Characterization of carbohydrate based vaccines

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Characterization of carbohydrate based vaccines

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CHARACTERIZATION OF CARBOHYDRATE BASED VACCINES

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1 Introduction

1.1 Brief history of vaccination

Infectious diseases have been an important cause of mortality throughout the history of mankind and have always played a key role in regulating the numerousness of humans. Vaccination is considered one of the most powerful means to save lives and to increase the level of health of humans. [1]

Vaccination is the active immunization with an immunogen (the vaccine) administered so that the host develops specific antibodies and B and T memory cells that can act against the natural immunogen. These cells stand ready to be activated should the host later be exposed to the pathogen bearing the natural immunogen (Figure 1).
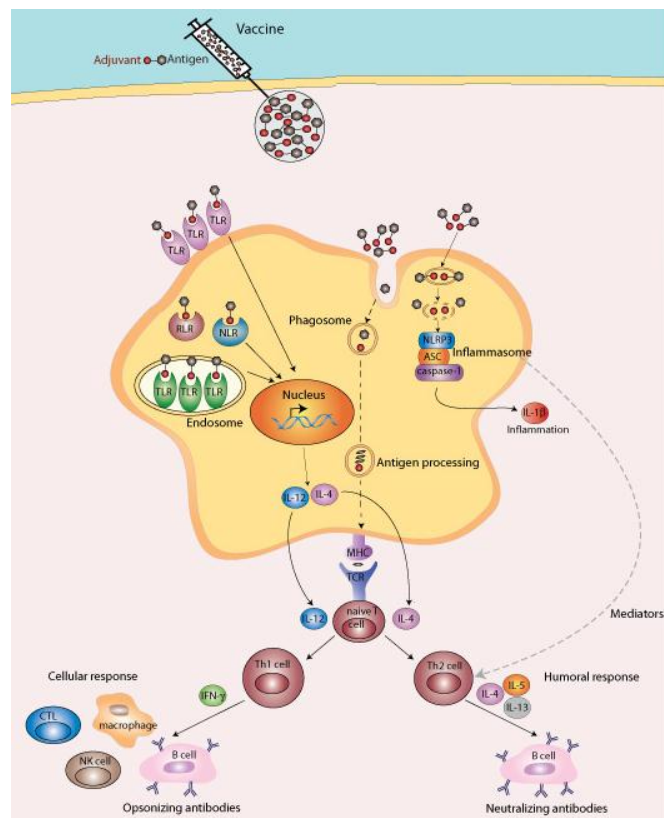
Figure 1: Active immunization after vaccination. Vaccination induces activation by cytokines, B cells differentiate into memory B cells (long-lived antigen-specific B cells) or plasma cells (effector B cells that secrete large quantities of antibodies). Most antigens activate B cells using activated T helper (Th) cells, primarily Th1 and Th2 cells. The Th1 response leads mainly to a cell-mediated immunity (cellular response) instead Th2 cells generally induce a humoral response (antibody)[2].

Vaccines are prophylactic in the sense that they are administered to healthy individuals to prevent a disease. Nevertheless, there is a growing trend to use vaccines to alleviate the suffering of those

already with disease. Preventive vaccines carry immunologic specificity for individual infective disease agents and provoke an immune response against them. Prophylactic vaccines represent the possibility to prevent global epidemics of infectious diseases. Historically vaccination was born with the work of Edward Jenner when, in 1796, he inoculated an eight years old boy, the son of his gardener, with pus scraped from cowpox blisters. The young boy was then protected from smallpox when he was challenged with variolous material. After subsequent experimentation vaccination was eventually accepted by British government and offered to the population in 1840 free of charge replacing variolation, an older practice, introduced in England by Lady Mary Wortley Montagu on her return from Costantinople, based on immunization of patients by infecting them with substance from the pustules of patients with a mild form of the smallpox. The worldwide implementation of vaccination campaigns against smallpox allowed the World Health Organization (WHO) to declare its eradication in 1980. During the 1900s many successes and discoveries have been registered on vaccine development and the effectiveness of vaccination has been proved on human health. Figure 2 shows how the incidence of serogroup C meningococcal disease decreased after the introduction of the glycoconjugate vaccine.
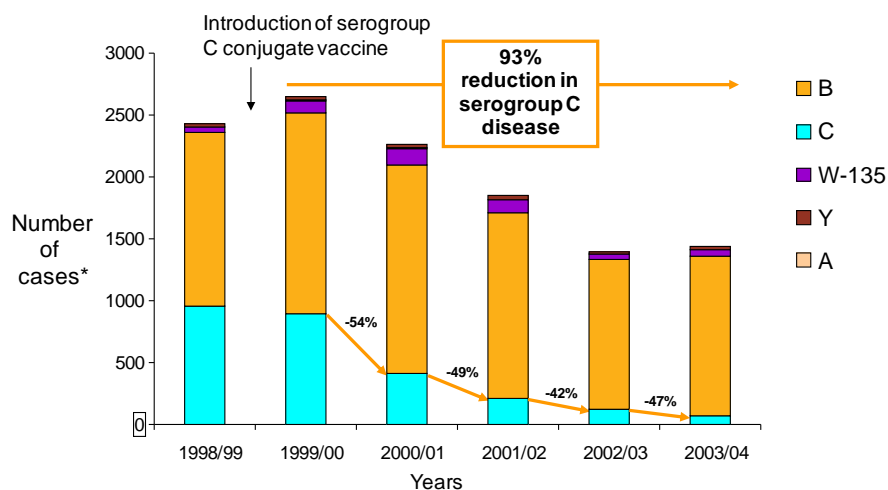
Figure 2: Example of the Effectiveness of Vaccine Coverage. The graph shows how disease incidence, in this case serogroup C meningococcal disease, can fall dramatically with increasing vaccine coverage.*Laboratory-confirmed cases. [3]

As reported in Table 1, different types of approaches have been studied so far for vaccine development and some of them became a reality such as: traditional whole pathogens live attenuated (polio oral vaccine), killed or inactivated (inactivate polio, inactivated whole virus influenza), toxins isolated from the pathogen and subsequently inactivated (diphtheria and tetanus vaccines), other subunit vaccines such as proteins produced by recombinant DNA technologies or from genetically detoxified microorganisms and bacterial polysaccharides (CPS) and glycoconjugates .

Type of vaccine	Description
Live, attenuated	Whole pathogen (polio)
Killed, inactivated	Whole pathogen killed (inactivated polio, influenza whole virus)
Toxoid	Formalin inactivated toxin of pathogen (tetanus and diphtheria)
Proteins, peptide	Proteins derived from recombinant DNA technologies or from genetically detoxified microorganisms (Hepatitis B, Pertussis)
Polysaccharide	Natural capsular polysaccharide (CPS) purified from bacterial pathogens
Glycoconjugate	Carbohydrate antigen covalently bounded to a protein carrier (serogroup C meningococcus)

Table 1: Types of vaccines.

1.2 Polysaccharide Vaccines

Polysaccharides are important virulence factor especially for encapsulated bacteria that present on their surface complex carbohydrate structures. Surface polysaccharides have several functions: they protect microorganisms from desiccation when they are exposed to the external environment, in some cases, for example the hyaluronic capsule of group A Streptococcus, their adhesive properties help the pathogen to invade the host; other capsular polysaccharides prevent the activation of the alternate complement pathway; sometimes they mimic molecules produced by human cells so that the pathogen is not recognized as foreign by our immune system (serogroup B meningococcal capsular polysaccharide, hyaluronic acid). [4]

Around 1930s the protective role of antibodies (Abs) induced by pneumococcal polysaccharide started to be investigated and in 1945 the first vaccine composed by purified polysaccharide from selected pneumococcal serotypes was tested in man.[5] [6]

The research on vaccines development was subsequently slowed down by the introduction of antibiotics, however with the emergence of drug resistant strains the development of polysaccharide vaccines started again and a number of them have been studied in large clinical studies.[7] [8] Polysaccharide vaccines against meningococcus serogroup ACWY, *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib) were licensed between the seventies and eighties.

Polysaccharide vaccines however did not completely solve the problem of bacterial diseases caused by encapsulated microorganisms. Bacterial polysaccharides are made by repeated units constituted of monosaccharides linked together by glycosidic linkages, and one of their main feature emerged from clinical trials, is that they are poorly immunogenic in children less than two years of age.[9] [10] [11]

In addition, the antibody response to bacterial polysaccharides is weakly affected by adjuvants, IgM represents the major class of antibodies induced and since their immune response does not induce memory, it is not boosted by subsequent immunizations.

These characteristics are due to the fact that, unlike proteins that are T-cell dependent (TD) antigens, polysaccharides are T-cell independent (TI) antigens. Polysaccharide antigens directly activate polysaccharide-specific B cells which differentiate then into plasma cells to produce antibodies, but memory B cells are not formed.

Proteins instead are TD antigens; following interaction with antigen-presenting cells (APC) like dendritic cells, macrophages and B-cells, protein antigens are internalized and processed into small peptides which are then re-exposed and presented to T lymphocytes in association with the major histocompatibility complex (MHC) class II molecules. Interaction with T cells induces B cells to differentiate into plasma cells and memory B cells. Unlike TI antigens, TD antigens are immunogenic early in infancy, the immune response induced can be boosted, enhanced by

adjuvants, and is characterized by antibody class switch and production of antigen-specific IgG.[11] [12] [13] [14]

1.3 Glycoconjugate vaccines

The limitation of polysaccharides vaccines has been overcome by covalent conjugation to a carrier protein as source of T-cell epitopes. Since 1929 Avery and Goebel have demonstrated that non-immunogenic sugars after conjugation to a carrier protein become able to induce antibodies in the animal model.[15]

However the first application of this concept to a vaccine for human use started only in 1980 with the development of the first conjugate vaccine against *Haemophilus influenzae* type b (Hib) that was later on licensed vaccine between 1987 and 1990.[16 17 18 19]

Many other glycoconjugate vaccines have been developed against bacterial pathogens such as *Neisseriae meningitidis*, *Streptococcus pneumoniae* and group B *Streptococcus*.[20 21 22 23 24 25 26 27]
Today glycoconjugate vaccines are among the safest and most efficacious vaccines developed during the last 30 years and they are currently used in the immunization schedules of different countries like for example the United States (US) as reported in Table 2.

Recommended Immunization Schedule for Persons Aged 0 Through 6 Years - United States 2011											
Vaccine ↓ Age →	Birth	1 month	2 months	4 months	6 months	12 months	15 months	18 months	19-23 months	2-3 years	4-6 years
Hepatitis B	HepB	HepB				HepB					
Rotavirus			RV	RV	RV						
Difteria, Tetanus, Pertussis			DTaP	DTaP	DTaP	DTaP					DTaP
Haemophilus influenzae type b			Hib	Hib	Hib	Hib					
Pneumococcal			PVC	PVC	PVC	PVC				PPSV	
Inactivated Poliovirus			IPV	IPV	IPV						IPV
Influenza					Influenza yearly						
Mealses, Mumps, Rubella						MMR					MMR
Varicella						Varicella					Varicella
Hepatitis A						HepaA (2 doses)				HepaA series	
Meningococcal										MCV4	
Recommended schedule for all children	Recommended schedule for high-risk groups										

Recommended Immunization Schedule for Persons Aged 7 Through 18 Years - United States 2011			
Vaccine ↓ Age →	7-10 years	11-12 years	15-18 years
Difteria, Tetanus, Pertussis		Tdap	Tdap
Human Papillomavirus		HPV	HPV
Meningococcal	MCV4	MCV4	MCV4
Influenza		Influenza yearly	
Pneumococcal		Pneumococcal	
Hepatitis A		HepA	
Hepatitis B		HepB	
Inactivated Poliovirus		IPV	
Mealses, Mumps, Rubella		MMR	
Varicella		Varicella	
Recommended schedule for all children	Recommended schedule for high-risk groups	Recommended schedule for catch-up immunization	

Table 2: Schedules of immunization currently used in US.[28]

Currently licensed glycoconjugate vaccines are reported on the Table 3.

Vaccine indication	Type of conjugate	Manufacturer
Haemophilus influenzae type b	PRP-TT	Sanofi-Pasteur
	PRP-OMPC	Merck
	PRP-CRM197	Pfizer
	Hib-CRM197	Novartis Vaccines
Haemophilus influenzae type b/*Neisseria meningitidis* group C	MenC/Hib-TT	GSK
Neisseria meningitidis serogroups ACW$_{135}$Y	MenA-TT A	Serum Institute India
	MenC-CRM197	Pfizer, Novartis Vaccines
	MenC-TT	Baxter
	MenACWY-DT	Sanofi-Pasteur
	MenACWY-CRM197	Novartis Vaccines
	MenACWY-TT	GSK
Streptococcus pneumoniae serotypes	7 valent-CRM197 (4, 6B, 9V, 14, 18C, 19F, 23F)	Pfizer
	13 valent-CRM197 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F)	Pfizer
	10 valent-DT/TT Protein D (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F)	GSK

Table 3: Licensed glycoconjugate vaccine.

Many studies have been performed to understand the mechanisms of internalization and processing of the glycoconjugate molecules by the immune system and many data have been published.[14 29 30 31 32 33]

Chemical conjugation of polysaccharides to protein carriers allows processing of the protein carrier by polysaccharide-specific B cells and presentation, on their surface, of the resulting peptides or glycopeptides in association with MHC class II. Further interaction with carrier-specific T cells then induces polysaccharide-specific B cells differentiation (Figure 4). As a consequence, a conjugate vaccine induces a T-cell-dependent response already early in life which leads to immunological memory and boosting of the response by further doses of the vaccine.
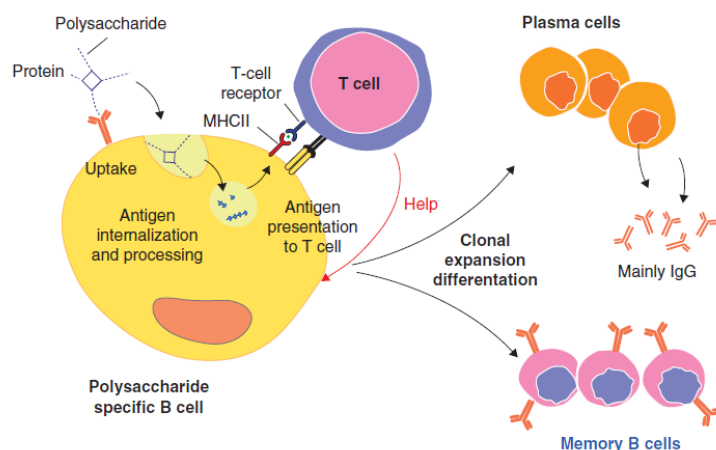
Figure 4: Schematic diagram of mechanism of action for conjugate vaccines.[34]

Recently a new working model for glycoconjugate vaccines has been proposed where T-cell populations that recognize carbohydrate epitopes derived by APC processing of conjugate vaccines and that, when presented by MHCII, these epitopes recruit T-cell help for the induction of adaptive immune responses to these vaccines.[35]

To prepare glycoconjugate vaccines, two main approaches based on different chemistry of conjugation, have been used so far. One is based on the random chemical activation of the saccharide chain followed by covalent binding with the protein carrier obtaining a cross-linked structure between the polysaccharide and the protein. A second approach is based on the generation by controlled fragmentation of the native polysaccharide, of appropriately sized oligosaccharides which are then activated at their terminal groups, usually with a linker molecule, and subsequently conjugated to the carrier protein obtaining a radial structure (Figure 5). Depending on the conjugation chemistry employed, a chemical spacer can be used in order to facilitate the coupling of the protein to the saccharide antigens and, in some cases, conjugation chemistry also requires prior derivatization of the protein carriers.
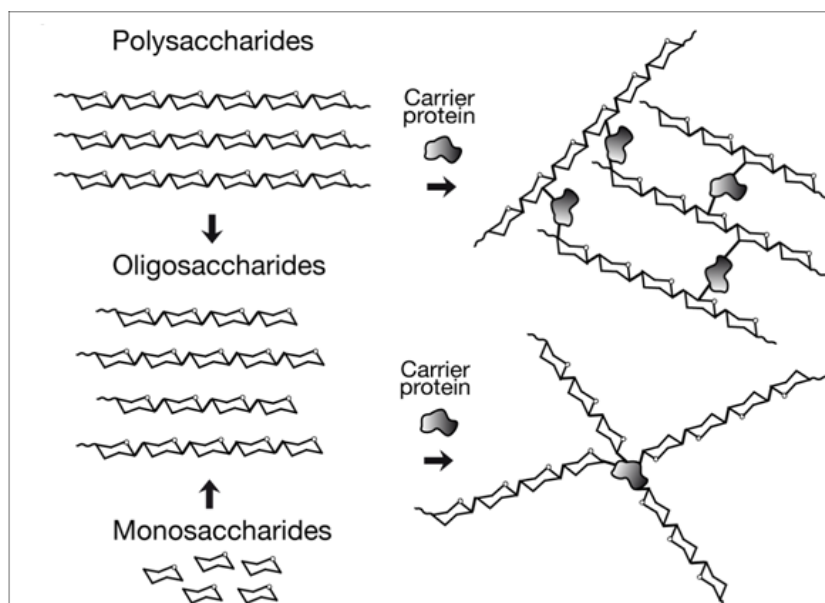
Figure 5: Structures of glycoconjugates.

The synthetic approach which, starting from suitable monosaccharides, can provide pure and well-defined oligosaccharides for coupling to protein carriers, is increasingly attractive.[36 37 38 39 40]

A new approach called bioconjugation is based on glyco-engineering the bacterial N-glycosylation pathway in bacteria such as *Escherichia coli*. The polysaccharide, encoded by the inserted genes, is produced on a polyisoprenoid carrier and then is transferred to an asparagine residue of the carrier protein which has to contain at least one (native or engineered) N-glycosylation site. [41 42 43 44]

A chemistry which favors the formation of cross-linked structures was used for Hib conjugate vaccines by Sanofi-Pasteur, Merck and Pfizer (Table 3).

Novartis Vaccine has instead developed a conjugation chemistry which results in conjugates having oligosaccharides oriented radially from the carrier protein.[34 45]

In particular Hib and meningococcal Novartis Vaccines conjugate vaccines are made starting from purified capsular polysaccharides which have been size reduced by acid hydrolysis and fractionated to select an intermediate chain length population. The oligosaccharides were then derivatized at their reducing termini introducing first a primary amino group by reductive amination followed by a 6-carbon atom flexible hydrocarbon spacer having a terminal active ester ready for conjugation.[20 23 46] The same technology was used by Novartis Vaccine to develop its quadrivalent A, C, W135, Y anti-meningococcal conjugate vaccine.[47]

1.4 Variables influencing the immunogenicity and physicochemical properties of glycoconjugate vaccines

Many aspects can influence the immunogenicity of conjugate vaccines and the main variables investigated so far are the size of the saccharide moiety, the saccharide: protein ratio in the purified conjugate, the conjugation strategy, the nature of the spacer and the protein carrier.

The size of the saccharide moiety and saccharide/protein ratio were investigated in different works such as Seppälä and Mäkelä in one of the first studies on the effect of size and chemistry on the immunogenicity of dextrans-protein conjugates found that dextrans of low molecular weight conjugated to chicken serum albumin, induced strong anti-dextran responses in mice, while increasing the dextrans' size resulted in reduced immunogenicity.[48] Peeters et al. showed that a synthetic tetramer of Hib capsular polysaccharide repeating unit, conjugated to a protein carrier, induced in adult mice and non-human primates antibody levels comparable to a commercial Hib conjugate and higher than those induced by a trimer, indicating that for Hib a minimum of eight sugars is needed for a proper immunological response.[49] Laferriere et al. found little influence of the carbohydrate chain length on the immunogenicity of pneumococcal conjugate vaccines in mice.[50] Pozsgay et al. studied the immunogenicity in mice of synthetic *Shigella dysenteriae* type 1 LPS oligosaccharides conjugated to human serum albumin (HSA). The authors found that octa-, dodeca-, and hexadecasaccharide fragments induced high levels of lipopolysaccharide binding IgG antibodies in mice after three injections and were superior to a tetrasaccharide conjugate. The influence of the carbohydrate/protein ratio was different for the three conjugates. The octasaccharide-HSA conjugate with the highest density evoked a good immune response, while in the case of dodeca- and hexadecasaccharide conjugates, the median density was optimal.[51]

These studies suggest that oligosaccharide chain length and hapten loading might be interconnected in determining the immunogenicity of glycoconjugate vaccines.

The spacer is a short linear molecule that is generally linked to the polysaccharide chain or to the protein or to both moieties, depending on the chemistry, used to facilitate the coupling between the protein and sugar. There are evidences in the literature which suggest that rigid, constrained spacers like cyclohexyl maleimide, elicit a significant amount of undesirable antibodies, with the risk of driving the immune response away from the targeted epitope on the hapten.[52 53] The use of a flexible alkyl type maleimido spacer has been reported as a way to overcome the previous observed immunogenicity of cyclic maleimide linkers.[54]

A number of protein carriers have been used so far in preclinical and clinical evaluation of conjugate vaccines.[55 56 57 58 59 60 61]

Proteins such as diphtheria and tetanus toxoids, which derive from the respective toxins after chemical detoxification with formaldehyde, were initially selected as carriers because of the safety track record accumulated with tetanus and diphtheria vaccination. CRM197, a non-toxic mutant of diphtheria toxin[62] which instead does not need chemical detoxification, has been extensively used as carrier for licensed Hib, pneumococcal, meningococcal conjugate vaccines and for other vaccines being developed. An outer membrane protein complex of serogroup B meningococcus has been used by Merck as carrier for their Hib conjugate vaccine.[63]

GSK in their multivalent pneumococcal conjugate vaccine introduced the use of the Hib-related protein D as carrier for most of the polysaccharides included into the vaccine.[64 65]

The team of John Robbins made extensive use of the recombinant non toxic form of *Pseudomonas aeruginosa* exo-toxin as carrier for *Staphylococcus aureus* type 5 and 8 as well as for *Salmonella typhi* Vi conjugates.[66 67]

A number of clinical trials have been conducted to compare the immunogenicity of different conjugate vaccines with different carrier proteins.[68 69 70 71 72]

It is however very difficult a direct comparison of the effect of different protein carriers, due to the coexistence of other variables as conjugation chemistry, saccharide chain length, adjuvant, formulation technology, and previous or concomitant vaccination with other antigens.

