogenic composition of any one of claims 60-66 wherein the at least two further bacterial saccharide conjugates comprise a *S. typhi* Vi capsular saccharide.
68. The immunogenic composition of any one of claims 60-67 wherein the dose of the Hib saccharide conjugate is between 0.1 and 9 $\mu$ g, 1 and 5 $\mu$ g or 2 and 3 $\mu$ g of saccharide.
- 10
69. The immunogenic composition of any one of claims 60-68 wherein the dose of each of the at least two further saccharide conjugates is between 2 and 20 $\mu$ g, 3 and 10 $\mu$ g or 4 and 7 $\mu$ g of saccharide.
- 15
70. The immunogenic composition of any one of claims 60-69 wherein the saccharide dose of the Hib saccharide conjugate is less than 90%, 75% or 60% , between 20% and 60% or around 50% of the mean saccharide dose of the at least two further saccharide conjugates.
- 20
71. The immunogenic composition of any one of claims 60-70 wherein the saccharide dose of the Hib saccharide conjugate is less than 90%, 75% or 60% or is between 20-60% or is around 50% of the saccharide dose of each of the at least two further saccharide conjugates.
- 25
72. The immunogenic composition of any one of claims 60-71 wherein the same carrier protein is used in the Hib conjugate and two or more of the at least two further bacterial saccharide conjugates.
- 30
73. The immunogenic composition of any one of claims 1-72 comprising a *N. meningitidis* serogroup B outer membrane vesicle preparation or capsular saccharide.
- 35
74. A vaccine comprising the immunogenic composition of any one of claims 1-73 and a pharmaceutically acceptable excipient.
- 40
75. A vaccine kit for concomitant or sequential administration comprising two multi-valent immunogenic compositions for conferring protection in a host against disease caused by *Bordetella pertussis*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Haemophilus influenzae* and *Neisseria meningitidis*, said kit comprising a first container comprising :
- tetanus toxoid (TT),  
diphtheria toxoid (DT), and

wholecell or acellular pertussis components

and a second container comprising:

5 the immunogenic composition of any one of claims 1-73.

76. A process for making the vaccine of claim 74 comprising the step of mixing the immunogenic composition of any one of claims 1-73 with a pharmaceutically acceptable excipient.

10

77. A method of immunising a human host against disease caused by *Neisseria meningitidis* infection comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine of claims 1-75.

15

78. The immunogenic composition of claims 1-73 for use in the treatment or prevention of disease caused by *Neisseria meningitidis* infection.

79. The use of the immunogenic composition of any one of claims 1-73 in the manufacture of a medicament for the treatment or prevention of diseases caused by *Neisseria meningitidis* infection.

20

80. An immunogenic composition comprising at least 2 different saccharides conjugated separately to the same type of carrier protein, wherein one or more saccharide(s) is/are conjugated to the carrier protein via a first type of chemical group on the protein carrier, and one or more saccharide(s) is/are conjugated to the carrier protein via a second type of chemical group on the protein carrier.

25

81. The immunogenic composition of claim 80, wherein the one or more saccharide(s) conjugated to the carrier protein via the first type of chemical group on the protein carrier, are different to the one or more saccharide(s) conjugated to the carrier protein via the second type of chemical group on the protein carrier.

30

82. An immunogenic composition of claim 80 or 81 comprising at least 2 different saccharides conjugated separately to the same carrier protein, wherein one or more saccharide(s) is/are conjugated to the carrier protein via a carboxyl group on the protein carrier, and one or more saccharide(s) is/are conjugated to the carrier protein via an amino group on the protein carrier.

35

83. An immunogenic composition of claims 80-82, wherein the first and second type of chemical group on the protein carrier are present on separate B- and/or T-cell epitopes on the carrier protein.

40

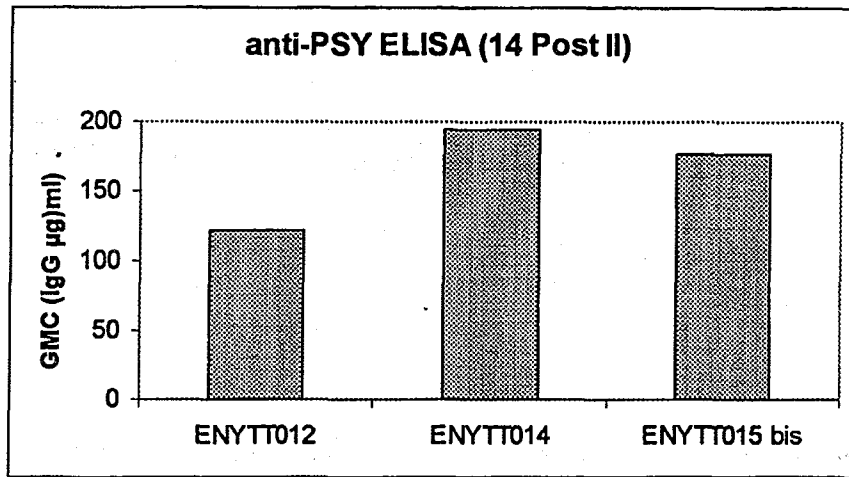
- 5 84. An immunogenic composition of claims 80-83, wherein the saccharides are selected from a group consisting of: *N. meningitidis* serogroup A capsular saccharide (MenA), *N. meningitidis* serogroup C capsular saccharide (MenC), *N. meningitidis* serogroup Y capsular saccharide (MenY), *N. meningitidis* serogroup W capsular saccharide (MenW), Group B Streptococcus group I capsular saccharide, Group B Streptococcus group II capsular saccharide, Group B Streptococcus group III capsular saccharide, Group B Streptococcus group IV capsular saccharide, Group B Streptococcus group V capsular saccharide, 10 *Staphylococcus aureus* type 5 capsular saccharide, *Staphylococcus aureus* type 8 capsular saccharide, Vi saccharide from *Salmonella typhi*, *N. meningitidis* LPS (such as L3 and/or L2), *M. catarrhalis* LPS, *H. influenzae* LPS, and from any of the capsular pneumococcal saccharides such as from serotype: 1, 2, 3, 4, 5, 6A, 15 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F or 33F.
- 20 85. The immunogenic composition of claims 80-84 wherein the carrier protein is selected from the group consisting of: TT, DT, CRM197, fragment C of TT and protein D.
- 25 86. The immunogenic composition of claims 80-84 wherein the carrier protein is TT.
- 30 87. An immunogenic composition of claims 80-86 comprising at least 2 different *N. meningitidis* capsular saccharides, wherein one or more is/are selected from a first group consisting of MenA and MenC which is/are conjugated to the carrier protein via the first type of chemical group on the protein carrier, and one or more different saccharides is/are selected from a second group consisting of MenC, MenY and MenW which is/are conjugated to the carrier protein via the second type of chemical group on the protein carrier.
- 35 88. An immunogenic composition of claim 87, wherein the first type of chemical group is a carboxyl group on the protein carrier, and the second type of chemical group is an amino group on the protein carrier.
- 40 89. The immunogenic composition of claims 87-88 comprising MenA conjugated via the first type of chemical group, and MenC conjugated via the second type of chemical group.
90. The immunogenic composition of claims 87-88 comprising MenC conjugated via the first type of chemical group, and MenY conjugated via the second type of chemical group.

91. The immunogenic composition of claims 87-88 comprising MenA conjugated via the first type of chemical group, and MenC, MenY and MenW conjugated via the second type of chemical group.
- 5 92. The immunogenic composition of claims 87-88 comprising MenA and MenC conjugated via the first type of chemical group, and MenY and MenW conjugated via the second type of chemical group.
- 10 93. The immunogenic composition of claims 87-92 further comprising a capsular saccharide from *H. influenzae* type b (Hib) conjugated to a carrier protein.
94. The immunogenic composition of claim 93, wherein Hib is conjugated to the same type of carrier protein as the *N. meningitidis* saccharides.
- 15 95. The immunogenic composition of claim 94, wherein Hib is conjugated to the carrier protein via either the first or second type of chemical group.
- 20 96. The immunogenic composition of claims 80-95, wherein the saccharides conjugated via the first type of chemical group do so through a linker, and the saccharides conjugated via the second type of chemical group do so directly, or wherein the saccharides conjugated via the second type of chemical group do so through a linker, and the saccharides conjugated via the first type of chemical group do so directly.
- 25 97. The immunogenic composition of claim 96 wherein the linker is bifunctional.
98. The immunogenic composition of claim 96 or 97, wherein the linker has between 4 and 12 carbon atoms.
- 30 99. The immunogenic composition of claims 96-98 wherein the linker has two reactive amino groups.
100. The immunogenic composition of claims 96-98 wherein the linker has two reactive carboxylic acid groups.
- 35 101. The immunogenic composition of claim claims 96-98 wherein the linker has a reactive amino group at one end and a reactive carboxylic acid group at the other end.
- 40 102. The immunogenic composition of claims 96-98 wherein the linker is ADH.

103. The immunogenic composition of claims 96-102, wherein the or each capsular saccharide(s) conjugated via a linker is conjugated to the linker with CDAP chemistry.
- 5 104. The immunogenic composition of claims 96-103, wherein the carrier protein is conjugated to the linker using carbodiimide chemistry, optionally using EDAC.
- 10 105. The immunogenic composition of claims 96-104, wherein the or each capsular saccharide is conjugated to the linker before the carrier protein is conjugated to the linker, or the linker is conjugated to the saccharide before it is conjugated to the carrier protein.
- 15 106. The immunogenic composition of claims 96-105, which is an immunogenic composition of claims 1-74.



**A**



**B**

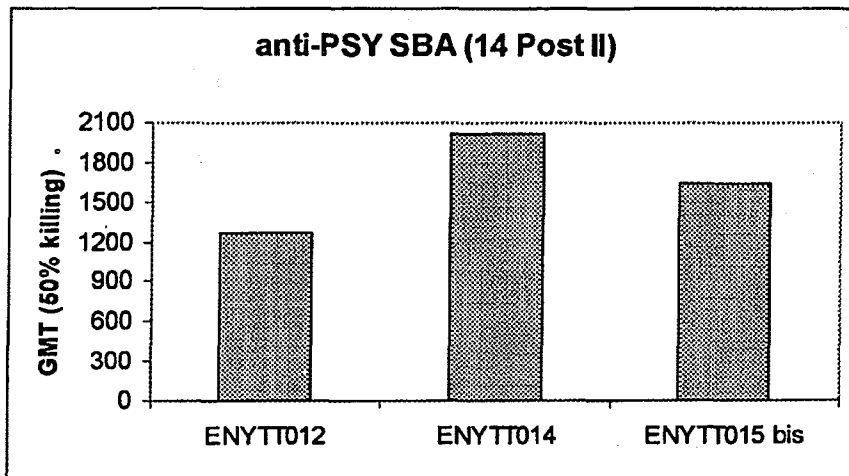


Figure 1

**Field of the Invention**

The present invention relates to an improved *Streptococcus pneumoniae* vaccine.

5

**Background of the Invention**

Children less than 2 years of age do not mount an immune response to most polysaccharide vaccines, so it has been necessary to render the polysaccharides immunogenic by chemical conjugation to a protein carrier. Coupling the polysaccharide, a T-independent antigen, to a protein, a T-dependent antigen, confers upon the polysaccharide the properties of T dependency including isotype switching, affinity maturation, and memory induction.

However, there can be issues with repeat administration of polysaccharide-protein conjugates, or the combination of polysaccharide-protein conjugates to form multivalent vaccines. For example, it has been reported that a *Haemophilus influenzae* type b polysaccharide (PRP) vaccine using tetanus toxoid (TT) as the protein carrier was tested in a dosage-range with simultaneous immunization with (free) TT and a pneumococcal polysaccharide-TT conjugate vaccine following a standard infant schedule. As the dosage of the pneumococcal vaccine was increased, the immune response to the PRP polysaccharide portion of the Hib conjugate vaccine was decreased, indicating immune interference of the polysaccharide, possibly via the use of the same carrier protein (Dagan *et al.*, Infect Immun. (1998); 66: 2093-2098)

The effect of the carrier-protein dosage on the humoral response to the protein itself has also proven to be multifaceted. In human infants it was reported that increasing the dosage of a tetravalent tetanus toxoid conjugate resulted in a decreased response to the tetanus carrier (Dagan *et al. supra*). Classical analysis of these effects of combination vaccines have been described as carrier induced epitopic suppression, which is not fully understood, but believed to result from an excess amount of carrier protein (Fattom, Vaccine 17: 126 (1999)). This appears to result in competition for Th-cells, by the B-cells to the carrier protein, and B-cells to the polysaccharide. If the B-cells to the carrier protein predominate, there are not enough Th-cells available to provide the necessary help for the B-cells specific to the polysaccharide. However, the observed immunological effects have been inconsistent, with the total amount of carrier protein in some instances increasing the immune response, and in other cases diminishing the immune response.

Hence there remain technical difficulties in combining multiple polysaccharide conjugates into a single, efficacious, vaccine formulation.

*Streptococcus pneumoniae* is a Gram-positive bacterium responsible for considerable morbidity and mortality (particularly in the young and aged), causing invasive diseases such as pneumonia, bacteraemia and meningitis, and diseases associated with colonisation, such as acute Otitis media. The rate of pneumococcal pneumonia in the US for persons over 60 years of age is estimated to be 3 to 8 per 100,000. In 20% of cases this leads to bacteraemia, and other manifestations such as meningitis, with a mortality rate close to 30% even with antibiotic treatment.

Pneumococcus is encapsulated with a chemically linked polysaccharide which confers serotype specificity. There are 90 known serotypes of pneumococci, and the capsule is the principle virulence determinant for pneumococci, as the capsule not only protects the inner surface of the bacteria from complement, but is itself poorly immunogenic. Polysaccharides are T-independent antigens, and can not be processed or presented on MHC molecules to interact with T-cells. They can however, stimulate the immune system through an alternate mechanism which involves cross-linking of surface receptors on B cells.

It was shown in several experiments that protection against invasive pneumococci disease is correlated most strongly with antibody specific for the capsule, and the protection is serotype specific.

*Streptococcus pneumoniae* is the most common cause of invasive bacterial disease and Otitis media in infants and young children. Likewise, the elderly mount poor responses to pneumococcal vaccines [Roghmann *et al.*, (1987), *J. Gerontol.* 42:265-270], hence the increased incidence of bacterial pneumonia in this population [Verghese and Berk, (1983) *Medicine (Baltimore)* 62:271-285].

The major clinical syndromes caused by *S. pneumoniae* are widely recognized and discussed in all standard medical textbooks (Fedson DS, Muscher DM. In: Plotkin SA, Orenstein WA, editors. *Vaccines*. 4th edition. PhiladelphiaWB Saunders Co, 2004a: 529-588). For instance, Invasive pneumococcal disease (IPD) is defined as any infection in which *S. pneumoniae* is isolated from the blood or another normally sterile site (Musher DM. *Streptococcus pneumoniae*. In Mandell GL, Bennett JE, Dolin R (eds). *Principles and Practice of Infectious diseases* (5th ed). New York, Churchill Livingstone, 2001, p2128-2147). Chronic obstructive pulmonary disease (COPD) is recognised as encompassing several conditions (airflow obstruction, chronic bronchitis, bronchiolitis or small airways disease and emphysema) that often coexist. Patients suffer exacerbations of their

condition that are usually associated with increased breathlessness, and often have increased cough that may be productive of mucus or purulent sputum (Wilson, Eur Respir J 2001 17:995-1007). COPD is defined physiologically by the presence of irreversible or partially reversible airway obstruction in patients with chronic bronchitis and/or  
5 emphysema (Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society. Am J Respir Crit Care Med. 1995 Nov;152(5 Pt 2):S77-121). Exacerbations of COPD are often caused by bacterial (e.g. pneumococcal) infection (Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clin Microbiol Rev. 2001  
10 Apr;14(2):336-63).

It is thus an object of the present invention to develop an improved formulation of a multiple serotype *Streptococcus pneumoniae* polysaccharide conjugate vaccine.

#### 15 **Brief description of Figures**

**Figure 1** Bar chart showing 11 valent conjugate immunogenicity in elderly Rhesus monkeys. The lighter bars represent the GMC after two inoculations with 11 valent conjugate in aluminium phosphate adjuvant. The darker bars represent the GMC after two inoculations with 11 valent conjugate in adjuvant C.

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**Figure 2** Bar chart showing memory B cells for PS3 after inoculation with the 11 valent conjugate in adjuvant C or aluminium phosphate adjuvant.

**Figure 3** Bar chart showing anti polysaccharide 19F immunogenicity in Balb/C mice for the 4-valent plain polysaccharides and the 4-valent dPly conjugates.

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**Figure 4** Bar chart showing anti polysaccharide 22F immunogenicity in Balb/C mice for the 4-valent plain polysaccharides and the 4-valent PhtD conjugates.

30 **Figure 5** Bar chart showing anti-22F IgG response in Balb/c mice

**Figure 6** Bar chart showing anti-22F opsono-phagocytosis titres in Balb/c mice.

**Figure 7** Bar chart comparing IgG responses induced in young C57B1 mice after  
35 immunisation with 13 Valent conjugate vaccine formulated in different adjuvants.

**Figure 8.** Bar chart showing the protective efficacy of different vaccine combinations in a monkey pneumonia model.

5 **Figure 9** Bar chart showing anti PhtD IgG response in Balb/c mice after immunisation with 22F-PhtD or 22F-AH-PhtD conjugates.

**Figure 10** Protection against type 4 pneumococcal challenge in mice after immunisation with 22F-PhtD or 22F-AH-PhtD.

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#### **Description of the Invention**

The present invention provides an improved *Streptococcus pneumoniae* vaccine comprising 10 or more (e.g. 11, 12, 13, 14, or 15 or more) capsular saccharides from different *S. pneumoniae* serotypes conjugated to 2 or more carrier proteins, wherein the vaccine comprises serotype 19F capsular saccharide conjugated to diphtheria toxoid or CRM197, and wherein the vaccine optionally further comprises protein D from *Haemophilus influenzae* as free protein or as a further carrier protein or both.

For the purposes of this invention, "immunizing a human host against exacerbations of COPD" or "treatment or prevention of exacerbations of COPD" or "reduction in severity of COPD exacerbations" refers to a reduction in incidence or rate of COPD exacerbations (for instance a reduction in rate of 0.1, 0.5, 1, 2, 5, 10, 20% or more) or a reduction in severity of COPD exacerbations as defined above, for instance within a patient group immunized with the compositions or vaccines of the invention.

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Typically the *Streptococcus pneumoniae* vaccine of the present invention will comprise capsular saccharide antigens (preferably conjugated), wherein the saccharides are derived from at least ten serotypes of *S. pneumoniae*. The number of *S. pneumoniae* capsular saccharides can range from 10 different serotypes (or "V", valences) to 23 different serotypes (23V). In one embodiment there are 10, 11, 12, 13, 14 or 15 different serotypes. In another embodiment of the invention, the vaccine may comprise conjugated *S. pneumoniae* saccharides and unconjugated *S. pneumoniae* saccharides. Preferably, the total number of saccharide serotypes is less than or equal to 23. For example, the invention may comprise 10 conjugated serotypes and 13 unconjugated saccharides. In a

similar manner, the vaccine may comprise 11, 12, 13, 14, 15 or 16 conjugated saccharides and 12, 11, 10, 9, 8 or 7, respectively, unconjugated saccharides.

In one embodiment the multivalent pneumococcal vaccine of the invention will be selected from the following serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, although it is appreciated that one or two other serotypes could be substituted depending on the age of the recipient receiving the vaccine and the geographical location where the vaccine will be administered, e.g. serotype 6A may be included on the list. For example, an 10-valent vaccine may comprise polysaccharides from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. An 11-valent vaccine may also include saccharides from serotype 3. A 12 or 13-valent paediatric (infant) vaccine may also include the 10 or 11 valent formulation supplemented with serotypes 6A and 19A, or 6A and 22F, or 19A and 22F, or 6A and 15B, or 19A and 15B, or 22F and 15B, whereas a 13-valent elderly vaccine may include the 11 valent formulation supplemented with serotypes 19A and 22F, 8 and 12F, or 8 and 15B, or 8 and 19A, or 8 and 22F, or 12F and 15B, or 12F and 19A, or 12F and 22F, or 15B and 19A, or 15B and 22F. A 14 valent paediatric vaccine may include the 10 valent formulation described above supplemented with serotypes 3, 6A, 19A and 22F; serotypes 6A, 8, 19A and 22F; serotypes 6A, 12F, 19A and 22F; serotypes 6A, 15B, 19A and 22F; serotypes 3, 8, 19A and 22F; serotypes 3, 12F, 19A and 22F; serotypes 3, 15B, 19A and 22F; serotypes 3, 6A, 8 and 22F; serotypes 3, 6A, 12F and 22F; or serotypes 3, 6A, 15B and 22F.

The composition in one embodiment includes capsular saccharides derived from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F (preferably conjugated). In a further embodiment of the invention at least 11 saccharide antigens (preferably conjugated) are included, for example capsular saccharides derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. In a further embodiment of the invention, at least 12 or 13 saccharide antigens are included, for example a vaccine may comprise capsular saccharides derived from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F or capsular saccharides derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F, although further saccharide antigens, for example 23 valent (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention.

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The vaccine of the present invention may comprise protein D (PD) from *Haemophilus influenzae* (see e.g. EP 0594610). *Haemophilus influenzae* is a key causative organism of otitis media, and the present inventors have shown that including this protein in a *Streptococcus pneumoniae* vaccine will provide a level of protection against *Haemophilus influenzae* related otitis media (reference POET publication). In one embodiment, the vaccine composition comprises protein D. In one aspect, PD is present as a carrier protein for one or more of the saccharides. In another aspect, protein D could be present in the vaccine composition as a free protein. In a further aspect, protein D is present both as a carrier protein and as free protein. Protein D may be used as a full length protein or as a fragment (WO0056360). In a further aspect, protein D is present as a carrier protein for the majority of the saccharides, for example 6, 7, 8, 9 or more of the saccharides may be conjugated to protein D. In this aspect, protein D may also be present as free protein.

The vaccine of the present invention comprises two or more different types of carrier protein. Each type of carrier protein may act as carrier for more than one saccharide, which saccharides may be the same or different. For example, serotypes 3 and 4 may be conjugated to the same carrier protein, either to the same molecule of carrier protein or to different molecules of the same carrier protein. In one embodiment, two or more different saccharides may be conjugated to the same carrier protein, either to the same molecule of carrier protein or to different molecules of the same carrier protein.

Each *Streptococcus pneumoniae* capsular saccharide may be conjugated to a carrier protein independently selected from the group consisting of TT, DT, CRM197, fragment C of TT, PhtD, PhtDE fusions (particularly those described in WO 01/98334 and WO 03/54007), detoxified pneumolysin and protein D, other than saccharide from serotype 19F which is always conjugated to DT or CRM 197, preferably DT. A more complete list of protein carriers that may be used in the conjugates of the invention is presented below

If the protein carrier is the same for 2 or more saccharides in the composition, the saccharides could be conjugated to the same molecule of the protein carrier (carrier molecules having 2 more different saccharides conjugated to it) [see for instance WO 04/083251]. Alternatively the saccharides may each be separately conjugated to different molecules of the protein carrier (each molecule of protein carrier only having one type of saccharide conjugated to it).

The carrier protein conjugated to one or more of the *S. pneumoniae* capsular saccharides in the conjugates present in the immunogenic compositions of the invention is optionally a member of the polyhistidine triad family (Pht) proteins, fragments or fusion proteins thereof. The PhtA, PhtB, PhtD or PhtE proteins may have an amino acid sequence sharing 80%, 85%, 90%, 95%, 98%, 99% or 100% identity with a sequence disclosed in WO 00/37105 or WO 00/39299 (e.g. with amino acid sequence 1-838 or 21-838 of SEQ ID NO: 4 of WO 00/37105 for PhtD). For example, fusion proteins are composed of full length or fragments of 2, 3 or 4 of PhtA, PhtB, PhtD, PhtE. Examples of fusion proteins are PhtA/B, PhtA/D, PhtA/E, PhtB/A, PhtB/D, PhtB/E, PhtD/A, PhtD/B, PhtD/E, PhtE/A, PhtE/B and PhtE/D, wherein the proteins are linked with the first mentioned at the N-terminus (see for example WO01/98334).

Where fragments of Pht proteins are used (separately or as part of a fusion protein), each fragment optionally contains one or more histidine triad motif(s) and/or coiled coil regions of such polypeptides. A histidine triad motif is the portion of polypeptide that has the sequence HxxHxH where H is histidine and x is an amino acid other than histidine. A coiled coil region is a region predicted by "Coils" algorithm Lupus, A et al (1991) Science 252; 1162-1164. In an embodiment the or each fragment includes one or more histidine triad motif as well as at least one coiled coil region. In an embodiment, the or each fragment contains exactly or at least 2, 3, 4 or 5 histidine triad motifs (optionally, with native Pht sequence between the 2 or more triads, or intra-triad sequence that is more than 50, 60, 70, 80, 90 or 100 % identical to a native pneumococcal intra-triad Pht sequence – e.g. the intra-triad sequence shown in SEQ ID NO: 4 of WO 00/37105 for PhtD). In an embodiment, the or each fragment contains exactly or at least 2, 3 or 4 coiled coil regions. In an embodiment a Pht protein disclosed herein includes the full length protein with the signal sequence attached, the mature full length protein with the signal peptide (for example 20 amino acids at N-terminus) removed, naturally occurring variants of Pht protein and immunogenic fragments of Pht protein (e.g. fragments as described above or polypeptides comprising at least 15 or 20 contiguous amino acids from an amino acid sequence in WO00/37105 or WO00/39299 wherein said polypeptide is capable of eliciting an immune response specific for said amino acid sequence in WO00/37105 or WO00/39299).

In particular, the term "PhtD" as used herein includes the full length protein with the signal sequence attached, the mature full length protein with the signal peptide (for example 20



amino acids at N-terminus) removed, naturally occurring variants of PhtD and immunogenic fragments of PhtD (e.g. fragments as described above or polypeptides comprising at least 15 or 20 contiguous amino acids from a PhtD amino acid sequence in WO00/37105 or WO00/39299 wherein said polypeptide is capable of eliciting an immune response specific for said PhtD amino acid sequence in WO00/37105 or WO00/39299 (e.g. SEQ ID NO: 4 of WO 00/37105 for PhtD).

If the protein carrier is the same for 2 or more saccharides in the composition, the saccharides could be conjugated to the same molecule of the protein carrier (carrier molecules having 2 more different saccharides conjugated to it) [see for instance WO 04/083251]. Alternatively the saccharides may each be separately conjugated to different molecules of the protein carrier (each molecule of protein carrier only having one type of saccharide conjugated to it).

Examples of carrier proteins which may be used in the present invention are DT (Diphtheria toxoid), TT (tetanus toxid) or fragment C of TT, DT CRM197 (a DT mutant) other DT point mutants, such as CRM176, CRM228, CRM 45 (Uchida et al J. Biol. Chem. 218; 3838-3844, 1973); CRM 9, CRM 45, CRM102, CRM 103 and CRM107 and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in US 4709017 or US 4950740; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in US 5917017 or US 6455673; or fragment disclosed in US 5843711, pneumococcal pneumolysin (Kuo et al (1995) Infect Immun 63; 2706-13) including ply detoxified in some fashion for example dPLY-GMBS (WO 04081515, PCT/EP2005/010258) or dPLY-formol, PhtX, including PhtA, PhtB, PhtD, PhtE and fusions of Pht proteins for example PhtDE fusions, PhtBE fusions (WO 01/98334 and WO 03/54007), (Pht A-E are described in more detail below) OMPC (meningococcal outer membrane protein – usually extracted from *N. meningitidis* serogroup B – EP0372501), PorB (from *N. meningitidis*), PD (Haemophilus Influenzae protein D – see, e.g., EP 0 594 610 B), or immunologically functional equivalents thereof, synthetic peptides (EP0378881, EP0427347), heat shock proteins (WO 93/17712, WO 94/03208), pertussis proteins (WO 98/58668, EP0471177), cytokines, lymphokines, growth factors or hormones (WO 91/01146), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen derived antigens (Falugi et al (2001) Eur J Immunol 31; 3816-3824) such as

N19 protein (Baraldoi et al (2004) *Infect Immun* 72; 4884-7) pneumococcal surface protein PspA (WO 02/091998), iron uptake proteins (WO 01/72337), toxin A or B of *C. difficile* (WO 00/61761).