1.5 Aim of the thesis

The aim of my PhD thesis is to investigate the role that different variables have on the immunogenicity and physicochemical properties of glycoconjugate vaccines.

In particular in this work three main aspects have been investigated: the carrier protein, the sugar moiety and the linker region.

Concerning the protein, a screening of new candidate carrier proteins were investigated and a comparative study on three common carriers, CRM197, TT and DT, has been done.

The role of the sugar moiety has been investigated through two different studies: the first was conducted on glycoconjugate of the biologically derived capsular polysaccharide from meningococcus serogroup A. Different chain length oligosaccharides and glycosylation degrees were tested. The second study was about synthetic antigens and their ability to represent the native epitope.

The influence of the linker on the immunogenicity of glycoconjugate vaccines in animal model was studied using four different linker molecules.

Moreover studies on the mechanism of antigen uptake by antigen presenting cells have been conducted.

2 Results

2.1 The influence of the carrier protein

In this part of my PhD project I have carried out two investigations, one directed to discover new proteins that could be used as carriers in future glycoconjugate vaccines, and another focused on the immunological and physicochemical comparison of glycoconjugates made with three commonly used protein carriers.

As model carbohydrate antigens I have used Laminarin (Lam) and the serogroups A, C, W_{135} and Y meningococcal oligosaccharides (MenA, MenC, MenW and MenY).

Laminarin (Lam) is a β-(1, 3) glucan with sporadic β-(1, 6) branches (Figure 6), extracted from the brown alga *Laminaria digitata* that has been studied as vaccine candidate against infections induced by *C. albicans*.[39][73][74]
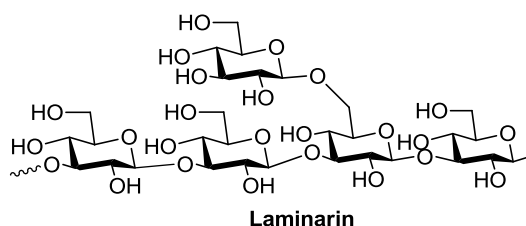
Laminarin

Figure 6: Laminarin structure.

Meningococcal saccharides from serogroups A, C, W_{135} and Y have been used as conjugate vaccines against *Neisseriae meningitidis* by different companies. The chemical structure of the different capsular polysaccharides is reported in the Figure 7.

MenA

→6)-α-D-Man*p*NAc(3/4OAc)-(1→OPO$_3$→

MenC

→9)-α-D-Neu5Ac(7/8OAc)-(2→

MenW

→6)-α-D-Gal*p*-(1→4)-α-D-Neu5Ac(7/9OAc)-(2→

MenY

→6)-α-D-Glc*p*-(1→4)-α-D-Neu5Ac(7/9OAc)-(2→
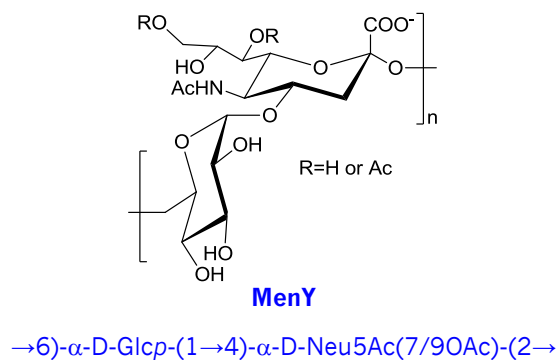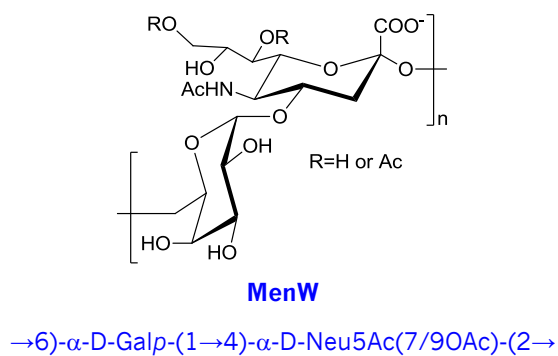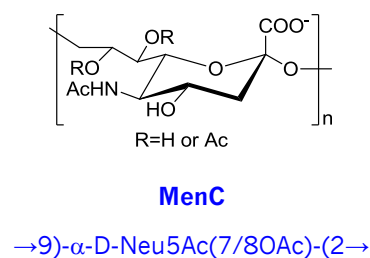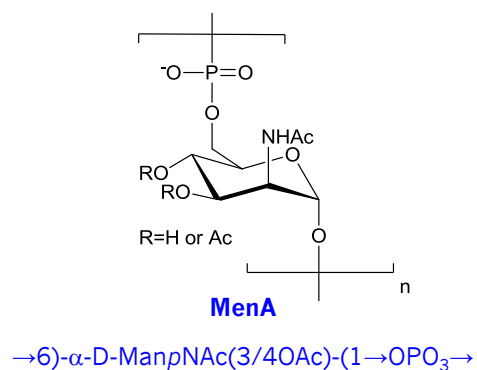
Figure 7: Repeating unit structure of *Neisseriae meningitidis* serogroups A, C, W$_{135}$ and Y saccharides.

2.2 Screening of new carrier proteins

In this study 31 proteins derived from different bacteria have been studied for their capacity to work as carrier for different saccharide antigens. The proteins used derived from different bacteria like Extraintestinal Pathogenic *Escherichia coli* (ExPEC), *N. Meningitidis* serogroup B, *Group A streptococcus* (GAS), *Group B streptococcus* (GBS) and *Streptococcus pneumoniae*. The proteins were selected by five main criteria: molecular weigh between 40 and 100kDa, non-enzymatic activity, non-toxicity, water solubility, availability by recombinant expression and appropriate number of lysine residues for conjugation reaction. All the proteins tested as new carrier candidate are reported in the following Table 4.

Protein	Micro organism	MW (kDa)
Ex 1	Extraintestinal Pathogenic *Escherichia coli*	54
Ex 2	Extraintestinal Pathogenic *Escherichia coli*	165
MB 1	*N. Meningitidis* serogroup B	27
MB 2	*N. Meningitidis* serogroup B	47,4
MB 3	*N. Meningitidis* serogroup B	65
MB 4	*N. Meningitidis* serogroup B	34,5
MB 5	*N. Meningitidis* serogroup B	68
GAS 1	*Group A streptococcus*	60
GAS 2	*Group A streptococcus*	89,4
GBS 3	*Group B streptococcus*	30
PNEU 1	*Streptococcus pneumoniae*	48,3
PNEU 2	*Streptococcus pneumoniae*	41,8
PNEU 3	*Streptococcus pneumoniae*	72,5
PNEU 4	*Streptococcus pneumoniae*	72,6
PNEU 5	*Streptococcus pneumoniae*	46,7

PNEU 6	*Streptococcus pneumoniae*	49,1
PNEU 7	*Streptococcus pneumoniae*	115,4
PNEU 8	*Streptococcus pneumoniae*	194
PNEU 9	*Streptococcus pneumoniae*	194
PNEU 10	*Streptococcus pneumoniae*	35,5
PNEU 11	*Streptococcus pneumoniae*	92
PNEU 12	*Streptococcus pneumoniae*	38,9
PNEU 13	*Streptococcus pneumoniae*	36,5
PNEU 14	*Streptococcus pneumoniae*	70,4
PNEU 15	*Streptococcus pneumoniae*	52
PNEU 16	*Streptococcus pneumoniae*	38
PNEU 17	*Streptococcus pneumoniae*	122
PNEU 18	*Streptococcus pneumoniae*	15
PNEU 19	*Streptococcus pneumoniae*	27
PNEU 20	*Streptococcus pneumoniae*	61

Table 4: Bacterial protein selected to be studied as new candidate carrier proteins for glycoconjugate vaccines

2.2.1 Conjugates preparation and characterization

The different Lam conjugates have been obtained by chemical derivatization of Lam at the reducing end with an adipic acid linker having a terminal succinimido ester.

The activated oligosaccharide was then reacted with the different protein carriers using the same active ester/protein molar ratio.

A conjugate between Lam and CRM197 was also prepared to be used as control during the immunological studies in animals.

To obtain the glycoconjugates, Laminarin has been activated as reported in Mat&Met

A schematic description of the chemical reactions to obtain Lam-CRM conjugate is reported on the Figure 8.
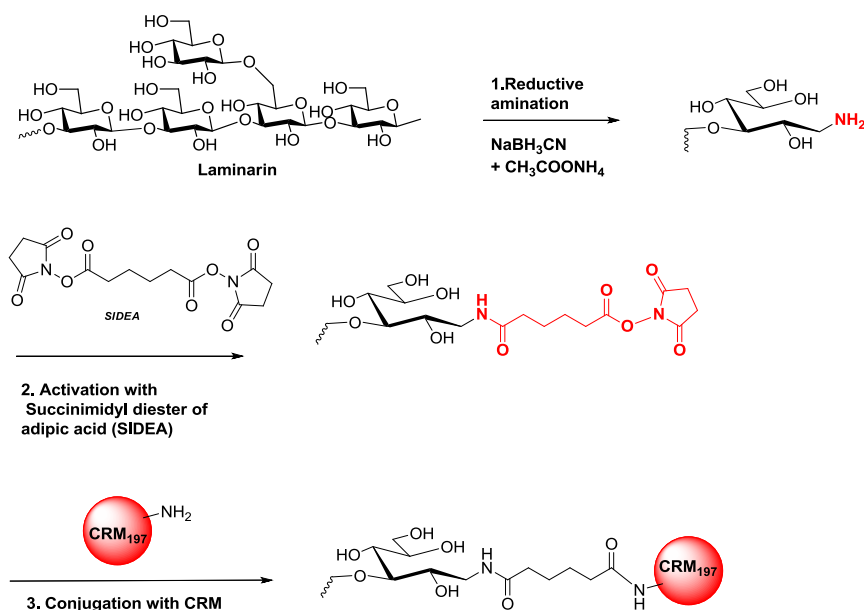
Figure 8: scheme of reaction to obtain Laminarin conjugate.

Laminarin conjugates have been characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-page) to asses that conjugation took place. Figure 9 shows the electrophoretic profiles of the selected lam conjugates. After conjugation the typical smear of a glycoconjugates could be observed on the SDS-page, the smear is due to the variable number of sugar chains bound to the protein and to the variable chain lenght of the sugar.
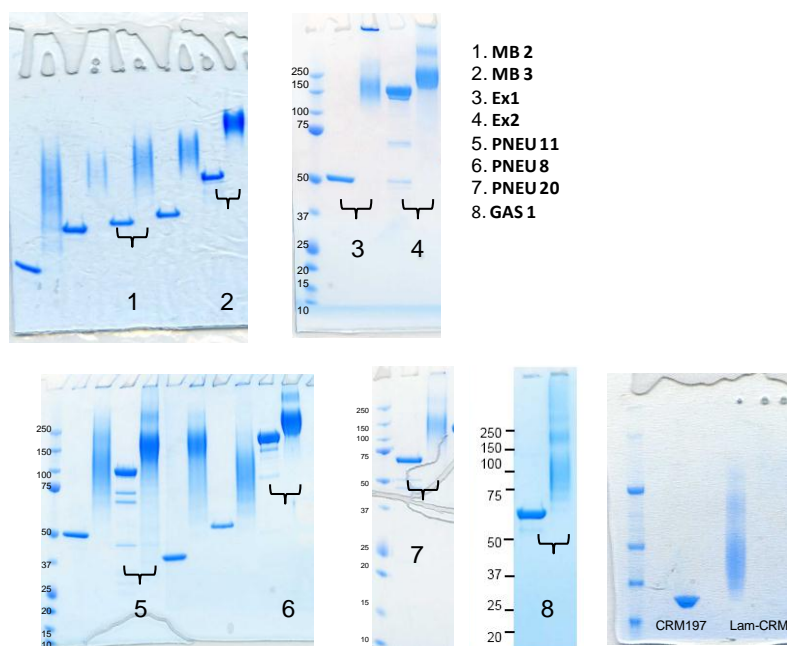
Figure 9: SDS-page of Laminarin glycoconjugates with new candidate carrier proteins

After purification Laminarin conjugates have been characterized on the saccharide and protein content. The glycosylation degree of the conjugates has been calculated weight/weight (w/w) as reported in the Table 5.

Laminarin conjugates	Saccharide/protein w/w
Ex 1	0.4
Ex 2	0.3
MB 1	0.5
MB 2	0.4
MB 3	0.6
MB 4	1.7
MB 5	0.6
MB 6	0.6
GAS 1	0.9
GAS 2	0.2
GBS 3	0.7
PNEU 1	0.6
PNEU 2	0.4
PNEU 3	0.6
PNEU 4	0.4
PNEU 5	0.6
PNEU 6	0.7
PNEU 7	0.1
PNEU 8	0.1
PNEU 9	0.1
PNEU 10	0.6

PNEU 11	0.2
PNEU 12	0.4
PNEU 13	0.6
PNEU 14	0.2
PNEU 15	0.4
PNEU 16	0.4
PNEU 17	0.3
PNEU 18	0.8
PNEU 19	1.2
PNEU 20	0.5
CRM197	0.7

Table 5: Laminarin glycoconjugates characterization by saccharide/protein ratio w/w.

The glycosylation degree of the different glycoconjugates varies in a range from 0.1 and 1.7 w/w. This difference is in accordance with the different molecular weight of the proteins since for example PNEU19 of 27kDa had reached a saccharide/protein w/w ratio of 1.2 while PNEU7, PNEU8 and PNEU9 of 115 and 194kDa have reached a ratio of 0.1. Moreover the different glycosylation degree could be due also to a different number of lysine on each protein and/or to differences in the conjugation efficiencies.

2.2.2 Evaluation of Laminarin glycoconjugates in mice

The Lam conjugates with the different candidate protein carriers have been tested in mice and the anti laminarin response compared to that provided by lam-CRM conjugates used as control. Ten different studies without adjuvant have been performed with an immunization dose of 5µg in term of saccharide. The anti Laminarin antibodies response after the third immunization has been evaluated and among the proteins tested, eight of them, namely Ex 1, Ex 2, MB 2, MB 3, GAS 1, PNEU 8, PNEU 11, PNEU 20, were selected as new candidate carriers since they were able to induce a response against the saccharide antigen comparable to that induced by CRM197 (Figure 10). In particular the conjugate with the PNEU 20 protein induced the highest anti laminarin antibodies titer.
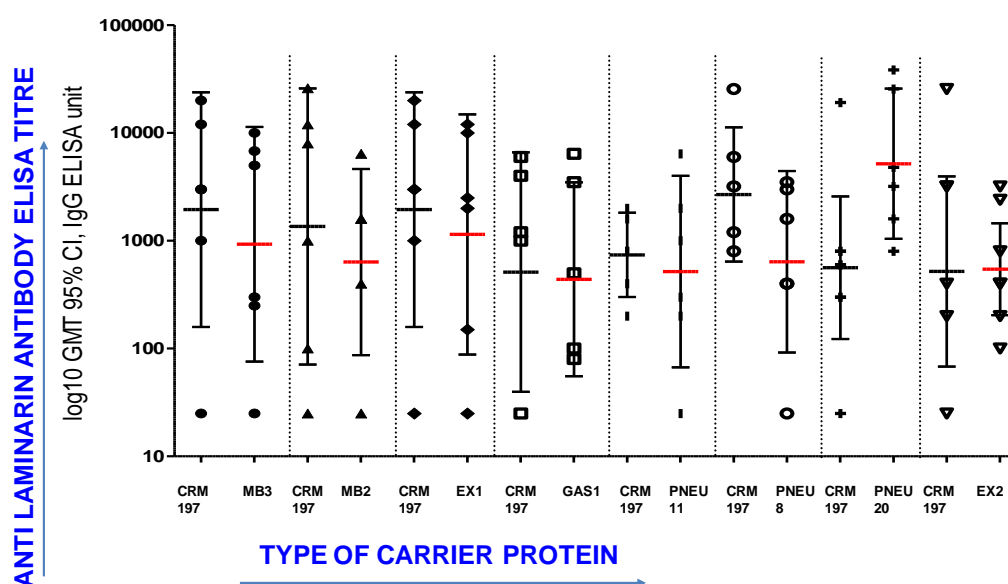
Figure 10: anti-Laminarin Abs level induced by new candidate carrier proteins in comparison with the positive control Lam-CRM. Each spot indicate a single mouse ELISA titer, the horizontal bar refers to the geometric mean of the group, while the vertical bar shows the 95% CI.

2.2.3 MenC conjugation with selected new candidate carrier proteins: conjugates preparation and characterization

Among the new candidate carrier proteins, seven have been conjugated to the serogroup C meningococcal oligosaccharide (MenC) to study their carrier functionality with a second saccharide antigen. PNEU 19 has been used as negative control since it didn't work as carrier for Laminarin, while as positive control MenC-CRM conjugate was used.

The preparation of the MenC conjugates was performed according to the scheme reported in Figure 11. Activated MenC oligosaccharides were obtained from Novartis V&D manufacturing department.
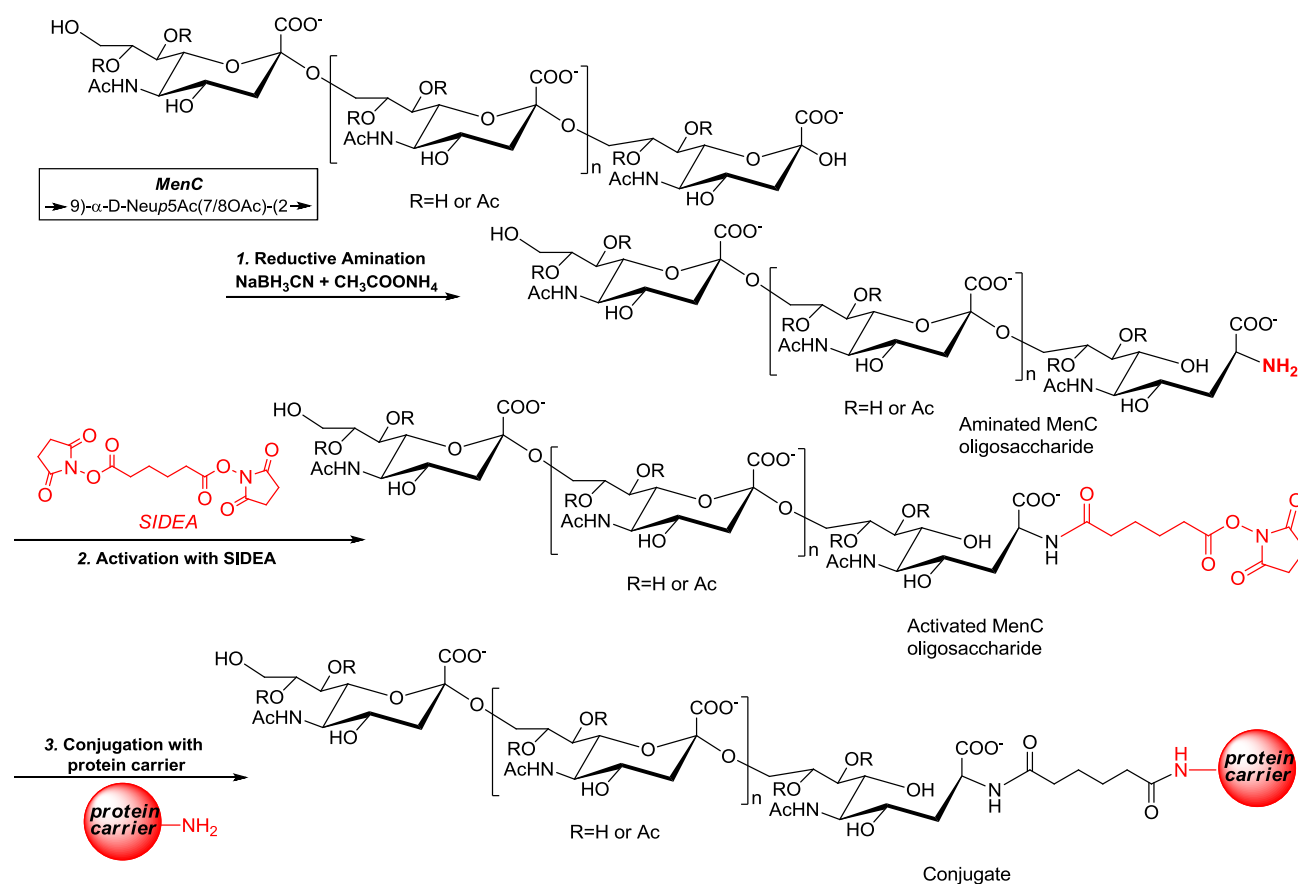
Figure 11: Scheme of reactions to obtain the MenC conjugates.

After purification conjugates have been characterized by SDS-page, as reported in the Figure 12, to asses that conjugation took place. For protein Ex2 we observed a double band profile corresponding to two protein isoforms always present on the purified batch. The two isoforms are equally involved in the conjugation process.

1. GAS 1
2. Ex 2
3. MB 2
4. PNEU 8
5. PNEU 11
6. PNEU 20
7. PNEU 19
8. Ex1
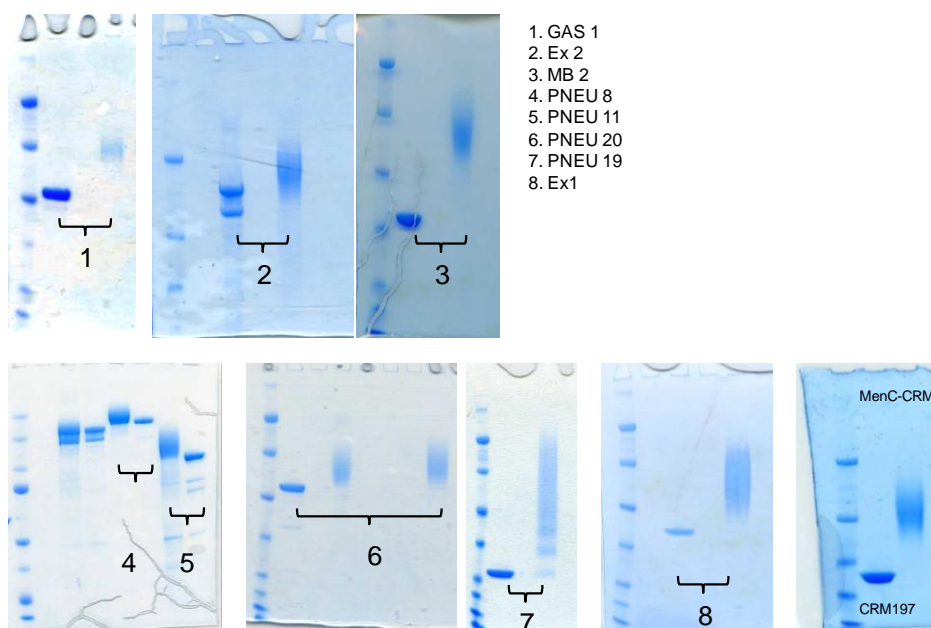
Figure 12: SDS-page of MenC glycoconjugates with new candidate carrier proteins.