5 Nurkka et al *Pediatric Infectious Disease Journal*. 23(11):1008-14, 2004 Nov. described an 11 valent pneumococcal vaccine with all serotypes conjugated to PD. However, the present inventors have shown that opsonophagocytic activity was improved for antibodies induced with conjugates having 19F conjugated to DT compared with 19F conjugated to PD. In addition, the present inventors have shown that a greater cross  
10 reactivity to 19A is seen with 19F conjugated to DT. It is therefore a feature of the composition of the present invention that serotype 19F is conjugated to DT or CRM 197. In one aspect, serotype 19F is conjugated to DT. The remaining saccharide serotypes of the immunogenic composition may all be conjugated to one or more carrier proteins that are not DT (i.e. only 19F is conjugated to DT), or may be split between one or more  
15 carrier proteins that are not DT and DT itself. In one embodiment, 19F is conjugated to DT or CRM 197 and all of the remaining serotypes are conjugated to PD. In a further embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are split between PD, and TT or DT or CRM 197. In a further embodiment, 19F is conjugated to DT or CRM 197 and no more than one saccharide is conjugated to TT. In one aspect of  
20 this embodiment, said one saccharide is 18C or 12F. In a further embodiment, 19F is conjugated to DT or CRM 197 and no more than two saccharides are conjugated to TT. In a further embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are split between PD, TT and DT or CRM 197. In a further embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are split between PD, TT and  
25 pneumolysin. In a further embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are split between PD, TT and CRM 197. In a further embodiment, 19F is conjugated to DT or CRM197 and the remaining serotypes are split between PD, TT, pneumolysin and optionally PhtD or PhtD/E fusion protein. In a further embodiment, 19F is conjugated to DT or CRM197, 19A is conjugated to pneumolysin or TT, one (two  
30 or three) further saccharide(s) is conjugated to TT, one further saccharide is conjugated to PhtD or PhtD/E and all further saccharides are conjugated to PD. In a further embodiment 19F is conjugated to DT or CRM197, 19A is conjugated to pneumolysin, one (two or three) further saccharide(s) is conjugated to TT, one further saccharide is conjugated to pneumolysin, 2 further saccharides are conjugated to PhtD or PhtD/E and all further  
35 saccharides are conjugated to PD.

In one embodiment, the immunogenic composition of the invention comprises protein D from *Haemophilus influenzae*. Within this embodiment, if PD is not one of the carrier proteins used to conjugate any saccharides other than 19F, for example 19F is conjugated to DT whilst the other serotypes are conjugated to one or more different carrier proteins which are not PD, then PD will be present in the vaccine composition as free protein. If PD is one of the carrier proteins used to conjugate saccharides other than 19F, then PD may optionally be present in the vaccine composition as free protein.

- 10 The term "saccharide" throughout this specification may indicate polysaccharide or oligosaccharide and includes both. Polysaccharides are isolated from bacteria and may be sized to some degree by known methods (see for example EP497524 and EP497525) and preferably by microfluidisation. Polysaccharides can be sized in order to reduce viscosity in polysaccharide samples and/or to improve filterability for conjugated products.
- 15 Oligosaccharides have a low number of repeat units (typically 5-30 repeat units) and are typically hydrolysed polysaccharides

Capsular polysaccharides of *Streptococcus pneumoniae* comprise repeating oligosaccharide units which may contain up to 8 sugar residues. For a review of the oligosaccharide units for the key *Streptococcus pneumoniae* serotypes see JONES, Christopher. **Vaccines based on the cell surface carbohydrates of pathogenic bacteria.** *An. Acad. Bras. Ciênc.*, June 2005, vol.77, no.2, p.293-324. ISSN 0001-3765. In one embodiment, a capsular saccharide antigen may be a full length polysaccharide, however in others it may be one oligosaccharide unit, or a shorter than native length saccharide chain of repeating oligosaccharide units. In one embodiment, all of the saccharides present in the vaccine are polysaccharides. Full length polysaccharides may be "sized" i.e. their size may be reduced by various methods such as acid hydrolysis treatment, hydrogen peroxide treatment, sizing by emulsiflex® followed by a hydrogen peroxide treatment to generate oligosaccharide fragments or microfluidization.

- 30
- The inventors have also noted that the focus of the art has been to use oligosaccharides for ease of conjugate production. The inventors have found that by using native or slightly sized polysaccharide conjugates, one or more of the following advantages may be realised: 1) a conjugate having high immunogenicity which is filterable, 2) the ratio of polysaccharide to protein in the conjugate can be altered such that the ratio of
- 35

polysaccharide to protein (w/w) in the conjugate may be increased (which can have an effect on the carrier suppression effect), 3) immunogenic conjugates prone to hydrolysis may be stabilised by the use of larger saccharides for conjugation. The use of larger polysaccharides can result in more cross-linking with the conjugate carrier and may  
5 lessen the liberation of free saccharide from the conjugate. The conjugate vaccines described in the prior art tend to depolymerise the polysaccharides prior to conjugation in order to improve conjugation. The present inventors have found that saccharide conjugate vaccines retaining a larger size of saccharide can provide a good immune response against pneumococcal disease.

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The immunogenic composition of the invention may thus comprise one or more saccharide conjugates wherein the average size (e.g. weight-average molecular weight;  $M_w$ ) of each saccharide before conjugation is above 80kDa, 100kDa, 200kDa, 300kDa, 400kDa, 500kDa or 1000kDa. In one embodiment one or more saccharide conjugates of  
15 the invention should have an average size of saccharide pre-conjugation of 50-1600, 80-1400, 100-1000, 150-500, or 200-400 kDa (note that where average size is  $M_w$ , 'kDa' units should be replaced herein with ' $\times 10^3$ '). In one embodiment the conjugate post conjugation should be readily filterable through a 0.2 micron filter such that a yield of more than 50, 60, 70, 80, 90 or 95% is obtained post filtration compared with the pre filtration  
20 sample.

For the purposes of the invention, "native polysaccharide" refers to a saccharide that has not been subjected to a process (e.g. post-purification), the purpose of which is to reduce the size of the saccharide. A polysaccharide can become slightly reduced in size during  
25 normal purification procedures. Such a saccharide is still native. Only if the polysaccharide has been subjected to sizing techniques would the polysaccharide not be considered native.

For the purposes of the invention, "sized by a factor up to x2" means that the saccharide  
30 is subject to a process intended to reduce the size of the saccharide but to retain a size more than half the size of the native polysaccharide. X3, x4 etc. are to be interpreted in the same way i.e. the saccharide is subject to a process intended to reduce the size of the polysaccharide but to retain a size more than a third, a quarter etc. the size of the native polysaccharide.

In an aspect of the invention, the immunogenic composition comprises *Streptococcus pneumoniae* saccharides from at least 10 serotypes conjugated to a carrier protein, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or each *S. pneumoniae* saccharide is native polysaccharide.

5

In an aspect of the invention, the immunogenic composition comprises *Streptococcus pneumoniae* saccharides from at least 10 serotypes conjugated to a carrier protein, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or each *S. pneumoniae* saccharide is sized by a factor up to x2, x3, x4, x5, x6, x7, x8, x9 or x10. In one embodiment of this aspect, the majority of the saccharides, for example 6, 7, 8 or more of the saccharides are sized by a factor up to x2, x3, x4, x5, x6, x7, x8, x9 or x10.

10

The molecular weight or average molecular weight (or size) of a saccharide herein refers to the weight-average molecular weight ( $M_w$ ) of the saccharide measured prior to conjugation and is measured by MALLS.

15

The MALLS technique is well known in the art and is typically carried out as described in example 2. For MALLS analysis of pneumococcal saccharides, two columns (TSKG6000 and 5000PWxl) may be used in combination and the saccharides are eluted in water. Saccharides are detected using a light scattering detector (for instance Wyatt Dawn DSP equipped with a 10mW argon laser at 488nm) and an interferometric refractometer (for instance Wyatt Otilab DSP equipped with a P100 cell and a red filter at 498nm).

20

In an embodiment the *S. pneumoniae* saccharides are native polysaccharides or native polysaccharides which have been reduced in size during a normal extraction process.

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In an embodiment, the *S. pneumoniae* saccharides are sized by mechanical cleavage, for instance by microfluidisation or sonication. Microfluidisation and sonication have the advantage of decreasing the size of the larger native polysaccharides sufficiently to provide a filterable conjugate. Sizing is by a factor of no more than x20, x10, x8, x6, x5, x4, x3 or x2.

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In an embodiment, the immunogenic composition comprises *S. pneumoniae* conjugates that are made from a mixture of native polysaccharides and saccharides that are sized by a factor of no more than x20. In one aspect of this embodiment, the majority of the

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saccharides, for example 6, 7, 8 or more of the saccharides are sized by a factor of up to x2, x3, x4, x5 or x6.

In an embodiment, the *Streptococcus pneumoniae* saccharide is conjugated to the carrier  
5 protein via a linker, for instance a bifunctional linker. The linker is optionally  
heterobifunctional or homobifunctional, having for example a reactive amino group and a  
reactive carboxylic acid group, 2 reactive amino groups or two reactive carboxylic acid  
groups. The linker has for example between 4 and 20, 4 and 12, 5 and 10 carbon atoms.  
A possible linker is ADH. Other linkers include B-propionamido (WO 00/10599),  
10 nitrophenyl-ethylamine (Gever et al (1979) Med. Microbiol. Immunol. 165; 171-288),  
haloalkyl halides (US4057685), glycosidic linkages (US4673574, US4808700), hexane  
diamine and 6-aminocaproic acid (US4459286). In an embodiment, ADH is used as a  
linker for conjugating saccharide from serotype 18C. In an embodiment, ADH is used as a  
linker for conjugating saccharide from serotype 22F.

15  
The saccharide conjugates present in the immunogenic compositions of the invention may  
be prepared by any known coupling technique. The conjugation method may rely on  
activation of the saccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate  
(CDAP) to form a cyanate ester. The activated saccharide may thus be coupled directly  
20 or via a spacer (linker) group to an amino group on the carrier protein. For example, the  
spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could  
be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-  
activated carrier protein (for example using GMBS) or a haloacetylated carrier protein (for  
example using iodoacetamide [e.g. ethyl iodoacetamide HCl] or N-succinimidyl  
25 bromoacetate or SIAB, or SIA, or SBAP). Preferably, the cyanate ester (optionally made  
by CDAP chemistry) is coupled with hexane diamine or ADH and the amino-derivatised  
saccharide is conjugated to the carrier protein using carbodiimide (e.g. EDAC or EDC)  
chemistry via a carboxyl group on the protein carrier. Such conjugates are described in  
PCT published application WO 93/15760 Uniformed Services University and WO  
30 95/08348 and WO 96/29094

Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-  
nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU. Many are described in WO  
98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of  
35 a free hydroxyl group of the saccharide with CDI (Bethell et al J. Biol. Chem. 1979, 254;

2572-4, Hearn et al J. Chromatogr. 1981. 218; 509-18) followed by reaction of with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group' reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

The conjugates can also be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide (or CDAP) activated saccharide derivatised with adipic acid dihydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256), for example using EDAC.

In an embodiment, a hydroxyl group (preferably an activated hydroxyl group for example a hydroxyl group activated to make a cyanate ester [e.g. with CDAP]) on a saccharide is linked to an amino or carboxylic group on a protein either directly or indirectly (through a linker). Where a linker is present, a hydroxyl group on a saccharide is preferably linked to an amino group on a linker, for example by using CDAP conjugation. A further amino group in the linker for example ADH) may be conjugated to a carboxylic acid group on a protein, for example by using carbodiimide chemistry, for example by using EDAC. In an embodiment, the pneumococcal capsular saccharide(s) is conjugated to the linker first before the linker is conjugated to the carrier protein. Alternatively the linker may be conjugated to the carrier before conjugation to the saccharide.

A combination of techniques may also be used, with some saccharide-protein conjugates being prepared by CDAP, and some by reductive amination.

In general the following types of chemical groups on a protein carrier can be used for coupling / conjugation:

A) Carboxyl (for instance via aspartic acid or glutamic acid). In one embodiment this group is linked to amino groups on saccharides directly or to an amino group on a linker with carbodiimide chemistry e.g. with EDAC.

5 B) Amino group (for instance via lysine). In one embodiment this group is linked to carboxyl groups on saccharides directly or to a carboxyl group on a linker with carbodiimide chemistry e.g. with EDAC. In another embodiment this group is linked to hydroxyl groups activated with CDAP or CNBr on saccharides directly or to such groups on a linker; to saccharides or linkers having an aldehyde group; to saccharides or linkers  
10 having a succinimide ester group.

C) Sulphydryl (for instance via cysteine). In one embodiment this group is linked to a bromo or chloro acetylated saccharide or linker with maleimide chemistry. In one  
15 embodiment this group is activated/modified with bis diazobenzidine.

D) Hydroxyl group (for instance via tyrosine). In one embodiment this group is activated/modified with bis diazobenzidine.

E) Imidazolyl group (for instance via histidine). In one embodiment this group is  
20 activated/modified with bis diazobenzidine.

F) Guanidyl group (for instance via arginine).

G) Indolyl group (for instance via tryptophan).  
25

On a saccharide, in general the following groups can be used for a coupling: OH, COOH or NH<sub>2</sub>. Aldehyde groups can be generated after different treatments known in the art such as: periodate, acid hydrolysis, hydrogen peroxide, etc.

30 *Direct coupling approaches:*

Saccharide-OH + CNBr or CDAP ----> cyanate ester + NH<sub>2</sub>-Prot --> conjugate

Saccharide-aldehyde + NH<sub>2</sub>-Prot ----> Schiff base + NaCNBH<sub>3</sub> ----> conjugate

Saccharide-COOH + NH<sub>2</sub>-Prot + EDAC --> conjugate

35 Saccharide-NH<sub>2</sub> + COOH-Prot + EDAC --> conjugate



*Indirect coupling via spacer (linker) approaches:*

Saccharide-OH + CNBr or CDAP  $\rightarrow$  cyanate ester + NH<sub>2</sub>---NH<sub>2</sub>  $\rightarrow$  saccharide---NH<sub>2</sub> + COOH-Prot + EDAC  $\rightarrow$  conjugate

Saccharide-OH + CNBr or CDAP  $\rightarrow$  cyanate ester + NH<sub>2</sub>---SH  $\rightarrow$  saccharide---SH + SH-Prot (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance)  $\rightarrow$  saccharide-S-S-Prot

Saccharide-OH + CNBr or CDAP  $\rightarrow$  cyanate ester + NH<sub>2</sub>---SH  $\rightarrow$  saccharide---SH + maleimide-Prot (modification of amino groups)  $\rightarrow$  conjugate

Saccharide-OH + CNBr or CDAP  $\rightarrow$  cyanate ester + NH<sub>2</sub>---SH  $\rightarrow$  Saccharide-SH + haloacetylated-Prot  $\rightarrow$  Conjugate

Saccharide-COOH + EDAC + NH<sub>2</sub>---NH<sub>2</sub>  $\rightarrow$  saccharide---NH<sub>2</sub> + EDAC + COOH-Prot  $\rightarrow$  conjugate

Saccharide-COOH + EDAC+ NH<sub>2</sub>---SH  $\rightarrow$  saccharide---SH + SH-Prot (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance)  $\rightarrow$  saccharide-S-S-Prot

Saccharide-COOH + EDAC+ NH<sub>2</sub>---SH  $\rightarrow$  saccharide---SH + maleimide-Prot (modification of amino groups)  $\rightarrow$  conjugate

Saccharide-COOH + EDAC + NH<sub>2</sub>---SH  $\rightarrow$  Saccharide-SH + haloacetylated-Prot  $\rightarrow$  Conjugate

Saccharide-Aldehyde + NH<sub>2</sub>---NH<sub>2</sub>  $\rightarrow$  saccharide---NH<sub>2</sub> + EDAC + COOH-Prot  $\rightarrow$  conjugate

Note: instead of EDAC above, any suitable carbodiimide may be used.

In summary, the types of protein carrier chemical group that may be generally used for coupling with a saccharide are amino groups (for instance on lysine residues), COOH groups (for instance on aspartic and glutamic acid residues) and SH groups (if accessible) (for instance on cysteine residues).

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Preferably the ratio of carrier protein to *S. pneumoniae* saccharide is between 1:5 and 5:1; e.g. between 1:0.5-4:1, 1:1-3.5:1, 1.2:1-3:1, 1.5:1-2.5:1; e.g. between 1:2 and 2.5:1; 1:1 and 2:1 (w/w). In an embodiment, the majority of the conjugates, for example 6, 7, 8, 9 or more of the conjugates have a ratio of carrier protein to saccharide that is greater than  
10 1:1, for example 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1 or 1.6:1.

In an embodiment, at least one *S. pneumoniae* saccharide is conjugated to a carrier protein via a linker using CDAP and EDAC. For example, 18C or 22F may be conjugated to a protein via a linker (for example those with two hydrazino groups at its ends such as  
15 ADH) using CDAP and EDAC as described above. When a linker is used, CDAP may be used to conjugate the saccharide to a linker and EDAC may then be used to conjugate the linker to a protein or, alternatively EDAC may be used first to conjugate the linker to the protein, after which CDAP may be used to conjugate the linker to the saccharide.

20 In general, the immunogenic composition of the invention may comprise a dose of each saccharide conjugate between 0.1 and 20 $\mu$ g, 1 and 10 $\mu$ g or 1 and 3 $\mu$ g of saccharide.

In an embodiment, the immunogenic composition of the invention contains each *S. pneumoniae* capsular saccharide at a dose of between 0.1-20 $\mu$ g; 0.5-10 $\mu$ g; 0.5- 5 $\mu$ g or 1-  
25 3 $\mu$ g of saccharide. In an embodiment, capsular saccharides may be present at different dosages, for example some capsular saccharides may be present at a dose of exactly 1 $\mu$ g or some capsular saccharides may be present at a dose of exactly 3 $\mu$ g. In an embodiment, saccharides from serotypes 3, 18C and 19F (or 4, 18C and 19F) are present at a higher dose than other saccharides. In one aspect of this embodiment, serotypes 3,  
30 18C and 19F (or 4, 18C and 19F) are present at a dose of around or exactly 3  $\mu$ g whilst other saccharides in the immunogenic composition are present at a dose of around or exactly 1 $\mu$ g.

"Around" or "approximately" are defined as within 10% more or less of the given figure for  
35 the purposes of the invention.

In an embodiment, at least one of the *S. pneumoniae* capsular saccharides is directly conjugated to a carrier protein (e.g. using one of the chemistries described above). Preferably the at least one of the *S. pneumoniae* capsular saccharides is directly  
5 conjugated by CDAP. In an embodiment, the majority of the capsular saccharides for example 5, 6, 7, 8, 9 or more are directly linked to the carrier protein by CDAP (see WO 95/08348 and WO 96/29094)

The immunogenic composition may comprise *Streptococcus pneumoniae* proteins, herein  
10 termed *Streptococcus pneumoniae* proteins of the invention. Such proteins may be used as carrier proteins, or may be present as free proteins, or may be present both as carrier proteins and as free proteins. The *Streptococcus pneumoniae* proteins of the invention are either surface exposed, at least during part of the life cycle of the pneumococcus, or are proteins which are secreted or released by the pneumococcus. Preferably the  
15 proteins of the invention are selected from the following categories, such as proteins having a Type II Signal sequence motif of LXXC (where X is any amino acid, e.g., the polyhistidine triad family (PhtX)), choline binding proteins (CbpX), proteins having a Type I Signal sequence motif (e.g., Sp101), proteins having a LPXTG motif (where X is any amino acid, e.g., Sp128, Sp130), and toxins (e.g., Ply). Preferred examples within these  
20 categories (or motifs) are the following proteins, or immunologically functional equivalents thereof.

In one embodiment, the immunogenic composition of the invention comprises at least 1 protein selected from the group consisting of the Poly Histidine Triad family (PhtX),  
25 Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133. In a further embodiment, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX  
30 family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, and Sp128. In one more embodiment, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions),  
35 pneumolysin (Ply), and Sp128.

The Pht (Poly Histidine Triad) family comprises proteins PhtA, PhtB, PhtD, and PhtE. The family is characterized by a lipidation sequence, two domains separated by a proline-rich region and several histidine triads, possibly involved in metal or nucleoside binding or enzymatic activity, (3-5) coiled-coil regions, a conserved N-terminus and a heterogeneous C terminus. It is present in all strains of pneumococci tested. Homologous proteins have also been found in other Streptococci and Neisseria. In one embodiment of the invention, the Pht protein of the invention is PhtD. It is understood, however, that the terms Pht A, B, D, and E refer to proteins having sequences disclosed in the citations below as well as naturally-occurring (and man-made) variants thereof that have a sequence homology that is at least 90% identical to the referenced proteins. Preferably it is at least 95% identical and most preferably it is 97% identical.

With regards to the PhtX proteins, PhtA is disclosed in WO 98/18930, and is also referred to Sp36. As noted above, it is a protein from the polyhistidine triad family and has the type II signal motif of LXXC. PhtD is disclosed in WO 00/37105, and is also referred to Sp036D. As noted above, it also is a protein from the polyhistidine triad family and has the type II LXXC signal motif. PhtB is disclosed in WO 00/37105, and is also referred to Sp036B. Another member of the PhtB family is the C3-Degrading Polypeptide, as disclosed in WO 00/17370. This protein also is from the polyhistidine triad family and has the type II LXXC signal motif. A preferred immunologically functional equivalent is the protein Sp42 disclosed in WO 98/18930. A PhtB truncate (approximately 79kD) is disclosed in WO99/15675 which is also considered a member of the PhtX family. PhtE is disclosed in WO00/30299 and is referred to as BVH-3. Where any Pht protein is referred to herein, it is meant that immunogenic fragments or fusions thereof of the Pht protein can be used. For example, a reference to PhtX includes immunogenic fragments or fusions thereof from any Pht protein. A reference to PhtD or PhtB is also a reference to PhtDE or PhtBE fusions as found, for example, in WO0198334.

Pneumolysin is a multifunctional toxin with a distinct cytolytic (hemolytic) and complement activation activities (Rubins et al., Am. J. Respir. Crit Care Med, 153:1339-1346 (1996)). The toxin is not secreted by pneumococci, but it is released upon lysis of pneumococci under the influence of autolysin. Its effects include e.g., the stimulation of the production of inflammatory cytokines by human monocytes, the inhibition of the beating of cilia on human respiratory epithelial, and the decrease of bactericidal activity and migration of

neutrophils. The most obvious effect of pneumolysin is in the lysis of red blood cells, which involves binding to cholesterol. Because it is a toxin, it needs to be detoxified (i.e., non-toxic to a human when provided at a dosage suitable for protection) before it can be administered *in vivo*. Expression and cloning of wild-type or native pneumolysin is known  
5 in the art. See, for example, Walker et al. (Infect Immun, 55:1184-1189 (1987)), Mitchell et al. (Biochim Biophys Acta, 1007:67-72 (1989) and Mitchell et al (NAR, 18:4010 (1990)). Detoxification of ply can be conducted by chemical means, e.g., subject to formalin or glutaraldehyde treatment or a combination of both (WO 04081515, PCT/EP2005/010258). Such methods are well known in the art for various toxins. Alternatively, ply can be  
10 genetically detoxified. Thus, the invention encompasses derivatives of pneumococcal proteins which may be, for example, mutated proteins. The term "mutated" is used herein to mean a molecule which has undergone deletion, addition or substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method. For example, as described above, a mutant ply protein may be  
15 altered so that it is biologically inactive whilst still maintaining its immunogenic epitopes, see, for example, WO90/06951, Berry et al. (Infect Immun, 67:981-985 (1999)) and WO99/03884.

As used herein, it is understood that the term "Ply" refers to mutated or detoxified  
20 pneumolysin suitable for medical use (i.e., non toxic).

Concerning the Choline Binding Protein family (CbpX), members of that family were originally identified as pneumococcal proteins that could be purified by choline-affinity chromatography. All of the choline-binding proteins are non-covalently bound to  
25 phosphorylcholine moieties of cell wall teichoic acid and membrane-associated lipoteichoic acid. Structurally, they have several regions in common over the entire family, although the exact nature of the proteins (amino acid sequence, length, etc.) can vary. In general, choline binding proteins comprise an N terminal region (N), conserved repeat regions (R1 and/or R2), a proline rich region (P) and a conserved choline binding region  
30 (C), made up of multiple repeats, that comprises approximately one half of the protein. As used in this application, the term "Choline Binding Protein family (CbpX)" is selected from the group consisting of Choline Binding Proteins as identified in WO97/41151, PbcA, SpsA, PspC, CbpA, CbpD, and CbpG. CbpA is disclosed in WO97/41151. CbpD and CbpG are disclosed in WO00/29434. PspC is disclosed in WO97/09994. PbcA is  
35 disclosed in WO98/21337. SpsA is a Choline binding protein disclosed in WO 98/39450.

Preferably the Choline Binding Proteins are selected from the group consisting of CbpA, PbcA, SpsA and PspC.

Another preferred embodiment is CbpX truncates wherein "CbpX" is defined above and "truncates" refers to CbpX proteins lacking 50% or more of the Choline binding region (C). Preferably such proteins lack the entire choline binding region. More preferably, the such protein truncates lack (i) the choline binding region and (ii) a portion of the N-terminal half of the protein as well, yet retain at least one repeat region (R1 or R2). More preferably still, the truncate has 2 repeat regions (R1 and R2). Examples of such preferred 5  
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embodiments are NR1xR2 and R1xR2 as illustrated in WO99/51266 or WO99/51188, however, other choline binding proteins lacking a similar choline binding region are also contemplated within the scope of this invention.

The LytX family is membrane associated proteins associated with cell lysis. The N-terminal domain comprises choline binding domain(s), however the LytX family does not have all the features found in the CbpA family noted above and thus for the present invention, the LytX family is considered distinct from the CbpX family. In contrast with the CbpX family, the C-terminal domain contains the catalytic domain of the LytX protein family. The family comprises LytA, B and C. With regards to the LytX family, LytA is disclosed in Ronda et al., Eur J Biochem, 164:621-624 (1987). LytB is disclosed in WO 98/18930, and is also referred to as Sp46. LytC is also disclosed in WO 98/18930, and is also referred to as Sp91. A preferred member of that family is LytC.

Another preferred embodiment are LytX truncates wherein "LytX" is defined above and "truncates" refers to LytX proteins lacking 50% or more of the Choline binding region. Preferably such proteins lack the entire choline binding region. Yet another preferred embodiment of this invention are CbpX truncate-LytX truncate chimeric proteins (or fusions). Preferably this comprises NR1xR2 (or R1xR2) of CbpX and the C-terminal portion (Cterm, i.e., lacking the choline binding domains) of LytX (e.g., LytCCterm or Sp91Cterm). More preferably CbpX is selected from the group consisting of CbpA, PbcA, SpsA and PspC. More preferably still, it is CbpA. Preferably, LytX is LytC (also referred to as Sp91). Another embodiment of the present invention is a PspA or PsaA truncates lacking the choline binding domain (C) and expressed as a fusion protein with LytX. Preferably, LytX is LytC.

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With regards to PsaA and PspA, both are known in the art. For example, PsaA and transmembrane deletion variants thereof have been described by Berry & Paton, *Infect Immun* 1996 Dec;64(12):5255-62. PspA and transmembrane deletion variants thereof have been disclosed in, for example, US 5804193, WO 92/14488, and WO 99/53940.

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Sp128 and Sp130 are disclosed in WO00/76540. Sp125 is an example of a pneumococcal surface protein with the Cell Wall Anchored motif of LPXTG (where X is any amino acid). Any protein within this class of pneumococcal surface protein with this motif has been found to be useful within the context of this invention, and is therefore considered a further protein of the invention. Sp125 itself is disclosed in WO 98/18930, and is also known as ZmpB – a zinc metalloproteinase. Sp101 is disclosed in WO 98/06734 (where it has the reference # y85993). It is characterized by a Type I signal sequence. Sp133 is disclosed in WO 98/06734 (where it has the reference # y85992). It is also characterized by a Type I signal sequence.

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Examples of preferred *Moraxella catarrhalis* protein antigens which can be included in a combination vaccine (especially for the prevention of otitis media) are: OMP106 [WO 97/41731 (Antex) & WO 96/34960 (PMC)]; OMP21 or fragments thereof (WO 0018910); LbpA &/or LbpB [WO 98/55606 (PMC)]; TbpA &/or TbpB [WO 97/13785 & WO 97/32980 (PMC)]; CopB [Helminen ME, *et al.* (1993) *Infect. Immun.* 61:2003-2010]; UspA1 and/or UspA2 [WO 93/03761 (University of Texas)]; OmpCD; HasR (PCT/EP99/03824); PilQ (PCT/EP99/03823); OMP85 (PCT/EP00/01468); lipo06 (GB 9917977.2); lipo10 (GB 9918208.1); lipo11 (GB 9918302.2); lipo18 (GB 9918038.2); P6 (PCT/EP99/03038); D15 (PCT/EP99/03822); OmpIA1 (PCT/EP99/06781); Hly3 (PCT/EP99/03257); and OmpE.

Examples of non-typeable *Haemophilus influenzae* antigens or fragments thereof which can be included in a combination vaccine (especially for the prevention of otitis media) include: Fimbrin protein [(US 5766608 - Ohio State Research Foundation)] and fusions comprising peptides therefrom [eg LB1(f) peptide fusions; US 5843464 (OSU) or WO 99/64067]; OMP26 [WO 97/01638 (Cortecs)]; P6 [EP 281673 (State University of New York)]; TbpA and/or TbpB; Hia; Hsf; Hin47; Hif; Hmw1; Hmw2; Hmw3; Hmw4; Hap; D15 (WO 94/12641); P2; and P5 (WO 94/26304).

The proteins of the invention may also be beneficially combined. By combined is meant that the immunogenic composition comprises all of the proteins from within the following combinations, either as carrier proteins or as free proteins or a mixture of the two. For

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example, in a combination of two proteins as set out hereinafter, both proteins may be used as carrier proteins, or both proteins may be present as free proteins, or both may be present as carrier and as free protein, or one may be present as a carrier protein and a free protein whilst the other is present only as a carrier protein or only as a free protein, or one may be present as a carrier protein and the other as a free protein. Where a combination of three proteins is given, similar possibilities exist. Preferred combinations include, but are not limited to, PhtD + NR1xR2, PhtD + NR1xR2-Sp91Cterm chimeric or fusion proteins, PhtD + Ply, PhtD + Sp128, PhtD + PsaA, PhtD + PspA, PhtA + NR1xR2, PhtA + NR1xR2-Sp91Cterm chimeric or fusion proteins, PhtA + Ply, PhtA + Sp128, PhtA + PsaA, PhtA + PspA, NR1xR2 + LytC, NR1xR2 + PspA, NR1xR2 + PsaA, NR1xR2 + Sp128, R1xR2 + LytC, R1xR2 + PspA, R1xR2 + PsaA, R1xR2 + Sp128, R1xR2 + PhtD, R1xR2 + PhtA. Preferably, NR1xR2 (or R1xR2) is from CbpA or PspC. More preferably it is from CbpA. Other combinations include 3 protein combinations such as PhtD + NR1xR2 + Ply, and PhtA + NR1xR2 + PhtD. In one embodiment, the vaccine composition comprises detoxified pneumolysin and PhtD or PhtDE as carrier proteins. In a further embodiment, the vaccine composition comprises detoxified pneumolysin and PhtD or PhtDE as free proteins.

The present invention further provides a vaccine containing the immunogenic compositions of the invention and a pharmaceutically acceptable excipient.

The vaccines of the present invention may be adjuvanted, particularly when intended for use in an elderly population but also for use in infant populations. Suitable adjuvants include an aluminum salt such as aluminum hydroxide gel or aluminum phosphate or alum, but may also be a salt of calcium, magnesium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatized saccharides, or polyphosphazenes.

It is preferred that the adjuvant be selected to be a preferential inducer of a TH1 type of response. Such high levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to a given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

The distinction of Th1 and Th2-type immune response is not absolute. In reality an individual will support an immune response which is described as being predominantly



Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. (Annual Review of Immunology, 7, p145-173). Traditionally, Th1-type responses are associated with the production of the INF- $\gamma$  and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of IL-4, IL-5, IL-6, IL-10. Suitable adjuvant systems which promote a predominantly Th1 response include: Monophosphoryl lipid A or a derivative thereof, particularly 3-de-O-acylated monophosphoryl lipid A (3D-MPL) (for its preparation see GB 2220211 A); and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with either an aluminum salt (for instance aluminum phosphate or aluminum hydroxide) or an oil-in-water emulsion. In such combinations, antigen and 3D-MPL are contained in the same particulate structures, allowing for more efficient delivery of antigenic and immunostimulatory signals. Studies have shown that 3D-MPL is able to further enhance the immunogenicity of an alum-adsorbed antigen [Thoelen *et al.* Vaccine (1998) 16:708-14; EP 689454-B1].

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative, particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO 95/17210. In one embodiment the immunogenic composition additionally comprises a saponin, which may be QS21. The formulation may also comprise an oil in water emulsion and tocopherol (WO 95/17210). Unmethylated CpG containing oligonucleotides (WO 96/02555) and other immunomodulatory oligonucleotides (WO0226757 and WO03507822) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

Particular adjuvants are those selected from the group of metal Salts, oil in water emulsions, Toll like receptors agonist, (in particular Toll like receptor 2 agonist, Toll like receptor 3 agonist, Toll like receptor 4 agonist, Toll like receptor 7 agonist, Toll like receptor 8 agonist and Toll like receptor 9 agonist), saponins or combinations thereof.

An adjuvant that can be used with the vaccine compositions of the invention are bleb or outer membrane vesicle preparations from Gram negative bacterial strains such as those taught by WO02/09746 – particularly *N. meningitidis* blebs. Adjuvant properties of blebs  
5 can be improved by retaining LOS (lipooligosaccharide) on its surface (e.g. through extraction with low concentrations of detergent [for instance 0-0.1% deoxycholate]). LOS can be detoxified through the *msbB(-)* or *htrB(-)* mutations discussed in WO02/09746. Adjuvant properties can also be improved by retaining PorB (and optionally removing PorA) from meningococcal blebs. Adjuvant properties can also be improved by truncating  
10 the outer core saccharide structure of LOS on meningococcal blebs – for instance via the *lgtB(-)* mutation discussed in WO2004/014417. Alternatively, the aforementioned LOS (e.g. isolated from a *msbB(-)* and/or *lgtB(-)* strain) can be purified and used as an adjuvant in the compositions of the invention.

15 A further adjuvant which may be used with the compositions of the invention may be selected from the group: a saponin, lipid A or a derivative thereof, an immunostimulatory oligonucleotide, an alkyl glucosaminide phosphate, an oil in water emulsion or combinations thereof. A further preferred adjuvant is a metal salt in combination with another adjuvant. It is preferred that the adjuvant is a Toll like receptor agonist in  
20 particular an agonist of a Toll like receptor 2, 3, 4, 7, 8 or 9, or a saponin, in particular Qs21. It is further preferred that the adjuvant system comprises two or more adjuvants from the above list. In particular the combinations preferably contain a saponin (in particular Qs21) adjuvant and/or a Toll like receptor 9 agonist such as a CpG containing immunostimulatory oligonucleotide. Other preferred combinations comprise a saponin (in  
25 particular QS21) and a Toll like receptor 4 agonist such as monophosphoryl lipid A or its 3 deacylated derivative, 3 D – MPL, or a saponin (in particular QS21) and a Toll like receptor 4 ligand such as an alkyl glucosaminide phosphate.

Particularly preferred adjuvants are combinations of 3D-MPL and QS21 (EP 0 671 948  
30 B1), oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210, WO 98/56414), or 3D-MPL formulated with other carriers (EP 0 689 454 B1). Other preferred adjuvant systems comprise a combination of 3 D MPL , QS21 and a CpG oligonucleotide as described in US6558670, US6544518.

In an embodiment the adjuvant is (or comprises) a Toll like receptor (TLR) 4 ligand, preferably an agonist such as a lipid A derivative particularly monophosphoryl lipid A or more particularly 3 Deacylated monophosphoryl lipid A (3 D - MPL).

5 3 D -MPL is available from GlaxoSmithKline Biologicals North America and primarily promotes CD4+ T cell responses with an IFN-g (Th1) phenotype . It can be produced according to the methods disclosed in GB 2 220 211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. Preferably in the compositions of the present invention small particle 3 D- MPL is used. Small particle 3 D  
 10 -MPL has a particle size such that it may be sterile-filtered through a 0.22µm filter. Such preparations are described in International Patent Application No. WO 94/21292. Synthetic derivatives of lipid A are known and thought to be TLR 4 agonists including, but not limited to:

15 OM174 (2-deoxy-6-o-[2-deoxy-2-[(R)-3-dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9(R)-[(R)-3-hydroxytetradecanoylamino]-α-D-glucopyranosylidihydrogenphosphate), (WO 95/14026)

OM 294 DP (3S, 9 R) -3-[(R)-dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9(R)-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1,10-bis(dihydrogenophosphate) (WO99 /64301 and WO 00/0462 )

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OM 197 MP-Ac DP ( 3S-, 9R) -3-[(R) -dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1 -dihydrogenophosphate 10-(6-aminohexanoate) (WO 01/46127)

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Other TLR4 ligands which may be used are alkyl Glucosaminide phosphates (AGPs) such as those disclosed in WO9850399 or US6303347 (processes for preparation of AGPs are also disclosed), or pharmaceutically acceptable salts of AGPs as disclosed in  
 30 US6764840. Some AGPs are TLR4 agonists, and some are TLR4 antagonists. Both are thought to be useful as adjuvants.

Another preferred immunostimulant for use in the present invention is Quil A and its derivatives. Quil A is a saponin preparation isolated from the South American tree Quilaja Saponaria Molina and was first described as having adjuvant activity by Dalsgaard et al.  
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in 1974 ("Saponin adjuvants", Archiv. für die gesamte Virusforschung, Vol. 44, Springer Verlag, Berlin, p243-254). Purified fragments of Quil A have been isolated by HPLC which retain adjuvant activity without the toxicity associated with Quil A (EP 0 362 278), for example QS7 and QS21 (also known as QA7 and QA21). QS-21 is a natural saponin  
5 derived from the bark of Quillaja saponaria Molina which induces CD8+ cytotoxic T cells (CTLs), Th1 cells and a predominant IgG2a antibody response and is a preferred saponin in the context of the present invention.

Particular formulations of QS21 have been described which are particularly preferred,  
10 these formulations further comprise a sterol (WO96/33739). The saponins forming part of the present invention may be separate in the form of micelles, mixed micelles (preferentially, but not exclusively with bile salts) or may be in the form of ISCOM matrices (EP 0 109 942 B1), liposomes or related colloidal structures such as worm-like or ring-like  
15 multimeric complexes or lipidic/layered structures and lamellae when formulated with cholesterol and lipid, or in the form of an oil in water emulsion (for example as in WO 95/17210). The saponins may preferably be associated with a metallic salt, such as aluminium hydroxide or aluminium phosphate (WO 98/15287).

Preferably, the saponin is presented in the form of a liposome, ISCOM or an oil in water emulsion.

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An enhanced system involves the combination of a monophosphoryl lipid A (or detoxified lipid A) and a saponin derivative, particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739. A particularly potent adjuvant  
25 formulation involving tocopherol with or without QS21 and/or 3D-MPL in an oil in water emulsion is described in WO 95/17210. In one embodiment the immunogenic composition additionally comprises a saponin, which may be QS21.

Immunostimulatory oligonucleotides or any other Toll-like receptor (TLR) 9 agonist may  
30 also be used. The preferred oligonucleotides for use in adjuvants or vaccines of the present invention are CpG containing oligonucleotides, preferably containing two or more dinucleotide CpG motifs separated by at least three, more preferably at least six or more nucleotides. A CpG motif is a Cytosine nucleotide followed by a Guanine nucleotide. The CpG oligonucleotides of the present invention are typically deoxynucleotides. In a  
35 preferred embodiment the internucleotide in the oligonucleotide is phosphorodithioate, or

more preferably a phosphorothioate bond, although phosphodiester and other internucleotide bonds are within the scope of the invention. Also included within the scope of the invention are oligonucleotides with mixed internucleotide linkages. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in  
5 US5,666,153, US5,278,302 and WO95/26204.

Examples of preferred oligonucleotides have the following sequences. The sequences preferably contain phosphorothioate modified internucleotide linkages.

OLIGO 1 (SEQ ID NO:1): TCC ATG ACG TTC CTG ACG TT (CpG 1826)

10 OLIGO 2 (SEQ ID NO:2): TCT CCC AGC GTG CGC CAT (CpG 1758)

OLIGO 3 (SEQ ID NO:3): ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG

OLIGO 4 (SEQ ID NO:4): TCG TCG TTT TGT CGT TTT GTC GTT (CpG 2006)

OLIGO 5 (SEQ ID NO:5): TCC ATG ACG TTC CTG ATG CT (CpG 1668)

OLIGO 6 (SEQ ID NO:6): TCG ACG TTT TCG GCG CGC GCC G (CpG 5456)

15

Alternative CpG oligonucleotides may comprise the preferred sequences above in that they have inconsequential deletions or additions thereto.

The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (for example see EP 468520). Conveniently, such  
20 oligonucleotides may be synthesized utilising an automated synthesizer.

The adjuvant may be an oil in water emulsion or may comprise an oil in water emulsion in combination with other adjuvants. The oil phase of the emulsion system preferably comprises a metabolisable oil. The meaning of the term metabolisable oil is well known in  
25 the art. Metabolisable can be defined as "being capable of being transformed by metabolism" (Dorland's Illustrated Medical Dictionary, W.B. Sanders Company, 25<sup>th</sup> edition (1974)). The oil may be any vegetable oil, fish, oil, animal or synthetic oil, which is not toxic to the recipient and is capable of being transformed by metabolism. Nuts, seeds, and grains are common sources of vegetable oils. Synthetic oils are also part of this  
30 invention and can include commercially available oils such as NEOBEE® and others. Squalene (2,6,10,15,19, 23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene) is an unsaturated oil which is found in large quantities in shark-liver oil, and in lower quantities in olive oil, wheat germ oil, rice bran oil, and yeast, and is a particularly preferred oil for use in this invention. Squalene is a metabolisable oil by virtue of the fact that it is an  
35 intermediate in the biosynthesis of cholesterol (Merck index, 10<sup>th</sup> Edition, entry no.8619).

Tocols (e.g. vitamin E) are also often used in oil emulsions adjuvants (EP 0 382 271 B1; US5667784; WO 95/17210). Tocols used in the oil emulsions (preferably oil in water emulsions) of the invention may be formulated as described in EP 0 382 271 B1, in that  
5 the tocols may be dispersions of tocol droplets, optionally comprising an emulsifier, of preferably less than 1 micron in diameter. Alternatively, the tocols may be used in combination with another oil, to form the oil phase of an oil emulsion. Examples of oil emulsions which may be used in combination with the tocol are described herein, such as the metabolisable oils described above.

10

Oil in water emulsion adjuvants per se have been suggested to be useful as adjuvant compositions (EP 0 399 843B), also combinations of oil in water emulsions and other active agents have been described as adjuvants for vaccines (WO 95/17210; WO 98/56414; WO 99/12565; WO 99/11241). Other oil emulsion adjuvants have been  
15 described, such as water in oil emulsions (US 5,422,109; EP 0 480 982 B2) and water in oil in water emulsions (US 5,424,067; EP 0 480 981 B). All of which form preferred oil emulsion systems (in particular when incorporating tocols) to form adjuvants and compositions of the present invention.

20 Most preferably the oil emulsion (for instance oil in water emulsions) further comprises an emulsifier such as TWEEN 80 and/or a sterol such as cholesterol.

A preferred oil emulsion (preferably oil-in-water emulsion) comprises a metabolisable, non-toxic oil, such as squalane, squalene or a tocopherol such as alpha tocopherol (and preferably both squalene and alpha tocopherol) and optionally an emulsifier (or surfactant)  
25 such as Tween 80. A sterol (preferably cholesterol) may also be included.

The method of producing oil in water emulsions is well known to the man skilled in the art. Commonly, the method comprises mixing the tocol-containing oil phase with a surfactant such as a PBS/TWEEN80™ solution, followed by homogenisation using a homogenizer, it would be clear to a man skilled in the art that a method comprising passing the mixture  
30 twice through a syringe needle would be suitable for homogenising small volumes of liquid. Equally, the emulsification process in microfluidiser (M110S Microfluidics machine, maximum of 50 passes, for a period of 2 minutes at maximum pressure input of 6 bar (output pressure of about 850 bar)) could be adapted by the man skilled in the art to produce smaller or larger volumes of emulsion. The adaptation could be achieved by

routine experimentation comprising the measurement of the resultant emulsion until a preparation was achieved with oil droplets of the required diameter.

In an oil in water emulsion, the oil and emulsifier should be in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

5

The size of the oil droplets found within the stable oil in water emulsion are preferably less than 1 micron, may be in the range of substantially 30-600nm, preferably substantially around 30-500nm in diameter, and most preferably substantially 150-500nm in diameter, and in particular about 150 nm in diameter as measured by photon  
10 correlation spectroscopy. In this regard, 80% of the oil droplets by number should be within the preferred ranges, more preferably more than 90% and most preferably more than 95% of the oil droplets by number are within the defined size ranges. The amounts of the components present in the oil emulsions of the present invention are conventionally in the range of from 0.5-20% or 2 to 10% oil (of the total dose volume), such as squalene;  
15 and when present, from 2 to 10% alpha tocopherol; and from 0.3 to 3% surfactant, such as polyoxyethylene sorbitan monooleate. Preferably the ratio of oil (preferably squalene): tocol (preferably  $\alpha$ -tocopherol) is equal or less than 1 as this provides a more stable emulsion. An emulsifier, such as Tween80 or Span 85 may also be present at a level of about 1%. In some cases it may be advantageous that the vaccines of the present  
20 invention will further contain a stabiliser.

Examples of preferred emulsion systems are described in WO 95/17210, WO 99/11241 and WO\*99/12565 which disclose emulsion adjuvants based on squalene,  $\alpha$ -tocopherol, and TWEEN 80, optionally formulated with the immunostimulants QS21 and/or 3D-MPL.

25 Thus in a particularly, preferred embodiment of the present invention, the adjuvant of the invention may additionally comprise further immunostimulants, such as LPS or derivatives thereof, and/or saponins. Examples of further immunostimulants are described herein and in "Vaccine Design – The Subunit and Adjuvant Approach" 1995, Pharmaceutical  
30 Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X.

In a preferred aspect the adjuvant and immunogenic compositions according to the invention comprise a saponin (preferably QS21) and/or an LPS derivative (preferably 3D-MPL) in an oil emulsion described above, optionally with a sterol (preferably  
35 cholesterol). Additionally the oil emulsion (preferably oil in water emulsion) may contain

span 85 and/or lecithin and/or tricaprylin. Adjuvants comprising an oil-in-water emulsion, a sterol and a saponin are described in WO 99/12565.

Typically for human administration the saponin (preferably QS21) and/or LPS derivative (preferably 3D-MPL) will be present in a human dose of immunogenic composition in the range of 1µg – 200µg, such as 10-100µg, preferably 10µg - 50µg per  
5 dose. Typically the oil emulsion (preferably oil in water emulsion) will comprise from 2 to 10% metabolisable oil. Preferably it will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% (preferably 0.4 – 2%) emulsifier (preferably tween 80 [polyoxyethylene sorbitan monooleate]). Where both squalene and alpha tocopherol are  
10 present, preferably the ratio of squalene: alpha tocopherol is equal to or less than 1 as this provides a more stable emulsion. Span 85 (Sorbitan trioleate) may also be present at a level of 0.5 to 1% in the emulsions used in the invention. In some cases it may be advantageous that the immunogenic compositions and vaccines of the present invention will further contain a stabiliser, for example other emulsifiers/surfactants, including caprylic  
15 acid (merck index 10<sup>th</sup> Edition, entry no. 1739), of which Tricaprylin is particularly preferred.

Where squalene and a saponin (preferably QS21) are included, it is of benefit to also include a sterol (preferably cholesterol) to the formulation as this allows a reduction in the total level of oil in the emulsion. This leads to a reduced cost of manufacture,  
20 improvement of the overall comfort of the vaccination, and also qualitative and quantitative improvements of the resultant immune responses, such as improved IFN-γ production. Accordingly, the adjuvant system of the present invention typically comprises a ratio of metabolisable oil:saponin (w/w) in the range of 200:1 to 300:1, also the present invention can be used in a "low oil" form the preferred range of which is 1:1 to 200:1,  
25 preferably 20:1 to 100:1, and most preferably substantially 48:1, this vaccine retains the beneficial adjuvant properties of all of the components, with a much reduced reactogenicity profile. Accordingly, the particularly preferred embodiments have a ratio of squalene:QS21 (w/w) in the range of 1:1 to 250:1, also a preferred range is 20:1 to 200:1, preferably 20:1 to 100:1, and most preferably substantially 48:1. Preferably a sterol (most  
30 preferably cholesterol) is also included present at a ratio of saponin:sterol as described herein.

The emulsion systems of the present invention preferably have a small oil droplet size in the sub-micron range. Most preferably the oil droplet sizes will be in the range 120 to 750 nm, and most preferably from 120-600nm in diameter.



A particularly potent adjuvant formulation (for ultimate combination with AlPO<sub>4</sub> in the immunogenic compositions of the invention) involves a saponin (preferably QS21), an LPS derivative (preferably 3D-MPL) and an oil emulsion (preferably squalene and alpha tocopherol in an oil in water emulsion) as described in WO 95/17210 or in WO 99/12565  
5 (in particular adjuvant formulation 11 in Example 2, Table 1).

Examples of a TLR 2 agonist include peptidoglycan or lipoprotein. Imidazoquinolines, such as Imiquimod and Resiquimod are known TLR7 agonists. Single stranded RNA is also a known TLR agonist (TLR8 in humans and TLR7 in mice), whereas double stranded  
10 RNA and poly IC (polyinosinic-polycytidylic acid - a commercial synthetic mimetic of viral RNA). are exemplary of TLR 3 agonists. 3D-MPL is an example of a TLR4 agonist whilst CPG is an example of a TLR9 agonist.

The immunogenic composition may comprise an antigen and an immunostimulant  
15 adsorbed onto a metal salt. Aluminium based vaccine formulations wherein the antigen and the immunostimulant 3-de-O-acylated monophosphoryl lipid A (3D-MPL), are adsorbed onto the same particle are described in EP 0 576 478 B1, EP 0 689 454 B1, and EP 0 633 784 B1. In these cases then antigen is first adsorbed onto the aluminium salt followed by the adsorption of the immunostimulant 3D-MPL onto the same aluminium salt  
20 particles. Such processes first involve the suspension of 3D-MPL by sonication in a water bath until the particles reach a size of between 80 and 500 nm. The antigen is typically adsorbed onto aluminium salt for one hour at room temperature under agitation. The 3D-MPL suspension is then added to the adsorbed antigen and the formulation is incubated at room temperature for 1 hour, and then kept at 4°C until use.

25 In another process, the immunostimulant and the antigen are on separate metal particles, as described in EP 1126876. The improved process comprises the adsorption of immunostimulant, onto a metallic salt particle, followed by the adsorption of the antigen onto another metallic salt particle, followed by the mixing of the discrete metallic particles  
30 to form a vaccine. The adjuvant for use in the present invention may be an adjuvant composition comprising an immunostimulant, adsorbed onto a metallic salt particle, characterised in that the metallic salt particle is substantially free of other antigen. Furthermore, vaccines are provided by the present invention and are characterised in that the immunostimulant is adsorbed onto particles of metallic salt which are substantially free

from other antigen, and in that the particles of metallic salt which are adsorbed to the antigen are substantially free of other immunostimulant.

Accordingly, the present invention provides an adjuvant formulation comprising immunostimulant which has been adsorbed onto a particle of a metallic salt, characterised  
5 in the composition is substantially free of other antigen. Moreover, this adjuvant formulation can be an intermediate which, if such an adjuvant is used, is required for the manufacture of a vaccine. Accordingly there is provided a process for the manufacture of a vaccine comprising admixing an adjuvant composition which is one or more immunostimulants adsorbed onto a metal particle with an antigen. Preferably, the antigen  
10 has been pre-adsorbed onto a metallic salt. Said metallic salt may be identical or similar to the metallic salt which is adsorbed onto the immunostimulant. Preferably the metal salt is an aluminium salt, for example Aluminium phosphate or Aluminium hydroxide.

The present invention further provides for a vaccine composition comprising immunostimulant adsorbed onto a first particle of a metallic salt, and antigen adsorbed  
15 onto a metallic salt, characterised in that first and second particles of metallic salt are separate particles.

LPS or LOS derivatives or mutations or lipid A derivatives described herein are designed to be less toxic (e.g. 3D-MPL) than native lipopolysaccharides and are interchangeable  
20 equivalents with respect to any uses of these moieties described herein. They may be TLR4 ligands as described above. Other such derivatives are described in WO020786737, WO9850399, WO0134617, WO0212258, WO03065806.

In one embodiment the adjuvant used for the compositions of the invention comprises a  
25 liposome carrier (made by known techniques from a phospholipids (such as dioleoyl phosphatidyl choline [DOPC]) and optionally a sterol [such as cholesterol]). Such liposome carriers may carry lipid A derivatives [such as 3D-MPL – see above] and/or saponins (such as QS21 – see above). In one embodiment the adjuvant comprises (per 0.5 mL dose) 0.1-10mg, 0.2-7, 0.3-5, 0.4-2, or 0.5-1 mg (e.g. 0.4-0.6, 0.9-1.1, 0.5 or 1 mg)  
30 phospholipid (for instance DOPC), 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol), 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) lipid A derivative (for instance 3D-MPL), and 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) saponin (for instance QS21).

35

This adjuvant is particularly suitable for elderly vaccine formulations. In one embodiment the vaccine composition comprising this adjuvant comprises saccharide conjugates derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F (and may also comprise one or more from serotypes 3, 6A, 19A, and 22F), wherein the GMC antibody titre induced against one or more (or all) the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

In one embodiment the adjuvant used for the compositions of the invention comprises an oil in water emulsion made from a metabolisable oil (such as squalene), an emulsifier (such as Tween 80) and optionally a tocol (such as alpha tocopherol). In one embodiment the adjuvant comprises (per 0.5 mL dose) 0.5-15, 1-13, 2-11, 4-8, or 5-6mg (e.g. 2-3, 5-6, or 10-11 mg) metabolisable oil (such as squalene), 0.1-10, 0.3-8, 0.6-6, 0.9-5, 1-4, or 2-3 mg (e.g. 0.9-1.1, 2-3 or 4-5 mg) emulsifier (such as Tween 80) and optionally 0.5-20, 1-15, 2-12, 4-10, 5-7 mg (e.g. 11-13, 5-6, or 2-3 mg) tocol (such as alpha tocopherol).

This adjuvant may optionally further comprise 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) lipid A derivative (for instance 3D-MPL).

These adjuvants are particularly suitable for infant or elderly vaccine formulations. In one embodiment the vaccine composition comprising this adjuvant comprises saccharide conjugates derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F (and may also comprise one or more from serotypes 3, 6A, 19A, and 22F), wherein the GMC antibody titre induced against one or more (or all) the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

This adjuvant may optionally contain 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol), 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) lipid A derivative (for instance 3D-MPL), and 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) saponin (for instance QS21).

This adjuvant is particularly suitable for elderly vaccine formulations. In one embodiment the vaccine composition comprising this adjuvant comprises saccharide conjugates

derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F (and may also comprise one or more from serotypes 3, 6A, 19A, and 22F), wherein the GMC antibody titre induced against one or more (or all) the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Plevnar® vaccine in human vaccinees.

In one embodiment the adjuvant used for the compositions of the invention comprises aluminium phosphate and a lipid A derivative (such as 3D-MPL). This adjuvant may comprise (per 0.5 mL dose) 100-750, 200-500, or 300-400 µg Al as aluminium phosphate, and 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) lipid A derivative (for instance 3D-MPL).

This adjuvant is particularly suitable for elderly or infant vaccine formulations. In one embodiment the vaccine composition comprising this adjuvant comprises saccharide conjugates derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F (and may also comprise one or more from serotypes 3, 6A, 19A, and 22F), wherein the GMC antibody titre induced against one or more (or all) the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Plevnar® vaccine in human vaccinees.

The vaccine preparations containing immunogenic compositions of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Intranasal administration of vaccines for the treatment of pneumonia or otitis media is preferred (as nasopharyngeal carriage of pneumococci can be more effectively prevented, thus attenuating infection at its earliest stage). Although the vaccine of the invention may be administered as a single dose, components thereof may also be co-administered together at the same time or at different times (for instance pneumococcal saccharide conjugates could be administered separately, at the same time or 1-2 weeks after the administration of the any bacterial protein component of the vaccine for optimal coordination of the immune responses with respect to each other). For co-administration, the optional Th1 adjuvant may be present in any or all of the different administrations. In addition to a single route of administration, 2 different routes of administration may be

used. For example, saccharides or saccharide conjugates may be administered IM (or ID) and bacterial proteins may be administered IN (or ID). In addition, the vaccines of the invention may be administered IM for priming doses and IN for booster doses.

- 5 The content of protein antigens in the vaccine will typically be in the range 1-100 $\mu$ g, preferably 5-50 $\mu$ g, most typically in the range 5 - 25 $\mu$ g. Following an initial vaccination, subjects may receive one or several booster immunizations adequately spaced.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).  
10 Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The vaccines of the present invention may be stored in solution or lyophilized. Preferably the solution is lyophilized in the presence of a sugar such as sucrose or lactose. It is still  
15 further preferable that they are lyophilized and extemporaneously reconstituted prior to use. Lyophilizing may result in a more stable composition (vaccine) and may possibly lead to higher antibody titers in the presence of 3D-MPL and in the absence of an aluminum based adjuvant.

20 In one aspect of the invention is provided a vaccine kit, comprising a vial containing an immunogenic composition of the invention, optionally in lyophilised form, and further comprising a vial containing an adjuvant as described herein. It is envisioned that in this aspect of the invention, the adjuvant will be used to reconstitute the lyophilised immunogenic composition.