Following purification, MenC conjugates were characterized for their saccharide and protein content. The calculated carbohydrate to protein ratios is reported in Table 6 and varied between 0.1 and 0.6 w/w for the different conjugates.

MenC Conjugates	Saccharide/protein w/w
Ex 1	0.3
Ex 2	0.1
MB 2	0.5
GAS 1	0.3
PNEU 8	0.1
PNEU 11	0.1
PNEU 19	0.6
PNEU 20	0.3
CRM197	0.6

Table 6: MenC glycoconjugates characterization by saccharide/protein ratio w/w

2.2.4 Evaluation of MenC glycoconjugates in mouse animal model

MenC glycoconjugates with the selected carrier proteins have been tested in mice without adjuvant with an immunization dose of 1µg in term of saccharide. The antibody response against MenC was evaluated on post third dosage immunization sera. The conjugates of four protein carriers elicited an

antibody response comparable to that induced by MenC-CRM197: PNEU20, EX1, EX2 and GAS1 (Figure 13).
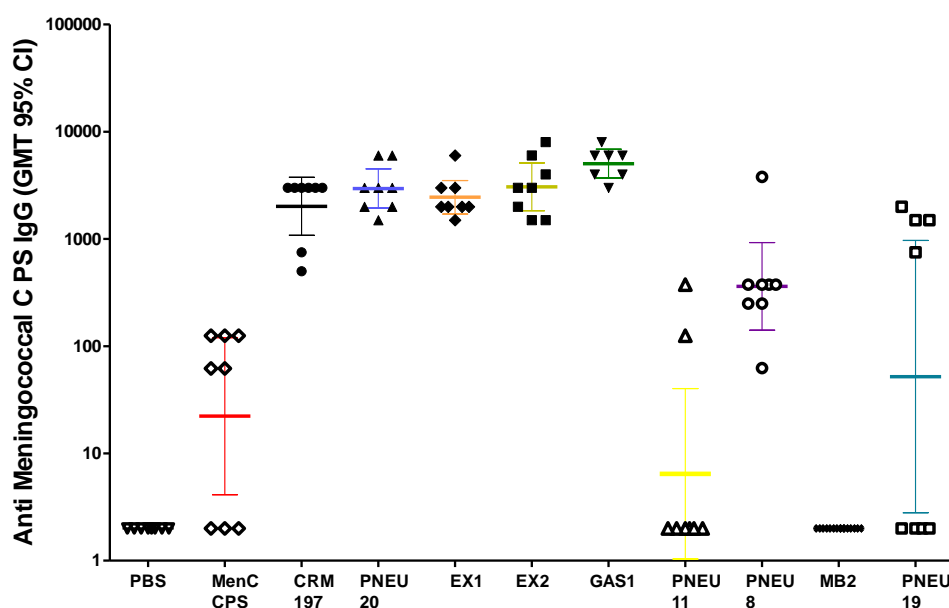
Figure 13: Anti-MenC Abs levels induced by new candidate carrier proteins in comparison with the positive control MenC-CRM. Each spot indicate single mouse ELISA titer, the horizontal bar was the geometric mean of the group, instead the vertical bar shows the statistical 95% CI.

2.2.5 PNEU 20 evaluation in MenAWY glycoconjugate: conjugation as well as products characterization

Since PNEU 20 induced the best antibody response against Laminarin and a good response against MenC, this protein was considered for future experiment and tested also in MenAWY glycoconjugates.

The conjugation of PNEU20 to the meningococcal oligosaccharides has been performed according to the scheme reported below (Figure 14). The activated oligosaccharides were provided by Novartis V&D manufacturing department. In brief the individual oligosaccharides were first reacted with an excess of ammonium acetate in the presence of sodium cyanoborohydride to provided amino-oligosaccharides which were subsequently derivatized to active esters by reaction with an excess of disuccinimidyl adipate. The resulting activated oligosaccharides, after purification, were then reacted with PNEU20 using an active ester/protein (mol/mol) ration of 12:1 in 10-100mM NaH_2PO_4 pH 7, as described in Materials and Methods.
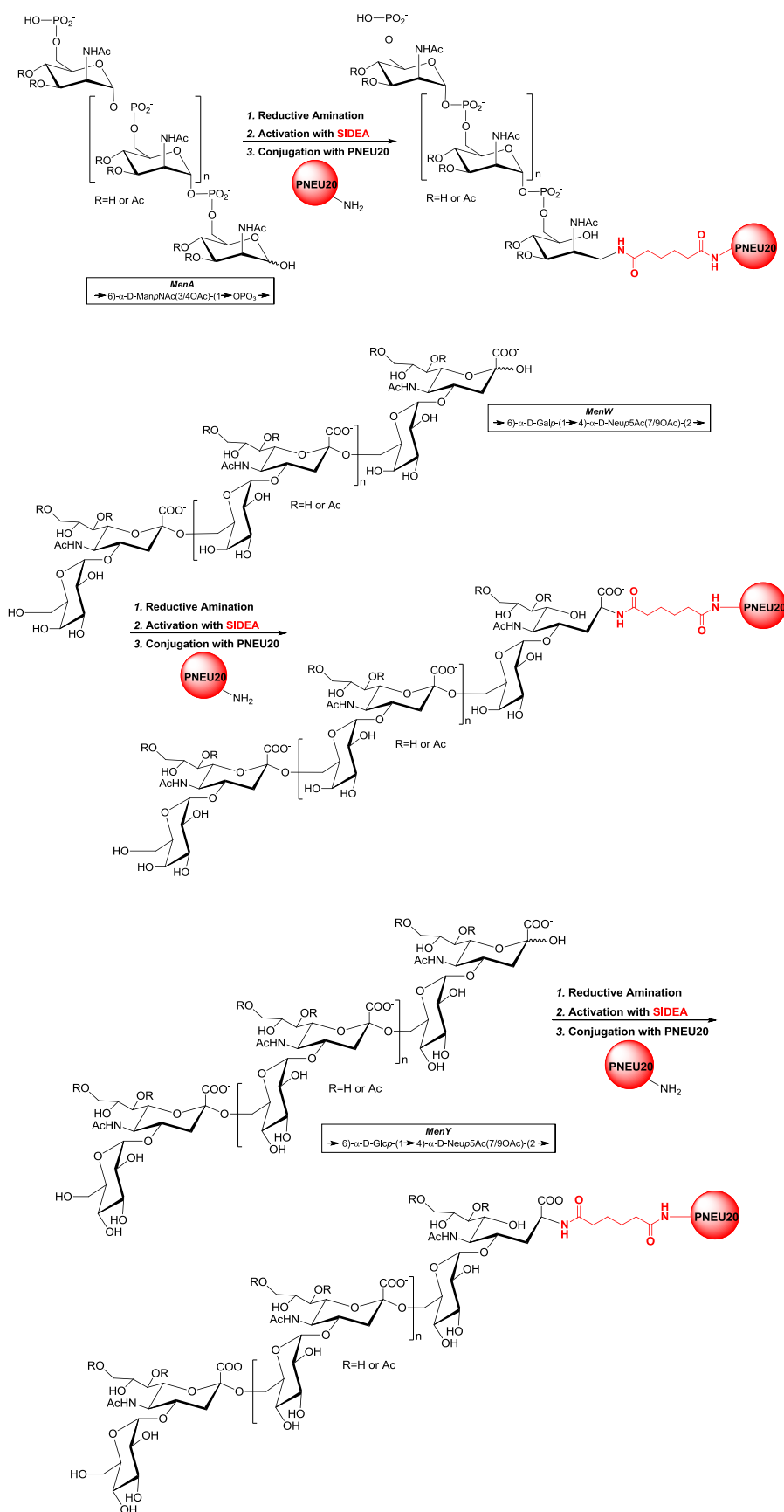
Figure 14: Scheme of the reaction to obtain MenAWY conjugates with PNEU20.

After purification, the conjugates were characterized by SDS-page, as reported in the Figure 15, to confirm that conjugation took place.
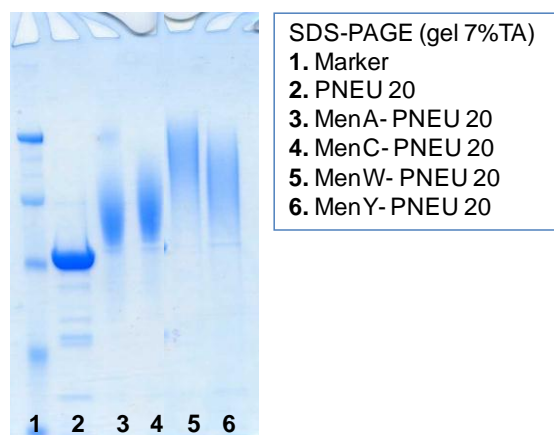
Figure 15: SDS-page of MenACWY glycoconjugates with PNEU20.

The purified conjugates have been characterized by their saccharide and protein content. The glycosylation degree (w/w) of each conjugate was then calculated. Glycosylation degrees are between 0.3 and 0.7 w/w (Table 7). Despite the fact that the same stechiometry was used, a different glycocylation degree was obtained for MenW. In particular as compared with MenY which has a similar structure, the conjugation reaction appears to proceed with higher yelds. This is consistent also with previous observation.[27]

PNEU 20 conjugates	Saccharide/protein ratio w/w
MenA	0.3
MenW	0.7
MenY	0.4

Table 7: MenAWY-PNEU20 glycoconjugates characterization by saccharide/protein ratio w/w.

2.2.6 Evaluation of PNEU20 glycoconjugates in mouse animal model

The individual meningoccus A, C, W, and Y conjugates with PNEU20 have been evaluated in mouse animal model without adjuvant with an immunization dose of 2 μg for MenA and 1 μg for MenCWY in term of saccharide. After the third immunization anti polysaccharides antibody response was evaluated by coating the different CPS on the ELISA plates.

The protein PNEU20 protein was able to induce a level of anti-saccharide antibody comparable with that of CRM197 when conjugated with the different meningococcal oligosaccharides (Figure 16).
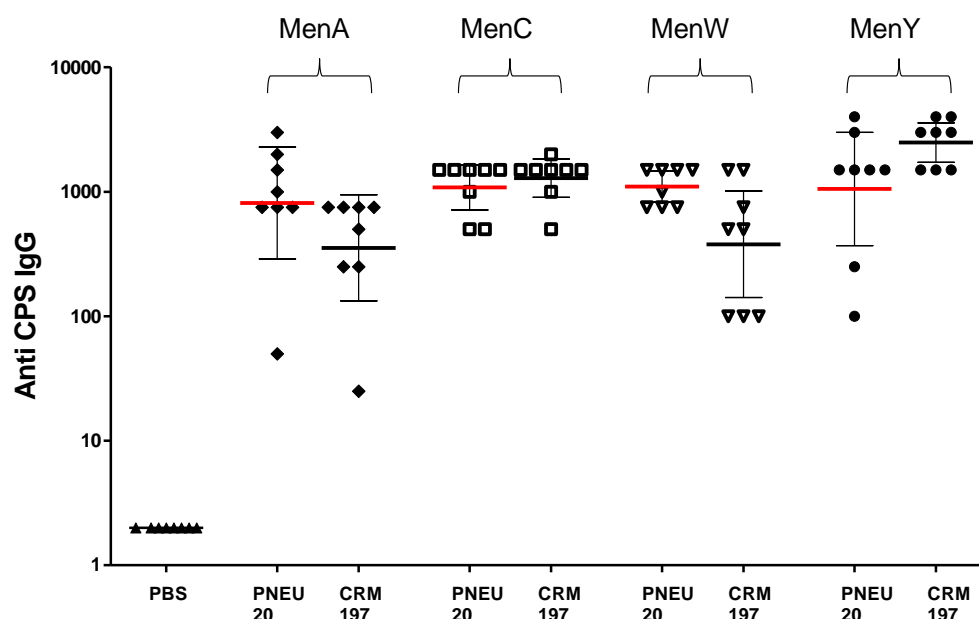
Figure 16: Anti-MenACWY Abs levels induced by PNEU20 glycoconjugates in comparison with the positive control MenACWY-CRM conjugates. Each spot indicate a single mouse ELISA titer, the horizontal bar was the geometric mean of the group, instead the vertical bar shows the statistical 95% CI.

To better investigate if some differences were present between the response induced by CRM197 and PNEU20 as carriers, sera after the 2nd immunization were analyzed by complement mediated bactericidal activity using rabbit complement (rSBA). The antibodies elicited by PNEU20 glycoconjugates have shown functional activities against each meningococcal bacterial strain (Table 8), confirming the capability of this protein to work as carrier for different glycoconjugate vaccines.

rSBA titers post 2nd dose		
Conjugates	CRM197	PNEU20
MenA (Men A - F8238)	2048	4096
MenC (Men C - 60E)	1024	2048
MenW (Men W - 240070)	128	512
MenY (Men Y - 860800)	2048	1024

Table 8: rSBA titers induced by sera post 2nd dose of immunization with MenACWY-CRM and MenACWY-PNEU20 glycoconjugates.

2.3 Comparison study of three common carrier proteins in MenACWY glycoconjugates

In this section of my PhD a comparative study of glycoconjugates obtained with three different carrier proteins currently used in vaccines has been performed. The carrier proteins taken into consideration were: tetanus toxoid (TT), diphtheria toxoid (DT) and CRM197.

The proteins TT and DT derive from their homologous toxins after chemical detoxification with formaldehyde.[75]

CRM197 is a non-toxic mutant of diphtheria toxin and does not need detoxification treatment. In CRM197 the glycine residue of diphtheria toxin in position 52 it is substituted by a glutamic acid residue (Figure 17).[76]
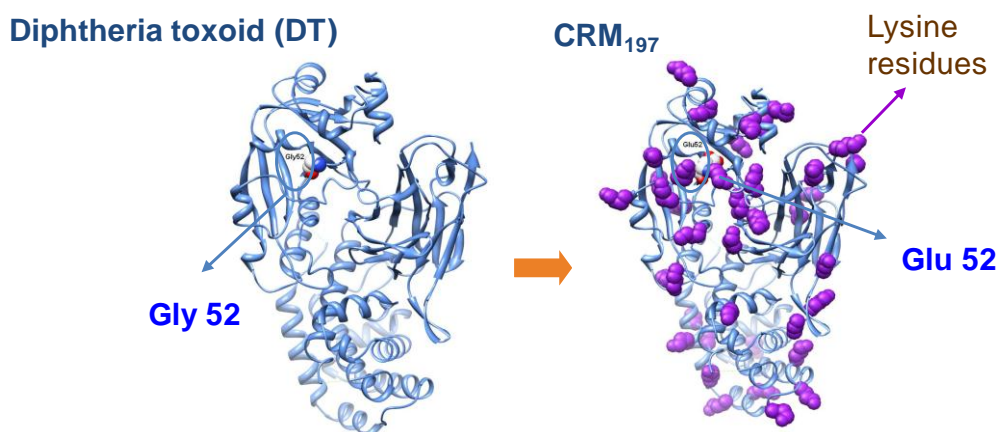
Figure 17: CRM197 and Diphtheria toxoid structures comparison.[46]

Recently the crystal structure of CRM197 has been solved investigating the structural basis for lack of toxicity of the protein.[77] The proteins have been used to conjugate with MenACWY oligosaccharides and products have been compared in their physicochemical characteristics and in their immunogenicity.

2.3.1 Serogroup A, C, W, Y meningococcal oligosaccharides conjugation with CRM197, TT and DT as carrier proteins: conjugates preparation and characterization

Conjugates of CRM, TT and DT with the meningococcal A, C, W, Y oligosaccharides were prepared following the same chemistry described in paragraphs 2.2.3 and 2.2.5 (Figure 11 and 14).

Activated oligosaccharides obtained from Novartis V&D manufacturing department were reacted with the different protein carriers in 100mM NaH_2PO_4 pH 7 using an active ester/protein (mol/mol) ratio of 12:1. The different conjugates were purified by precipitation with ammonium sulphate as described in Materials and Methods.

The three proteins CRM, TT and DT have a molecular weight of 58, 60 and 150 kDa respectively.

Before conjugation, TT was purified to select the monomeric form. Monomer selection has been performed by gel filtration on Sephacryl S300. The chromatogram obtained is reported on the Figure 18A. The monomer has been selected by collecting the fractions to the peak at lower molecular weight. TT monomer was characterized by HPLC in comparison with the starting material. (Figure 18B)
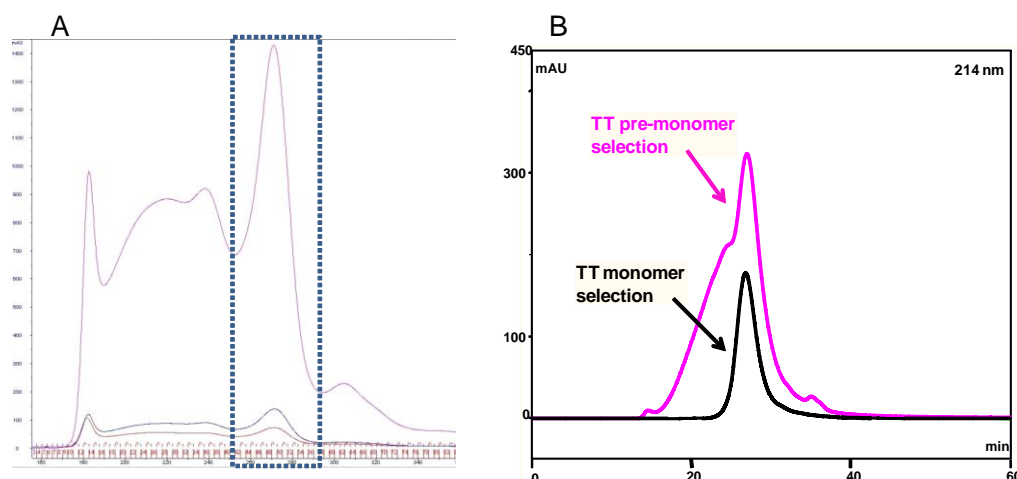
Figure 18: A) Chromatographic profile of TT protein obtained by gel filtration on Sephacryl S300.

B) SEC- HPLC profile obtained on TSK 4000 Gel SW of TT protein before and after monomer selection.

Each protein has been conjugated with the oligosaccharides of the four serogroups using the chemistry of conjugation previously reported before for MenACWY (paragraphs 2.2.3 and 2.2.5).

Conjugates have been characterized by SDS-page, as shown in Figure 19, to confirm that conjugation took place. Depending on the type of saccharide, conjugates with different electrophoretic profile were observed. The tendency of MenW to give higher glycosylated products was confirmed.
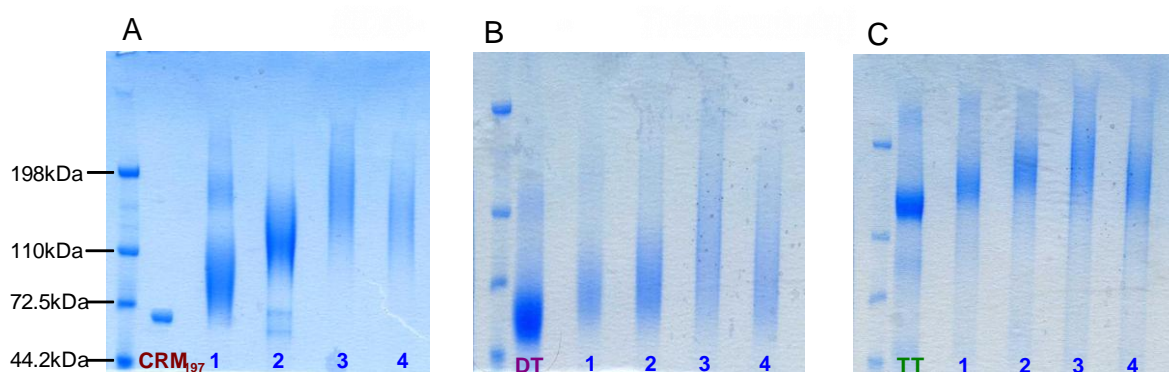
Figure 19: Electrophoretic profiles of glycoconjugates with different carrier proteins: CRM197 panel A, DT panel B and TT panel C. Lanes 1 to 4 have been loaded with MenA, MenC, MenW and MenY conjugates respectively.

Further characterization has been done by HPLC analysis as reported in Figure 20. Both SDS-page and HPLC show that with CRM197 better characterized conjugates have been obtained.
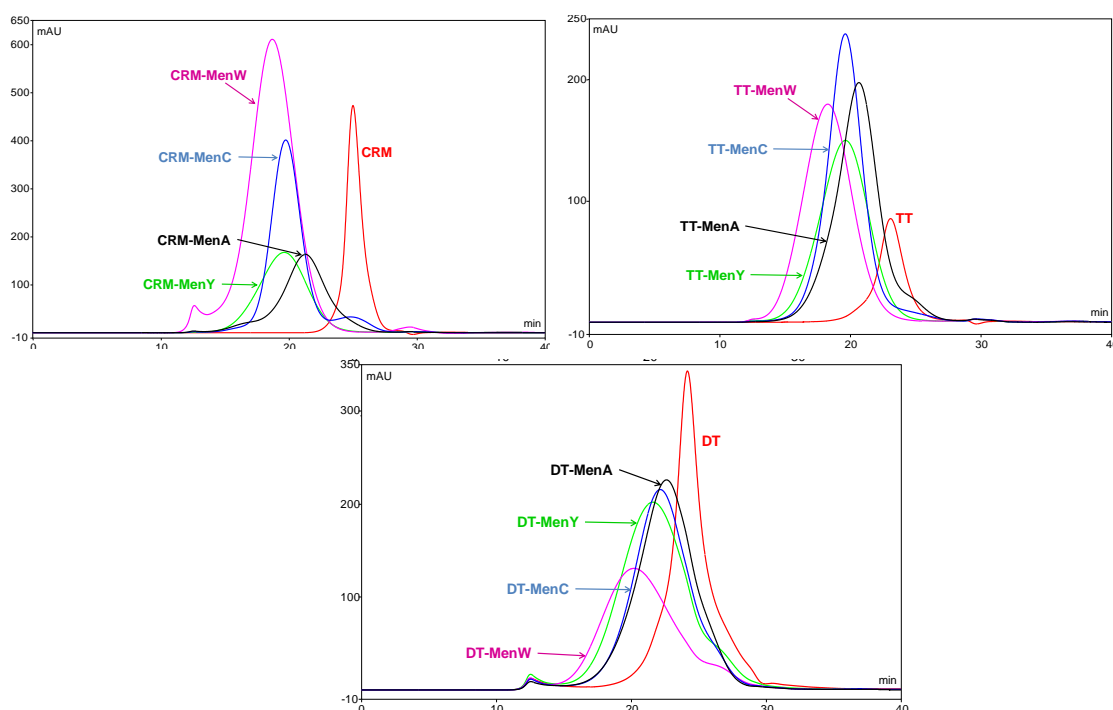
Figure20: SEC-HPLC profiles of the conjugates obtained TSK 4000 Gel SW.

Purified conjugates have been also characterized for their saccharide content, protein content and residual unconjugated saccharide (free saccharide %). The saccharide/protein ratio w/w and the free saccharide % for each conjugate are reported in Table 9. Glycosylation degrees obtained

ranged between 0.1 and 0.7 w/w depending on the saccharide protein combination. The free saccharide % varied from 3.7 to 22. This difference on the free saccharide content could derive from a different behaviour of the conjugates, depending on the protein/saccharide combination, during the ammonium sulphate precipitation applied for the purificationIn any case the 0.4 w/w ratio of MenA-TT conjugate will become 0.31 considering the 22% of free saccharide and doesn't affect considerably the immunization dosage content in term of conjugated saccharide.

Conjugates	Sacch/Prot (w/w)	Free Sacch (%)
MenA-CRM	0,5	16,3
MenC-CRM	0,6	17,7
MenW-CRM	0,7	10,7
MenY-CRM	0,4	3,7
MenA-TT	0,4	22,1
MenC-TT	0,2	14,1
MenW-TT	0,2	7,9
MenY-TT	0,1	16,2
MenA-DT	0,2	13,7
MenC-DT	0,1	10,3
MenW-DT	0,3	6,2
MenY-DT	0,1	11,8

Table 9: MenACWY glycoconjugates with CRM, TT and DT characterization by saccharide/protein ratio w/w.

2.3.2 Evaluation of glycoconjugates in mouse animal model

The meningococcal oligosaccharides conjugates with the different carrier proteins have been evaluated for their immunogenicity in Balb/c mice, with and without alum phosphate ($AlPO_4$) as adjuvant.

The adjuvanted formulations have been characterized concerning the absorption degree to $AlPO_4$ in PBS buffer pH 7-7.5. After centrifugation and supernatant separation from the pellet containing the $AlPO_4$, the protein content was determined in the supernatant and compared to that of a control constituted by the formulation not exposed to the adjuvant.

Conjugates	% absorption
MenA-CRM	23
MenC-CRM	13
MenW-CRM	19
MenY-CRM	14
MenA-TT	12
MenC-TT	11
MenW-TT	0
MenY-TT	0
MenA-DT	10
MenC-DT	18
MenW-DT	6
MenY-DT	7
MenACWY-CRM	21
MenACWY-TT	0
MenACWY-DT	14

Table 10: % of absorption on $AlPO_4$ adjuvant of MenACWY-CRM, TT and DT vaccine formulations.

As shown in the Table 10, with these buffer and pH conditions used, none of the conjugates significantly absorbed to $AlPO_4$.

All immunizations were done with the same schedule of injection and bleedings as reported on the Figure 21.
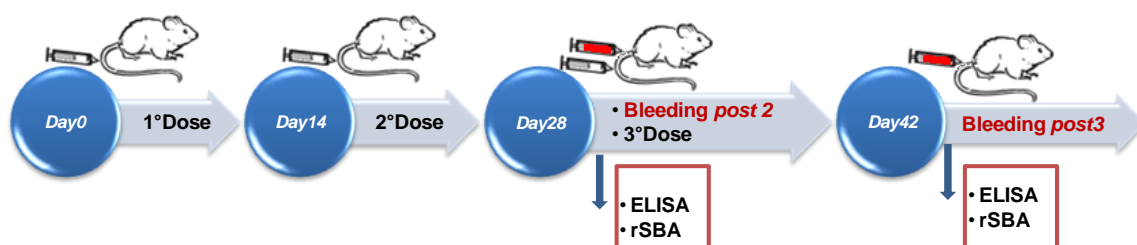
Figure 21: Mice immunization schedule.

Conjugates	Immunization Sacch dosage (µg/mice)	Immunization Protein dosage (µg/mice)
MenA-CRM	2	4
MenC-CRM	1	1,6
MenW-CRM	1	1,4
MenY-CRM	1	2,5
ACWY-CRM	2,1,1,1	9,5
MenA-TT	2	5
MenC-TT	1	5
MenW-TT	1	5
MenY-TT	1	10
ACWY-TT	2,1,1,1	25
MenA-DT	2	10
MenC-DT	1	10
MenW-DT	1	3,3
MenY-DT	1	10
ACWY-DT	2,1,1,1	33,3

Table 11: Conjugates immunization dosages.

Initially we tested in Balb/c mice formulations without adjuvant of individual meningococcal conjugates with CRM, TT and DT. Sera post 2nd and 3rd immunization were analyzed by ELISA and complement mediated bactericidal activity using rabbit complement (rSBA). In all cases after the 2nd immunization the antibodies response against the individual unconjugated polysaccharides was low and increased with the 3rd immunization. However the high variability of the response did not allow to measuring any statistical difference between the different conjugates (Figure 22). In term of bactericidal activity all the conjugates elicited low levels of rSBA titer after the second dose which increased after the third (Table 12).
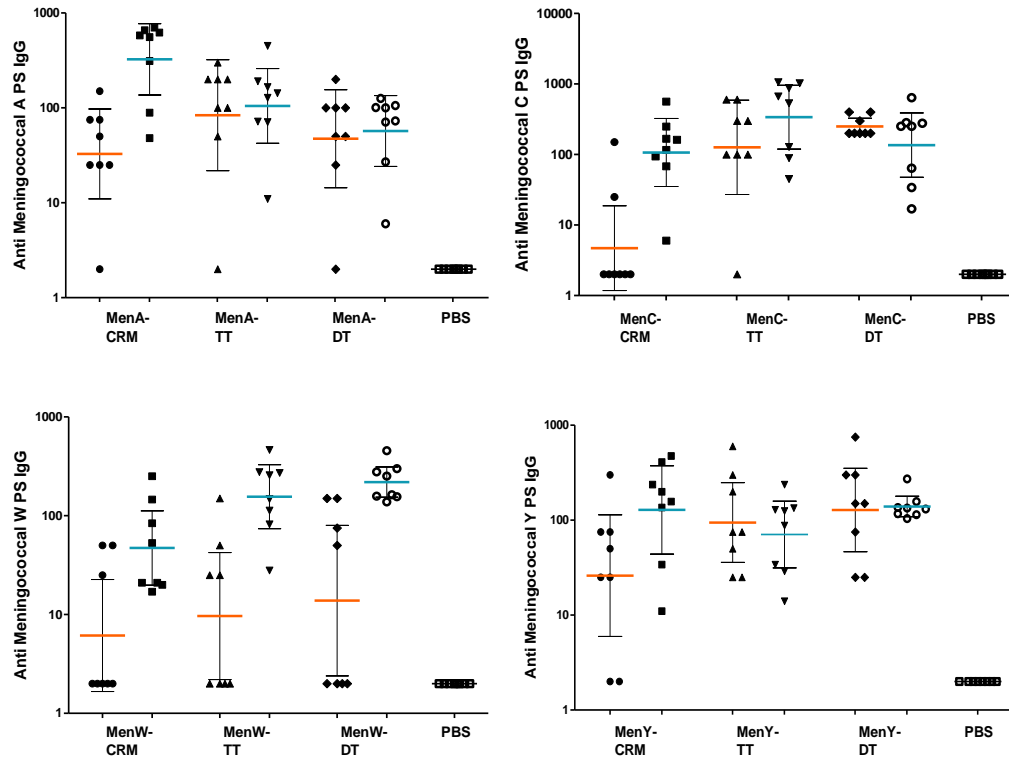
Figure 22: Antibody response induced in mice by the individual meningococcal conjugates formulated without adjuvant. Each spot indicate single mouse ELISA titer, the horizontal bar was the geometric mean of the group, while the vertical bar shows the statistical 95% CI.

Bacteria strain	A		C		W		Y	
Dose	2nd	3rd	2nd	3rd	2nd	3rd	2nd	3rd
Monovalent formulation								
CRM conjugates	64	2048	8	32	8	128	64	2048
TT conjugates	64	4096	32	128	8	128	128	512
DT conjugates	32	128	128	2048	64	256	128	2048
PBS	8	8	8	8	8	8	8	8

Table 12: Rabbit complement SBA titers determined in pool sera from mice immunized with individual meningococcal conjugates without adjuvant.

Due to the high variability of the response without adjuvant we performed the next studies using $AlPO_4$ as adjuvant. Two different studies with the individual meningococcal conjugates have been conducted with $AlPO_4$ as adjuvant and the same trend of response has been observed inside the immunization groups, for this reason the titers have been grouped to calculate the GMT (CI 95%) on 16 mice. Only one study was performed comparing three tetravalent formulations containing the conjugates of the four meningococcal oligosaccharides with CRM197, DT and TT respectively and in this case the immunization groups were composed of eight mice.

Each carrier protein was able to induce a specific response against each saccharide antigen, vaccination induced a significant level of antibodies in comparison with PBS alone (Table 13).

All groups showed a significant booster effect following the third dose.

To compare the different vaccines a statistical analysis using a non parametric t test has been done on ELISA titers and highly significant differences have been obtained between MenA-CRM vs MenA-TT (***[3] P= 0.0002) at post 3 and MenA-DT vs MenA-TT at post 3 (**[3] P< 0.008) for monovalent vaccines. In tetravalent formulation the difference between MenA-CRM and MenA-TT is maintained at post 3 (**[3] P< 0.008).

Bacteria strain	A		C		W		Y	
Monovalent formulation								
Dose	2nd	3rd *2	2nd	3rd*2	2nd	3rd*2	2nd	3rd*2
CRM conjugates	488 (266; 896)	5406 ***3 (4278; 6831)	301 (166; 45)	3201 (2157; 4752)	208 (120.0; 359.1)	1659 (932.4; 2953)	461 (244.9; 866.3)	1733 (1409; 2131)
TT conjugates	163 (86; 309)	1234 (797; 1911)	800 (547.8; 169)	4227 (3270; 5464)	73 (46.14; 114.6)	680 (454.1; 1019)	548 (291.2; 1030)	1533 (1118; 2102)
DT conjugates	254 (121; 530)	3963 **3 (2681; 5858)	619 (450.0; 52.0)	5327 (4341; 6537)	203 (137.0; 300.5)	1203 (753.3; 1922)	464 (298.7; 720.8)	1410 (922.8; 2155)
Tetravalent formulation								
CRM conjugates	507 (382; 674.1)	6683 **3 (5114; 8735)	993 (727.1; 1357)	2016 (1514; 2686)	479.9 (297.9; 772.9)	3942 (2150; 7229)	958.4 (743.5; 1235)	2913 (2107; 4027)
TT conjugates	236 (141.8; 394.2)	1849 (871.8; 3922)	944.4 (775.9; 1149)	1889 (1309; 2726)	243.1 (107.0; 552.2)	1612 (952.4; 2728)	739.0 (556.3; 981.8)	3000 (2201; 4089)
DT conjugates	249 (124.3; 496.9)	2711 (1497; 4908)	766.1 (568.7; 1032)	2228 (1067; 4651)	371.9 (256.4; 539.6)	2769 (1447; 5298)	944.4 (561.5; 1588)	4559 (3039; 6839)
PBS/AlPO$_4$	2	2	2	2	2	2	2	2

Table 13: Antibody response induced in mice by the individual meningococcal conjugates and their combinations formulated with AlPO$_4$. (GMT 95%CI) **[1] P< 0.0014; *[2] P< 0.05; ***[3] P= 0.0002; **[3] P< 0.008.

The analysis of immunological response was completed by analyzing the functional activity of the induced antibodies. This was achieved by performing the rSBA assay on pools of mice sera immunized with the different conjugates. In general a similar trend of response was observed when comparing rSBA and ELISA titers (Table 14). However, interestingly, rSBA revealed a decreased of bactericidal titer against serogroup A meningococcus when the MenA conjugates were tested in tetravalent formulation. This difference was not evident with ELISA titers and might be related to the maturation of anti-meningococcal antibodies affinity which might proceed in a different way when MenA conjugates are in tetravalent formulation.

Bacteria strain	A		C		W		Y	
Dose	2nd	3rd	2nd	3rd	2nd	3rd	2nd	3rd
Monovalent formulation								
CRM conjugates	8192	24453	1024	4096	256	1536	2048	6144
TT conjugates	256	3072	1024	6144	64	384	512	5120
DT conjugates	2048	12165	2048	12165	256	1536	1024	6144
Tetravalent formulation (ACWY)								
CRM conjugates	515*	8192	2048	16138	256	1024	1024	16138
TT conjugates	48*	2048	512	2048	64	256	256	4096
DT conjugates	8*	4096	2048	8192	256	512	2048	8192
PBS/AlPO4	8	8	8	8	8	8	8	8

Table 14: Rabbit complement SBA titers determined in pool of sera from mice immunized with individual meningococcal conjugates and their combinations formulated with $AlPO_4$. Values for monovalent formulations are the average of rSBA titers obtained in two different studies. *Average of three replicates.

Additional information on the immunological response of the meningococcal conjugates with the three different carrier proteins was obtained by analyzing the level of anti-carrier antibody. Each conjugate induced a specific antibody response against the protein moiety. As opposed to CRM197,

TT and DT conjugates seem to have a similar behavior in inducing high levels of anti-carrier antibodies already after the second dose. A similar trend has been observed also for the tetravalent formulations (Figure 23 A and B). Due to the lower glycosylation degree of the TT and DT conjugates, the amount of protein per dose that mice received is generally higher for these two types of conjugates (Table 11) which could explain the higher anti carrier antibody titer. However MenA-CRM and MenA-TT have a similar carbohydrate to protein ratio indicating that CRM197 has the tendency to drive the response towards the carbohydrate moiety which is the role of the protein carrier in a conjugate.
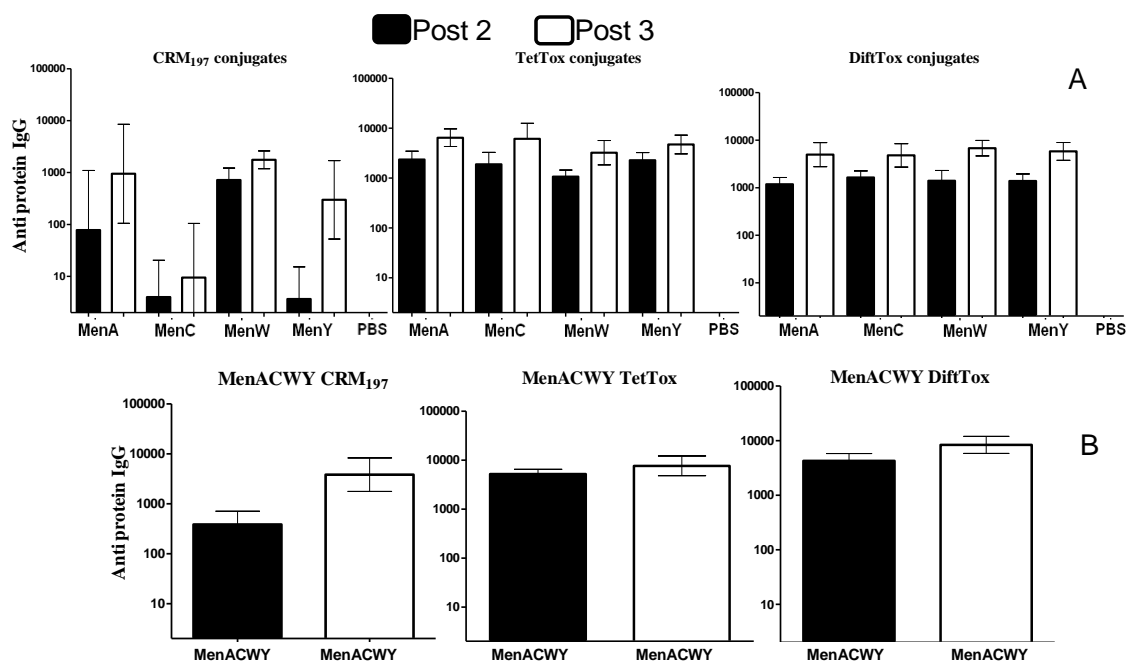
Figure 23: Anti-protein IgG detection on sera post 2nd and post 3rd immunization from the study with adjuvant. A) monovalent formulations; B) tetravalent formulations. Data have been grouped for type of protein.

2.4 Characterization of the influence of the sugar moiety on glycoconjugate immunogenicity

To investigate how the saccharide moiety could influence the glycoconjugate immunogenicity two different studies have been performed. In the first study a natural antigen, MenA oligosaccharide, has been taken as model to study how the saccharide chain length and glycosylation degree can influence the immunogenicity of a glycoconjugate in mouse animal model.

A second investigation has been carried out on a synthetic antigen, the synthetic *β-glucan* oligosaccharides, to evaluate if this antigen could be able to represent the epitope of the natural sugar.

2.5 Saccharide chain length and protein loading of a natural carbohydrate antigen

This investigation has been performed using serogroup A meningococcus oligosaccharides as model of carbohydrate antigen and CRM197 as model of carrier protein. The MenA capsular polysaccharide (CPS) has been hydrolyzed and, using ad hoc purification conditions, oligosaccharides with different chain length have been obtained. The oligosaccharides have been conjugated to CRM197 with different conjugation conditions to obtain conjugates at different glycosylation degree.

Hydrolysis of MenA CPS has been performed as reported in Mat&Met. Different purification steps were applied to select oligosaccharides with average degree of polymerization (avDP) of 6/10, 15 and 30/40 (Figure 24).
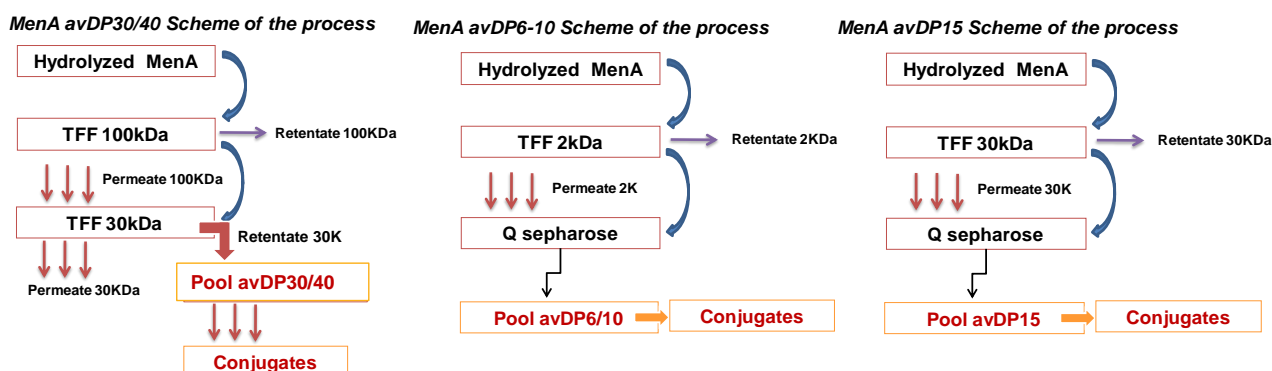
Figure 24: Purification steps applied to hydrolyzed MenA to select avDP 6/10, 15 and 30/40.

In order to obtain oligosaccharides with *av*DP 30/40 the MenA hydrolysis mixture was firstly processed by tangential ultrafiltration (UF) using a 100kDa cut off membrane. The permeate was

thne concentrated on a 30kDa membrane and after diafiltration the retentate was recovered and used for conjugation with CRM197.

AvDP15 and avDP6/10 oligosaccharides were obtained firstly processing the hydrolyzed material trough UF30K or 2K respectively; subsequently the two permeate were further purified by anionic exchange chromatography column which was eluted with a sodium chloride gradient. Fractions containing the desired chain length oligosaccharides were identified by HPAEC-PAD analysis and pooled (Figure 25).
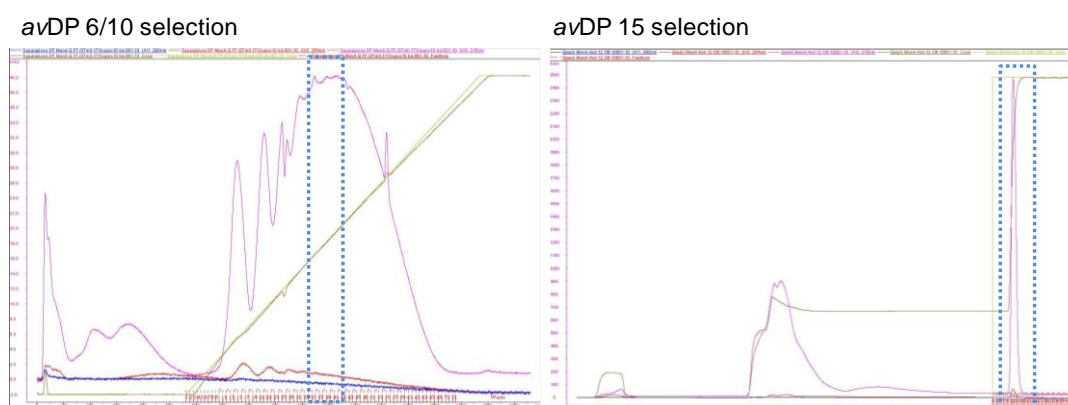
Figure 25: Chromatogram obtained by anionic exchange chromatography column to select avDP 6/10 and 15. Blue box indicates the fractions collected.