25

Although the vaccines of the present invention may be administered by any route, administration of the described vaccines into the skin (ID) forms one embodiment of the present invention. Human skin comprises an outer "horny" cuticle, called the stratum corneum, which overlays the epidermis. Underneath this epidermis is a layer called the  
30 dermis, which in turn overlays the subcutaneous tissue. Researchers have shown that injection of a vaccine into the skin, and in particular the dermis, stimulates an immune response, which may also be associated with a number of additional advantages. Intradermal vaccination with the vaccines described herein forms a preferred feature of the present invention.

35

The conventional technique of intradermal injection, the "mantoux procedure", comprises steps of cleaning the skin, and then stretching with one hand, and with the bevel of a narrow gauge needle (26-31 gauge) facing upwards the needle is inserted at an angle of between 10-15°. Once the bevel of the needle is inserted, the barrel of the needle is  
5 lowered and further advanced whilst providing a slight pressure to elevate it under the skin. The liquid is then injected very slowly thereby forming a bleb or bump on the skin surface, followed by slow withdrawal of the needle.

More recently, devices that are specifically designed to administer liquid agents into or  
10 across the skin have been described, for example the devices described in WO 99/34850 and EP 1092444, also the jet injection devices described for example in WO 01/13977; US 5,480,381, US 5,599,302, US 5,334,144, US 5,993,412, US 5,649,912, US 5,569,189, US 5,704,911, US 5,383,851, US 5,893,397, US 5,466,220, US 5,339,163, US 5,312,335, US 5,503,627, US 5,064,413, US 5,520, 639, US 4,596,556, US 4,790,824, US  
15 4,941,880, US 4,940,460, WO 97/37705 and WO 97/13537. Alternative methods of intradermal administration of the vaccine preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (WO 99/27961), or transdermal patches (WO 97/48440; WO 98/28037); or applied to the surface of the skin (transdermal or transcutaneous delivery WO 98/20734 ; WO 98/28037).

20

When the vaccines of the present invention are to be administered to the skin, or more specifically into the dermis, the vaccine is in a low liquid volume, particularly a volume of between about 0.05 ml and 0.2 ml.

25 The content of antigens in the skin or intradermal vaccines of the present invention may be similar to conventional doses as found in intramuscular vaccines (see above). However, it is a feature of skin or intradermal vaccines that the formulations may be "low dose". Accordingly the protein antigens in "low dose" vaccines are preferably present in as little as 0.1 to 10 $\mu$ g, preferably 0.1 to 5  $\mu$ g per dose; and the saccharide (preferably  
30 conjugated) antigens may be present in the range of 0.01-1 $\mu$ g, and preferably between 0.01 to 0.5  $\mu$ g of saccharide per dose.

As used herein, the term "intradermal delivery" means delivery of the vaccine to the region of the dermis in the skin. However, the vaccine will not necessarily be located exclusively  
35 in the dermis. The dermis is the layer in the skin located between about 1.0 and about 2.0

mm from the surface in human skin, but there is a certain amount of variation between individuals and in different parts of the body. In general, it can be expected to reach the dermis by going 1.5 mm below the surface of the skin. The dermis is located between the stratum corneum and the epidermis at the surface and the subcutaneous layer below.

5 Depending on the mode of delivery, the vaccine may ultimately be located solely or primarily within the dermis, or it may ultimately be distributed within the epidermis and the dermis.

The present invention further provides an improved vaccine for the prevention or amelioration of Otitis media caused by *Haemophilus influenzae* by the addition of

10 *Haemophilus influenzae* proteins, for example protein D in free or conjugated form. In addition, the present invention further provides an improved vaccine for the prevention or amelioration of pneumococcal infection in infants (e.g., Otitis media), by relying on the addition of one or two pneumococcal proteins as free or conjugated protein to the

15 *S. pneumoniae* conjugate compositions of the invention. Said pneumococcal free proteins may be the same or different to any *S. pneumoniae* proteins used as carrier proteins. One or more *Moraxella catarrhalis* protein antigens can also be included in the combination vaccine in a free or conjugated form. Thus, the present invention is an improved method to elicit a (protective) immune response against Otitis media in infants.

20 In another embodiment, the present invention is an improved method to elicit a (protective) immune response in infants (defined as 0-2 years old in the context of the present invention) by administering a safe and effective amount of the vaccine of the invention [a paediatric vaccine]. Further embodiments of the present invention include the

25 provision of the antigenic *S. pneumoniae* conjugate compositions of the invention for use in medicine and the use of the *S. pneumoniae* conjugates of the invention in the manufacture of a medicament for the prevention (or treatment) of pneumococcal disease.

In yet another embodiment, the present invention is an improved method to elicit a

30 (protective) immune response in the elderly population (in the context of the present invention a patient is considered elderly if they are 50 years or over in age, typically over 55 years and more generally over 60 years) by administering a safe and effective amount of the vaccine of the invention, preferably in conjunction with one or two *S. pneumoniae* proteins present as free or conjugated protein, which free *S. pneumoniae* proteins may be

35 the same or different as any *S. pneumoniae* proteins used as carrier proteins.

A further aspect of the invention is a method of immunising a human host against disease caused by *S. pneumoniae* and optionally *Haemophilus influenzae* infection comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine or kit of the invention.

A further aspect of the invention is an immunogenic composition of the invention for use in the treatment or prevention of disease caused by *S.pneumoniae* and optionally *Haemophilus influenzae* infection.

A further aspect of the invention is use of the immunogenic composition or vaccine or kit of the invention in the manufacture of a medicament for the treatment or prevention of diseases caused by *S. pneumoniae* and optionally *Haemophilus influenzae* infection.

The terms "comprising", "comprise" and "comprises" herein are intended by the inventors to be optionally substitutable with the terms "consisting of", "consist of" and "consists of", respectively, in every instance.

Embodiments herein relating to "vaccine compositions" of the invention are also applicable to embodiments relating to "immunogenic compositions" of the invention, and vice versa.

All references or patent applications cited within this patent specification are incorporated by reference herein.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

### Examples

#### **Example 1: EXPRESSION OF PROTEIN D**

##### ***Haemophilus influenzae* protein D**

##### **Genetic construction for protein D expression**



## Starting materials

### The Protein D encoding DNA

Protein D is highly conserved among *H. influenzae* of all serotypes and non-typeable strains. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical  
5 Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) Infect. Immun. 59: 119-125.

### 10 The expression vector pMG1

The expression vector pMG1 is a derivative of pBR322 (Gross *et al.*, 1985) in which bacteriophage  $\lambda$  derived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman *et al.*, 1983). In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene.

15

### The *E. coli* strain AR58

The *E. coli* strain AR58 was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil' cl857  $\Delta$ H1). N99 and SA500 are *E. coli* K12 strains derived from Dr. Martin Rosenberg's laboratory at the  
20 National Institute of Health.

### The expression vector pMG 1

For the production of protein D, the DNA encoding the protein has been cloned into the expression vector pMG 1. This plasmid utilises signals from lambda daphage DNA to drive the transcription and translation of inserted foreign genes. The vector contains  
25 the promoter PL, operator OL and two utilisation sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross *et al.*, 1985). Vectors containing the PL promoter, are introduced into an *E. coli* lysogenic host to stabilise the plasmid DNA. Lysogenic host strains contain replication-defective lambda daphage DNA integrated into the genome (Shatzman *et al.*, 1983). The chromosomal lambda daphage DNA directs the synthesis of the cl repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promoter and thereby  
30 transcription of the inserted gene. The cl gene of the expression strain AR58 contains a temperature sensitive mutant so that PL directed transcription can be regulated by  
35 temperature shift, i.e. an increase in culture temperature inactivates the repressor and

synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimatata & Rosenberg, 1981).

5 **The E. coli strain AR58**

The AR58 lysogenic *E. coli* strain used for the production of the protein D carrier is a derivative of the standard NIH *E. coli* K12 strain N99 (F<sup>-</sup> su<sup>+</sup> galK2, lacZ<sup>-</sup> thr<sup>+</sup>). It contains a defective lysogenic lambdaphage (galE::TN10, lambdaKil<sup>-</sup> cI857 ΔH1). The Kil<sup>-</sup> phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation  
 10 confers a temperature sensitive lesion to the cI repressor. The ΔH1 deletion removes the lambdaphage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil<sup>-</sup> cI857 ΔH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon  
 15 coding for tetracyclin resistance in the adjacent galE gene.

**Construction of vector pMGMDPPrD**

The pMG 1 vector which contains the gene encoding the non-structural S1 protein of Influenzae virus (pMGNS1) was used to construct pMGMDPPrD. The protein D gene  
 20 was amplified by PCR from the pHIC348 vector (Janson *et al.* 1991 Infect. Immun. 59:119-125) with PCR primers containing NcoI and XbaI restriction sites at the 5' and 3' ends, respectively. The NcoI/XbaI fragment was then introduced into pMGNS1 between NcoI and XbaI thus creating a fusion protein containing the N-terminal 81 amino acids of the NS1 protein followed by the PD protein. This vector was labelled pMGNS1PrD.

25 Based on the construct described above the final construct for protein D expression was generated. A BamHI/BamHI fragment was removed from pMGNS1PrD. This DNA hydrolysis removes the NS1 coding region, except for the first three N-terminal residues. Upon religation of the vector a gene encoding a fusion protein with the following N-terminal amino acid sequence has been generated:

30

-----MDP SSHSSNMANT-----

NS1

Protein D

The protein D does not contain a leader peptide or the N-terminal cysteine to which lipid chains are normally attached. The protein is therefore neither excreted into the periplasm nor lipidated and remains in the cytoplasm in a soluble form.

The final construct pMG-MDPPrD was introduced into the AR58 host strain by  
5 heat shock at 37 °C. Plasmid containing bacteria were selected in the presence of Kanamycin. Presence of the protein D encoding DNA insert was demonstrated by digestion of isolated plasmid DNA with selected endonucleases. The recombinant *E. coli* strain is referred to as ECD4.

Expression of protein D is under the control of the lambda P<sub>L</sub> promoter/ O<sub>L</sub>  
10 Operator. The host strain AR58 contains a temperature-sensitive *ci* gene in the genome which blocks expression from lambda P<sub>L</sub> at low temperature by binding to O<sub>L</sub>. Once the temperature is elevated *ci* is released from O<sub>L</sub> and protein D is expressed.

#### *Small-scale preparation*

15 At the end of the fermentation the cells are concentrated and frozen.

The extraction from harvested cells and the purification of protein D was performed as follows. The frozen cell culture pellet is thawed and resuspended in a cell disruption solution (Citrate buffer pH 6.0) to a final OD<sub>660</sub> = 60. The suspension is passed twice through a high pressure homogenizer at P = 1000 bar. The cell culture homogenate  
20 is clarified by centrifugation and cell debris is removed by filtration. In the first purification step the filtered lysate is applied to a cation exchange chromatography column (SP Sepharose Fast Flow). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer.

In a second purification step impurities are retained on an anionic exchange matrix  
25 (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated by ultrafiltration.

30 The protein D containing ultrafiltration retentate is finally passed through a 0.2 µm membrane.

#### *Large Scale Preparation*

The extraction from harvested cells and the purification of protein D was performed as follows. The harvested broth is cooled and directly passed twice through a high pressure homogenizer at a Pressure of around 800 bars.

In the first purification step the cell culture homogenate is diluted and applied to a cation exchange chromatography column (SP Sepharose Big beads). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer and filtrated.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated and diafiltrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 µm membrane.

#### Example 1b: EXPRESSION OF PhtD

The PhtD protein is a member of the pneumococcal histidine-triad (Pht) protein family characterized by the presence of histidine-triads (HXXHXXH motif). PhtD is a 838 aa-molecule and carries 5 histidine triads (see MedImmune WO00/37105 SEQ ID NO: 4 for amino acid sequence and SEQ ID NO: 5 for DNA sequence). PhtD also contains a proline-rich region in the middle (amino acid position 348-380). PhtD has a 20 aa-N-terminal signal sequence with a LXXC motif.

#### 25 Genetic construct

The gene sequence of the mature MedImmune PhtD protein (from aa 21 to aa 838) was transferred recombinantly to *E. coli* using the in-house pTCMP14 vector carrying the pλ promoter. The *E. coli* host strain is AR58, which carries the cI857 thermosensitive repressor, allowing heat-induction of the promoter.

30 Polymerase chain reaction was realized to amplify the *phtD* gene from a MedImmune plasmid (carrying the *phtD* gene from *Streptococcus pneumoniae* strain Norway 4 (serotype 4) – SEQ ID NO: 5 as described in WO 00/37105). Primers, specific for the *phtD* gene only, were used to amplify the *phtD* gene in two fragments. Primers carry either the *NdeI* and *KpnI* or the *KpnI* and *XbaI* restriction sites. These primers do not hybridize with any nucleotide from the vector but only with *phtD* specific gene sequences. An artificial ATG start codon was inserted using the first primer carrying the *NdeI* restriction site. The generated PCR products were then inserted into the pGEM-T cloning vector (Promega),

and the DNA sequence was confirmed. Subcloning of the fragments in the TCMP14 expression vector was then realized using standard techniques and the vector was transformed into *AR58 E. coli*.

### PhtD Purification

5 PhtD purification is achieved as follows:

- 10     ▪ Growth of *E. coli* cells in the presence of Kanamycin: growth 30 hours at 30 °C then induction for 18 hours at 39.5 °C
- 15     ▪ Breakage of the *E. coli* cells from whole culture at OD ±115 in presence of EDTA 5 mM and PMSF 2 mM as protease inhibitors: Rannie, 2 passages, 1000 bars.
- 15     ▪ Antigen capture and cells debris removal on expanded bed mode Streamline Q XL chromatography at room temperature (20°C); the column is washed with NaCl 150 mM + Empigen 0.25% pH 6.5 and eluted with NaCl 400 mM + Empigen 0.25% in 25 mM potassium phosphate buffer pH 7.4.
- 20     ▪ Filtration on Sartobran 150 cartridge (0.45 + 0.2 µm)
- 20     ▪ Antigen binding on Zn<sup>++</sup> Chelating Sepharose FF IMAC chromatography at pH 7.4 in presence of 5 mM imidazole at 4°C; the column is washed with Imidazole 5 mM and Empigen 1% and eluted with 50 mM imidazole, both in 25 mM potassium phosphate buffer pH 8.0.
- 25     ▪ Weak anion exchange chromatography in positive mode on Fractogel EMD DEAE at pH 8.0 (25 mM potassium phosphate) at 4°C; the column is washed with 140 mM NaCl and eluted at 200 mM NaCl while contaminants (proteins and DNA) remain adsorbed on the exchanger.
- 30     ▪ Concentration and ultrafiltration with 2 mM Na/K phosphate pH 7.15 on 50 kDa membrane.
- 30     ▪ Sterilising filtration of the purified bulk on a Millipak-20 0.2 µm filter cartridge.

### Example 1c: EXPRESSION OF PNEUMOLYSIN

35 Pneumococcal pneumolysin was prepared and detoxified as described in WO2004/081515 and WO2006/032499.

### Example 2:

40     **Preparation of conjugates**

It is well known in the art how to make purified pneumococcal polysaccharides. For the purposes of these examples the polysaccharides were made essentially as

described in EP072513 or by closely-related methods. Before conjugation the polysaccharides may be sized by microfluidisation as described below.

The activation and coupling conditions are specific for each polysaccharide. These are given in Table 1. Sized polysaccharide (except for PS5, 6B and 23F) was dissolved in NaCl 2M, NaCl 0.2M or in water for injection (WFI). The optimal polysaccharide concentration was evaluated for all the serotypes. All serotypes except serotype 18C were conjugated directly to the carrier protein as detailed below. Two alternative serotype 22F conjugates were made; one conjugated directly, one through an ADH linker.

From a 100 mg/ml stock solution in acetonitrile or acetonitrile/water 50%/50% solution, CDAP (CDAP/PS ratio 0.5-1.5 mg/mg PS) was added to the polysaccharide solution. 1.5 minute later, 0.2M-0.3M NaOH was added to obtain the specific activation pH. The activation of the polysaccharide was performed at this pH during 3 minutes at 25 °C. Purified protein (protein D, PhtD, pneumolysin or DT) (the quantity depends on the initial PS/carrier protein ratio) was added to the activated polysaccharide and the coupling reaction was performed at the specific pH for up to 2 hour (depending upon serotype) under pH regulation. In order to quench un-reacted cyanate ester groups, a 2M glycine solution was then added to the mixture. The pH was adjusted to the quenching pH (pH 9.0). The solution was stirred for 30 minutes at 25 °C and then overnight at 2-8 °C with continuous slow stirring.

#### 20 Preparation of 18C:

18C was linked to the carrier protein via a linker – Adipic acid dihydrazide (ADH) Polysaccharide serotype 18C was microfluidized before conjugation.

#### **Derivatization of tetanus toxoid with EDAC**

For derivatization of the tetanus toxoid, purified TT was diluted at 25 mg/ml in 0.2M NaCl and the ADH spacer was added in order to reach a final concentration of 0.2M. When the dissolution of the spacer was complete, the pH was adjusted to 6.2. EDAC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide) was then added to reach a final concentration of 0.02M and the mixture was stirred for 1 hour under pH regulation. The reaction of condensation was stopped by increasing pH up to 9.0 for at least 30 minutes at 25°C.

30 Derivatized TT was then diafiltrated (10 kDa CO membrane) in order to remove residual ADH and EDAC reagent.

TT<sub>AH</sub> bulk was finally sterile filtered until coupling step and stored at -70°C.

#### **Chemical coupling of TT<sub>AH</sub> to PS 18C**

Details of the conjugation parameters can be found in Table 1.

2 grams of microfluidized PS were diluted at the defined concentration in water and adjusted to 2M NaCl by NaCl powder addition.

CDAP solution (100 mg/ml freshly prepared in 50/50 v/v acetonitrile/WFI) was added to reach the appropriate CDAP/PS ratio.

- 5 The pH was raised up to the activation pH 9.0 by the addition of 0.3M NaOH and was stabilised at this pH until addition of TT<sub>AH</sub>.

After 3 minutes, derivatized TT<sub>AH</sub> (20 mg/ml in 0.2 M NaCl) was added to reach a ratio TT<sub>AH</sub>/PS of 2; the pH was regulated to the coupling pH 9.0. The solution was left one hour under pH regulation.

- 10 For quenching, a 2M glycine solution, was added to the mixture PS/TT<sub>AH</sub>/CDAP.

The pH was adjusted to the quenching pH (pH 9.0).

The solution was stirred for 30 min at 25 °C, and then left overnight at 2-8°C with continuous slow stirring.

- 15 PS22F<sub>AH</sub>-PhtD conjugate

In a second conjugation method for this saccharide (the first being the direct PS22-PhtD conjugation method shown in Table 1), 22F was linked to the carrier protein via a linker – Adipic acid dihydrazide (ADH). Polysaccharide serotype 22F was microfluidized before conjugation.

20

#### **PS 22F derivatization**

Activation and coupling are performed at 25°C under continuous stirring in a temperature-controlled waterbath.

- 25 Microfluidized PS22F was diluted to obtain a final PS concentration of 6 mg/ml in 0.2M NaCl and the solution was adjusted at pH 6.05 ± 0.2 with 0.1N HCl.

CDAP solution (100 mg/ml freshly prepared in acetonitrile/WFI, 50/50) was added to reach the appropriate CDAP/PS ratio (1.5/1 ww).

- 30 The pH was raised up to the activation pH 9.00 ± 0.05 by the addition of 0.5M NaOH and was stabilised at this pH until addition of ADH.

After 3 minutes, ADH was added to reach the appropriate ADH/PS ratio (8.9/1 w/w); the pH was regulated to coupling pH 9.0. The solution was left for 1 hour under pH regulation.

The PS<sub>AH</sub> derivative was concentrated and diafiltrated.

35

Coupling.

PhtD at 10 mg/ml in 0.2M NaCl was added to the PS22F<sub>AH</sub> derivative in order to reach a PhtD/PS22F<sub>AH</sub> ratio of 4/1 (w/w). The pH was adjusted to 5.0 ± 0.05 with HCl. The EDAC solution (20 mg/ml in 0.1M Tris-HCl pH 7.5) was added manually in 10 min (250 µl / min) to reach 1 mg EDAC/mg PS22F<sub>AH</sub>. The resulting solution was incubated for 150 min (though 60 mins was also used) at 25°C under stirring and pH regulation. The solution was neutralized by addition of 1M Tris-HCl pH 7.5 (1/10 of the final volume) and let 30 min at 25°C.

Prior to the elution on Sephacryl S400HR, the conjugate was clarified using a 5µm Minisart filter.

The resulting conjugate has a final PhtD/PS ratio of 4.1 (w/w), a free PS content below 1% and an antigenicity (α-PS/α-PS) of 36.3% and anti-PhtD antigenicity of 7.4%.

#### 15 Purification of the conjugates:

The conjugates were purified by gel filtration using a Sephacryl S400HR gel filtration column equilibrated with 0.15M NaCl (S500HR for 18C) to remove small molecules (including DMAP) and unconjugated PS and protein. Based on the different molecular sizes of the reaction components, PS-PD, PS-TT, PS-PhtD, PS-pneumolysin or PS-DT conjugates are eluted first, followed by free PS, then by free PD or free DT and finally DMAP and other salts (NaCl, glycine).

Fractions containing conjugates are detected by UV<sub>280 nm</sub>. Fractions are pooled according to their K<sub>d</sub>, sterile filtered (0.22µm) and stored at +2-8°C. The PS/Protein ratios in the conjugate preparations were determined.

#### 25 Specific activation/coupling/quenching conditions of PS S. pneumoniae-Protein D/TT/DT/PhtD/Plyconjugates

Where "µfluid" appears in a row header, it indicates that the saccharide was sized by microfluidisation before conjugation. Sizes of saccharides following microfluidisation are given in table 2.

35 **Table 1** Specific activation/coupling/quenching conditions of PS S. pneumoniae-Protein D/TT/DT/PhtD/Plyconjugates

Serotype	1 µfluid	4 µfluid	5	6A	6B	7F µfluid
PS conc.(mg/ml)	2.5	2.5	7.1	5.0	5.0	5.0



<b>PS dissolution</b>	WFI	WFI	WFI	NaCl 2M	NaCl 2M	NaCl 2M
<b>PD conc.(mg/ml)</b>	10.0	10.0	5.0	5.0	5.0	10.0
<b>Initial PD/PS Ratio (w/w)</b>	1.5/1	1.5/1	1/1	1/1	1.1/1	1.2/1
<b>CDAP conc. (mg/mg PS)</b>	0.50	0.50	0.79	0.83	0.83	0.75
<b>pH<sub>a</sub>=pH<sub>c</sub>=pH<sub>q</sub></b>	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.5/9.5/9.0	9.5/9.5/9.0

<b>Serotype</b>	<b>9V µfluid</b>	<b>14 µfluid</b>	<b>18C µfluid</b>	<b>19A µfluid</b>	<b>19F µfluid</b>	<b>22F µfluid</b>	<b>23F</b>
<b>PS conc.(mg/ml)</b>	5.0	5.0	4.5	15.0	9.0	6.0	2.38
<b>PS dissolution</b>	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 0.2M	NaCl 2M
<b>Carrier protein conc.(mg/ml)</b>	10.0	10.0	20.0 (TT)	10.0 (Ply)	20.0 (DT)	10.0 (PhtD)	5.0
<b>Initial carrier protein/PS Ratio (w/w)</b>	1.2/1	1.2/1	2/1	2.5/1	1.5/1	3/1	1/1
<b>CDAP conc. (mg/mg PS)</b>	0.50	0.75	0.75	1.5	1.5	1.5	0.79
<b>pH<sub>a</sub>=pH<sub>c</sub>=pH<sub>q</sub></b>	9.5/9.5/9.0	9.5/9.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0

Note: pH<sub>a</sub>,c,q corresponds to the pH for activation, coupling and quenching, respectively

## 5 Characterisation:

Each conjugate was characterised and met the specifications described in Table 2. The polysaccharide content ( $\mu\text{g/ml}$ ) was measured by the Resorcinol test and the protein content ( $\mu\text{g/ml}$ ) by the Lowry test. The final PS/PD ratio (w/w) is determined by the ratio of the concentrations.

5

**Free polysaccharide content (%):**

The free polysaccharide content of conjugates kept at 4°C or stored 7 days at 37°C was determined on the supernatant obtained after incubation with  $\alpha$ -carrier protein antibodies and saturated ammonium sulfate, followed by a centrifugation.

10 An  $\alpha$ -PS/ $\alpha$ -PS ELISA was used for the quantification of free polysaccharide in the supernatant. The absence of conjugate was also controlled by an  $\alpha$ -carrier protein/ $\alpha$ -PS ELISA.

**Antigenicity:**

15 The antigenicity on the same conjugates was analyzed in a sandwich-type ELISA wherein the capture and the detection of antibodies were  $\alpha$ -PS and  $\alpha$ -Protein respectively.

**Free protein content (%):**

20 Unconjugated carrier protein can be separated from the conjugate during the purification step. The content of free residual protein was determined using size exclusion chromatography (TSK 5000-PWXL) followed by UV detection (214 nm). The elution conditions allowed separating the free carrier protein and the conjugate. Free protein content in conjugate bulks was then determined versus a calibration curve (from 0 to 50  
25  $\mu\text{g/ml}$  of carrier protein). Free carrier protein in % was obtained as follows: % free carrier = (free carrier ( $\mu\text{g/ml}$ )/ (Total concentration of corresponding carrier protein measured by Lowry ( $\mu\text{g/ml}$ ) \* 100%).

**Stability:**

30 Molecular weight distribution ( $K_{av}$ ) and stability was measured on a HPLC-SEC gel filtration (TSK 5000-PWXL) for conjugates kept at 4°C and stored for 7 days at 37°C.

The 10/11/13/14-valent characterization is given in Table 2 (see comment thereunder).

The protein conjugates can be adsorbed onto aluminium phosphate and pooled to form the final vaccine.

### Conclusion:

- 5 Immunogenic conjugates have been produced, that have since been shown to be components of a promising vaccine.

TABLE 2 – characteristics of the conjugates

Conjugates	PS size (Dax10 <sup>*</sup> )	Carrier/PS Ratio	Free PS (Elisa)	Free Carrier	PS Antigenicity (Elisa)	Conj. Size (kDa)
PS1-PD	349-382*	1.5-1.6	1.0%-1.2%	3.9%-4.8%	87%-95%	1499 - 1715
PS4-PD	93-100*	1.5-1.6	4.7-6.5%	3.2%-4.0%	90%-96%	1303 - 1606
PS5-PD***	367-443	0.80	8.7-11.2%	2.2%-3.8%	93%-108%	1998-2352
PS6A-PD	1100-1540	0.61	4.5%	Not done	45.9%	Not done
PS6B-PD***	1069-1391	0.7-0.8	1.3-1.6%	<2.0%	68%-75%	4778-5235
PS7F-PD	255-264*	1.1-1.2	<1%	<1.4%	58%	3907-4452
PS9V-PD	258-280*	1.3-1.5	<1%	<1.3%	67%-69%	9073-9572
PS14-PD	232-241*	1.4	<1%	<1.5%	70%	3430-3779
PS18C-TT	89-97*	2.2-2.4	1.5-2.2%	<4%	46%-56%	5464-6133
PS19A-Ply*	151	3.2	<1%		29%	
PS19F-DT	133-143*	1.4-1.5	4.1%-5.9%	<1.2%-<1.3%	82%-88%	2059-2335
PS22F-PhtD*	159-167	2.17	5.8	Not done	37%	Not done
PS22F-AHPhtD*	159-167	3.66-4.34	<1%	Not done	28-31%	Not done
PS23F-PD***	914-980	0.5	1.4-1.9%	3.7%-4.9%	137%-154%	2933-3152

\* PS size following microfluidization of the native PS

- 10 A 10 valent vaccine was made by mixing serotype 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F conjugates (e.g. at a dose of 1, 3, 1, 1, 1, 1, 1, 3, 3, 1 µg of saccharide, respectively per human dose). An 11 valent vaccine was made by further adding the serotype 3 conjugate from Table 5 (e.g. at 1 µg of saccharide per human dose). A 13 valent vaccine was made by further adding the serotypes 19A and 22F conjugates above (with 22F either directly linked to PhtD, or alternatively through an ADH linker) [e.g. at a dose of 3
- 15 µg each of saccharide per human dose]. A 14 valent vaccine may be made by further

adding the serotype 6A conjugate above [e.g. at a dose of 1 µg of saccharide per human dose.

**Example 3: Evidence that inclusion of *Haemophilus influenzae* protein D in an immunogenic composition of the invention can provide improved protection against acute otitis media (AOM).**

Study design.