The purified oligosaccharides have been characterized by HPAEC-PAD profiling, ^{31}P NMR and ^{1}H NMR. HPAEC-PAD profile gave information about the polydispersion of the oligosaccharides and the range of DPs present in each pool (Figure 26). ^{1}H NMR allowed to asses the structural identity of the oligosaccharides pool by the presence of characteristic signals of MenA repeating unit as evidenced in Figure 27. ^{31}P NMR allowed the determination of the avDP of the oligosaccharide by integration of the signals related to the phosphodiester (in chain) and the monoester signal (end group). The avDPs calculated by the formula [(P_{de}/P_{me}) +1] as reported also in Mat&Met are reported on the Table 15. The ^{31}P NMR minor peak observed in the P_{de} region (up-field) was assigned to the de-O-acetylated repeating units, which don't contain OAc groups both at C_3 and C_4.

Chain length oligosaccharide	^{31}P NMR avDP
6/10	9
15	12
30/40	36

Table 15: avDP calculation by ^{31}P NMR.
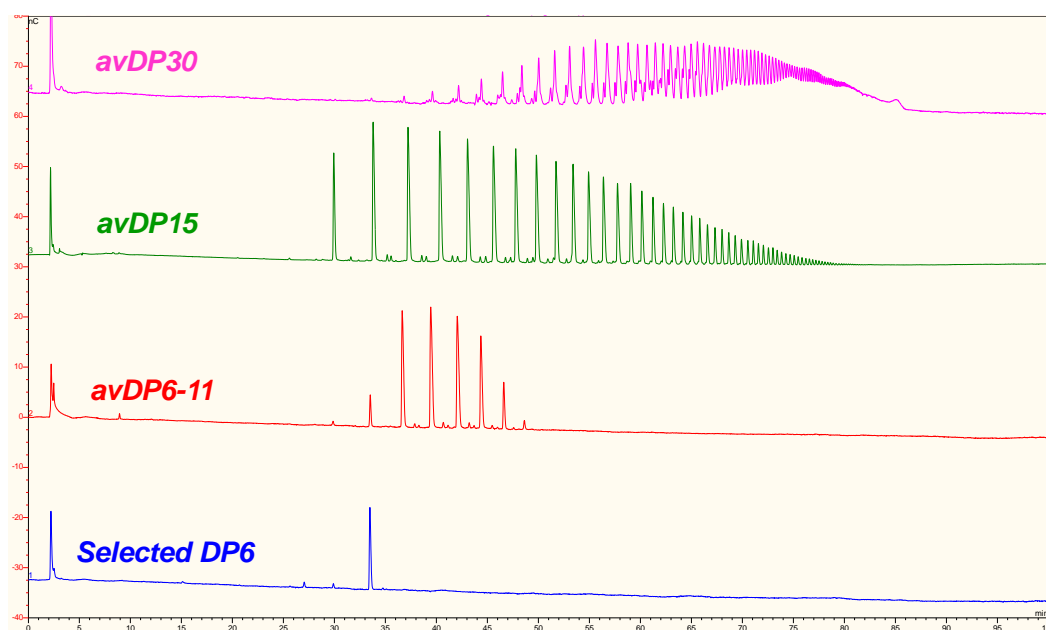
Figure 26: HPAEC-PAD profiles obtained for each avDP oligosaccharides.
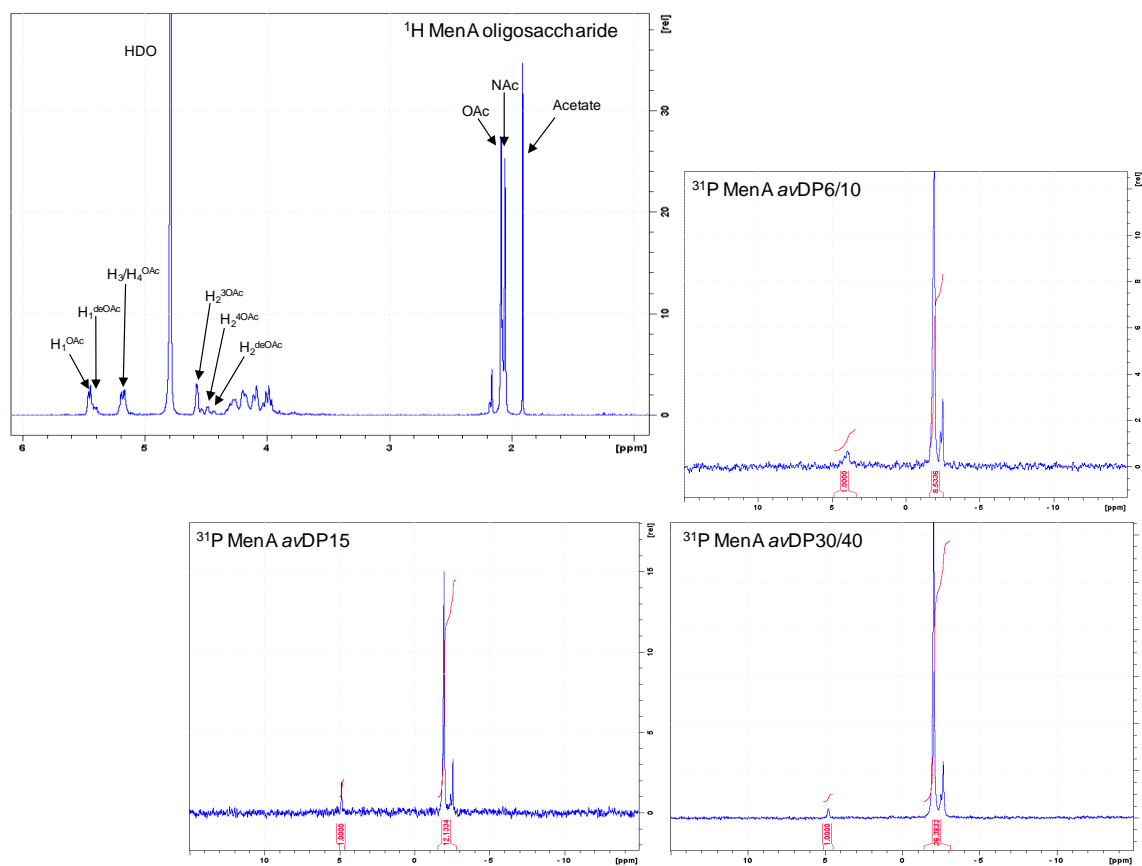
Figure 27: ¹H NMR and ³¹P NMR of MenA oligosaccharides.

All oligosaccharides have been chemically activated according to the following scheme of reaction (Figure 28) and using the experimental conditions reported in Mat&Met for oligosaccharides activation with succinimidyl diester of adipic acid (SIDEA).

2.5.1 Conjugates preparation and characterization

Using different conjugation conditions conjugates with different saccharide/protein ration have been obtained which have been characterized by SDS-page, as reported on the Figure 29, to confirm that conjugation took place. The excess of oligosaccharide used was sufficient to conjugate all the CRM197 protein in reation.
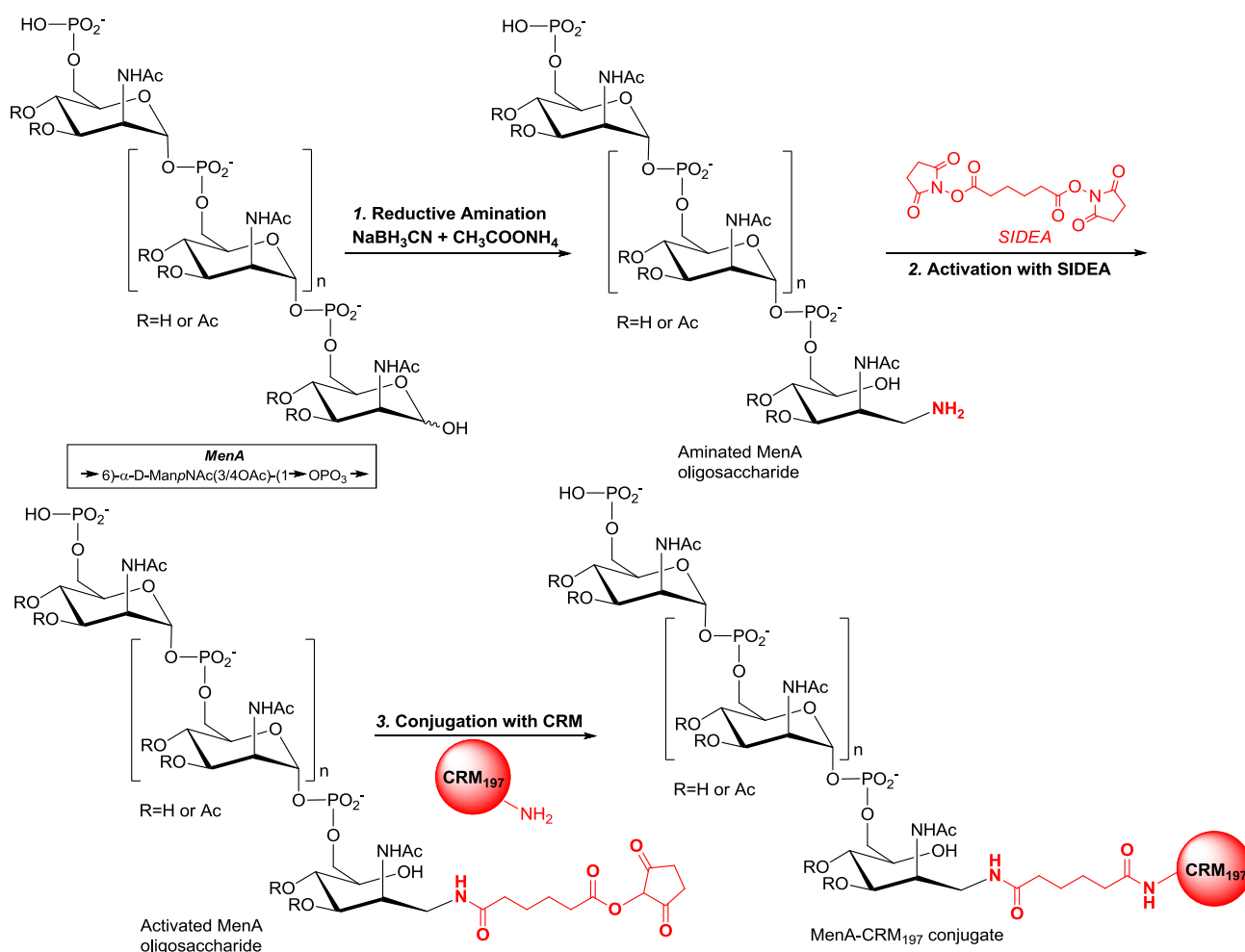
Figure 28: MenA-CRM conjugation scheme after amination and activation of the oligosaccairde.

1. **Marker**
2. **CRM$_{197}$**
3. ***av*DP15 (5mol/mol) conjugate**
4. ***av*DP15 (9mol/mol) conjugate**
5. ***av*DP15 (3mol/mol) conjugate**
6. ***av*DP30/40 (5mol/mol) conjugate**
7. ***av*DP30/40 (3mol/mol) conjugate**
8. ***av*DP6/11 (4mol/mol) conjugate**

1 2 3   4   5   6 7   8
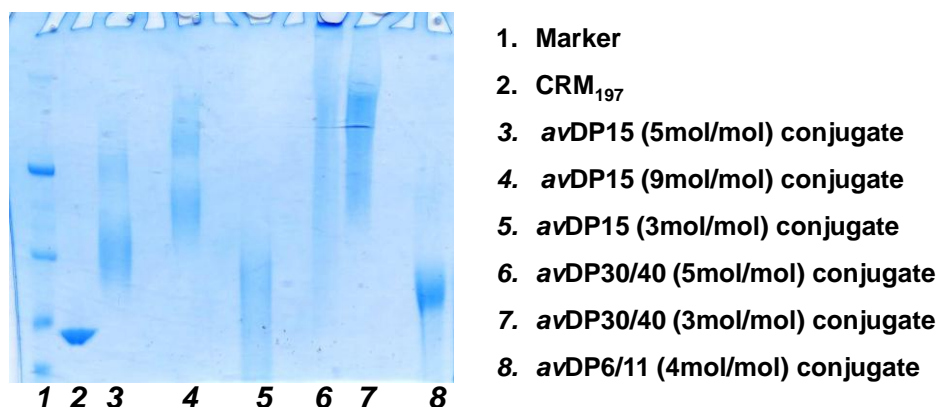
Figure 29: SDS-page of MenA-CRM conjugates with avDPs saccharide antigens and different glycosylation degree.

The different glycoconjugates were purified by gel filtration chromatography in order to remove the unreacted oligosaccharides. After purification the conjugates were characterized for their protein and saccharide content. As described before the conjugation conditions were varied in order to obtain products with different glycosylation degree which are reported in the following table (Table 16).

Conjugate	Sacch/Prot (w/w)	Sacch/Prot (mol/mol)
avDP6/10		
ML (M13)	0.15	4
HL (M15)	0.34	8
avDP15		
LL (M10)	0.23	3
ML (GMP)	0.42	5
HL (M5)	0.8	9
avDP30/40		
LL (MT2)	0.46	3
LL (M8)	0.56	3
ML (M11)	0.88	5

Table 16: Saccharide/protein ratio of MenA-CRM conjugates obtained with saccharide antigen at different avDP.

2.5.2 Evaluation of conjugates in mice

Both monovalent (MenA-CRM) and tetravalent (ACWY-CRM) formulations were evaluated using $AlPO_4$ as adjuvant. As far as the tetravalent formulation is concerned the immunization dosage was 2µg for MenA conjugates and 1µg for the other conjugates expressed on saccharide basis.

The immunological response against serogroup A meningococcal polysaccharide was evaluated by ELISA, the functional activity of the induced antibodies was evaluated by rSBA. The results are reported in Figure 30 (A, B, C) and Table 17 (A1, B1, C1).

Within the three categories of avDP we did not observe significant immunogenicity differences in the range of glycosylation degree studied. However both ELISA titers and rSBA titers showed a trend of higher response induced by the conjugates with lower glycosylation degree. This behavior is particularly evident in tetravalent formulations. The reasons for this trend are not clear; however we should note that conjugates with low glycosylation degree corresponded to a higher amount of molecules (moles) injected into the animals per dose.

avDP 30/40 conjugates

MenA-CRM Conjugates loading	Sacc/Prot (w/w)	Sacc/Prot (mol/mol)	umol tot cj per dose
ML	0.88	5	3.9*10-5
LL	0.6-0.5	3	6.1*10-5 – 7.4*10-4
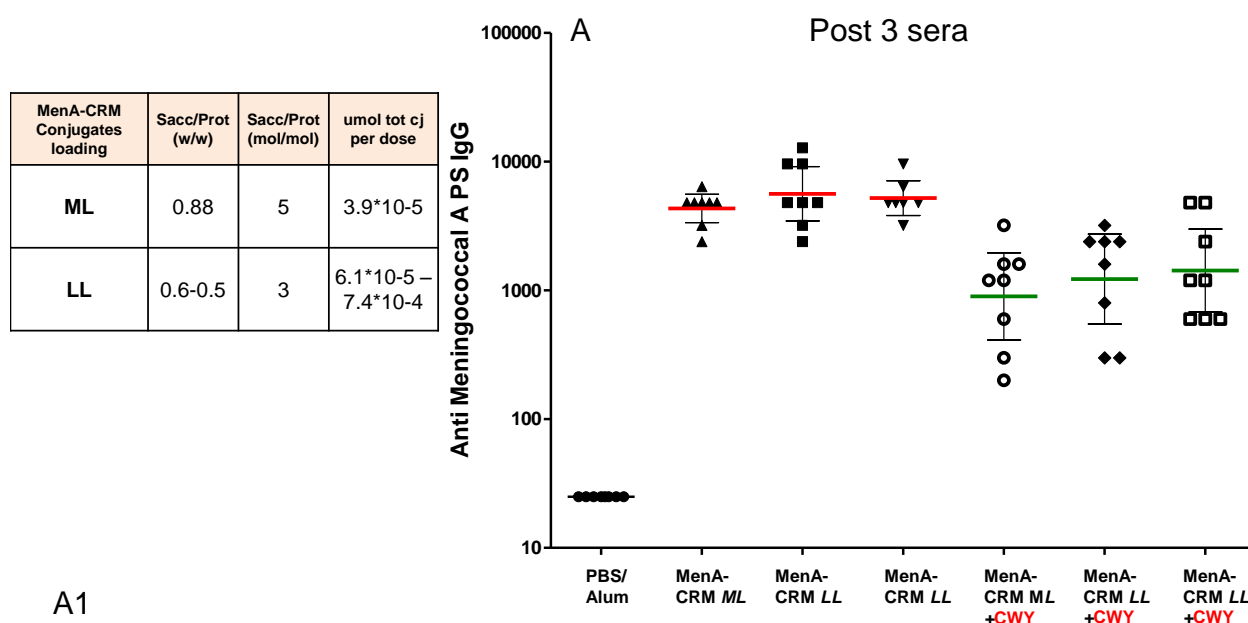
A1

rSBA titres (MenA F8238)		
	Post 2	Post 3
PBS + AlumPhosphate	<16	<16
MenA-CRM ML	4096	8192
MenA-CRM LL	8192 - 4096	8192 - 8192
MenA-CRM ML +CWY	512	4096
MenA-CRM LL +CWY	1024 - 512	2048 - 4096

avDP 15 conjugates

MenA-CRM Conjugates loading	Sacc/Prot (w/w)	Sacc/Prot (mol/mol)	umol tot cj per dose
HL	0.80	9	4.3*10-5
ML	0.42	5	8.2*10-5
LL	0.23	3	1.5*10-4

B   Post 3 sera

B1

rSBA titres (MenA F8238)		
	Post 2	Post 3
PBS + AlumPhosphate	<16	<16
MenA-CRM HL	1024	8192
MenA-CRM ML	1024	8192
MenA-CRM LL	8192	16384
MenA-CRM HL +CWY	128	2048
MenA-CRM ML +CWY	256	4096-2048
MenA-CRM LL +CWY	2048	8192

avDP 6/11 conjugates

MenA-CRM Conjugates loading	Sacc/Prot (w/w)	Sacc/Prot (mol/mol)	umol tot cj per dose
HL	0.34	8	1*10-4
ML	0.15	4	2.2*10-4

C1

rSBA titres (MenA F8238)		
	Post 2	Post 3
PBS + AlumPhosphate	<16	<16
MenA-CRM HL	1024	4096
MenA-CRM ML	2048	8192
MenA-CRM HL +CWY	1024	4096
MenA-CRM ML +CWY	<16	4096

Figure 30: Antibody (ELISA) response of monovalent and tetravalent MenA-CRM formulations. A) avDP 30/40 MenA conjugates; B) avDP15 MenA conjaugates; C) avDP6/10 MenA conjugates.

Table 17: rSBA titers of monovalent and tetravalent MenA-CRM formulations. A1) avDP 30/40 MenA conjugates; B1) avDP15 MenA conjugates; C1) avDP6/10 MenA conjugates.

2.6   Epitope mapping of a synthetic carbohydrate antigen

Another aspect that we wanted to investigate, concerning the influence of the carbohydrate moiety on the immunogenicity of glycoconjugate vaccines, is the ability of synthetic oligosaccharides to represent the corresponding epitope of the native polysaccharide structure. We focused our attention on synthetic *β-glucan* oligosaccharides.

The synthetic *β-glucans* represent the cell wall β-glucans produced by pathogenic fungi like *C. albicans.* It has been already shown that Laminarin a *β-glucans* derived from the alga *Laminaria digitata,* when conjugated with CRM197 and injected in mice, induce antibodies that react with the cell wall *β-glucans* of *C. albicans* and protect mice from the challenge with this fungus. It has also been shown that conjugates with synthetic *β-glucans* made of 15 repeating unit can also induce

protecting antibodies. We wanted therefore perform additional studies to understand the minimal chain length that could induce antibodies against Laminarin and therefore against *C.albicans* cell wall; moreover we designed experiments to understand the role of branching on the immunogenicity and specificity of the induced antibodies. With this goal in mind our carbohydrate chemistry team synthesized five *β-glucan* oligosaccharides whose structure is reported in Figure 31.
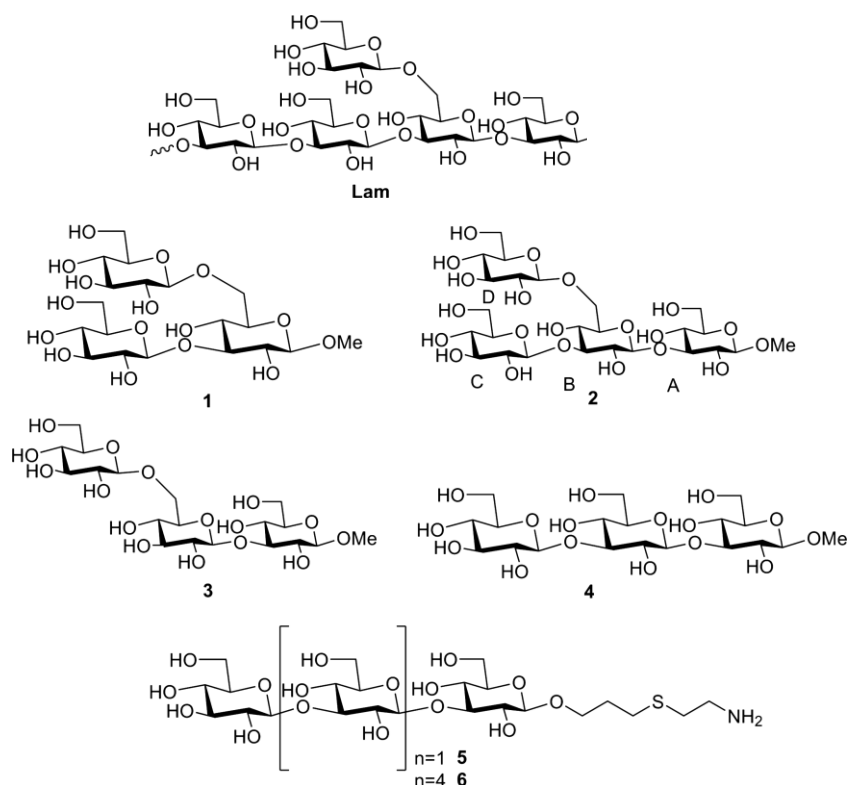
Figure 31: Chemical structure of Laminarin and synthetic fragments.