The study used an 11Pn-PD vaccine – comprising serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F each conjugated to protein D from *H. influenzae* (refer to Table 5 in Example 4). Subjects were randomized into two groups to receive four doses of either the 11Pn-PD vaccine or *Havrix* at approximately 3, 4, 5 and 12-15 months of age. All subjects received GSK Biologicals' *Infanrix-hexa* (DTPa-HBV-IPV/Hib) vaccine concomitantly at 3, 4 and 5 months of age. *Infanrix-hexa* is a combination of *Pediarix* and Hib mixed before administration. Efficacy follow-up for the "According-to-Protocol" analysis started 2 weeks after administration of the third vaccine dose and continued until 24-27 months of age. Nasopharyngeal carriage of *S. pneumoniae* and *H. influenzae* was evaluated in a selected subset of subjects.

Parents were advised to consult the investigator if their child was sick, had ear pain, spontaneous perforation of the tympanic membrane or spontaneous ear discharge. If the investigator suspected an episode of AOM, the child was immediately referred to an Ear, Nose and Throat (ENT) specialist for confirmation of the diagnosis.

A clinical diagnosis of AOM was based on either the visual appearance of the tympanic membrane (i.e. redness, bulging, loss of light reflex) or the presence of middle ear fluid effusion (as demonstrated by simple or pneumatic otoscopy or by microscopy). In addition, at least two of the following signs or symptoms had to be present: ear pain, ear discharge, hearing loss, fever, lethargy, irritability, anorexia, vomiting, or diarrhea. If the ENT specialist confirmed the clinical diagnosis, a specimen of middle ear fluid was collected by tympanocentesis for bacteriological testing.

For subjects with repeated sick visits, a new AOM episode was considered to have started if more than 30 days had elapsed since the beginning of the previous episode. In addition, an AOM episode was considered to be a new bacterial episode if the isolated bacterium/serotype differed from the previous isolate whatever the interval between the two consecutive episodes.

35

### Trial results

A total of 4968 infants were enrolled, 2489 in the 11Pn-PD group and 2479 in the control group. There were no major differences in the demographic characteristics or risk factors between the two groups.

#### 5 Clinical episodes and AOM case definition

During the per protocol follow-up period, a total of 333 episodes of clinical AOM were recorded in the 11Pn-PD group and 499 in the control group.

Table 3 presents the protective efficacy of the 11Pn-PD vaccine and both 7-valent vaccines previously tested in Finland (Eskola *et al* N Engl J Med 2001; 344: 403 – 409 and Kilpi *et al*/Clin Infect Dis 2003 37:1155-64) against any episode of AOM and AOM  
10 caused by different pneumococcal serotypes, *H. influenzae*, *NTHi* and *M. catarrhalis*.

Statistically significant and clinically relevant reduction by 33.6% of the overall AOM disease burden was achieved with 11Pn-PD, irrespective of the etiology (table 3).

The overall efficacy against AOM episodes due to any of the 11 pneumococcal serotypes  
15 contained in the 11Pn-PD vaccine was 57.6% (table 3).

Another important finding in the current study is the 35.6% protection provided by the 11Pn-PD vaccine against AOM caused by *H. influenzae* (and specifically 35.3% protection provided by *NTHi*). This finding is of major clinical significance, given the  
20 increased importance of *H. influenzae* as a major cause of AOM in the pneumococcal conjugate vaccine era. In line with the protection provided against AOM, the 11Pn-PD vaccine also reduced nasopharyngeal carriage of *H. influenzae* following the booster dose in the second year of life. These findings are in contrast with previous observations in Finland where, for both 7-valent pneumococcal conjugate vaccines, an increase in AOM  
25 episodes due to *H. influenzae* was observed, (Eskola *et al* and Kilpi *et al*) as evidence of etiological replacement.

A clear correlation between protection against AOM episodes due to Hi and antibody levels against the carrier Protein D could not be established, as post-primary anti-PD IgG  
30 antibody concentrations in 11Pn-PD vaccinees, that remained Hi AOM episode-free, were essentially the same as post-primary anti-PD IgG antibody levels measured in 11Pn-PD vaccinees that developed at least one Hi AOM episode during the efficacy follow-up period. However, although no correlation could be established between the biological impact of the vaccine and the post-primary IgG anti-PD immunogenicity, it is reasonable

to assume that the PD carrier protein, which is highly conserved among *H. influenzae* strains, has contributed to a large extent in the induction of the protection against Hi.

5 The effect on AOM disease was accompanied by an effect on nasopharyngeal carriage that was of similar magnitude for vaccine serotype pneumococci and *H. influenzae* (Figure 1). This reduction of the nasopharyngeal carriage of *H. influenzae* in the PD-conjugate vaccinees supports the hypothesis of a direct protective effect of the PD-conjugate vaccine against *H. influenzae*, even if the protective efficacy could not be correlated to the anti-PD IgG immune responses as measured by ELISA.

10

In a following experiment a chinchilla otitis media model was used with serum pools from infants immunised with the 11 valent formulation of this example or with the 10 valent vaccine of Example 2 (see also Table 1 and 2 and comments thereunder). Both pools induce a significant reduction of the percentage of animals with otitis media versus the pre-immune serum pool. There is no significant difference between the 10 and 11 valent immune pools. This demonstrates that both vaccines have a similar potential to induce protection against otitis media caused by non typeable *H. influenzae* in this model.

15  
20

VB61783

Table 3

Type of AOM episode	11Pn-PD					Pneumar In FinOM (Esteban et al)					7v-OMP in FinOM (Klip et al)				
	11Pn-PD	n		VE		7v-CRM	n		VE		7v-OMP	n		VE	
		Control	%	LL	95%CI-UL		Control	%	LL	95%CI-UL		Control	%	LL	95%CI-UL
N	2455	2452			786	794			805	794					
Any AOM	333	499	33.6	20.8	1251	1345	6	4	1384	1345	-1	-12	10	10	
Any AOM with MEF	322	474	32.4	19.0	1177	1267	7	-5	1279	1267	0	-12	10	10	
Culture confirmed pneumococcus	92	189	51.5	36.8	271	414	34	21	314	414	25	11	37	37	
Vaccine pneumococcal serotypes*	60	141	57.6	41.4	107	250	57	44	110	250	56	44	66	66	
Other bacterial pathogens															
<i>H. influenzae</i>	44	68	35.6	3.8	315	287	-11	-34	8	287	-9	-32	10	10	
Non-typeable <i>H. influenzae</i> (NTHI)	41	63	35.3	1.8	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	
<i>M. catarrhalis</i>	31	34	9.4	-52.5	379	381	-1	-19	15	381	-16	-36	2	2	

NP = Not published; N = number of subjects in ATP efficacy cohort; n = number of episodes  
 \*Vaccine pneumococcal serotypes: for 11Pn-PD = 11 serotypes, for Pneumar and 7v-OMP = 7 serotypes  
 MEF = Middle ear fluid

**Example 4:****Selection of carrier protein for serotype 19F****ELISA assay used**

5 The 22F inhibition ELISA method was essentially based on an assay proposed in 2001 by Concepcion and Frasch and was reported by Henckaerts et al., 2006, *Clinical and Vaccine Immunology* 13:356-360. Briefly, purified pneumococcal polysaccharides were mixed with methylated human serum albumin and adsorbed onto Nunc Maxisorp™ (Roskilde, DK) high binding microtitre plates overnight at 4°C. The plates were blocked  
10 with 10% fetal bovine serum (FBS) in PBS for 1 hour at room temperature with agitation. Serum samples were diluted in PBS containing 10% FBS, 10 µg/mL cell-wall polysaccharide (SSI) and 2 µg/mL of pneumococcal polysaccharide of serotype 22F (ATCC), and further diluted on the microtitre plates with the same buffer. An internal reference calibrated against the standard serum 89-SF using the serotype-specific IgG  
15 concentrations in 89-SF was treated in the same way and included on every plate. After washing, the bound antibodies were detected using peroxidase-conjugated anti-human IgG monoclonal antibody (Stratech Scientific Ltd., Soham, UK) diluted in 10% FBS (in PBS), and incubated for 1 hour at room temperature with agitation. The color was developed using ready-to-use single component tetramethylbenzidine peroxidase enzyme  
20 immunoassay substrate kit (BioRad, Hercules, CA, US) in the dark at room temperature. The reaction was stopped with H<sub>2</sub>SO<sub>4</sub> 0.18 M, and the optical density was read at 450 nm. Serotype-specific IgG concentrations (in µg/mL) in the samples were calculated by referencing optical density points within defined limits to the internal reference serum curve, which was modeled by a 4-parameter logistic log equation calculated with  
25 SoftMax Pro™ (Molecular Devices, Sunnyvale, CA) software. The cut-off for the ELISA was 0.05 µg/mL IgG for all serotypes taking into account the limit of detection and the limit of quantification.

**Opsonophagocytosis assay**

30 At the WHO consultation meeting in June 2003, it was recommended to use an OPA assay as set out in Romero-Steiner *et al* *Clin Diagn Lab Immunol* 2003 **10** (6): pp1019-1024. This protocol was used to test the OPA activity of the serotypes in the following tests.

**35 Preparation of conjugates**



In studies 11Pn-PD&Di-001 and 11Pn-PD&Di-007, three 11-valent vaccine formulations (Table 4) were included in which 3µg of the 19F polysaccharide was conjugated to diphtheria toxoid (19F-DT) instead of 1µg polysaccharide conjugated to protein D (19F-PD). Conjugation parameters for the studies 11Pn-PD, 11 Pn-PD&Di-001 and 11 Pn-PD&Di-007 are disclosed in Tables 5, 6 and 7 respectively.

Anti-pneumococcal antibody responses and OPA activity against serotype 19F one month following primary vaccination with these 19F-DT formulations are shown in Table 8 and 9 respectively.

Table 10 shows 22F-ELISA antibody concentrations and percentages of subjects reaching the 0.2 µg/mL threshold before and after 23-valent plain polysaccharide booster vaccination.

The opsonophagocytic activity was shown to be clearly improved for antibodies induced with these 19F-DT formulations as demonstrated by higher seropositivity rates (opsonophagocytic titers  $\geq$  1:8) and OPA GMTs one month following primary vaccination (Table 9). One month after 23-valent plain polysaccharide booster vaccination, opsonophagocytic activity of 19F antibodies remained significantly better for children primed with 19F-DT formulations (Table 11).

Table 12 presents immunogenicity data following a 11Pn-PD booster dose in toddlers previously primed with 19F-DT or 19F-PD conjugates compared to a 4<sup>th</sup> consecutive dose of *Prevnar*®. Given the breakthrough cases reported after the introduction of *Prevnar*® in the US, the improved opsonophagocytic activity against serotype 19F when conjugated to the DT carrier protein may be an advantage for the candidate vaccine.

Table 13 provides ELISA and OPA data for the 19F-DT conjugate with respect to the cross-reactive serotype 19A. It was found that 19F-DT induces low but significant OPA activity against 19A.

**Table 4 Pneumococcal conjugate vaccine formulations used in clinical studies.**

Formulation	Pneumococcal serotype $\mu\text{g}/\text{carrier protein}$											Al <sup>3+</sup> mg
	1	3	4	5	6B	7F	9V	14	18C	19F	23F	
11Pn-PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	< 0.8
19F-DT Form 1	3/PD	3/PD	3/PD	3/PD	10/DT	3/PD	3/PD	3/PD	3/PD	3/DT	5/DT	$\leq 0.35$
19F-DT Form 2	3/PD	2/PD	2/PD	3/PD	5/DT	3/PD	2/PD	2/PD	2/PD	3/DT	5/DT	$\leq 0.35$
19F-DT Form 3	3/PD	3/PD	3/PD	3/PD	3/PD	3/PD	3/PD	3/PD	3/PD	3/DT	3/PD	= 0.5

**Table 5 Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D/TT/DTconjugates**

5

Serotype	1 Native	3 $\mu\text{fluid}$	4 Native	5 Native	6B Native	7F Native
PS conc.(mg/ml)	1.5	2	2.0	7.5	5.5	3.0
PS dissolution	NaCl 150mM	NaCl 2M	WFI	WFI	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.7	1/1	1/1	1/1	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
$\text{pH}_a=\text{pH}_c=\text{pH}_q$	9.0/9.0/9.0	9.5/9.5/9.0	8.8/8.8/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0
Coupling time	60 mins	60 mins	45 mins	40 mins	60 mins	60 mins

Serotype	9V Native	14 Native	18C Native	19F Native	23F Native
PS conc.(mg/ml)	1.75	2.5	1.75	4.0	2.5
PS dissolution	NaCl 2M	NaCl 2M	WFI	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.75	1/0.75	1/1.2	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75
$\text{pH}_a=\text{pH}_c=\text{pH}_q$	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.5/9.5/9.0
Coupling time	60 mins	60 mins	45 mins	30 mins	60 mins

**Table 6** Specific activation/coupling/quenching conditions of PS *S.pneumoniae*-Protein D/DTconjugates for the 11 Pn-PD&Di-001 study

5

Serotype	1 µfluid	3 µfluid	4 µfluid	5 µfluid	6B µfluid	7F Native
PS conc.(mg/ml)	4	2.0	2.5	7.5	10	3.0
PS dissolution	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	10.0	5.0	5.0	5.0 NaCl 2M	20 (DT) NaCl 2M	5.0
Initial PD/PS Ratio (w/w)	1.2/1	1/1	1/1	1/1	1.5/1	1/1
CDAP conc. (mg/mg PS)	1.50	0.75	1.5	2	1.5	0.75
pH <sub>a</sub> =pH <sub>c</sub> =pH <sub>o</sub>	9.0/9.0/9.0	9.5/9.5/9.0	9.5/9.5/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9/9/9
Coupling time	60 mins	60 mins	60 mins	60 mins	60 mins	60 mins

Serotype	9V Native	14 Native	18C µfluid	19F µfluid	23F µfluid
PS conc.(mg/ml)	1.75	2.5	5.0	9.0	10
PS dissolution	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M
Carrier protein conc.(mg/ml)	5.0	5.0	5.0	20 (DT)	10 (DT)
Initial carrier protein/PS Ratio (w/w)	0.75/1	0.75/1	1.2/1	1.5/1	1.5/1
CDAP conc. (mg/mg PS)	0.75	0.75	1.5	1.5	0.75
pH <sub>a</sub> =pH <sub>c</sub> =pH <sub>o</sub>	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0
Coupling time	60 mins	60 mins	30 mins	60 mins	60 mins

**Table 7** Specific activation/coupling/quenching conditions of PS *S.pneumoniae*-Protein D/DTconjugates for the 11 Pn-PD&Di-007 study

10

Serotype	1 Native	3 µfluid	4 Native	5 Native	6B Native	7F µfluid
PS	1.5	2.0	2	7.5	5.5	5.0

conc.(mg/ml)						
PS dissolution	NaCl 150 mM	NaCl 2M	WFI	WFI	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5	10
Initial PD/PS Ratio (w/w)	0.7/1	1/1	1	1/1	1/1	1.2/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
pH <sub>a</sub> =pH <sub>c</sub> =pH <sub>q</sub>	9.0/9.0/9.0	9.5/9.5/9.0	8.8/8.8/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.5/9.5/9
Coupling time	60 mins	60 mins	45 mins	40 mins	60 mins	60 mins

Serotype	9V µfluid	14 µfluid	18C Native	19F µfluid	19F µfluid	23F µfluid
PS conc.(mg/ml)	5.0	5.0	1.75	9.0	10.0	9.5
PS dissolution	NaCl 2M	NaCl 2M	WFI	NaCl 2M	NaCl 2M	NaCl 2M
Carrier protein conc.(mg/ml)	10	10.0	5.0	20 (DT)	5.0 (PD)	10
Initial carrier protein/PS Ratio (w/w)	1.2/1	1.2/1	1.2/1	1.5/1	1.2/1	1/1
CDAP conc. (mg/mg PS)	0.5	0.75	0.75	1.5	0.75	0.75
pH <sub>a</sub> =pH <sub>c</sub> =pH <sub>q</sub>	9.5/9.5/9.0	9.5/9.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0
Coupling time	60 mins	60 mins	45 mins	120 mins	120 mins	60 mins

5

**Table 8** Percentage of subjects with 19F antibody concentration  $\geq 0.20 \mu\text{g/mL}$  and 19F antibody Geometric mean antibody concentrations (GMCs with 95% CI;  $\mu\text{g/mL}$ ) one month following  $1\mu\text{g}$  19F-PD,  $3\mu\text{g}$  19F-DT or *Prevnar* ( $2\mu\text{g}$  19F-CRM) primary vaccination (Total cohort)

Group	11Pn-PD&Di-001 (22F-ELISA)			11Pn-PD&Di-007 (22F-ELISA)		
	N	% $\geq 0.20 \mu\text{g/mL}$ (95% CI)	GMC ( $\mu\text{g/mL}$ ) (95% CI)	N	% $\geq 0.20 \mu\text{g/mL}$ (95% CI)	GMC ( $\mu\text{g/mL}$ ) (95% CI)

11Pn-PD	152	98.7 (95.3-99.8)	1.93 (1.67-2.22)	50	100 (92.9-100)	2.78 (2.31-3.36)
19F-DT Form 1 <sup>†</sup>	146	99.3 (96.2-100)	2.88 (2.45-3.38)	-	-	-
19F-DT Form 2 <sup>†</sup>	150	96.0 (91.5-98.5)	2.43 (2.01-2.94)	-	-	-
19F-DT Form 3 <sup>†</sup>	-	-	-	50	96.0 (86.3-99.5)	3.70 (2.58-5.30)
<i>Pprevnar</i>	148	98.6 (95.2-99.8)	2.98 (2.60-3.41)	41	97.6 (87.1-99.9)	2.91 (2.15-3.94)

<sup>†</sup> The composition of the different formulations is provided in table 4.

**Table 9** Percentage of subjects with 19F OPA titer  $\geq$  1:8 and 19F OPA GMTs one month following primary vaccination with 1 $\mu$ g 19F-PD, 3 $\mu$ g 19F-DT or *Pprevnar* (2 $\mu$ g 19F-CRM) (Total cohort)

5

Group	11Pn-PD&DI-001			11Pn-PD&DI-007		
	N	$\geq$ 1:8 (95% CI)	GMT (95% CI)	N	$\geq$ 1:8 (95% CI)	GMT (95% CI)
11Pn-PD	136	84.6 (77.4-90.2)	77.8 (58.1-104.4)	46	95.7 (85.2-99.5)	167.8 (118.1-238.6)
19F-DT Form 1 <sup>†</sup>	137	95.6 (90.7-98.4)	263.2 (209.4-330.7)	-	-	-
19F-DT Form 2 <sup>†</sup>	139	92.1 (86.3-96.0)	218.9 (166.5-287.9)	-	-	-
19F-DT Form 3 <sup>†</sup>	-	-	-	49	91.8 (80.4-97.7)	403.1 (225.7-719.9)
<i>Pprevnar</i>	131	86.3 (79.2-91.6)	82.6 (61.1-111.6)	38	81.6 (65.7-92.3)	65.0 (37.7-112.2)

<sup>†</sup> The composition of the different formulations is provided in Table4.

**Table 10** Percentage of subjects with 19F antibody concentration  $\geq$  0.20  $\mu$ g/mL and 19F antibody GMCs ( $\mu$ g/mL) prior to and one month following 23-valent plain polysaccharide booster in children primed with 1 $\mu$ g 19F-PD, 3 $\mu$ g 19F-DT or *Pprevnar* (2 $\mu$ g 19F-CRM) (Total cohort)

10

Primary group	11Pn-PD&DI-002 (22F ELISA)					
	Prior to booster vaccination			One month post 23-valent PS booster		
	N	% $\geq$ 0.20 $\mu$ g/mL (95% CI)	GMC ( $\mu$ g/ml) (95% CI)	N	% $\geq$ 0.20 $\mu$ g/mL (95% CI)	GMC ( $\mu$ g/ml) (95% CI)
11Pn-PD	70	77.1 (65.6-86.3)	0.67 (0.45-0.98)	67	94.0 (85.4-98.3)	11.50 (7.76-17.03)
19F-DT Form 1 <sup>†</sup>	68	91.2 (81.8-96.7)	0.71 (0.54-0.94)	69	98.6 (92.2-100)	14.50 (10.47-20.07)
19F-DT Form 2 <sup>†</sup>	74	81.1 (70.3-89.3)	0.59 (0.43-0.80)	72	95.8 (88.3-99.1)	9.90 (6.74-14.54)
<i>Pprevnar</i>	65	64.6 (51.8-76.1)	0.40 (0.27-0.60)	67	100 (94.6-100)	9.40 (6.95-12.71)

<sup>†</sup> The composition of the different formulations is provided in Table4.

**Table 11** Percentage of subjects with 19F OPA titer  $\geq$  1:8 and 19F OPA GMTs prior to and one month following 23-valent plain polysaccharide

521

**booster in children primed with 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) (Total cohort)**

Primary group	11Pn-PD&Di-002					
	Prior to booster vaccination			One month post 23-valent PS booster		
	N	% ≥ 1:8 (95% CI)	GMT (95% CI)	N	% ≥ 1:8 (95% CI)	GMT (95% CI)
11Pn-PD	29	27.6 (12.7-47.2)	10.9 (5.0-23.7)	28	82.1 (63.1-93.9)	408.0 (157.3-1058.3)
19F-DT Form 1 <sup>†</sup>	19	47.4 (24.4-71.1)	18.1 (7.2-45.7)	18	94.4 (72.7-99.9)	1063.8 (386.6-2927.5)
19F-DT Form 2 <sup>†</sup>	27	33.3 (16.5-54.0)	8.5 (4.7-15.3)	28	100 (87.7-100)	957.6 (552.8-1659.0)
<i>Prevnar</i>	24	12.5 (2.7-32.4)	8.1 (3.4-19.6)	23	82.6 (61.2-95.0)	380.9 (133.2-1089.5)

<sup>†</sup> The composition of the different formulations is provided in Table4.

- 5 **Table 12** Percentage of subjects with antibody concentrations ≥ 0.2 µg/mL, OPA ≥ 1:8 and GMCs/GMTs against 19F pneumococci one month following 11Pn-PD or *Prevnar* booster in children primed with 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) (Total cohort)

Primary group	11Pn-PD&Di-002					
	22F-ELISA assay			OPA assay		
	N	% ≥ 0.20 µg/mL (95% CI)	GMC (µg/ml) (95% CI)	N	% ≥ 1:8 (95% CI)	GMT (95% CI)
11Pn-PD	70	100 (94.9-100)	4.52 (3.7-5.5)	21	100 (83.9-100)	255.6 (135.5-481.9)
19F-DT Form 1 <sup>†</sup>	66	98.5 (91.8-100)	3.45 (2.8-4.3)	23	95.7 (78.1-99.9)	374.0 (192.6-726.2)
19F-DT Form 2 <sup>†</sup>	70	98.6 (92.3-100)	3.80 (2.9-4.9)	29	96.6 (82.2-99.9)	249.1 (144.7-428.7)
<i>Prevnar</i>	69	97.1 (89.9-99.6)	2.56 (2.0-3.3)	31	96.8 (83.3-99.9)	528.7 (319.4-875.2)

<sup>†</sup> The composition of the different formulations is provided in Table4.

- 10 **Table 13** Percentage of subjects with antibody concentrations ≥ 0.2 µg/mL, OPA ≥ 1:8 and GMCs/GMTs against 19A pneumococci one month following primary vaccination with 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) (Total cohort)

Group	11Pn-PD&DI-001					
	22F-ELISA assay			OPA assay		
	N	% ≥ 0.20 µg/mL (95% CI)	GMC (µg/mL) (95% CI)	N	% ≥ 1:8 (95% CI)	GMT (95% CI)
11Pn-PD	45	28.9 (16.4-44.3)	0.09 (0.07-0.11)	52	7.7 (2.1-18.5)	5.2 (4.0-6.8)
19F-DT Form 2 <sup>†</sup>	51	29.4 (17.5-43.8)	0.11 (0.08-0.16)	59	27.1 (16.4-40.3)	12.4 (7.6-20.3)
<i>Prevnar</i>	55	18.2 (9.1-30.9)	0.10 (0.08-0.12)	61	3.3 (0.4-11.3)	4.6 (3.8-5.6)

<sup>†</sup> The composition of the different formulations is provided in Table4

**Example 5: Adjuvant experiments in preclinical models: impact on the immunogenicity of pneumococcal 11-valent polysaccharide conjugates in elderly Rhesus monkeys**

5

To optimize the response elicited to conjugate pneumococcal vaccines in the elderly population, GSK formulated an 11-valent polysaccharide (PS) conjugate vaccine with a novel adjuvant Adjuvant C – see below.

10 Groups of 5 elderly Rhesus monkeys (14 to 28 years-old) were immunized intramuscularly (IM) at days 0 and 28 with 500 µl of either 11-valent PS conjugates adsorbed onto 315 µg of AIPO4 or 11-valent PS conjugates admixed with Adjuvant C.

15 In both vaccine formulations, the 11-valent PS conjugates were each composed of the following conjugates PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-PD, PS19F-PD, PS23F-DT and PS6B-DT. The vaccine used was 1/5 dose of of the human dose of the vaccine (5 µg of each saccharide per human dose except for 6B [10 µg]) conjugated according to Table 6 conditions (Example 4), except 19F was made according to the following CDAP process conditions: sized saccharide at 9 mg/ml, PD at 5  
20 mg/ml, an initial PD/PS ratio of 1.2/1, a CDAP concentration of 0.75 mg/mg PS, pHa=pHc=pHq 9.0/9.0/9.0 and a coupling time of 60 min.

25 Anti-PS ELISA IgG levels and opsono-phagocytosis titres were dosed in sera collected at day 42. Anti-PS3 memory B cell frequencies were measured by Elispot from peripheral blood cells collected at day 42.

30 According to the results shown here below, Adjuvant C significantly improved the immunogenicity of 11-valent PS conjugates versus conjugates with AIPO4 in elderly monkeys. The novel adjuvant enhanced the IgG responses to PS (Figure 1) and the opsono-phagocytosis antibody titres (Table 14). There was also supportive evidence that the frequency of PS3-specific memory B cells is increased by the use of Adjuvant C (Figure 2).

35 **Table 14, Conjugate Immunogenicity in elderly Rhesus monkeys (post-II opsono-phagocytosis titres)**

		PS1	PS3	PS4	PS5	PS6B	PS7F	PS9V	PS14	PS18C	PS19F	PS23F
11-valent AIPO4	Pre-immune	<8	5	<8	5	<8	16	<8	<8	<8	<8	<8
	day 14 post II	8	181	64	49	64	4096	42	37	169	64	<84
11 valent Adj-C	Pre-immune	5	9	<8	5	8	37	<8	<8	<8	<8	<8
	day 14 post II	776	1351	891	676	6208	16384	111	161	7132	2048	<64

### B Cell Elispot

5 The principle of the assay relies on the fact that memory B cells mature into plasma cells in vitro following cultivation with CpG for 5 days. In vitro generated antigen-specific plasma cells can be easily detected and therefore be enumerated using the B-cell elispot assay. The number of specific plasma cells mirrors the frequency of memory B cells at the onset of the culture.

10 Briefly, in vitro generated plasma cells are incubated in culture plates coated with antigen. Antigen-specific plasma cells form antibody/antigen spots, which are detected by conventional immuno-enzymatic procedure and enumerated as memory B cells. In the present study, Polysaccharides have been used to coat culture plates in order to enumerate respective memory B cells. Results are expressed as a frequency of PS specific  
15 memory B cells within a million of memory B cells.

The study shows that Adjuvant C may be able to alleviate the known problem of PS3 boostability (see 5th International Symposium on Pneumococci and Pneumococcal Diseases, April 2-6 2006, Alice Springs, Central Australia.

20 Specificities of immune responses against a serotype 3 pneumococcal conjugate. Schuerman L, Prymula R, Poolman J. Abstract book p 245, PO10.06).

#### **Example 6, Effectiveness of detoxified Pneumolysin (dPly) as a protein carrier to enhance the immunogenicity of PS 19F in young Balb/c mice**

25 Groups of 40 female Balb/c mice (4-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 4-valent plain PS or 4-valent dPly-conjugated PS, both admixed with Adjuvant C.

Both vaccine formulations were composed of 0.1 µg (quantity of saccharide) of each of the following PS: PS8, PS12F, PS19F and PS22F.

30 Anti-PS ELISA IgG levels were dosed in sera collected at day 42.

The anti-PS19F response, shown as an example in Figure 3, was strongly enhanced in mice given 4-valent dPly conjugates compared to mice immunized with the plain PS. The same improvement was observed for the anti-PS8, 12F and 22F IgG responses (data not  
35 shown).

40



5 **Example 7, Effectiveness of Pneumococcal Histidine Triad Protein D (PhtD) as a protein carrier to enhance the immunogenicity of PS 22F in young Balb/c mice**

Groups of 40 female Balb/c mice (4-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 4-valent plain PS or 4-valent PhtD-conjugated PS, both admixed with Adjuvant C.

10 Both vaccine formulations were composed of 0.1 µg (quantity of saccharide) of each of the following PS: PS8, PS12F, PS19F and PS22F.

Anti-PS ELISA IgG levels were dosed in sera collected at day 42.

The anti-PS22F response, shown as an example in Figure 4, was strongly enhanced in mice given 4-valent PhtD conjugates compared to mice immunized with the plain PS. The same improvement was observed for the anti-PS8, 12F and 19F IgG responses (data not shown).

20 **Example 8, Immunogenicity in elderly C57Bl mice of 13-valent PS conjugates containing 19A-dPly and 22F-PhtD**

Groups of 30 old C57Bl mice (>69-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 11-valent PS conjugates or 13-valent PS conjugates, both admixed with Adjuvant C (see below).

25 The 11-valent vaccine formulation was composed of 0.1 µg saccharide of each of the following conjugates: PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS6B-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-TT, PS19F-DT and PS23F-PD (see Table 1 and comment on 11 valent vaccine discussed under Table 2). The 13-valent vaccine formulation contained in addition 0.1 µg of PS19A-dPly and PS22F-PhtD conjugates (see Table 1 and comment on 13 valent vaccine discussed under Table 2 [using directly-conjugated 22F]). In group 2 and 4 the pneumolysin carrier was detoxified with GMBS treatment, in group 3 and 5 it was done with formaldehyde. In groups 2 and 3 PhtD was used to conjugate PS 22F, in Groups 4 and 5 a PhtD\_E fusion (the construct VP147 from WO 03/054007) was used. In group 6 19A was conjugated to diphtheria toxoid and 22F to protein D.

35 Anti-PS19A and 22F ELISA IgG levels were dosed in individual sera collected at day 42. The ELISA IgG response generated to the other PS was measured in pooled sera.

19A-dPly and 22F-PhtD administered within the 13-valent conjugate vaccine formulation were shown immunogenic in old C57Bl mice (Table 15). The immune response induced against the other PS was not negatively impacted in mice given the 13-valent formulation compared to those immunized with the 11-valent formulation.

40

**Table 15, PS Immunogenicity in old C57Bl mice (post-III IgG levels)**

<b>Old C57 Black mice</b>						
<b>ELISA</b>	<b>GROUP 1</b>	<b>GROUP 2</b>	<b>GROUP 3</b>	<b>GROUP 4</b>	<b>GROUP 5</b>	<b>GROUP 6</b>
	<b>11V</b> 0.1µg/50µl Adj C	<b>11V</b> <b>19A-dPly</b> <b>gmbs</b> <b>22F-PhtD</b> 0.1µg/50µl Adj C	<b>11V</b> <b>19A-dPly</b> <b>formol</b> <b>22F-PhtD</b> 0.1µg/50µl Adj C	<b>11V</b> <b>19A-dPly</b> <b>gmbs</b> <b>22F-PhtD-E</b> 0.1µg/50µl Adj C	<b>11V</b> <b>19A-dPly</b> <b>formol</b> <b>22F-PhtD-E</b> 0.1µg/50µl Adj C	<b>11V</b> <b>19A-DT</b> <b>22F-PD</b> 0.1µg/50µl Adj C
<b>1</b> average Pool	<b>19.30</b>	<b>20.20</b>	<b>24.40</b>	<b>12.80</b>	<b>12.10</b>	<b>13.60</b>
<b>3</b> average Pool	<b>6.32</b>	<b>4.84</b>	<b>5.21</b>	<b>6.74</b>	<b>2.38</b>	<b>2.54</b>
<b>4</b> average Pool	<b>60.9</b>	<b>67.1</b>	<b>51.4</b>	<b>47.4</b>	<b>45.5</b>	<b>41.1</b>
<b>5</b> average Pool	<b>1.34</b>	<b>3.81</b>	<b>3.06</b>	<b>2.75</b>	<b>1.26</b>	<b>1.23</b>
<b>6B</b> average Pool	<b>4.41</b>	<b>4.12</b>	<b>5.88</b>	<b>1.58</b>	<b>2.31</b>	<b>5.64</b>
<b>7F</b> average Pool	<b>0.83</b>	<b>0.81</b>	<b>1.65</b>	<b>1.98</b>	<b>0.89</b>	<b>0.99</b>
<b>9V</b> average Pool	<b>13.8</b>	<b>23.7</b>	<b>20.0</b>	<b>13.1</b>	<b>15.5</b>	<b>9.6</b>
<b>14</b> average Pool	<b>25.73</b>	<b>42.96</b>	<b>34.12</b>	<b>32.53</b>	<b>23.97</b>	<b>15.60</b>
<b>18C</b> average Pool	<b>13.4</b>	<b>20.1</b>	<b>11.9</b>	<b>9.1</b>	<b>8.3</b>	<b>8.4</b>
<b>19F</b> average Pool	<b>57.5</b>	<b>90.0</b>	<b>63.8</b>	<b>36.5</b>	<b>47.0</b>	<b>69.1</b>
<b>23F</b> average Pool	<b>NR</b>	<b>NR</b>	<b>NR</b>	<b>NR</b>	<b>NR</b>	<b>NR</b>
<b>19A</b> GMC	<b>0.06</b>	<b>0.09</b>	<b>0.25</b>	<b>0.08</b>	<b>0.23</b>	<b>0.19</b>

	IC %sero	0.04-0.1 33%	0.05-0.14 47%	0.15-0.41 83%	0.06-0.12 53%	0.14-0.38 80%	0.09-0.3 73%
<b>22F</b>	GMC	NR	<b>5.81</b>	<b>3.76</b>	<b>0.54</b>	<b>0.85</b>	<b>2.02</b>
	IC		3.2-10.6	1.8-7.9	0.3-1.1	0.4-1.7	1.2-3.4
	%sero	0%	97%	90%	77%	87%	97%

**Example 9, Immunogenicity in young Balb/c mice of 13-valent PS conjugates containing 19A-dPly and 22F-PhtD**

5 Groups of 30 young Balb/c mice (4-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 11-valent PS conjugates or 13-valent PS conjugates, both admixed with Adjuvant C (see below).

10 The 11-valent vaccine formulation was composed of 0.1 µg saccharide of each of the following conjugates: PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS6B-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-TT, PS19F-DT and PS23F-PD (see Table 1 and comment on 11 valent vaccine discussed under Table 2). The 13-valent vaccine formulation contained in addition 0.1 µg of PS19A-dPly and PS22F-PhtD conjugates (see Table 1 and comment on 13 valent vaccine discussed under Table 2 [using directly-conjugated 22F]). In group 2 and 4 the pneumolysin carrier was detoxified with GMBS treatment, in group 3 and 5 it was done with formaldehyde. In groups 2 and 3 PhtD was used to conjugate PS 22F, in Groups 4 and 5 a PhtD\_E fusion (the construct VP147 from WO 03/054007) was used. In group 6 19A was conjugated to diphtheria toxoid and 22F to protein D.

15 Anti-PS19A and 22F ELISA IgG levels were dosed in individual sera collected at day 42. The ELISA IgG response generated to the other PS was measured in pooled sera.

20 19A-dPly and 22F-PhtD administered within the 13-valent conjugate vaccine formulation were shown immunogenic in young Balb/c mice (Table 16). The immune response induced against the other PS was not negatively impacted in mice given the 13-valent formulation compared to those immunized with the 11-valent formulation.

25 **Table 16, PS immunogenicity in young Balb/c mice (post-III IgG levels)**

<b>BalbC mice</b>						
ELISA	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
	11V	11V 19A-dPly gmbs 22F-PhtD	11V 19A-dPly formol 22F-PhtD	11V 19A-dPly gmbs 22F-PhtD-E	11V 19A-dPly formol 22F-PhtD-E	11V 19A-DT 22F-PD
	0.1µg/50µl Adj C	0.1µg/50µl Adj C	0.1µg/50µl Adj C	0.1µg/50µl Adj C	0.1µg/50µl Adj C	0.1µg/50µl Adj C
<b>1</b>	<b>average</b>	<b>131.70</b>	<b>101.20</b>	<b>83.00</b>	<b>82.40</b>	<b>85.50</b>

	Pool						
14	average Pool	71.19	77.18	46.53	59.67	38.47	53.69
18C	average Pool	109.4	122.3	137.1	79.9	73.7	83.1
19F	average Pool	73.9	102.5	112.2	75.5	62.3	72.1
23F	average Pool	19.19	30.74	29.44	31.52	19.13	24.94
19A	GMC	0.4	25.58	41.49	14.25	27.49	6.74
	IC	0.24-0.68	12-54.5	24.4-70.5	5.9-34.6	16.6-45.4	4-11.3
	%sero	75%	100%	100%	100%	100%	100%
22F	GMC	0.12	2.51	3.67	45.74	30.68	96.38
	IC	0.09-0.16	0.94-6.73	1.59-8.42	29.3-71.4	17-53.3	73.5-126.4
	%sero	10%	95%	95%	100%	100%	100%

Example 11: Formulations being made and tested

5

a) The following formulations are made (using the 13 valent vaccine from table 1 and serotype 3 from table 5 – see comment on 14 valent vaccine discussed under Table 2 [using directly-conjugated 22F or through an ADH linker]). The saccharides are formulated with aluminium phosphate and 3D-MPL as shown below.

10

14V 25µg MPL						14V 10µg MPL							
Sum of BAC Aluminium content -> FF						Sum of BAC Aluminium content -> FF							
Per Dose:						Per Dose:							
PS	carrier	µg PS	µg MPL	ratio PS/Al 1/x	µg Al	PS	carrier	µg PS	µg MPL	ratio PS/Al 1/x	µg Al		
1	PD	1		10	10	1	PD	1		10	10		
3	PD	1		10	10	3	PD	1		10	10		
4	PD	3		10	30	4	PD	3		10	30		
5	PD	1		10	10	5	PD	1		10	10		
6A	PD	1		10	10	6A	PD	1		10	10		
6B	PD	1		10	10	6B	PD	1		10	10		
7F	PD	1		10	10	7F	PD	1		10	10		
9V	PD	1		10	10	9V	PD	1		10	10		
14	PD	1		10	10	14	PD	1		10	10		
18C	TT <sub>ADH</sub>	3		15	45	18C	TT <sub>ADH</sub>	3		15	45		
19A	dPly	3		10	30	19A	dPly	3		10	30		
19F	DT	3		10	30	19F	DT	3		10	30		
22F	PhuD	3		10	30	22F	PhuD	3		10	30		
23F	PD	1		10	10	23F	PD	1		10	10		
BAC MPL 50/200			25	4	100	BAC MPL 50/200			10	4	40		
FF Aluminium content					Sum =	355	FF Aluminium content					Sum =	295

b) The same saccharide formulation is adjuvanted with each of the following adjuvants:

- In the table herebelow the concentration of the emulsion components per 500µl dose is shown.

Ingredients	Adjuvant A1	Adjuvant A2	Adjuvant A3
	250µl o/w emulsion	125µl o/w emulsion	50µl o/w emulsion
alpha Tocopherol	11.88mg	5.94mg	2.38mg
Squalene	10.7mg	5.35mg	2.14mg
Tween 80	4.85mg	2.43mg	0.97mg

Ingredients	Adjuvant A4	Adjuvant A5	Adjuvant A6	Adjuvant A7
	250µl o/w emulsion	250µl o/w emulsion	125µl o/w emulsion	50µl o/w emulsion
alpha Tocopherol	11.88mg	11.88mg	5.94mg	2.38mg
Squalene	10.7mg	10.7mg	5.35mg	2.14mg
Tween 80	4.85mg	4.85mg	2.43mg	0.97mg
3D-MPL	50µg	25µg	25µg	10µg

10

c) The saccharides are also formulated with two liposome based adjuvants:

**Composition of Adjuvant B1**  
**Qualitative Quantitative (per 0.5 mL dose)**

15 Liposomes:  
 - DOPC 1 mg  
 - cholesterol 0.25 mg  
 3DMPL 50 µg  
 QS21 50 µg  
 20 KH<sub>2</sub>PO<sub>4</sub> 3.124 mg Buffer  
 Na<sub>2</sub>HPO<sub>4</sub> 0.290 mg Buffer  
 NaCl 2.922 mg  
 (100 mM)  
 WFI q.s. ad 0.5 ml Solvent  
 25 pH 6.1  
 1. Total PO<sub>4</sub> concentration = 50 mM

**Composition of Adjuvant B2**  
**Qualitative Quantitative (per 0.5 mL dose)**

30 Liposomes:  
 - DOPC 0.5 mg  
 - cholesterol 0.125 mg  
 3DMPL 25 µg  
 QS21 25 µg

- KH<sub>2</sub>PO<sub>4</sub> 3.124 mg Buffer  
 Na<sub>2</sub>HPO<sub>4</sub> 0.290 mg Buffer  
 NaCl 2.922 mg  
 (100 mM)  
 5 WFI q.s. ad 0.5 ml Solvent  
 pH 6.1

d) The saccharides are also formulated with Adjuvant C (see above for other compositions where this adjuvant has been used):

- 10 **Qualitative Quantitative (per 0.5 mL dose)**  
 Oil in water emulsion: 50 µl  
 - squalene 2.136 mg  
 - α-tocopherol 2.372 mg  
 15 - Tween 80 0.97 mg  
 - cholesterol 0.1 mg  
 3DMPL 50 µg  
 QS21 50 µg  
 KH<sub>2</sub>PO<sub>4</sub> 0.470 mg Buffer  
 20 Na<sub>2</sub>HPO<sub>4</sub> 0.219 mg Buffer  
 NaCl 4.003 mg  
 (137 mM)  
 KCl 0.101 mg  
 (2.7 mM)  
 25 WFI q.s. ad 0.5 ml Solvent  
 pH 6.8

**Example 12, Impact of conjugation chemistry on 22F-PhtD conjugate immunogenicity in Balb/c mice**

- 30 Groups of 30 female Balb/c mice were immunised by the intramuscular (IM) route at days 0, 14 and 28 with 13-valent PS formulations containing PS 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F (dose: 0.3 µg saccharide / PS for PS 4, 18C, 19A, 19F and 22F and 0.1 µg saccharide / PS for the other PS).

- 35 PS 18C was conjugated to Tetanus Toxoid, 19F to Diphtheria Toxoid, 19A to formol-detoxified Ply, 22F to PhtD and the other PS to PD.

- Two formulations, constituted of either 22F-PhtD prepared by direct CDAP chemistry or 22F-AH-PhtD (ADH-derivitized PS), were compared. See Example 2, Table 1 and comment under Table 2 for characteristics of 13 valent vaccine made either with 22F directly conjugated or via an ADH spacer. The vaccine formulations were supplemented  
 40 with adjuvant C.

Anti-PS22F ELISA IgG levels and opsono-phagocytosis titres were measured in sera collected at day 42.

22F-AH-PhtD was shown much more immunogenic than 22F-PhtD in terms of both IgG levels (figure 5) and opsono-phagocytic titres (figure 6).

5

**Example 13. Impact of new adjuvants on immunogenicity of *Streptococcus pneumoniae* capsule PS conjugates**

10 Groups of 40 female Balb/c mice were immunised by the IM route at days 0, 14 and 28 with 13-valent PS formulations containing PS 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F (dose: 0.3 µg / PS for PS 4, 18C, 19A, 19F and 22F and 0.1 µg / PS for the other PS).

15 PS 18C was conjugated to Tetanus Toxoid, 19F to Diphtheria Toxoid, 19A to formol-detoxified Ply, 22F to PhtD and the other PS to PD. See Example 2, Table 1 and comment under Table 2 for characteristics of 13 valent vaccine made with 22F directly conjugated.

Four formulations, supplemented with either AlPO<sub>4</sub>, adjuvant A1, adjuvant A4 or adjuvant A5, were compared.

20 Anti-PS, Ply, PhtD and PD ELISA IgG levels were measured in sera collected at day 42 and pooled per group. The following ratio was calculated for each antigen: IgG level induced with the new adjuvant tested / IgG level induced with AlPO<sub>4</sub>.

All the new adjuvants tested improved at least 2-fold the immune responses to 13-valent conjugates compared to the classical AlPO<sub>4</sub> formulation (figure 7).

25 **Example 14. protective efficacy of a PhtD/detoxified Ply combo in a pneumococcal monkey pneumonia model**

30 Groups of 6 Rhesus monkeys (3 to 8 years-old), selected as those having the lowest pre-existing anti-19F antibody levels, were immunized intramuscularly at days 0 and 28 with either 11-valent PS conjugates (i.e. 1 µg of PS 1, 3, 5, 6B, 7F, 9V, 14 and 23F, and 3 µg of PS 4, 18C and 19F) or PhtD (10 µg) + formol-detoxified Ply (10 µg) or the adjuvant alone.

PS 18C was conjugated to Tetanus Toxoid, 19F to Diptheria Toxoid and the other PS to PD. See Example 2, Table 1 and comment under Table 2 for characteristics of 11 valent vaccine. All formulations were supplemented with adjuvant C.

5 Type 19F pneumococci ( $5 \cdot 10^8$  cfu) were inoculated in the right lung at day 42. Colonies were counted in broncho-alveolar lavages collected at days 1, 3 and 7 post-challenge. The results were expressed as the number of animals per group either dead, lung colonized or cleared at day 7 after challenge.

As shown in figure 8, a good protection close to statistical significance (despite the low number of animals used) was obtained with 11-valent conjugates and the PhtD+dPly  
10 combo ( $p < 0.12$ , Fisher Exact test) compared to the adjuvant alone group.

**Example 15, impact of conjugation chemistry on the anti-PhtD antibody response and the protective efficacy against a type 4 challenge induced by 22F-PhtD conjugates**

15 Groups of 20 female OF1 mice were immunised by the intramuscular route at days 0 and 14 with 3  $\mu$ g of either 22F-PhtD (prepared by direct CDAP chemistry) or 22F-AH-PhtD (ADH-derivitized PS), or the adjuvant alone. Both monovalent 22F conjugates were made by the processes of Example 2 (see also Table 1 and Table 2). Each formulation was  
20 supplemented with adjuvant C.

Anti-PhtD ELISA IgG levels were measured in sera collected at day 27.

Mice were challenged intranasally with  $5 \cdot 10^6$  cfu of type 4 pneumococci at day 28 (i.e. a  
25 pneumococcal serotype not potentially covered by the PS present in the vaccine formulation tested). The mortality induced was monitored until day 8 post-challenge.

22F-AH-PhtD induced a significantly higher anti-PhtD IgG response and better protection  
30 against type 4 challenge than 22F-PhtD.



## CLAIMS

1. A *Streptococcus pneumoniae* immunogenic composition comprising 9 or more, 10 or more, 11 or more, 13 or more, or 14 or more capsular saccharides from different *S. pneumoniae* serotypes conjugated to 2 or more different carrier proteins, wherein the composition comprises serotype 19F capsular saccharide conjugated to diphtheria toxoid (DT) or CRM197, optionally wherein 19F is the only saccharide in the composition conjugated to diphtheria toxoid (DT) or CRM197
2. An immunogenic composition according to claim 1 wherein serotype 19F is conjugated to Diphtheria toxoid.
3. An immunogenic composition according to claim 1 or 2 wherein said composition further comprises protein D from *Haemophilus influenzae*.
4. An immunogenic composition according to any preceding claim wherein 19F capsular saccharide is directly conjugated to the carrier protein.
5. An immunogenic composition according to any of claims 1 to 3 wherein 19F capsular saccharide is conjugated to the carrier protein via a linker.
6. An immunogenic composition according to claim 5 wherein the linker is bifunctional.
7. The immunogenic composition of claim 5 or 6 wherein the linker is ADH.
8. The immunogenic composition of claims 5, 6, or 7 wherein the linker is attached to the carrier protein by carbodiimide chemistry, preferably using EDAC.
9. The immunogenic composition of any of claims 5 to 8 wherein the saccharide is conjugated to the linker before the carrier protein is conjugated to the linker.
10. The immunogenic composition of any of claims 5 to 8 wherein the carrier protein is conjugated to the linker before the saccharide is conjugated to the linker.
11. the immunogenic composition of any preceding claim wherein the 19F saccharide is conjugated to the carrier protein or to the linker using CDAP chemistry.
12. The immunogenic composition of any preceding claim wherein the ratio of carrier protein to 19F saccharide is between 5:1 and 1:5, 4:1 and 1:1 or 2:1 and 1:1, or 1.5:1 and 1.4:1 (w/w).
13. The immunogenic composition of any preceding claim wherein the average size (e.g.  $M_w$ ) of the 19F saccharide is above 100 kDa.
14. The immunogenic composition of claim 13 wherein the average size (e.g.  $M_w$ ) of the 19F saccharide is between 100-750, 110-500, 120-250, or 125 and 150 kDa.

15. The immunogenic composition of claim 13 or 14 wherein the 19F saccharide is either a native polysaccharide or is sized by a factor of no more than x5.
16. The immunogenic composition of claim 13, 14 or 15 wherein the 19F saccharide has been sized by microfluidization.
17. The immunogenic composition of any preceding claim wherein the dose of the 19F saccharide conjugate is between 1 and 10  $\mu\text{g}$ , 1 and 5  $\mu\text{g}$ , or 1 and 3  $\mu\text{g}$  of saccharide.
18. An immunogenic composition according to any preceding claim wherein at least 8 of the capsular saccharides are conjugated to the same carrier protein.
19. An immunogenic composition according to claim 18 wherein said carrier protein is not diphtheria toxoid and/or is not CRM197.
20. An immunogenic composition according to any preceding claim comprising 2 different carrier proteins.
21. An immunogenic composition according to any preceding claim comprising 3, 4, 5 or 6 different carrier proteins.
22. An immunogenic composition according to any preceding claim wherein one or more or all carrier proteins is selected from the group consisting of DT, CRM 197, TT, Fragment C, dPly, PhtA, PhyB, PhtD, PhtE, PhtDE OmpC, PorB and *Haemophilus influenzae* Protein D.
23. An immunogenic composition according to claim 22 wherein one carrier protein is protein D.
24. An immunogenic composition according to claim 22 wherein one carrier protein is TT.
25. An immunogenic composition according to any of claims 22, 23 or 24 wherein both TT and protein D are present as carrier proteins.
26. An immunogenic composition according to any of claims 22, 23, 24 or 25 wherein at least 8 of the capsular saccharides are conjugated to protein D.
27. An immunogenic composition according to any preceding claim wherein the composition comprises capsular saccharide 18C conjugated to TT, optionally wherein 18C is the only saccharide in the composition conjugated to tetanus toxoid (TT).
28. An immunogenic composition according to any preceding claim wherein 18C capsular saccharide is directly conjugated to the carrier protein.
29. An immunogenic composition according to any of claims 1 to 27 wherein 18C capsular saccharide is conjugated to the carrier protein via a linker.
30. An immunogenic composition according to claim 29 wherein the linker is bifunctional.
31. The immunogenic composition of claim 29 or 30 wherein the linker is ADH.

32. The immunogenic composition of claims 29, 30, or 31 wherein the linker is attached to the carrier protein by carbodiimide chemistry, preferably using EDAC.
33. The immunogenic composition of any of claims 29 to 31 wherein the saccharide is conjugated to the linker before the carrier protein is conjugated to the linker.
34. The immunogenic composition of any of claims 29 to 31 wherein the carrier protein is conjugated to the linker before the saccharide is conjugated to the linker.
35. the immunogenic composition of any preceding claim wherein the 18C saccharide is conjugated to the carrier protein or linker using CDAP chemistry.
36. The immunogenic composition of any preceding claim wherein the 18C saccharide is conjugated to the carrier protein or linker using reductive amination.
37. The immunogenic composition of any preceding claim wherein the ratio of carrier protein to 18C saccharide is between 0.5:1-5:1, 1:1-4:1, 1.5:1-3:1, or 2:1 and 2.5:1 (w/w).
38. The immunogenic composition of any preceding claim wherein the average size (e.g.  $M_w$ ) of the 18C saccharide is above 50 kDa.
39. The immunogenic composition of claim 38 wherein the average size (e.g.  $M_w$ ) of the 18C saccharide is between 50-500, 60-400, 70-300, 75-200, or 80 and 100 kDa.
40. The immunogenic composition of claim 39 wherein the 18C saccharide is either a native polysaccharide or is sized by a factor of no more than x5.
41. The immunogenic composition of claim 38, 39 or 40 wherein the 18C saccharide has been sized by microfluidization.
42. The immunogenic composition of any preceding claim wherein the dose of the 18C saccharide conjugate is between 1 and 10  $\mu\text{g}$ , 1 and 5  $\mu\text{g}$ , or 1 and 3  $\mu\text{g}$  of saccharide.
43. The immunogenic composition of claim 42 wherein the dose of the 18C saccharide conjugate is 3  $\mu\text{g}$  of saccharide.
44. An immunogenic composition according to any preceding claim wherein a capsular saccharide conjugate of serotype 6B is present, but is not conjugated to DT and/or CRM197.
45. An immunogenic composition according to any preceding claim wherein a capsular saccharide conjugate of serotype 23F is present, but is not conjugated to DT and/or CRM197.
46. An immunogenic composition according to any preceding claim wherein at least serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F are present as conjugated saccharides.

47. An immunogenic composition according to any preceding claim wherein serotypes 1, 4, 5, 6B, 7F, 9V, 14 and 23F are present and are all conjugated to protein D.
48. An immunogenic composition according to any preceding claim further comprising serotype 3 present as a conjugated saccharide.
49. An immunogenic composition according to claim 48 wherein serotype 3 is conjugated to protein D.
50. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 6A and/or 15B.
51. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 19A.
52. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 22F.
53. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 8.
54. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 12F.
55. An immunogenic composition according to any preceding claim wherein at least one of the capsular saccharides is directly conjugated to the carrier protein.
56. An immunogenic composition according to any preceding claim wherein at least one of the capsular saccharides is conjugated to the carrier protein via a linker.
57. An immunogenic composition according to claim 56 wherein the linker is bi-functional.
58. The immunogenic composition of claim 56 or 57 wherein the linker is ADH.
59. The immunogenic composition of claims 56, 57, or 58 wherein the linker is attached to the carrier protein by carbodiimide chemistry, preferably using EDAC.
60. The immunogenic composition of any of claims 56 to 59 wherein the saccharide is conjugated to the linker before the carrier protein is conjugated to the linker.
61. The immunogenic composition of any of claims 56 to 59 wherein the carrier protein is conjugated to the linker before the saccharide is conjugated to the linker.
62. The immunogenic composition of any preceding claim wherein at least one of the capsular saccharides is conjugated to the carrier protein and/or linker using CDAP chemistry.
63. The immunogenic composition of any preceding claim wherein at least one of the capsular saccharides is conjugated to the carrier protein and/or linker using reductive amination.

64. The immunogenic composition of claim 63 wherein capsular saccharide 3 is conjugated to the carrier protein and/or linker using reductive amination.
65. The immunogenic composition of claim 63 or 64 wherein capsular saccharide 1 is conjugated to the carrier protein and/or linker using reductive amination.
66. The immunogenic composition of any preceding claim wherein the ratio of carrier protein to saccharides is between 0.5:1-5:1, 1:2 and 2.5:1, 1:1-3.5:1, or 1.3:1-3.0:1.
67. The immunogenic composition of any preceding claim wherein the average size (e.g.  $M_w$ ) of the saccharides (in the saccharide conjugates) is above 50 kDa, e.g. 50-1600, 80-1400, 100-1000, 150-500, or 200-400 kDa.
68. The immunogenic composition of any preceding claim which comprises serotype 1 (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 100-1000, 200-800, 250-600, or 300 and 400 kDa.
69. The immunogenic composition of any preceding claim which comprises serotype 4 (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 50-500, 60-300, 70-200, or 75 and 125 kDa.
70. The immunogenic composition of any preceding claim which comprises serotype 5 (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 100-1000, 200-700, 300-500, or 350 and 450 kDa.
71. The immunogenic composition of any preceding claim which comprises serotype 6B (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 500-1600, 750-1500, or 1000 and 1400 kDa.
72. The immunogenic composition of any preceding claim which comprises serotype 7F (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 50-1000, 100-750, 150-500, or 200 and 300 kDa.
73. The immunogenic composition of any preceding claim which comprises serotype 9V (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 50-1000, 100-750, 150-500, 200-400, or 250 and 300 kDa.
74. The immunogenic composition of any preceding claim which comprises serotype 14 (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 50-1000, 100-750, 150-500, or 200 and 250 kDa.
75. The immunogenic composition of any preceding claim which comprises serotype 23F (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 500-1500, 700-1300, 800-1100, or 900 and 1000 kDa.
76. The immunogenic composition of any preceding claim which comprises serotype 19A (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 50 and 800kDa, 110 and 700 kDa, 110-300, 120-200, 130-180, or 140-160 kDa.