The oligosaccharides have been used as inhibitor molecules in the binding between anti-Lam Abs, induced in mice by immunization with Lam-CRM glycoconjugate, and Laminarin which were coated on the ELISA plates. The IC50 for each molecule was calculated (Figure 32). The hexasaccharide **6** was shown to be the best inhibitor, having an IC50 12-fold lower than trisaccharide **4** and 20-fold lower than tetrasaccharide **2**. Furthermore, compound **6** gave a 95% inhibition at **4** mMol concentration, while trisaccharide **4** at the same concentration reached an inhibition level of 85% and the *β*-(1,6)-branched tetrasaccharide **2** only 73%. The remaining two trisaccharides **1** and **3** exhibit no inhibition at the assay concentration.

Inhibitors	2	4	6	Laminarin
IC50	1×10^{-1}	6×10^{-2}	5×10^{-3}	7×10^{-6}
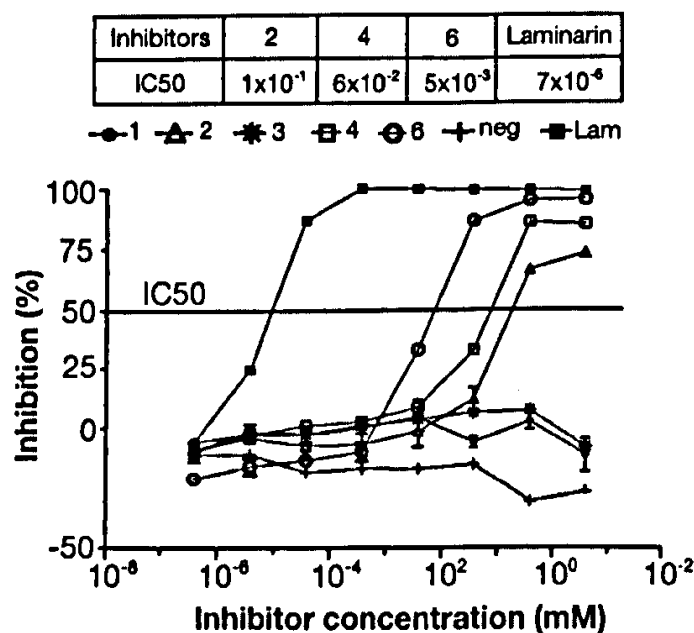
Figure 32: Competitive ELISA of synthetic glucans 1-4 and 6. Meningococcal C polysaccharide was used as negative control (neg).

From this competitive inhibition study we obtained the information that the linear *β-glucans* better represent the immunogenic epitope located on Laminarin and that a chain length of at least six sugars would be needed in order to efficiently fill the combining site of anti Laminarin antibodies. Accordingly we conjugated the hexasaccharide *6* to CRM197 and we evaluated its immunogenicity in mice. The β-(1,3) glucan hexasaccharide-CRM197 conjugate was tested in Balb/c mice for its ability to induce anti laminarin antibodies. For this purpose groups of 8 mice were immunized with 5 μg carbohydrate based dose of Lam- and Hexa-CRM197 conjugates, respectively. For Hexa-CRM197 a 1 μg dose was used as well, with the aim of studying the dose effect. Vaccines were formulated with the adjuvant MF59, an oil in water emulsion, which was shown to be effective in boosting both cellular and humoral immune response and is commonly used for seasonal flu vaccination.[78]

Adjuvant alone in phosphate buffer (PBS) was used as negative controls.

Sera were analyzed by ELISA for their content of anti laminarin IgG. After three injections, the hexasaccharide conjugate, at both 1 and 5 μg dosages, induced titers of specific anti laminarin IgG antibody significantly higher as compared to Lam-CRM197 (geometric mean titers 18101 vs 5191; p< 0.05). Interestingly, as shown in Figure 33, the immunological response of the hexasaccharide conjugate appeared to be more homogeneous in comparison with Lam-CRM.
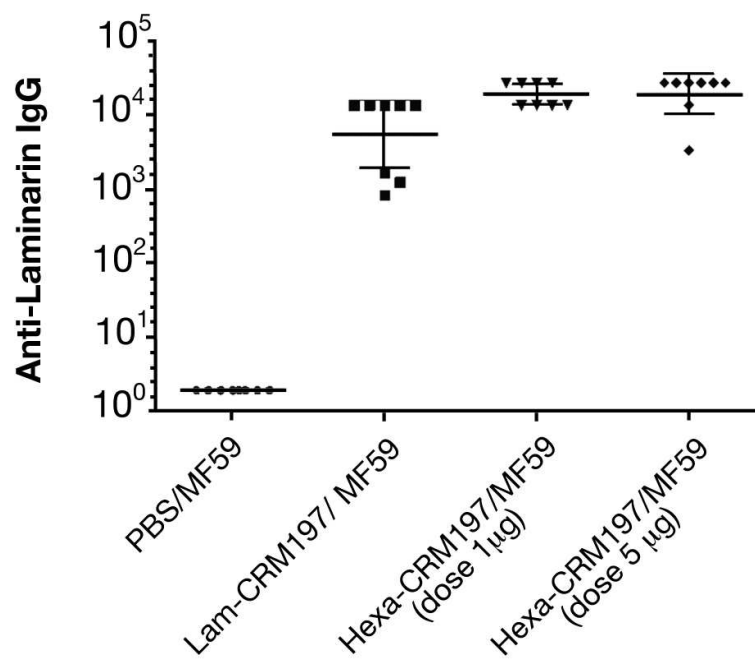
Fiure 33: Antibodies titers induced in mice by the synthetic β-(1, 3) glucan hexasaccharide-CRM197 conjugate formulated with adjuvant MF59. Horizontal and vertical bars refer to the geometric means and 95% confidence interval, respectively.

2.7 The nature of the spacer

In the manufacturing process of glycoconjugate vaccines a spacer molecule is frequently used in order to facilitate the coupling of the protein to the saccharide antigen. Usually the spacer, also called linker, is installed on the saccharide structure (Figure 34) but sometimes also on the protein or both moieties as was applied for PedvaxHIB vaccine production.[79]
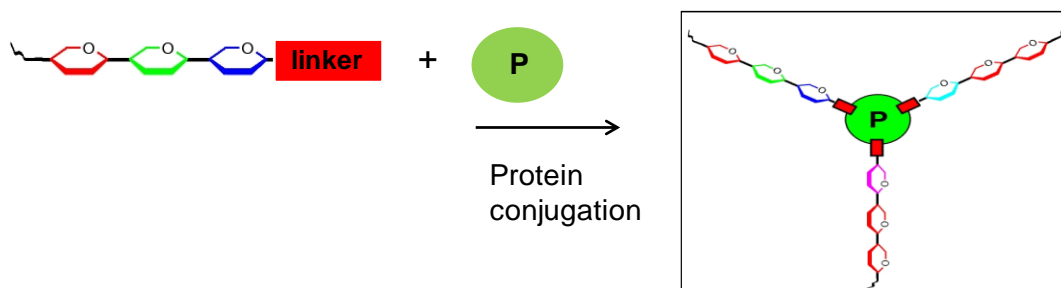
Figure 34: Schematic conjugation process with linker activation of the sugar moiety.

In this part of the PhD thesis I investigated the influence of the spacer structure on the immunogenicity of the glycoconjugate vaccine. As pilot carbohydrate antigen I have used again the serogroup A meningococcal oligosaccharides while concerning the linker I have considered four molecules with different physicochemical characteristics as reported in the Table 18. The linkers are bifunctional molecules able to react at both sides and some of these are homobifunctional while others are heterobifunctional.
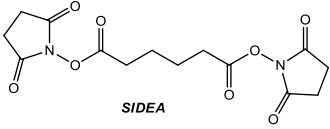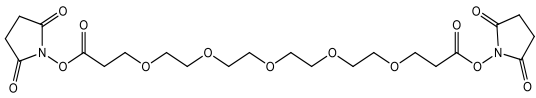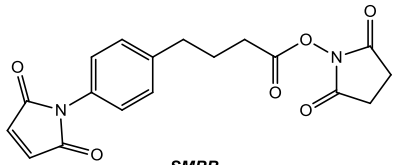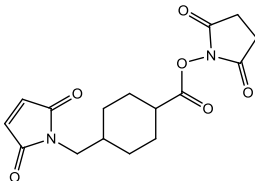
Linker	Chemical physical property	Structure
1. Succinimidyl diester of adipic acid (SIDEA)	Linear structure Hydrophobic Homobifunctional	*SIDEA*
2.Bis succinimidyl penta ethylene glycol (BisNHSPEG$_5$)	Linear structure Hydrophilic Homobifunctional	*Bis(NHS)PEG$_5$*
3.Succinimidyl4 p-maleimidophenyl butyrate (SMPB)	Rigid structure Heterobifunctional	*SMPB*
4.Succinimidyl4 N-maleimidomethyl cyclohexane-1-carboxylate (SMCC)	Rigid structure Heterobifunctional	*SMCC*

Table 18: Different linkers used to obtain MenA-CRM conjugates.

Accordingly four activated MenA oligosaccharides have been prepared with the different linker molecules, using reaction conditions which allowed the retention of a reducing group on one end of the linker thus enabling the subsequent coupling with the carrier protein CRM197.

The conjugates with the different linkers were then purified, characterized and tested in Balb/c mice for their immunogenicity.

2.7.1   Preparation and characterization of conjugates with different linkers

Serogroup A meningococcal oligosaccharide avDP15 was obtained as described in Mat&Met and briefly summarized in paragraph 2.5. The MenA-CRM conjugate with adipic acid residue spacer was obtained from the Manufacturing Department of Novartis V&D. MenA-CRM conjugate with "penta ethylene glycol" residue as spacer was prepared following the scheme reported in Figure 35A with a procedure very similar to that conventional used for MenA-CRM with adipic acid spacer in Novartis V&D.

MenA-CRM conjugate with SMPB as spacer has been prepared according to the Figure 35C. In this case the MenA has been firstly derivatized at its reducing ends with cystamine following by reduction with TCEP to provide a thiol group. The thiolated oligosaccharide was then allowed to react with the maleimido group of SMPB according to the conditions described in Mat&Met. The derived oligosaccharide was then purified by acetone precipitation, dried and tested for its content of active ester group.

The same procedure was adopted for MenA derivatization with SMCC spacer (Figure 35B).

In addition in the case of SMCC we have prepared also a conjugate where the protein was first derivatized by reaction of its lysine amino group with the active ester moiety of SMCC spacer (Figure 35D). The activation degree of CRM197 with SMCC spacer was determined by MALDI-TOF mass spectrometry and turned out to be an average 8 mol of linker /mol CRM197.

The three activated oligosaccharides were than conjugated with CRM197 in 50-100mM NaH_2PO_4 pH 7 using a molar ration 13:1 (active ester/protein).

CRM_{SMCC} was reacted with thiolated MenA oligosaccharides using a molar ratio of 10:1 SH:linker (mol/mol).

Conjugates were purified by size exclusion chromatography as described in Mat&Met.
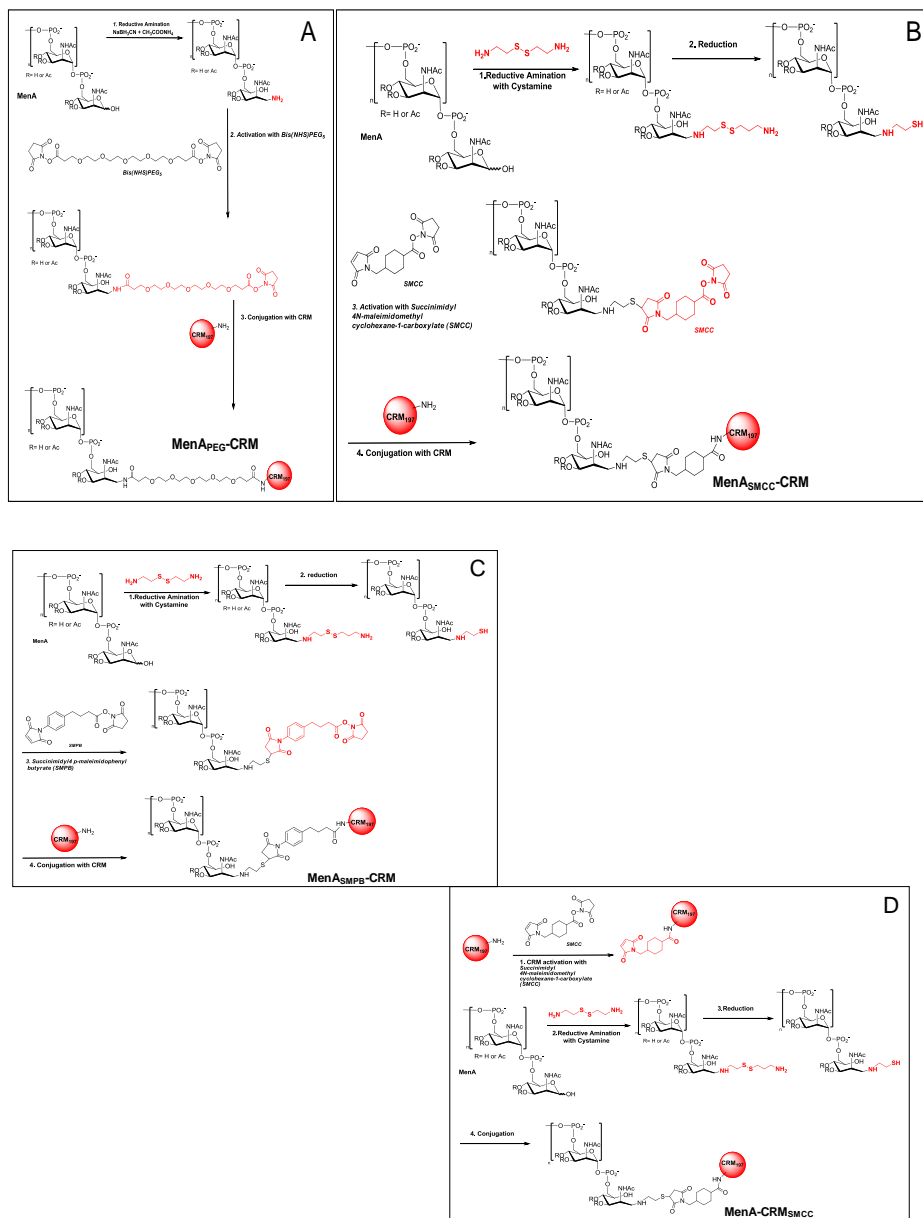
Figure 35: Scheme of reactions to obtain MenA-CRM conjugates with different linkers. A) MenA$_{PEG}$-CRM; B) MenA$_{SMCC}$-CRM; C) MenA$_{SMPB}$-CRM; D) MenA-CRM$_{SMCC}$

Conjugates with different linkers have been characterized by SDS-page and HPLC as shown on Figures 36 and 37. SDS-page analysis revealed in all cases the complete conjugation of CRM197 to the MenA oligosaccharides. HPLC analysis also confirmed the successful conjugation of CRM197, in addition this analysis indicate a lower molecular weight in the case of MenA-CRM$_{SMCC}$, MenA$_{PEG}$-CRM and MenA$_{SMPB}$-CRM as compared to MenA$_{SIDEA}$-CRM and MenA$_{SMCC}$-CRM, supporting a higher carbohydrate/protein ratio for the last two. This was also confirmed by the chemical analysis of carbohydrate and protein content of the conjugate as reported in Table 19. The higher glycosylation degree obtained for MenA$_{SIDEA}$-CRM as compare to MenA$_{SMCC}$-CRM implies that a lower number of conjugate moles are administered to mice during immunization.

1. CRM197   2. CRM197-linker   3. conjugate
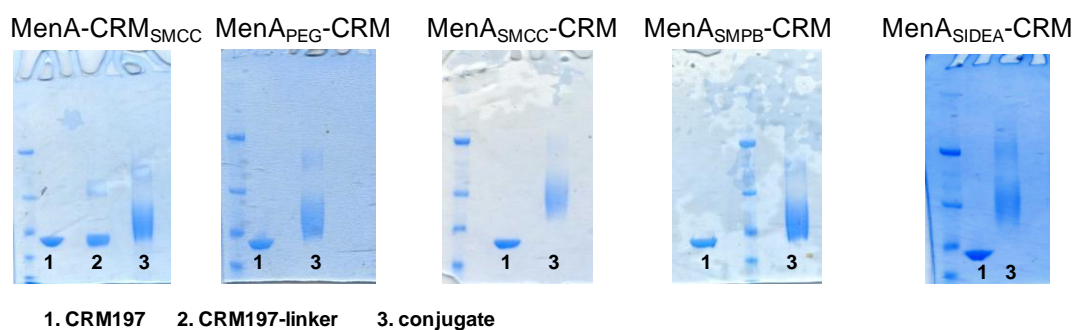
Figure 36: SDS-page of MenA-CRM conjugates obtained with different linkers.

HPLC: Size exclusion TSK3000
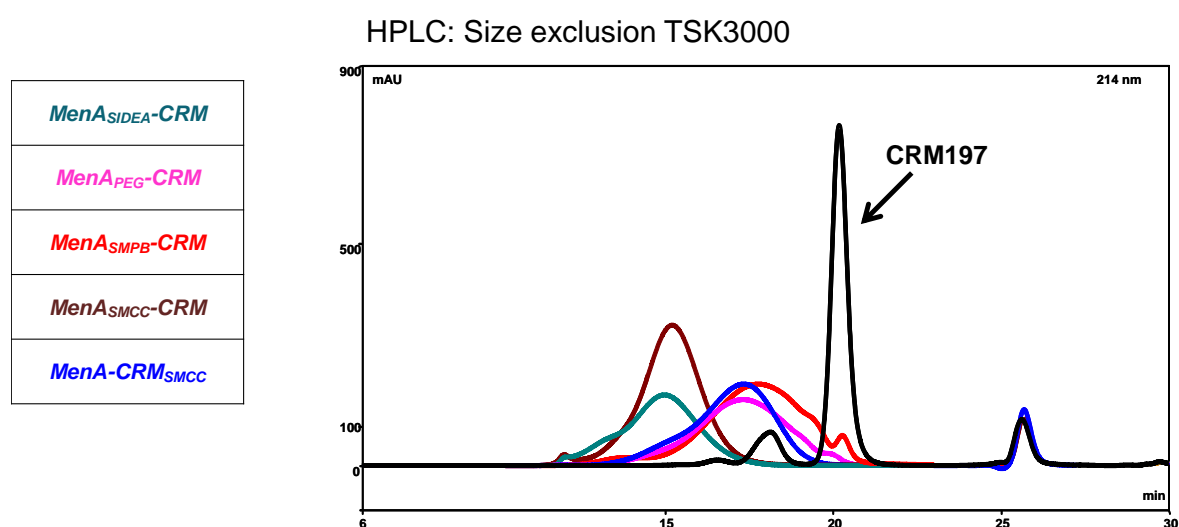
Figure 37: HPLC profile of MenA-CRM conjugates obtained with different linkers.

Conjugates	Sacc/Prot (w/w)	Sacc/Prot (mol/mol)
MenA$_{SIDEA}$-CRM	0.42	4.9
MenA$_{PEG}$-CRM	0.16	1.93
MenA$_{SMPB}$-CRM	0.15	1.84
MenA$_{SMCC}$-CRM	0.39	4.71
MenA-CRM$_{SMCC}$	0.16	1.94

Table 19: Chemical comparison of MenA-CRM conjugates obtained with different linkers.

2.7.2   Evaluation of conjugates in mouse animal model

Conjugates were evaluated in mice using the immunization schedule reported before (Figure 21, monovalent formulation with $AlPO_4$ as adjuvant).

Vaccines were used at 2µg saccharide/dose.

Anti-MenA and anti-protein response have been evaluated by ELISA and the functionality of anti-MenA antibody has been evaluated by rSBA.

Conjugates obtained with different chemistry of conjugation were able to induce a specific antibody response against the saccharide antigen MenA (Figure 38A) that was comparable between the different molecules, differences have been observed between MenA$_{SMPB}$-CRM that has induced the lower response and the two conjugates MenA$_{SIDEA}$-CRM and MenA$_{SMCC}$-CRM that induced the higher response, these differences after the third dose were also confirmed by statistical analysis using a non-parametric t test (P= 0.0041; P= 0.0034). For all vaccines a booster effect is present between the second and the third dose. The anti-MenA antibodies induced were bactericidal and while high comparability was observed at post 3, post 2 data shown that only MenA$_{SIDEA}$-CRM and MenA$_{SMCC}$-CRM were able to induce functional Ab after two doses (Table 20). Similar anti-carrier response (Figure 38B) was observed after three doses, instead at post 2 MenA$_{SIDEA}$-CRM and MenA$_{SMCC}$-CRM have induced a lower level of anti-CRM abs. MenA$_{SMPB}$-CRM that have induced the lowest anti-MenA response have induced an higher level of anti-protein Ab.
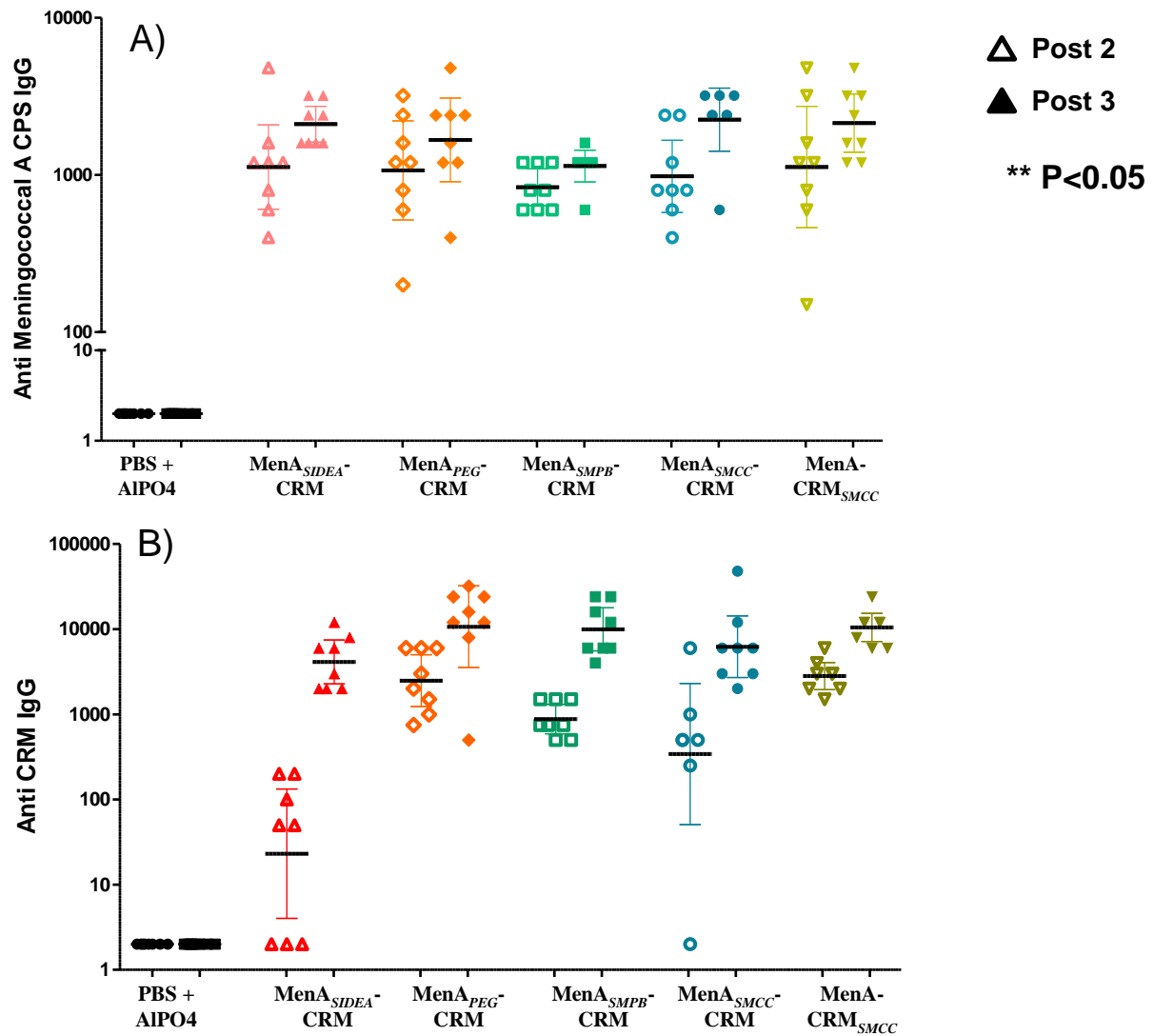
Figure 38: Antibodies response induced by MenA-CRM conjugates obtained with different linkers after the 2nd and the 3rd dose of vaccines. A) Anti-Serogroup A meningoccocus CPS IgG response; B) Anti-CRM IgG response.

rSBA (MenA)		
Conjugates	**Post 2**	**Post 3**
MenA$_{SIDEA}$-CRM	1024	8192
MenA$_{PEG}$-CRM	8	4096
MenA$_{SMPB}$-CRM	8	4096
MenA$_{SMCC}$-CRM	512	4096
MenA-CRM$_{SMCC}$	1024	4096
PBS\AlPO$_4$	8	8

Table 20: rSBA titers of the elicited antibodies with MenA-CRM conjugates with different linkers, post the 2nd and the 3rd dose of vaccines.

2.8   Labelling of glycoconjugate

Glycoconjugates are among the safest and most efficacious vaccines developed in the last 30 years. Many papers have been published concerning their mechanism of action; however a complete understanding of the uptake and processing by the immune system has not been reached yet. We decide therefore to perform some experiments in order to study some still poorly investigated aspects of their mechanism of action. In particular we focused our attention on: cell recruitment after glycoconjugate administration; type of cells involved in the uptake of the glycoconjugate antigen; intracellular localization of the antigen after uptake.

In order to carry out these interrogations we needed to track the antigen during uptake and internalization/processing events. Therefore we needed to label the conjugate antigen with a fluorescent dye suitable for fluorescence activated cell sorting analysis (FACS) and confocal microscopy. MenC-CRM was selected as glycoconjugate model and this molecule was double labeled in order to be able to track both the protein as well as the carbohydrate moiety.

To obtain the double labelled glycoconjugate, MenC oligosaccharides were activated and labelled with A488, as reported in Mat&Met and subsequently were conjugated with CRM197 as reported. After conjugation the second labelling with A647 has been done on the protein moiety. The scheme of reaction to obtain MenC-CRM with double labelling (MenC-CRM dl) is reported in Figure 39.
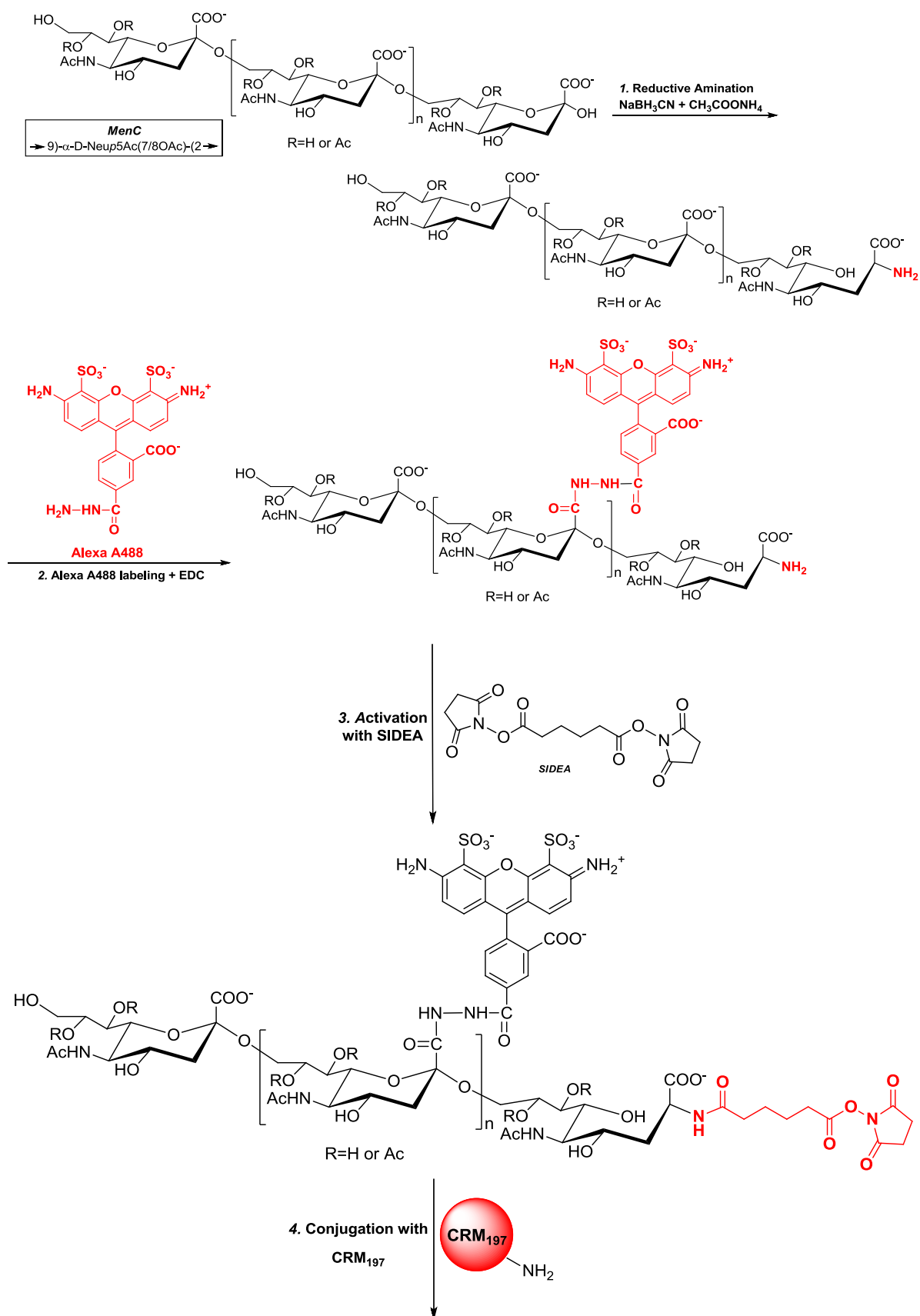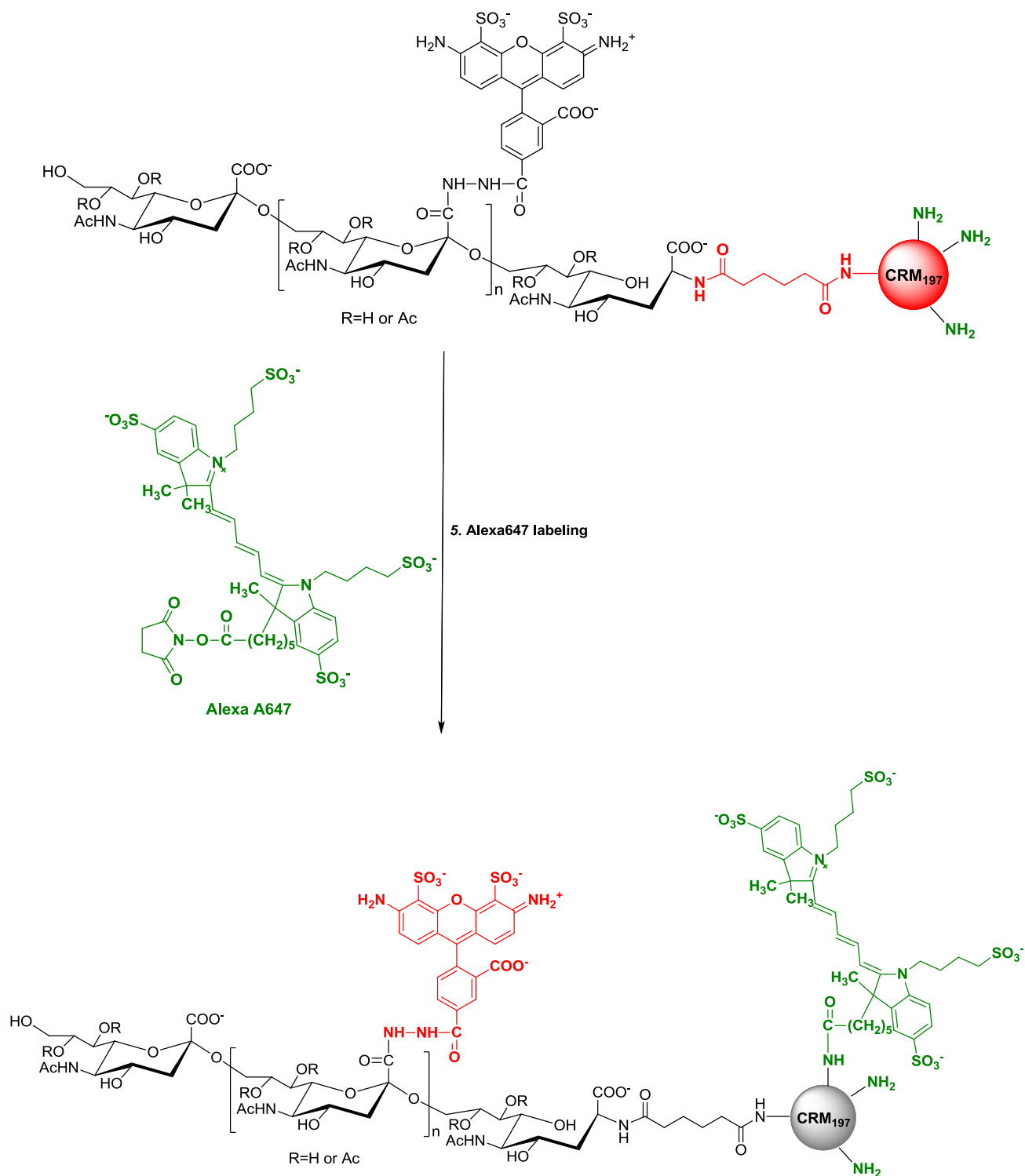
Figure 39: Scheme of reaction to obtain MenC-CRM conjugates with double labeling with A488 and A647.

Labelled glycoconjugate was characterized by SDS-page as reported in Figure 40: coomassie staining of MenC/A488-CRM and UV exposure of the double labelled MenC/A488-CRM/A647.

1. CRM$_{197}$
2. MenC/A488-CRM
3. MenC/A488-CRM/A647

MenC/A488-CRM   MenC/A488-CRM/A647   MenC/A488-CRM/A647 UV exposition
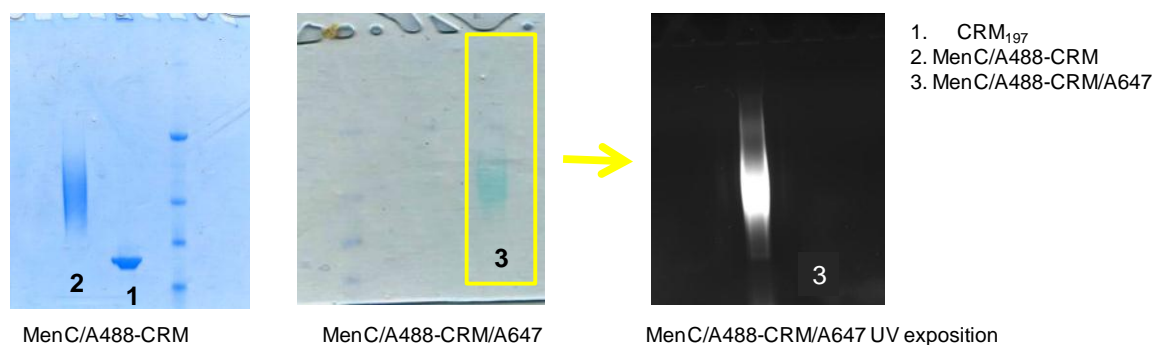
Figure 40: MenC-CRM dl SDS-page characterization.

Labelled conjugates have been characterized by spectrophotometric analysis between 200 and 800 nm and two peaks have been determined at 495 and 650 nm that are the typical wave lengths of the dyes A488 and A647 (Figure 41).
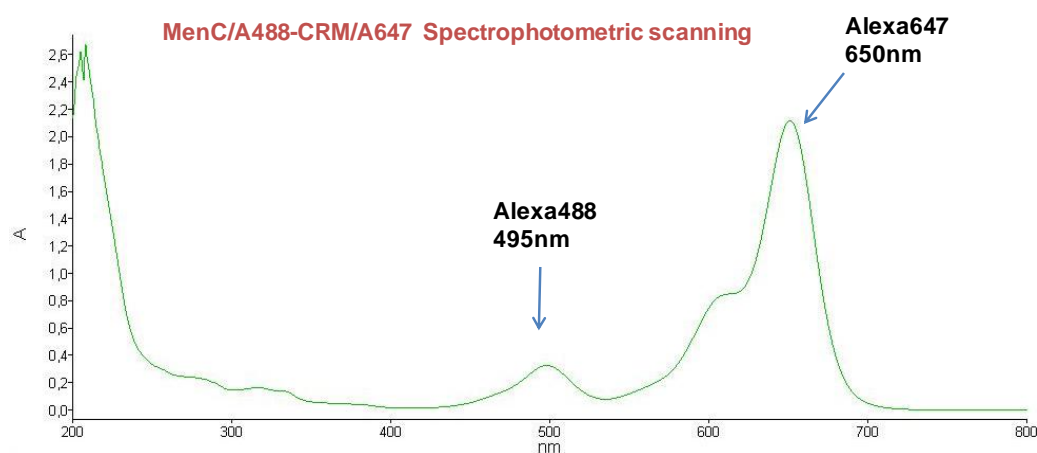
Figure 41: Spectrophotometric analysis of the labelled conjugates.

The double labelled conjugate was characterized after purification for the saccharide and protein content and degree of labelling as reported on the Table 21. A glycosylation degree of 0.4 w/w has been obtained, a degree of labelling (DOL) of 0.5 on the sugar moiety with A488 and of 7 on the protein moiety with A647. DOL has been calculated as reported in Mat&Met applying the equation reported below

DOL= (Amax * MW)/[sample] * $\varepsilon_{dey.}$

Conjugate	Sacch/prot w/w	DOL488	DOL647
MenC/A488-CRM/A647	0.4	0.5	7

Table 21: MenC-CRM dl characterization by saccharide/protein ratio w/w and DOL.

2.8.1 In- and ex- vivo antigen uptake characterization

We took advantage of the MenC-CRM dl glycoconjugate to investigate the internalization process in vitro and ex vivo, mainly focusing on the characterization of cell types involved in antigen uptake and internalization.

Briefly, Balb/c mice were treated intraperitoneally with MenC-CRM dl and peritoneal wash samples were collected at different time point post injection (0, 30 min, 6 h and 24 h). The cells in these samples were fixed and stained for the presence of specific cellular markers, enabling the identification of macrophages, B cells, monocytes, neutrophils and eosinophils.

In Figure 41 we show the cell recruitment into the peritoneum after GC injection. The cell type that is recruited at highest numbers are B cells, showing a massive influx immediately at 30 minutes p.i. This increase is maintained during the course of the analysis. Neutrophils show a peak of recruitment at 6 h and then decrease rapidly back to basal level. Macrophages slightly increase at 30 min p.i and then are maintained for up to 24 h.
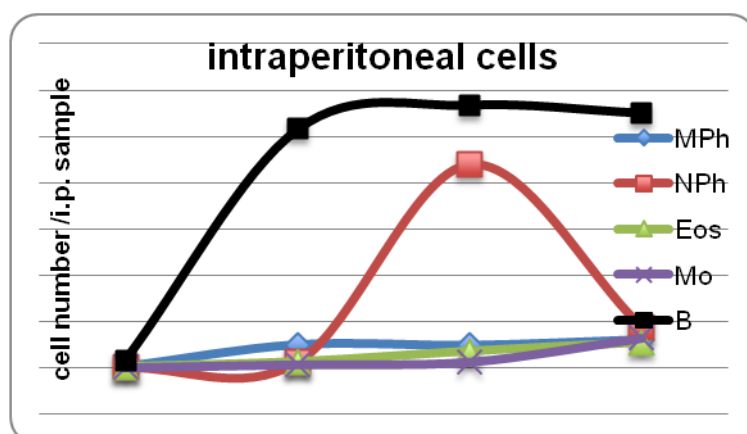
Figure 41: Graphical representation of cell recruitment after intraperitoneally mice treatment with glycoconjugate (GC).

The same samples were also analyzed for antigen uptake and the results are reported in Figure 42 A and B. Professional antigen presenting cells (APC) like macrophages and B cells rapidly take up the labeled glycoconjugate. Uptake by macrophages is generally fast, non-specific and antigen will most likely be processed and presented on MHC-II molecules to T cells. The fast uptake was also observed in our setting: macrophages were found to be Ag-positive at already 30 min post injection and remain positive up to 24 h. In contrast, antigen uptake by B cells is slower and generally mediated by ligation of the specific B cell receptor. Accordingly, only a minor cell fraction of B cells stains positive for the glycoconjugate at any time point. Also monocytes are recruited and become Ag positive between 30 min and 6 h post injection, but their number is quite low and does not contribute in a major way to antigen uptake.

Besides professional APC, some cells of the innate arm of the immune system like eosinophils or neutrophils can bind antigen on their surface and act as antigen transporters. Eosinophils are recruited and most cells become antigen positive, instead neutrophils were recruited but most cells did not become antigen positive.

In conclusion, the major contribution to antigen uptake in our system comes from macrophages.
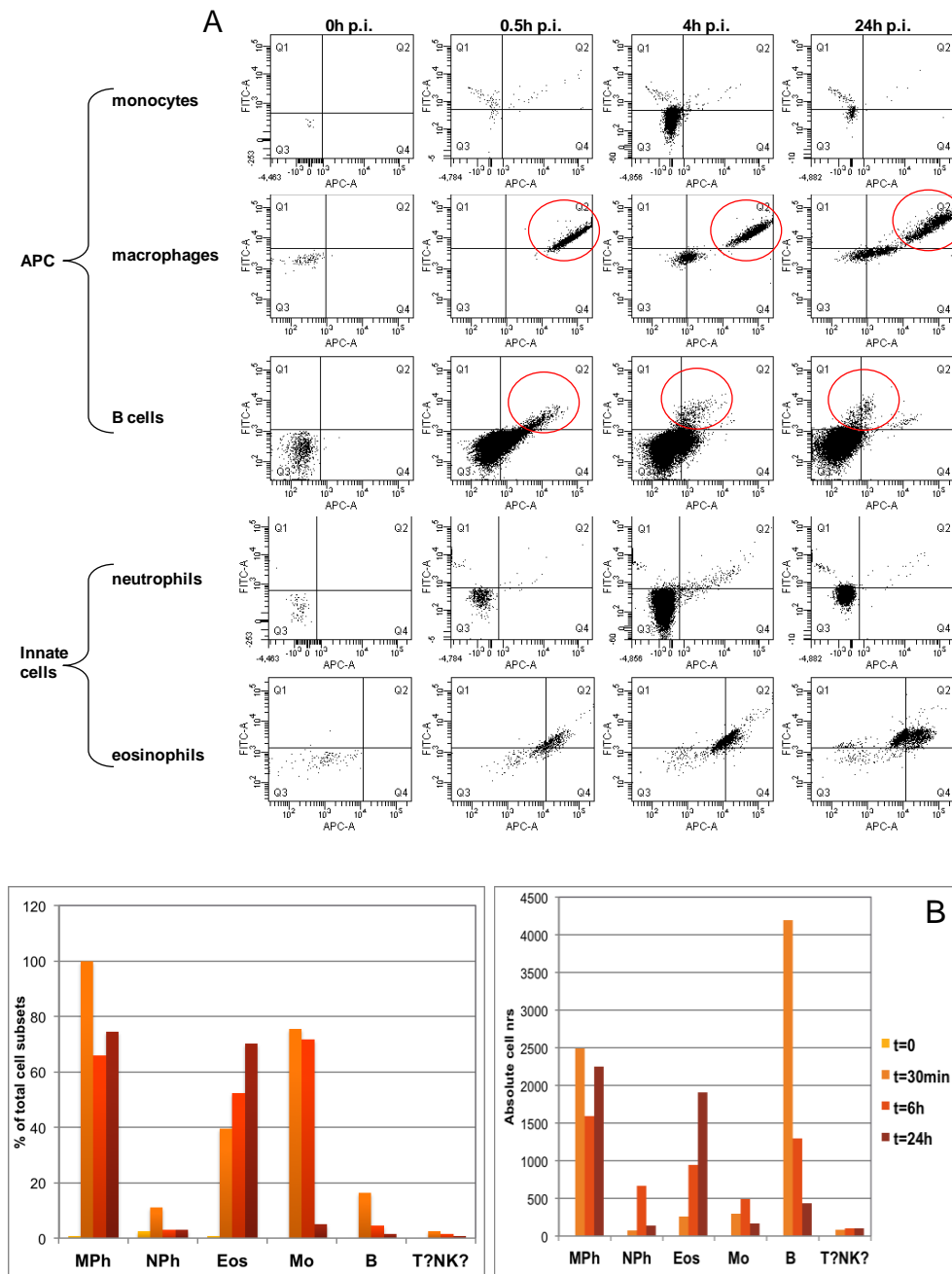
Figure 42 A and B: antigen uptake experiment by FACS. A) FACS analysis conducted at different time. B) Graphical representation of antigen uptake experiment, data were expressed both as % of total cell subset and as absolute cell number at different time.