77. The immunogenic composition of any preceding claim which comprises serotype 22F (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 50 and 800 kDa, 110 and 700 kDa, 110-300, 120-200, 130-180, or 150-170 kDa.
78. The immunogenic composition of any preceding claim which comprises serotype 6A (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 500 and 1600 kDa, or 1100 and 1540 kDa.
79. The immunogenic composition of any preceding claim which comprises serotype 3 (saccharide conjugate) having an average saccharide size (e.g.  $M_w$ ) of between 50 and 1000 kDa, 60 and 800, 70 and 600, 80 and 400, 100 and 300, or 150 and 250 kDa.
80. The immunogenic composition of any preceding claim which comprises serotypes 5, 6B and 23F as native saccharides.
81. The immunogenic composition of any preceding claim wherein the dose of the capsular saccharide conjugates is between 1 and 10  $\mu\text{g}$ , 1 and 5  $\mu\text{g}$ , or 1 and 3  $\mu\text{g}$  of saccharide per conjugate.
82. The immunogenic composition of any preceding claim which comprises conjugates of serotypes 4, 18C and 19F at dosages of 3  $\mu\text{g}$  of saccharide per conjugate.
83. The immunogenic composition of any preceding claim which comprises conjugates of serotypes 1, 5, 6B, 7F, 9V, 14 and 23F at dosages of 1  $\mu\text{g}$  of saccharide per conjugate.
84. The immunogenic composition of any preceding claim which comprises conjugates of serotypes 6A and/or 3 at dosages of 1  $\mu\text{g}$  of saccharide per conjugate.
85. The immunogenic composition of any preceding claim which comprises conjugates of serotypes 19A and/or 22F at dosages of 3  $\mu\text{g}$  of saccharide per conjugate.
86. The immunogenic composition of any preceding claim which further comprises unconjugated *S. pneumoniae* saccharides of serotypes different from those conjugated, such that the number of conjugated and unconjugated saccharide serotypes is less than or equal to 23.
87. The immunogenic composition of any preceding claim which further comprises one or more unconjugated or conjugated *S pneumoniae* proteins.
88. The immunogenic composition of claim 87 which comprises one or more unconjugated *S. pneumoniae* proteins.
89. The immunogenic composition of claim 87 or 88 wherein said one or more *S. pneumoniae* proteins are selected from Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family,

- LytX truncates, CbpX truncate-LytX truncate chimeric proteins, detoxified pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133.
90. The immunogenic composition of claims 87, 88 or 89 which comprises pneumolysin.
  91. The immunogenic composition of any of claims 87 to 90 which comprises a PhtX protein.
  92. An immunogenic composition according to any preceding claim which comprises pneumolysin as free or carrier protein.
  93. An immunogenic composition according to any preceding claim which comprises a PhtX protein as free or carrier protein.
  94. The immunogenic composition of claim 93 wherein said PhtX protein is PhtD or a PhtBD or PhtDE fusion protein.
  95. A immunogenic composition according to any preceding claim which further comprises an adjuvant.
  96. An immunogenic composition according to claim 95 wherein the adjuvant is a preferential inducer of a Th1 response.
  97. An immunogenic composition according to claim 95 or 96 wherein the adjuvant comprises one or more components selected from QS21, MPL® or an immunostimulatory oligonucleotide.
  98. An immunogenic composition according to any of claims 95 to 97 wherein the adjuvant comprises QS21 and MPL.
  99. The immunogenic composition of claim 95, wherein the adjuvant comprises a liposome carrier.
  100. The immunogenic composition of claim 99, wherein the adjuvant comprises (per 0.5 mL dose) 0.1-10mg, 0.2-7, 0.3-5, 0.4-2, or 0.5-1 mg (e.g. 0.4-0.6, 0.9-1.1, 0.5 or 1 mg) phospholipid (for instance DOPC).
  101. The immunogenic composition of claim 99 or 100, wherein the adjuvant comprises (per 0.5 mL dose) 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol).
  102. The immunogenic composition of claims 99-101, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) lipid A derivative (for instance 3D-MPL).
  103. The immunogenic composition of claims 99-102, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30 µg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 µg) saponin (for instance QS21).
  104. The immunogenic composition of claim 95, wherein the adjuvant comprises an oil in water emulsion.
  105. The immunogenic composition of claim 104, wherein the adjuvant comprises (per 0.5 mL dose) 0.5-15, 1-13, 2-11, 4-8, or 5-6mg (e.g. 2-3, 5-6, or 10-11 mg) metabolisable oil (such as squalene).

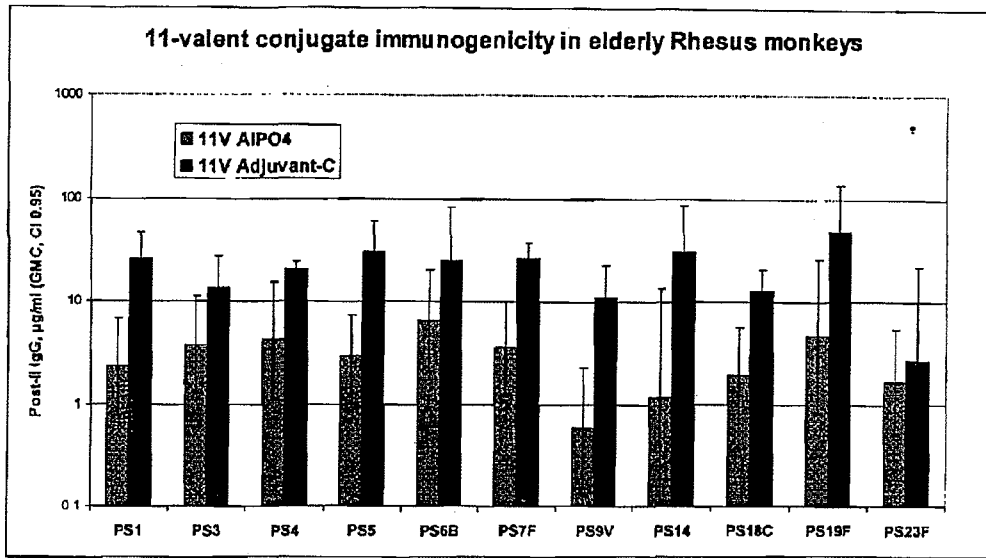
106. The immunogenic composition of claim 104 or 105, wherein the adjuvant comprises (per 0.5 mL dose) 0.1-10, 0.3-8, 0.6-6, 0.9-5, 1-4, or 2-3 mg (e.g. 0.9-1.1, 2-3 or 4-5 mg) emulsifier (such as Tween 80).
107. The immunogenic composition of claims 104-106, wherein the adjuvant comprises (per 0.5 mL dose) 0.5-20, 1-15, 2-12, 4-10, 5-7 mg (e.g. 11-13, 5-6, or 2-3 mg) tocol (such as alpha tocopherol).
108. The immunogenic composition of claims 104-107, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30  $\mu$ g (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50  $\mu$ g) lipid A derivative (for instance 3D-MPL).
109. The immunogenic composition of claims 104-108, wherein the adjuvant comprises (per 0.5 mL dose) 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol).
110. The immunogenic composition of claims 104-109, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30  $\mu$ g (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50  $\mu$ g) saponin (for instance QS21).
111. The immunogenic composition of claim 95, wherein the adjuvant comprises a metal salt and lipid A derivative.
112. The immunogenic composition of claim 111, wherein the adjuvant comprises (per 0.5 mL dose) 100-750, 200-500, or 300-400  $\mu$ g Al as aluminium phosphate.
113. The immunogenic composition of claim 111 or 112, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30  $\mu$ g (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50  $\mu$ g) lipid A derivative (for instance 3D-MPL).
114. A vaccine kit comprising an immunogenic composition according to any of claims 1 to 94 and further comprising for concomitant or sequential administration an adjuvant as defined in any of claims 96 to 113.
115. A vaccine comprising the immunogenic composition of any one of claims 1 to 113 and a pharmaceutically acceptable excipient.
116. A process for making the vaccine according to claim 116 which comprises the step of mixing the immunogenic composition of any of claims 1 to 113 with a pharmaceutically acceptable excipient.
117. A method of immunising a human host against disease caused by *Streptococcus pneumoniae* infection comprising administering to the host an immunoprotective dose of the immunogenic composition of any of claims 1 to 113 or vaccine of claim 115.
118. The method of claim 117, wherein the human host is elderly, and the disease is either or both of pneumonia or invasive pneumococcal disease (IPD).
119. The method of claim 117 or 118, wherein the human host is elderly, and the disease is exacerbations of chronic obstructive pulmonary disease (COPD).



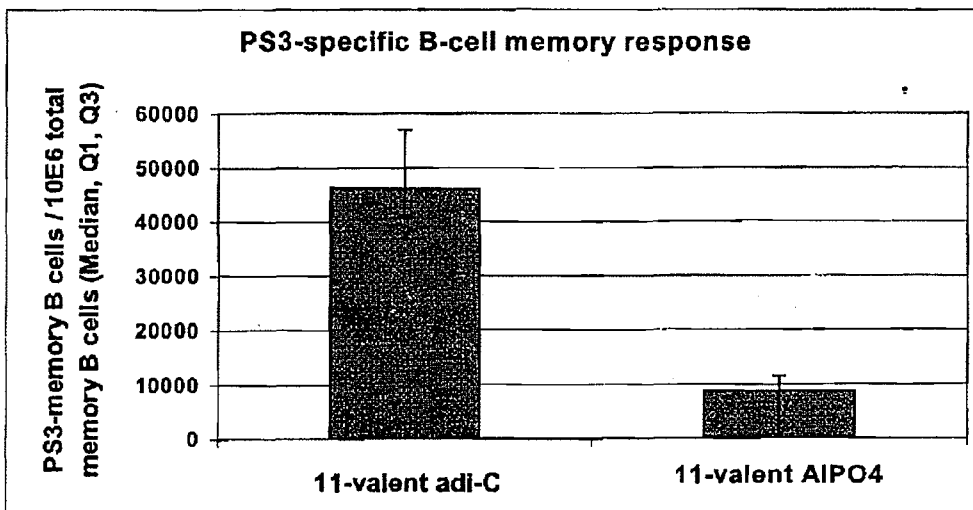
120. The method of claim 117, wherein the human host is infant, and the disease is otitis media.
121. The method of claim 117 or 120, wherein the human host is infant, and the disease is meningitis and/or bacteraemia.
122. The method of claims 117, 120 or 121, wherein the human host is infant, and the disease is pneumonia and/or conjunctivitis.
123. The immunogenic composition of claims 1 to 113 or vaccine of claim 115 for use in the treatment or prevention of disease caused by *Streptococcus pneumoniae* infection.
124. A use of the immunogenic composition of claims 1 to 113 or vaccine of claim 115 in the manufacture of a medicament for the treatment or prevention of diseases caused by *Streptococcus pneumoniae* infection.
125. The use of claim 124, wherein the disease is either or both of pneumonia or invasive pneumococcal disease (IPD) of elderly humans.
126. The use of claim 124 or 125, wherein the disease is exacerbations of chronic obstructive pulmonary disease (COPD) of elderly humans.
127. The use of claim 124, wherein the disease is otitis media of infant humans.
128. The use of claim 124 or 127, wherein the disease is meningitis and/or bacteraemia of infant humans.
129. The use of claims 124, 127 or 128, wherein the disease is pneumonia and/or conjunctivitis of infant humans.
130. A use of the immunogenic composition of claims 1 to 113 or vaccine of claim 115 comprising a capsular saccharide conjugate of serotype 19F but not comprising capsular saccharide from serotype 19A in the manufacture of a medicament for the treatment or prevention of diseases caused by *Streptococcus pneumoniae* infection by serotype 19A strains.
131. A method of immunising a human host against disease caused by *Streptococcus pneumoniae* serotype 19A infection comprising the steps of administering to the host an immunoprotective dose of the immunogenic composition of claims 1 to 113 or vaccine of claim 115 which comprises a capsular saccharide conjugate of serotype 19F but does not comprise capsular saccharide from serotype 19A.
132. A method of eliciting a protective immune response in infants against Otitis media comprising the administration as separate or combined components, sequentially or concomitantly (i) an immunogenic composition or vaccine according to any of claims 1 to 115 and (ii) Protein D from *Haemophilus influenzae* which protein D may be free and/or conjugated.
133. A method of eliciting a protective immune response to infants against *S. pneumonia* by administering the immunogenic composition or vaccine of any preceding claim.

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134. A method of eliciting a protective immune response to the elderly against *S. pneumonia* by administering in combination, sequentially or concomitantly (i) the immunogenic composition or vaccine of any preceding claim (ii) one or more *S. pneumoniae* surface proteins selected from the group consisting of the PhtX family and pneumolysin.
  135. A method of eliciting a protective immune response to infants against Otitis media by administering the immunogenic composition or vaccine of any preceding claim.
  136. A method of eliciting a protective immune response to the infants against Otitis media by administering as separate or combined components, sequentially or concomitantly (i) the vaccine of any preceding claim (ii) one or more *S. pneumoniae* surface proteins selected from the group consisting of the PhtX family and pneumolysin.
  137. The immunogenic composition of claims 1-113 or vaccine of claim 115, which comprises saccharide conjugates derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F wherein the GMC antibody titre induced against one or more of the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.
  138. The immunogenic composition of claim 137, wherein the GMC antibody titre induced against serotype 4 is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.
  139. The immunogenic composition of claim 137 or 138, wherein the GMC antibody titre induced against serotype 6B is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.
  140. The immunogenic composition of claims 137-139, wherein the GMC antibody titre induced against serotype 9V is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.
  141. The immunogenic composition of claims 137-140, wherein the GMC antibody titre induced against serotype 14 is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.
  142. The immunogenic composition of claims 137-141, wherein the GMC antibody titre induced against serotype 18C is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.
  143. The immunogenic composition of claims 137-142, wherein the GMC antibody titre induced against serotype 19F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.
  144. The immunogenic composition of claims 137-143, wherein the GMC antibody titre induced against serotype 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

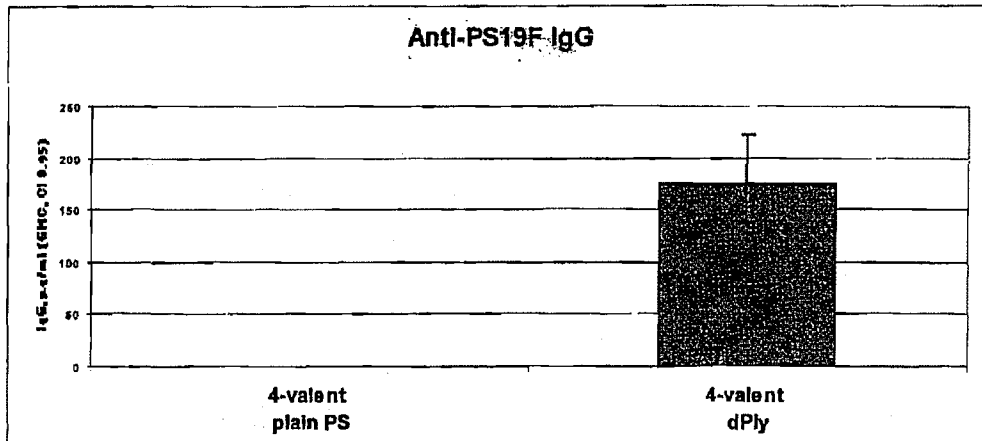
145. The immunogenic composition of claims 137-144 which comprises a serotype 3 saccharide conjugate.
146. The immunogenic composition of claims 137-145 which comprises a serotype 6A saccharide conjugate.
147. The immunogenic composition of claims 137-146 which comprises a serotype 19A saccharide conjugate.
148. The immunogenic composition of claims 137-147 which comprises a serotype 22F saccharide conjugate.



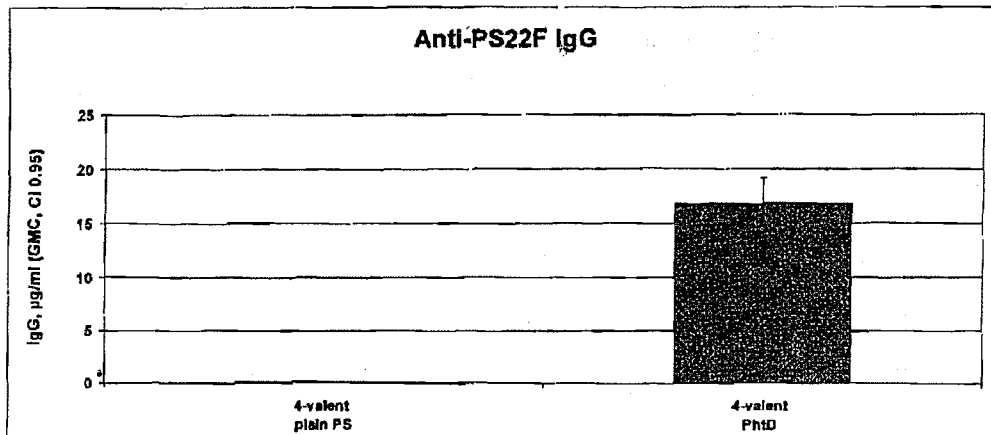
**Figure 1, Conjugate immunogenicity in elderly Rhesus monkeys (post-II anti-PS IgG levels)**



**Figure 2, Conjugate immunogenicity in elderly Rhesus monkeys (post-II anti-PS3 memory B cell frequencies)**



**Figure 3, PS19F immunogenicity in Balb/c mice (post-III IgG levels)**



**Figure 4, PS22F immunogenicity in Balb/c mice (post-III IgG levels)**

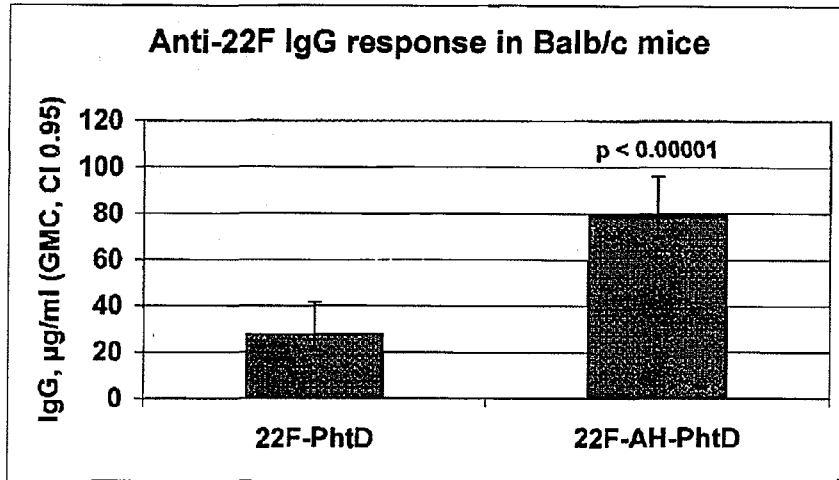


Figure 5, serum anti-PS IgG antibody levels

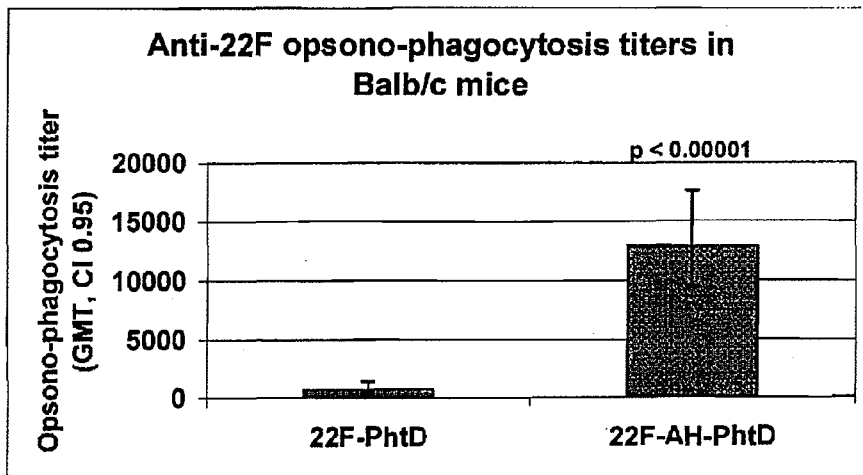


Figure 6, opsono-phagocytosis titres

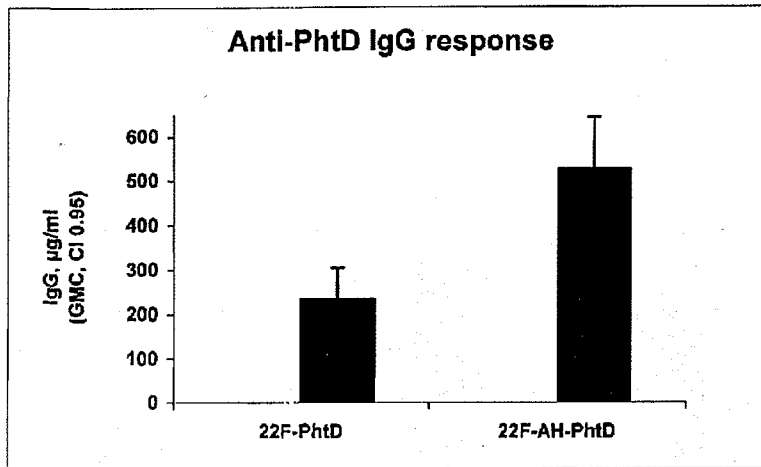


Figure 9 serum anti-PhtD IgG response

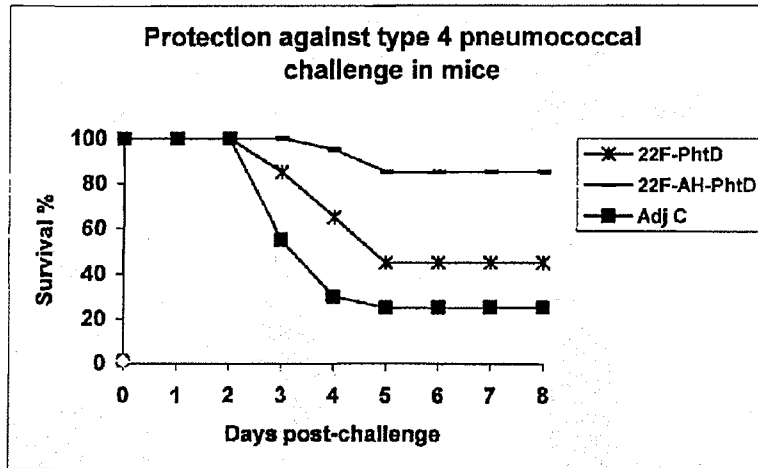


Figure 10  
Protection against type 4 pneumococcal challenge in mice

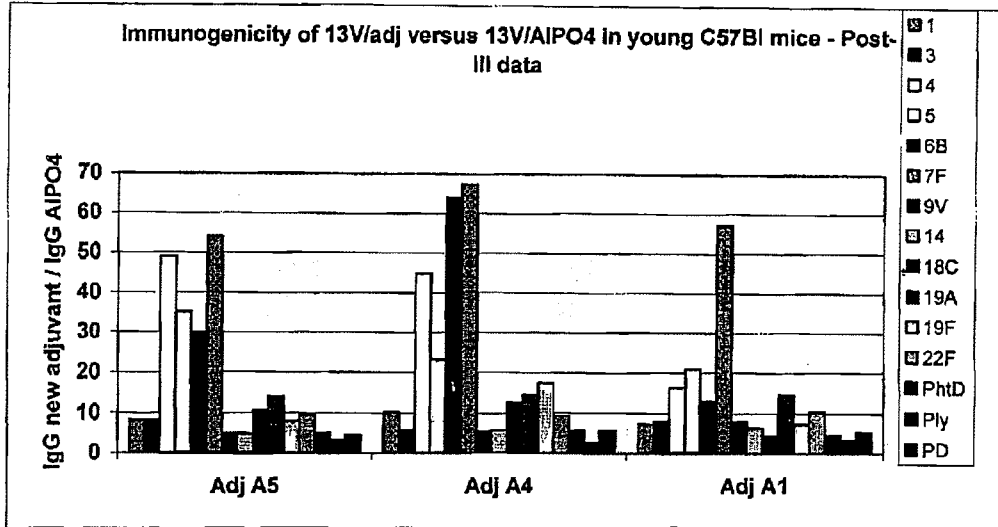


Figure 7, comparison of IgG responses induced with new adjuvants to the response elicited with AIPO<sub>4</sub>

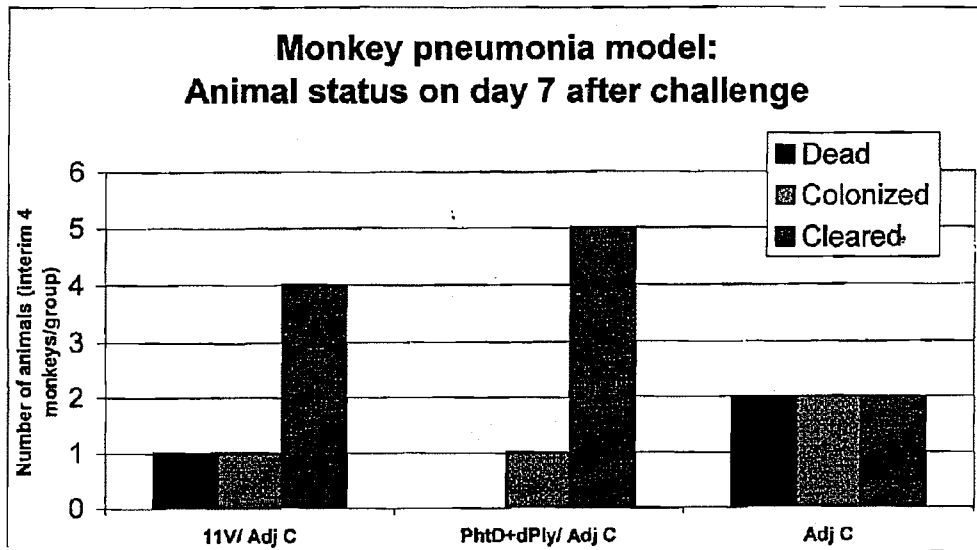


Figure 8, protective efficacy of PhtD + dPly protein combo against type 19F lung colonization in Rhesus monkeys



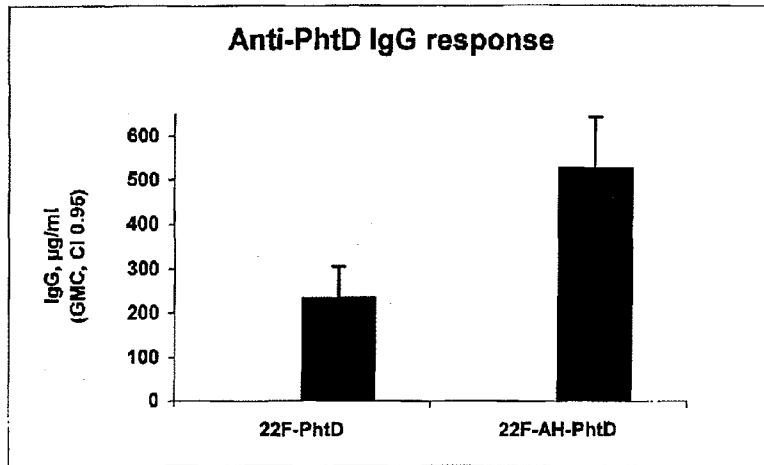


Figure 9 serum anti-PhtD IgG response

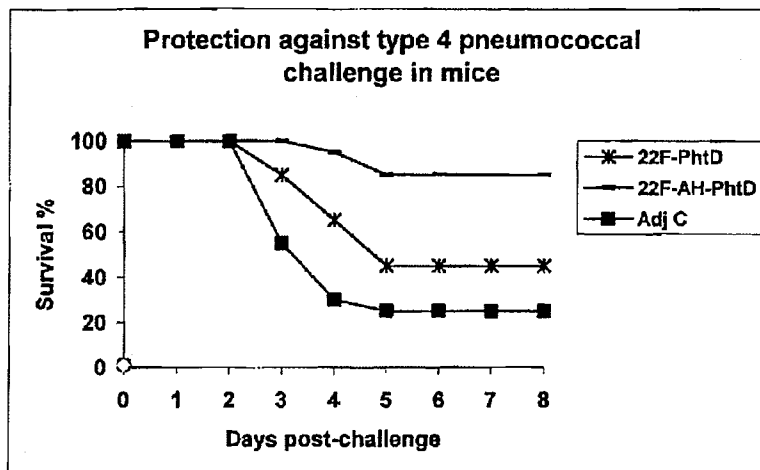



Figure 10  
Protection against type 4 pneumococcal challenge in mice

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Pediatr Infect Dis J. 2006 Apr;25(4):301-5.

# **Streptococcus pneumoniae serogroups 15 and 33: an increasing cause of pneumococcal infections in children in the United States after the introduction of the pneumococcal 7-valent conjugate vaccine.**

Gonzalez BE<sup>1</sup>, Hulten KG, Lamberth L, Kaplan SL, Mason EO Jr; U.S. Pediatric Multicenter Pneumococcal Surveillance Group.

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## **Abstract**

### **BACKGROUND:**

After the widespread use of the 7-valent pneumococcal conjugate vaccine, replacement serotypes have emerged. Serogroups 15 and 33 have emerged as nonvaccine serotypes causing invasive disease. We describe the clinical characteristics of children with infections caused by these serogroups and determined the genetic relationship of the strains.

### **MATERIALS AND METHODS:**

The United States Pediatric Multicenter Pneumococcal Surveillance Group has prospectively identified children with pneumococcal infections since 1993. Charts were reviewed retrospectively, isolates were serogrouped and serotyped and randomly selected strains were fingerprinted with the use of pulsed field gel electrophoresis. Selected strains were further characterized by multilocus sequence typing.

## RESULTS:

Between January 1994 and December 2004, 103 children had pneumococcal disease caused by serogroup 15, and 40 children had infections caused by serogroup 33. There was an increase from a mean of 7 cases per year for serogroup 15 in the prevaccine period to 14 cases per year in the postvaccine period and from 2 cases per year for serogroup 33 to 7 cases per year in the same periods. Isolates were susceptible to penicillin and ceftriaxone in both periods. A predominant clone was found in each serogroup representing 60% (30 of 50) of serogroup 15 strains and 83% (24 of 29) of the serotype 33F strains. The serogroup 15 clone comprised strains of serotypes 15B and 15C, whereas the 33 clone contained only serotype 33F strains. One isolate from each of the 15 and 33 clones was characterized by multilocus sequence typing and were found to be ST199 and 100, respectively.

## CONCLUSIONS:

Pneumococcal disease in children caused by penicillin-susceptible clones of serogroups 15 and 33 is increasing in the United States. Clinicians should consider replacement serotypes when encountered with invasive pneumococcal disease in vaccinated children.

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## MeSH Terms, Substances

### MeSH Terms

- Adolescent
- Child
- Child, Preschool
- Cluster Analysis
- DNA Fingerprinting/methods
- Electrophoresis, Gel, Pulsed-Field
- Humans
- Infant
- Meningococcal Vaccines/administration & dosage\*
- Pneumococcal Infections/epidemiology\*
- Pneumococcal Infections/microbiology\*
- Pneumococcal Vaccines/administration & dosage\*
- Serotyping
- Streptococcus pneumoniae/classification\*
- Streptococcus pneumoniae/genetics
- Streptococcus pneumoniae/pathogenicity
- United States/epidemiology
- Vaccination

### Substances

- Meningococcal Vaccines
- Pneumococcal Vaccines
- heptavalent pneumococcal conjugate vaccine

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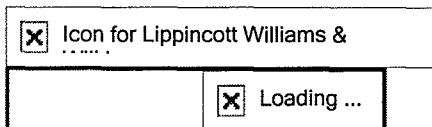
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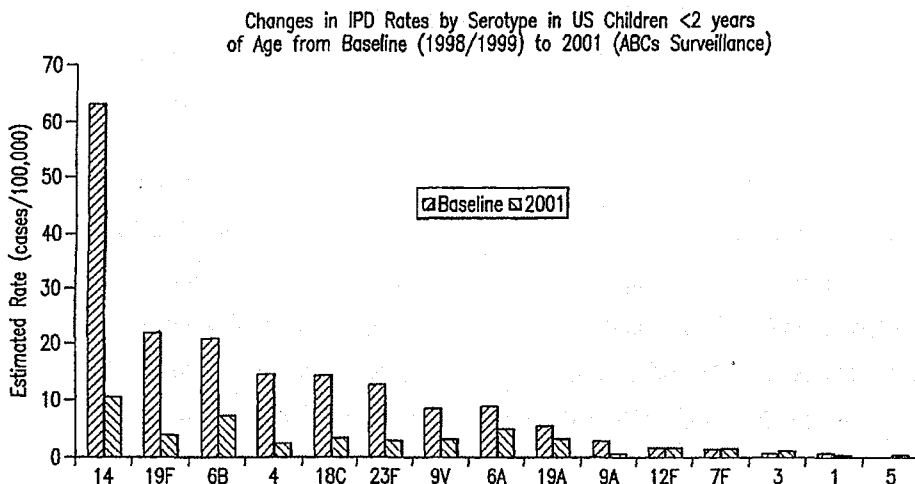
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(54) Title: MULTIVALENT PNEUMOCOCCAL POLYSACCHARIDE-PROTEIN CONJUGATE COMPOSITION

WO 2006/110381 A1



(57) Abstract: An immunogenic composition having 13 distinct polysaccharide-protein conjugates and optionally, an aluminum-based adjuvant, is described. Each conjugate contains a capsular polysaccharide prepared from a different serotype of Streptococcus pneumoniae (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) conjugated to a carrier protein. The immunogenic composition, formulated as a vaccine, increases coverage against pneumococcal disease in infants and young children globally, and provides coverage for serotypes 6A and 19A that is not dependent on the limitations of serogroup cross-protection.

## MULTIVALENT PNEUMOCOCCAL POLYSACCHARIDE-PROTEIN CONJUGATE COMPOSITION

### FIELD OF THE INVENTION

5           The present invention relates generally to the field of medicine, and specifically to microbiology, immunology, vaccines and the prevention of infection by a bacterial pathogen by immunization.

### BACKGROUND OF THE INVENTION

10           *Streptococcus pneumoniae* is a leading cause of meningitis, pneumonia, and severe invasive disease in infants and young children throughout the world. The multivalent pneumococcal polysaccharide vaccines have been licensed for many years and have proved valuable in preventing pneumococcal disease in elderly adults and high-risk patients. However, infants and young children respond poorly to most pneumococcal polysaccharides. The 7-valent pneumococcal conjugate vaccine (7vPnC, Prevnar®) was the first of its kind demonstrated to be highly immunogenic and effective against invasive disease and otitis media in infants and young children. This vaccine is now approved in many countries around the world. Prevnar contains the capsular polysaccharides from serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, each  
15           conjugated to a carrier protein designated CRM<sub>197</sub>. Prevnar covers approximately 80-90%, 60-80%, and 40-80% of invasive pneumococcal disease (IPD) in the US, Europe, and other regions of the world, respectively [1,2]. Surveillance data gathered in the years following Prevnar's introduction has clearly demonstrated a reduction of invasive pneumococcal disease in US infants as expected (FIG. 1) [3,4].

25           Surveillance of IPD conducted in US infants prior to the introduction of Prevnar demonstrated that a significant portion of disease due to serogroups 6 and 19 was due to the 6A (approximately one-third) and 19A (approximately one-fourth) serotypes [5,6]. Pneumococcal invasive disease surveillance conducted in the US after licensure of Prevnar suggests that a large burden of disease is still attributable  
30           to serotypes 6A and 19A (FIG. 1) [3]. Moreover, these two serotypes account for more cases of invasive disease than serotypes 1, 3, 5, and 7F combined (8.2 vs. 3.3 cases/100,000 children 2 years and under). In addition, serotypes 6A and 19A are

associated with high rates of antibiotic resistance (FIG. 2) [7,8,9]. While it is possible that serogroup cross-protection will result in a decline of serotype 6A and 19A disease as more children are immunized, there is evidence to suggest that there will be a limit to the decline, and a significant burden of disease due to these serotypes will remain (see below).

Given the relative burden and importance of invasive pneumococcal disease due to serotypes 1, 3, 5, 6A, 7F, and 19A, adding these serotypes to the Prevnar formulation would increase coverage for invasive disease to >90% in the US and Europe, and as high as 70%-80% in Asia and Latin America. This vaccine would significantly expand coverage beyond that of Prevnar, and provide coverage for 6A and 19A that is not dependent on the limitations of serogroup cross-protection.

#### SUMMARY OF THE INVENTION

Accordingly, the present invention provides generally a multivalent immunogenic composition comprising 13 distinct polysaccharide-protein conjugates, wherein each of the conjugates contains a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae* conjugated to a carrier protein, together with a physiologically acceptable vehicle. Optionally, an adjuvant, such as an aluminum-based adjuvant, is included in the formulation. More specifically, the present invention provides a 13-valent pneumococcal conjugate (13vPnC) composition comprising the seven serotypes in the 7vPnC vaccine (4, 6B, 9V, 14, 18C, 19F and 23F) plus six additional serotypes (1, 3, 5, 6A, 7F and 19A).

The present invention also provides a multivalent immunogenic composition, wherein the capsular polysaccharides are from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F of *Streptococcus pneumoniae* and the carrier protein is CRM<sub>197</sub>.

The present invention further provides a multivalent immunogenic composition, wherein the capsular polysaccharides are from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9v, 14, 18C, 19A, 19F and 23F of *Streptococcus pneumoniae*, the carrier protein is CRM<sub>197</sub>, and the adjuvant is an aluminum-based adjuvant, such as

aluminum phosphate, aluminum sulfate and aluminum hydroxide. In a particular embodiment of the invention, the adjuvant is aluminum phosphate.

5 The present invention also provides a multivalent immunogenic composition, comprising polysaccharide-protein conjugates together with a physiologically acceptable vehicle, wherein each of the conjugates comprises a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae* conjugated to a carrier protein, and the capsular polysaccharides are prepared from serotype 3 and at least one additional serotype.

10

In one embodiment of this multivalent immunogenic composition, the additional serotype is selected from the group consisting of serotypes 1, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F. In another embodiment, the carrier protein is CRM<sub>197</sub>. In yet another embodiment, the composition comprises an adjuvant, such as an aluminum-based adjuvant selected from aluminum phosphate, aluminum sulfate and aluminum hydroxide. In a particular embodiment, the adjuvant is aluminum phosphate.

15

20 The present invention also provides a multivalent immunogenic composition, comprising polysaccharide-protein conjugates together with a physiologically acceptable vehicle, wherein each of the conjugates comprises a capsular polysaccharide from a different serotype of *Streptococcus pneumoniae* conjugated to a carrier protein, and the capsular polysaccharides are prepared from serotypes 4, 6B, 9V, 14, 18C, 19F, 23F and at least one additional serotype.

25

In one embodiment of this multivalent immunogenic composition, the additional serotype is selected from the group consisting of serotypes 1, 3, 5, 6A, 7F, and 19A. In another embodiment, the carrier protein is CRM<sub>197</sub>. In yet another embodiment, the composition comprises an adjuvant, such as an aluminum-based adjuvant selected from aluminum phosphate, aluminum sulfate and aluminum hydroxide. In a particular embodiment, the adjuvant is aluminum phosphate.

30

The present invention also provides a method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide conjugate, comprising administering to a human an immunologically effective amount of any of the immunogenic compositions just described.

5

The present invention further provides that any of the immunogenic compositions administered is a single 0.5 mL dose formulated to contain: 2 µg of each saccharide, except for 6B at 4 µg; approximately 29 µg CRM<sub>197</sub> carrier protein; 0.125 mg of elemental aluminum (0.5 mg aluminum phosphate) adjuvant; and sodium chloride and sodium succinate buffer as excipients.

10

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 depicts the changes in IPD rates by serotype in US children <2 years of age from baseline (1998/1999) to 2001.

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FIG. 2 depicts the distribution of pneumococcal isolates with resistance to penicillin (PCN) in children <5 years of age (1998).

FIG. 3 depicts the reverse cumulative distribution curves (RCDC) of OPA post-third dose results from the D118-P16 Prevnar trial.

20

#### DETAILED DESCRIPTION OF THE INVENTION

##### ***Inclusion of Prevnar Serotypes 4, 6B, 9V, 14, 18C, 19F, 23F***

Data from IPD surveillance between 1995-1998 estimated that the seven serotypes in Prevnar were responsible for around 82% of IPD in children <2 years of age [5]. In Northern California, the site of the efficacy trial, the Prevnar serotypes accounted for 90% of all cases of IPD in infants and young children [10]. Since introduction of the Prevnar vaccine in 2000, there has been a significant decrease in the overall IPD rates due to a decrease in disease due to the vaccine serotypes [3,4]. Therefore, there is no justification at this time to remove any of the Prevnar serotypes from the next generation of pneumococcal conjugate vaccines but rather to add serotypes to obtain wider coverage.

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***Inclusion of Serotypes 1, 3, 5 and 7F***

In the US, the rate of IPD caused by serotype 1 in children under the age of 5 years is <2%, about the same as for each of types 3 and 7F [1,6]. Serotypes 1 and 5 account for higher rates of IPD in US populations at high risk for invasive pneumococcal disease. Specifically, serotype 1 causes 3.5% of IPD in Alaskan native children <2 years of age, and 18% in children 2-4 years of age [11]. Both serotype 1 and serotype 5 significantly cause disease in other parts of the world and in indigenous populations in developed countries [12,13,14].

Serotype 1 may also be associated with more severe disease as compared with other pneumococcal serotypes [15]. This observation is based on the difference in rates of case identification between the US and Europe, and the associated difference in medical practice. Overall, the incidence of IPD is lower in Europe than in the US. However, the percent of IPD caused by serotype 1 in Europe is disproportionately higher than in the US (6-7%, vs. 1-2%, respectively). In Europe, blood cultures are obtained predominantly from hospitalized children. In the US, it is routine medical practice to obtain blood cultures in an outpatient setting from children presenting with fever  $\geq 39^{\circ}\text{C}$  and elevated white blood cell counts. Given the difference in medical practice, it is postulated that the lower percent of disease caused by serotype 1 in the US may be diluted by higher rates of other serotypes causing milder disease, while the higher percent in Europe reflects more serious disease. In addition, seroepidemiology studies of children with complicated pneumonia demonstrate that serotype 1 is disproportionately represented [16,17,18]. This suggests that inclusion of serotype 1 may reduce the amount of severe pneumococcal disease, as well as, contribute to a total reduction in invasive pneumococcal disease.

The addition of serotypes 3 and 7F will increase coverage against IPD in most areas of the world by approximately 3%-7%, and in Asia by around 9%. Thus, an 11-valent vaccine would cover 50% in Asia and around 80% of IPD in all other regions [1,2]. These serotypes are also important with respect to otitis media coverage [19]. In a multinational study of pneumococcal serotypes causing otitis media, Hausdorff et al found serotype 3 to be the 8th most common middle ear fluid



isolate overall [20]. Serotype 3 accounted for up to 8.7% of pneumococcal serotypes associated with otitis media. Thus, the importance of types 3 and 7F in otitis media, as well as in IPD, warrants their inclusion in a pneumococcal conjugate vaccine.

5           However, attempts to produce a multivalent pneumococcal conjugate vaccine that exhibits significant immunogenicity with respect to serotype 3 polysaccharides have been unsuccessful. For example, in a study of the immunogenicity and safety of an 11-valent pneumococcal protein D conjugate vaccine (11-Pn-PD), no priming effect was observed for serotype 3 in infants who had received three doses of the  
10 vaccine followed by a booster dose of either the same vaccine or a pneumococcal polysaccharide vaccine (Nurkka *et al.* (2004) *Ped. Inf. Dis. J.*, 23:1008-1014). In another study, opsonophagocytic assay (OPA) results from infants who had received doses of 11-Pn-PD failed to show antibody responses for serotype 3 at levels comparable to other tested serotypes (Gatchalian *et al.*, 17<sup>th</sup> Annual Meeting of the  
15 Eur. Soc. Paed. Inf. Dis. (ESPID), Poster No. 4, P1A Poster Session 1, Istanbul Turkey, Mar. 27, 2001). In yet another study, which assessed the efficacy of an 11-Pn-PD in the prevention of acute otitis media, the vaccine did not provide protection against episodes caused by serotype 3 (Prymula *et al.* [www.thelancet.com](http://www.thelancet.com), Vol. 367: 740-748 (March 4, 2006)). Accordingly, a pneumococcal conjugate vaccine  
20 comprising capsular polysaccharides from serotype 3 and capable of eliciting an immunogenic response to serotype 3 polysaccharides provides a significant improvement over the existing state of the art.

#### **Inclusion of Serotypes 6A and 19A**

##### 25 a. *Epidemiology of Serotypes 6A and 19A*

Surveillance data in the literature suggest that serotypes 6A and 19A account for more invasive pneumococcal disease in US children <2 years of age than serotypes 1, 3, 5, and 7F combined (FIG. 1) [1,5]. In addition, these serotypes are commonly associated with antibiotic resistance (FIG. 2) and play an important role in  
30 otitis media [6,19,20]. The ability of the current Prevnar vaccine to protect against disease due to 6A and 19A is not clear. The rationale for inclusion of 6A and 19A components in a 13vPnC vaccine is discussed below.

b. *Responses to 6A and 19A Induced by 6B and 19F Polysaccharides*

The licensed unconjugated pneumococcal polysaccharide vaccines (for use in persons at least two years of age) have contained 6A or 6B capsular polysaccharide but not both [21]. Immunogenicity data generated at the time of formulation of the 23-valent pneumococcal polysaccharide vaccine demonstrated that a 6B monovalent vaccine induced antibody to both the 6A and 6B capsules. The data from several trials assessing IgG and opsonophagocytic assay (OPA) responses in a variety of populations with free polysaccharide and with pneumococcal conjugate vaccines suggested that IgG responses to 6A are induced by 6B antigens, but the responses are generally lower, and the OPA activity with 6A organisms is different than with 6B organisms [22,23,24,25]. In addition, subjects responding with high 6B antibody may have little or no activity against 6A.

In contrast to the chemical composition of the 6A and 6B capsular polysaccharides where there exists a high degree of similarity, the 19A and 19F capsules are quite different due to the presence of two additional side chains in the 19A polysaccharide. Not surprisingly, immune responses measured in human volunteers immunized with 19F polysaccharide vaccine showed that responses to 19F were induced in 80% of subjects, but only 20% of subjects had a response to 19A [26]. Low levels of cross-reactive IgG and OPA responses to serotype 19A after immunization with 19F polysaccharide have also been documented in trials with conjugate vaccines as well [24,26].

Internal data on cross-reactive OPA responses to 6A and 19A have been generated from the 7vPnC bridging trial (D118-P16) conducted in US infants (FIG. 3). These studies are consistent with the findings of others, and demonstrate induction of cross-reactive functional antibody to 6A polysaccharide after immunization with 6B polysaccharide, although at a lower level, and very little functional antibody to 19A after immunization with 19F.

***Impact of 6B and 19F Immunization on 6A and 19A in Animal Models***

Animal models have been used to evaluate the potential for cross-protection with polysaccharide immunization. In an otitis media model developed by Giebink et al., chinchillas were immunized with a tetravalent polysaccharide outer membrane protein (OMP) conjugate vaccine (containing 6B, 14, 19F, 23F saccharides) or placebo [27]. In this trial there appeared to be some cross-protection for 6A; however this did not reach statistical significance and the level of protection was lower than with 6B against otitis media. In this same model there was 100% protection against 19F otitis media, but only 17% protection against 19A otitis media.

Saeland et al. used sera from infants immunized with an 8-valent pneumococcal tetanus conjugate vaccine (containing 6B and 19F) to passively immunize mice prior to an intranasal challenge with 6A organisms, in a lung infection model [28]. Of the 59 serum samples, 53% protected mice against bacteremia with 6B and 37% protected against 6A. Mice passively immunized with sera from infants immunized with four doses of an 11-valent pneumococcal conjugate vaccine (containing 19F conjugated to tetanus toxoid) were given an intranasal challenge with 19A organisms in the same model [29]. Of 100 mice passively immunized and then challenged, 60 mice had no 19A organisms detected in lung tissue, whereas organisms were identified in all mice given saline placebo. However, passive immunization did not protect against challenge with 19F organisms in this model; therefore, the relevance of the model for serogroup 19 is questionable. In general these models provide evidence of some biological impact of 6B immunization on 6A organisms although the effect on the heterologous serotype was not as great as that observed with the homologous serotype. The impact of 19F immunization on 19A organisms is not well understood from these models.

***Impact of 6B and 19F Polysaccharide Conjugate Immunization on 6A and 19A Disease in Efficacy/Effectiveness Trials***

The number of cases of disease due to the 6B, 6A, 19F and 19A serotypes in 7vPnC and 9vPnC (7vPnC plus serotypes 1 and 5) efficacy trials is noted in Table 1 [30,10,31]. The numbers of invasive disease cases are too small to allow any conclusions to be drawn for serotypes 6A and 19A. However, the Finnish otitis

media trial generated a large number of pneumococcal isolates [32]. In the per protocol analysis 7vPnC was 84% (95% CI 62%, 93%) efficacious against otitis media due to serotype 6B and 57% (95% CI 24%, 76%) efficacious against otitis media due to serotype 6A (Table 1). In contrast, serotype-specific efficacy with the  
 5 7vPnC was not demonstrated for otitis media due to either 19F or 19A.

**Table 1. Cases of Pneumococcal Disease Due to Serotypes 6B, 6A, 19F, and 19A in Efficacy Trials with the 7vPnC and 9vPnC Vaccines**

	6B		6A		19F		19A	
	PnC	Contr.	PnC	Contr.	PnC	Contr.	PnC	Contr.
Kaiser Efficacy Trial – 7vPnC (ITT)	1	7	0	1	2*	13	0	1
Navajo Efficacy Trial – 7vPnC (ITT)	0	5	1	0	1	1	1	0
South African Efficacy Trial – 9vPnC HIV (-) (ITT)	1	2	1	0	0	1	3	1
South African Efficacy Trial – 9vPnC HIV (+) (ITT)	1	7	3	10	2	3	2	3
Finnish Otitis Media Trial – 7vPnC (PP)	9*	56	19*	45	43	58	17	26

\*Statistically significant efficacy demonstrated

From references 30, 10 and 33, and personal communications

Contr = control

ITT = Intention to treat analysis

PP = per protocol analysis

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Post-marketing IPD surveillance data is also available from a case-control trial conducted by the Centers for Disease Control to evaluate the effectiveness of Prevnar [33]. Cases of pneumococcal invasive disease occurring in children 3 to 23 months of age were identified in the surveillance laboratories and matched with three  
 20 control cases by age and zip code. After obtaining consent, medical and immunization history (subjects were considered immunized if they had received at least one dose of Prevnar) was obtained from parents and medical providers for cases and controls. The preliminary results were presented at the 2003 ICAAC meeting and a summary of the findings for 6B, 19F, 19A and 6A disease is presented  
 25 in Table 2. These data indicate that Prevnar is able to prevent disease due to 6A, although at a level that may be somewhat lower than serotype 6B disease. These data also indicate that the cross-protection for invasive disease due to 19A is limited.

**Table 2. Preliminary results of a Case Control Trial Performed by the CDC (presented at ICAAC, 2003)**

Serotype	Informative Sets, n	VE* (95% CI)
Vaccine Type, All	115	94 (87, 97)
Vaccine Related, All	36	70 (38, 86)
Non-Vaccine Type, All	43	-4 (-106, 48)
6B	27	94 (72, 99)
19F	19	73 (16, 92)
6A	15	87 (53, 97)
19A	16	40 (-87, 80)

\*Vaccine effectiveness comparing vaccinated ( $\geq 1$  dose) vs. unvaccinated, and adjusted for underlying conditions  
Reference 40 and personal/confidential communication

5

A published analysis [3] of the use of Prevnar also indicated that serotypes 6B and 19F conferred a moderate reduction in IPD caused by serotypes 6A and 19A among children under two years of age (Table 1 in [3]). Disease rates among unimmunized adults caused by serotypes 6A, 9A, 9L, 9N, 18A, 18B, 18F, 19A, 19B, 19C, 23A and 23B ("all vaccine-related serotypes") were somewhat reduced (Table 2 in [3]). These data establish that herd immunity from the use of Prevnar in children under two years of age was modest for serotypes 6A and 19A, and provide a basis for the inclusion of serotypes 6A and 19A in the 13vPnC vaccine of this invention.

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#### **Conclusion for addition of 6A and 19A**

The post-marketing surveillance data and the case-control study results noted in FIG. 1 and Table 2 with the 7vPnC vaccine suggest that, consistent with the other information on immune responses and performance in the animals models described above, there may be some cross-protection against 6A disease, but to a lesser extent than to 6B disease. Furthermore, it appears the protection against 19A is limited. Therefore, a 13vPnC vaccine containing serotypes 6A and 19A provides coverage that is not dependent on the limitations of serogroup cross-protection by serotypes 6B and 19F.

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Accordingly, the present invention provides a multivalent immunogenic composition comprising 13 distinct polysaccharide-protein conjugates, wherein each of the conjugates contains a different capsular polysaccharide conjugated to a carrier protein, and wherein the capsular polysaccharides are prepared from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F of *Streptococcus pneumoniae*, together with a physiologically acceptable vehicle. One such carrier protein is the diphtheria toxoid designated CRM<sub>197</sub>. The immunogenic composition may further comprise an adjuvant, such as an aluminum-based adjuvant, such as aluminum phosphate, aluminum sulfate and aluminum hydroxide.

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Capsular polysaccharides are prepared by standard techniques known to those skilled in the art. In the present invention, capsular polysaccharides are prepared from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F of *Streptococcus pneumoniae*. These pneumococcal conjugates are prepared by separate processes and formulated into a single dosage formulation. For example, in one embodiment, each pneumococcal polysaccharide serotype is grown in a soy-based medium. The individual polysaccharides are then purified through centrifugation, precipitation, ultra-filtration, and column chromatography. The purified polysaccharides are chemically activated to make the saccharides capable of reacting with the carrier protein.

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Once activated, each capsular polysaccharide is separately conjugated to a carrier protein to form a glycoconjugate. In one embodiment, each capsular polysaccharide is conjugated to the same carrier protein. In this embodiment, the conjugation is effected by reductive amination.

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The chemical activation of the polysaccharides and subsequent conjugation to the carrier protein are achieved by conventional means. See, for example, U.S. Pat. Nos. 4,673,574 and 4,902,506 [34,35].

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Carrier proteins are preferably proteins that are non-toxic and non-reactogenic and obtainable in sufficient amount and purity. Carrier proteins should be

amenable to standard conjugation procedures. In a particular embodiment of the present invention, CRM<sub>197</sub> is used as the carrier protein.

CRM<sub>197</sub> (Wyeth, Sanford, NC) is a non-toxic variant (i.e., toxoid) of diphtheria toxin isolated from cultures of *Corynebacterium diphtheria* strain C7 (β197) grown in casamino acids and yeast extract-based medium. CRM<sub>197</sub> is purified through ultra-filtration, ammonium sulfate precipitation, and ion-exchange chromatography. Alternatively, CRM<sub>197</sub> is prepared recombinantly in accordance with U.S. Patent No. 5,614,382, which is hereby incorporated by reference. Other diphtheria toxoids are also suitable for use as carrier proteins.

Other suitable carrier proteins include inactivated bacterial toxins such as tetanus toxoid, pertussis toxoid, cholera toxoid (e.g., as described in International Patent Application WO2004/083251 [38]), *E. coli* LT, *E. coli* ST, and exotoxin A from *Pseudomonas aeruginosa*. Bacterial outer membrane proteins such as outer membrane complex c (OMPC), porins, transferrin binding proteins, pneumolysin, pneumococcal surface protein A (PspA), pneumococcal adhesin protein (PsaA), C5a peptidase from Group A or Group B streptococcus, or *Haemophilus influenzae* protein D, can also be used. Other proteins, such as ovalbumin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or purified protein derivative of tuberculin (PPD) can also be used as carrier proteins.

After conjugation of the capsular polysaccharide to the carrier protein, the polysaccharide-protein conjugates are purified (enriched with respect to the amount of polysaccharide-protein conjugate) by a variety of techniques. These techniques include concentration/diafiltration operations, precipitation/elution, column chromatography, and depth filtration. See examples below.

After the individual glycoconjugates are purified, they are compounded to formulate the immunogenic composition of the present invention, which can be used as a vaccine. Formulation of the immunogenic composition of the present invention can be accomplished using art-recognized methods. For instance, the 13 individual pneumococcal conjugates can be formulated with a physiologically acceptable

vehicle to prepare the composition. Examples of such vehicles include, but are not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions.

5 In certain embodiments, the immunogenic composition will comprise one or more adjuvants. As defined herein, an "adjuvant" is a substance that serves to enhance the immunogenicity of an immunogenic composition of this invention. Thus, adjuvants are often given to boost the immune response and are well known to the skilled artisan. Suitable adjuvants to enhance effectiveness of the composition  
10 include, but are not limited to:

(1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.;

(2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (defined below) or bacterial cell  
15 wall components), such as, for example,

(a) MF59 (PCT Publ. No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below, although not required)) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA),

20 (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and

(c) Ribi™ adjuvant system (RAS), (Corixa, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the  
25 group consisting of 3-O-deacylated monophosphorylipid A (MPL™) described in U.S. Patent No. 4,912,094 (Corixa), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (Detox™);

(3) saponin adjuvants, such as Quil A or STIMULON™ QS-21 (Antigenics, Framingham, MA) (U.S. Patent No. 5,057,540) may be used or particles generated  
30 therefrom such as ISCOMs (immunostimulating complexes);

(4) bacterial lipopolysaccharides, synthetic lipid A analogs such as aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa, and which are described in U.S. Patent No. 6,113,918; one