To characterize the intracellular localization of the antigen at the cellular level, cells from IP wash samples at 24 h post injection. were analyzed by confocal microscopy. Briefly, cells were fixed, permeabilized and stained with specific cellular marker, MHCII and Lamp1. We were able to identify at least 2 distinct populations (Figure 43 A, B and C):

1. Cells with high antigen content, but poorly expressing MHCII on their surface (A). In these cells MenC-CRM dl is intracellular and co-localizes with Lamp1, a marker for the late endosomal compartment (B). These cells are generally larger than other Ag-positive cells and we speculate that they could represent macrophages that, referring to general mechanism of antigen uptake, they remain longer antigen positive and are very active in the uptake mechanism.

2. Cells with lower antigen content. In these cells MenC-CRM dl localizes on the cell surface with some regions of co-localization with MHCII. These cells could represent B cells, where antigens are loaded on MHCII molecules (C).
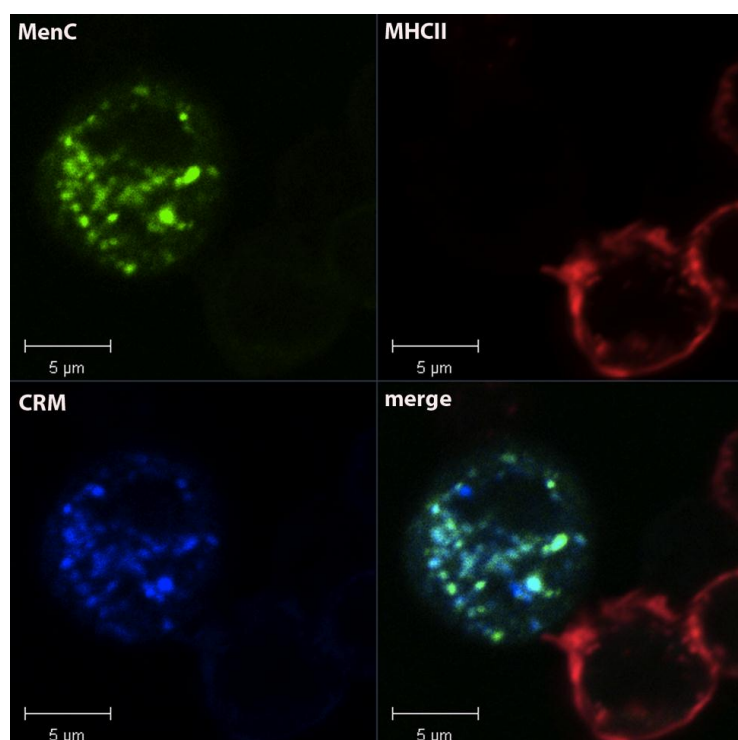
Figure 43A: Confocal microscopy experiment. Cells with high antigen content, but poorly expressing MHCII on their surface.
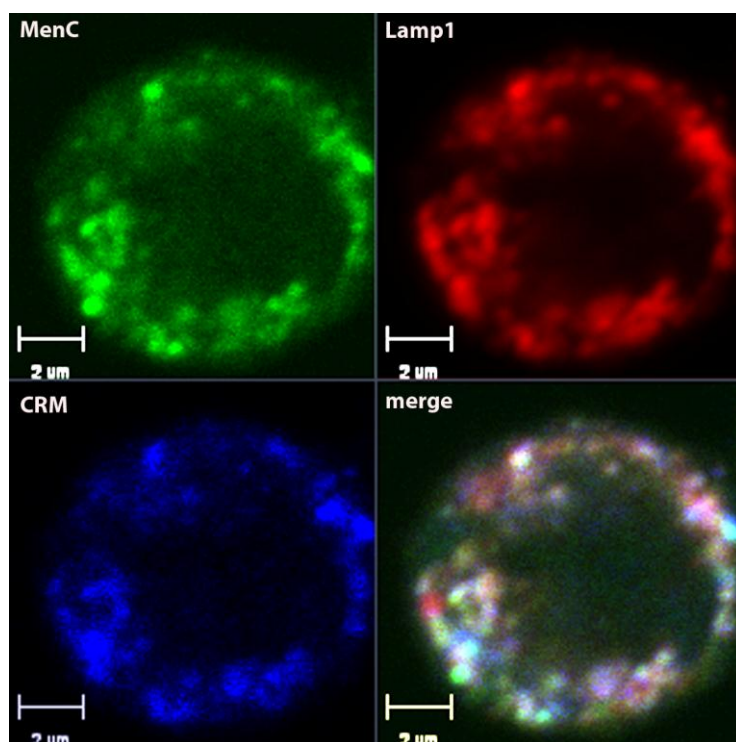
Figure 43B: Confocal microscopy experiment. Cells where the antigen is intracellular and co-localizes with Lamp1.
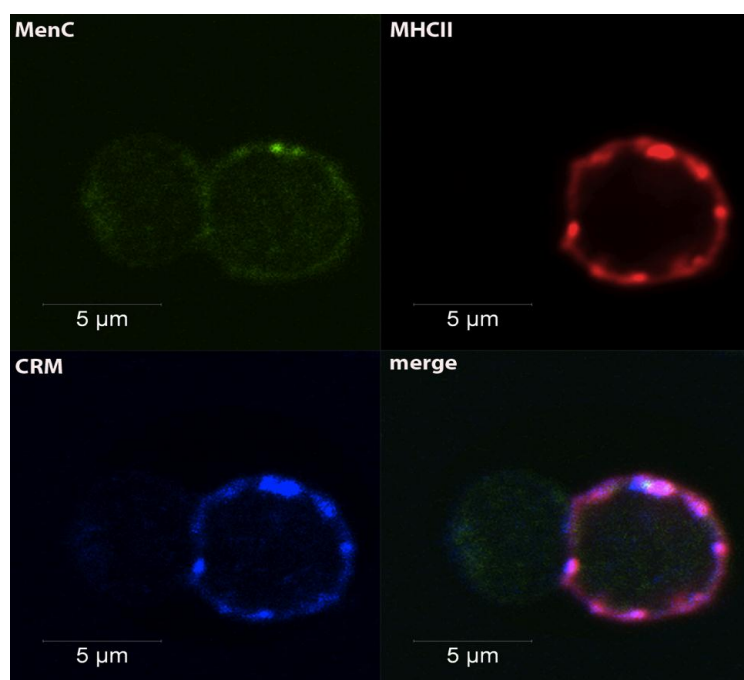
Figure 43C: Confocal microscopy experiment. Cells with lower antigen content where the antigen localizes on the cell surface with some regions of co-localization with MHCII.

Notably, in both cell types, we can observe a complete co-localization of protein with the sugar moieties, indicating that during the GC processing the two components, the protein and the sugar moiety, tend to stay in the same cellular compartments.

3 Discussion

Glycoconjugate vaccines are among the safest and more efficacious vaccines developed during the last 30 years. They have been developed to improve the immunogenicity of polysaccharide vaccines which are T-cell independent, poorly immunogenic in infants and young children < 2 years of age [56] [80] and do not induce immunological memory.[81]

There is no other example where the introduction of a new approach to vaccine development has had such a rapid and positive effect in preventing infections by a series of human pathogens.[31]

The Hib conjugate vaccines, the prototypes of successful conjugate vaccines, have resulted in the virtual eradication of disease due to Hib in the United States and much of the developed world.[82]

Given the success of Hib vaccine other glycoconjugate vaccines have been successfully developed against other pathogens such as *Neisseriae meningitidis*, *Streptococcus pneumoniae* and they are currently present on WHO schedule of immunization.

The introduction of anti-pneumococcal glycoconjugate vaccines has demonstrated the utility of these vaccines in pediatric and other high-risk populations, in reducing the carriage rates of pneumococci, and the prevalence of antimicrobial-resistant organisms.[83] [84] [85] [86] [87]

The introduction of a monovalent, group C meningococcal polysaccharide–protein conjugate vaccine in England in late 1999 led to an initial dramatic decrease in the incidence and mortality of meningococcal disease in targeted pediatric populations. Similar results were demonstrated elsewhere in Europe over the last decade.[88] [89] [90]

The development of glycoconjugate vaccines has allowed the investigation of their immunogenicity in preclinical and clinical studies in relation with their chemical physical properties. In addition glycoconjugates have been introduced in vaccines combinations that are now part of human immunization schedules.[34] [91] [92]

Moreover the success of glycoconjugates vaccines has triggered studies on new possible application such as therapeutic vaccines for cancer, Alzheimer and conjugates vaccines for drug abuse, which are currently under development.[93] [94]

Ideally, knowing the immunogenic properties of many conjugates vaccines that have been developed and the understanding of their mechanism of action, a rational design of a glycoconjugate could be possible. On the other hand data obtained until now show that many variables can affect the immunogenicity of a conjugate vaccine and they have to be tested and optimized whenever a new vaccine will be developed.

Glycoconjugate vaccines developed so far have different characteristics depending on their carbohydrate antigen, the carrier protein and the conjugation chemistry. All these aspects confer to the glycoconjugate different physic-chemical characteristics that may result in different immunological properties.

This PhD work is fitting into this framework by proposing an investigation of the three main moieties that could influence the immunogenicity of a glycoconjugate vaccine: the protein carrier, the sugar moiety and the chemistry of conjugation in particular studying the spacer moiety.

3.1 The influence of the carrier protein

A number of protein carriers have been used so far in preclinical and clinical evaluation of conjugate vaccines. Diphtheria and tetanus toxoids (DT and TT), CRM197, the outer membrane protein complex of serogroup B meningococcus (OMPC) and Hib-related protein D (PD) are the proteins used so far in licensed conjugate vaccines. OMPC has been used as carrier for *Haemophilus influenzae* (Hib) capsular polysaccharide (PRP) which is immunogenic in children as young as 2 month of age. DT has been used as carrier for the first Hib vaccine and subsequently to develop a quadrivalent meningococcal ACWY vaccine which was proven to be safe and immunogenic in children and adolescents. CRM197 has been successfully used as carrier for many conjugate vaccines against Hib, Pneumococcus (7 and 13 valent) and recently for a tetravalent (ACWY) meningococcal conjugate vaccine which prove to be immunogenic in all classes of age. TT, besides Hib and MenC, has been used also as carrier for a tetravalent meningococcal vaccine that induces equal or higher SBA persistence in adolescents when compared with the PS vaccine. PD has been used as carrier to develop the 10-valent pneumococcal nontypeable *Heamophilus influenzae* (PHiD-CV) and the usage of this protein as carrier might provide additional protection against acute otitis media due to pneumococcus in infants and children.[55][95] [96][97][98]

Studies to obtain an optimized carrier protein which contains selected human T cell epitopes have been published. A rationally designed recombinant protein containing strings of promiscuous human CD4+ T-cell epitopes derived from various pathogens including tetanus, influenza virus, *Plasmodium falciparum* and hepatitis B virus, proved to be a very good carrier for Hib and meningococcal oligosaccharides.[99][100]

Other carrier proteins such as keyhole limpet hemocyanin (KLH) and the virus like particle Qb are taken into consideration in developing conjugate vaccines for non-infectious diseases.[101]

We have conducted a screening study on different bacterial proteins to investigate their ability to behave as carrier for different saccharide antigens. Data obtained have shown that only 8 among 31 proteins tested were able to induce a specific IgG response against the model carbohydrate antigen

Laminarin. These proteins were additionally tested with a different saccharide antigen, the meningococcal serogroup C oligosaccharide, and four of them, PNEU20, EX1, EX2 and GAS1, confirmed their functionality as carrier. In particular the protein PNEU20 turned out to be the best carrier and when it was conjugated to meningococcal serogroups ACWY oligosaccharides, the resulting conjugates induced, in mice, a response comparable with that of CRM197 based meningococcal ACWY oligosaccharides. The antibodies elicited by PNEU20-MenACWY conjugates were also functional when tested by rSBA.

We were therefore able to identify a new candidate carrier protein, PNEU20, which works as carrier for different carbohydrate antigens, inducing high titers of anti-carbohydrate specific and functional antibodies.

A number of clinical trials have been conducted to compare the immunogenicity of different conjugate with different carrier proteins. Comparison of four Hib conjugate vaccines developed with four different carrier proteins resulted in differences of antibody response generated in infants: DT conjugate (PRP-D) was the less immunogenic when compared with TT and CRM197 PRP conjugates. PRP-CRM and PRP-TT were the most immunogenic and have induced a similar level of anti-PRP antibody. PRP-OMP was the only able to produce a significant immune response after one injection, it also performed well after two injection but a similar antibody levels was induced after three injection, with final levels one/third of those achieved by PRP-CRM and PRP-TT. Additional studies in infants residing in different geographic areas, confirmed that OMP conjugate induces significant antibody levels after a single injection at 2 months of age. Studies published to compare meningococcal tetravalent vaccines obtained using CRM or DT as carrier protein showed that in adolescents MenACWY-CRM generates a robust immune response agains all serogroups, with a response noninferior for serogroup C and a statistical significant superiority for serogroups AWY, compared with MenACWY-DT vaccine. Comparing the two vaccines also in children aged 2-10 years, the MenACWY-CRM vaccine was non inferior to MenCAWY-DT for all four serogroups, and met statistical superiority for groups CW and Y. [67 68 69 71]

But a direct comparison of the influence of different protein carriers on glycoconjugates immunogenicity is difficult due to the coexistence of other variables such as conjugation chemistry, saccharide chain length, adjuvant, formulation technology, and previous or concomitant vaccination with other antigens. [34]

We have therefore performed a preclinical comparison between the three main carrier proteins, CRM197, TT and DT which have been conjugated with the same conjugation chemistry to serogroups A, C, W135 and Y meningococcal oligosaccharides.

This study allowed to do a comparison of the physico-chemical characteristics between the conjugates obtained using the three different proteins firstly. Despite applying the same chemistry of conjugation and stoichiometry, we observed that using CRM197 we were able to obtain conjugates with higher glycosylation degree. This could be due to differences between the proteins derived from the manipulation that DT and TT undergo during chemical detoxification. In fact diphtheria and tetanus toxins are detoxified often directly in culture medium by treatment with formaldehyde and lysine. This complex reaction involves also the lysine residues of the toxins through the formation of Schiff bases which are further stabilized by the reaction with other residues like imidazole and tyrosine rings. The products are cross-linked and heterogeneous species which might be less reactive and contain reduced number of of lysines available for conjugation. [102]

Since CRM197 is a non-toxic protein and does not require the chemical detoxification, the protein has a well defined physico-chemical profile. Consequently CRM197 allows the preparation of defined glycoconjugates.

The immunogenicity evaluation in mice of the different conjugates has shown that all carriers were able to induce a specific antibodies response against each saccharide antigens. When tested without the adjuvant all responses were affected by high variability.

Immunogenicity studies with adjuvant showed that the proteins induced a comparable response against each saccharide antigen and for all vaccines we observed a significant booster effect after the third dose of immunization. No significant differences have been observed between the responses of the different vaccines except that for serogroup A meningococcus conjugates: in fact, when tested as monovalent vaccines, MenA-CRM conjugate induced, after the third dose, an antibody titer significantly higher than that of MenA-TT (***[3] P= 0.0002) and also MenA-DT (**[3] P< 0.008). Tetravalent formulations confirmed the significant difference between MenA-CRM and MenA-TT after three doses (**[3] P< 0.008). The elicited antibodies were also functional when tested by rSBA. When anti carrier antibodies were determined, CRM197 appeared to be the less immunogenic.

This study demonstrates that the three common carriers CRM197, DT and TT are comparable in inducing a specific response against four different saccharide antigens, in mice. At the same time CRM197 was confirmed an excellent carrier also considering its physico-chemical characteristics of high pure protein with non-cross linked structure and consequently amenable to better characterization.

3.2 The influence of the sugar moiety

The sugar moiety of glycoconjugate vaccines is key to the induction of pathogen specific functional antibodies.

Many studies have been published about the influence of the carbohydrate on the immunogenicity of glycoconjugate vaccines,[49] [50] [103] [104] however it has not been possible until now to identify predetermined characteristics for their optimal immunogenicity. Data published suggest that saccharide chain length and saccharide loading onto the protein could influence the glycoconjugate immunogenicity.

To further investigate these parameters we used meningococcal serogroup A saccharide, as model. Three different saccharide average chain lengths (avDP6/10, 15 and 30/40) were obtained applying ad hoc purification of the oligosaccharides. The oligosaccharides were activated and then conjugated to CRM197 using reaction stoichiometries which allowed to obtain products at average glycosylation degree varying from 9 to 3 moles of saccharide per mole of protein. Depending on the oligosaccharides length some limitations in the protein loading were found, for example loadings higher than 5 (mol/mol) could not be obtained when using the longer saccharides, while on the other hand, loadings lower than 4 (mol/mol) were not achievable with the shorter oligosaccharides. This behavior probably reflects a better reactivity of the activated short oligosaccharides which can be explained by a reduced steric hindrance and/or a higher diffusion rate as compared to the longer saccharide chains.

The immunological evaluation in mice of these conjugate in both, monovalent and tetravalent formulations, has showed that in all cases we have induced the specific response against the serogroup A meningococcus capsular polysaccharide, and the elicited antibodies were functional when tested by rSBA.

The immunogenic properties of the different vaccines seem not having been affected by the saccharide average chain length, although is difficult to draw conclusions due to the overlapping of the sugar population. Considering the carbohydrate loading, a trend to a lower response could be observed for the conjugate with high glycosylation degree and this might be related to the lower number of conjugate moles administered to mice using constant saccharide based dosages.

In this part of the PhD work we showed that in the case of serogroup A meningococcus, as already reported for other carbohydrate antigens[102], short chain length oligosaccharides (avDP6/10) are sufficient to induce high antibody titres, with bactericidal activity against the serogroup A meningococcus, in mice.

Short oligosaccharides antigens could therefore be attractive as candidate vaccines considering the possibility to obtain them via chemical synthesis.

Currently, most of the conjugate vaccines are produced from poly- or oligosaccharides extracted and purified from microbial cultures. They can be obtained with very high purity, although heterogeneity and traces of bacterial contaminants (endotoxins, variants containing non-protective epitopes, etc.) cannot be avoided. The chemical synthesis of oligosaccharides instead provides homogeneous, well defined and characterized molecules with built-in chemical terminal functionalities suitable for conjugation to carrier proteins.[34]

An attractive concept for future conjugate vaccines, although challenging, is based on fully synthetic glycopeptide vaccines where not only the hapten moiety is obtained by chemical synthesis but also the carrier is constituted by synthetic peptides representing CD4+ T-cell epitopes.[105] [106]

We investigated five synthetic *β-glucan* oligosaccharides for their ability to represent the natural epitope of Laminarin antigen. By competitive studies we showed that linear *β-glucans* better represent the immunogenic epitope located on Laminarin and that a chain length of at least six sugars would be needed in order to efficiently fill the combining site of anti Laminarin antibodies. Our observation was than confirmed by immunogenic study in mice were the hexasaccharide antigen conjugated with CRM197 have induced a comparable response with that induced by the native conjugate with Laminarin. Synthetic *β-glucan* hexasaccharide could be then considered as a new candidate vaccine for *C.albicans*.

3.3   The influence of the spacer molecule

Data present in the literature have shown that also the spacer molecule could have a role in driving the immunogenicity of the glycoconjugate vaccine.[51] [52] [53]

We studied therefore four different linker molecules having different chemical physical aspects, maintaining the strategy of conjugation and the carbohydrate antigen, the serogroup A meningococcus.

Conjugation strategy for each spacer molecule involved the saccharide end reducing group and conjugates with similar glycosylation degree have been obtained except that for SMCC when used to activate the sugar moiety and SIDEA that allow to obtain glycoconjugate with higher saccharide/protein ratio.

Immunogenicity data have shown that all conjugates were able to induce an anti-MenA polysaccharide response and the higher response has been obtained with MenA$_{SIDEA}$-CRM and MenA$_{SMCC}$-CRM that have induced bactericidal antibodies also after the second dose of vaccine. On

the contrary significant lower response has been induced by MenA$_{SMPB}$-CRM. Otherwise evaluating the anti-carrier antibodies induced by MenA$_{SMPB}$-CRM we can see that are comparable with those induced by the other conjugates after three doses. This observation could allow us to conclude that rigid molecules containing aromatic rings might not be the optimal linkers for conjugate vaccines. It has been already shown that the rigid linker containing the aromatic ring induces very high anti linker antibodies[51], this could cause a reduction on the anti-carbohydrate antibodies induction.

To study more in deep this aspect, future work should be focused on determination of anti-spacer antibodies induced by each type of conjugate to find whether a correlation exists between the anti-carbohydrate response and the intrinsic immunogenicity of the spacer molecule.

3.4   Investigation on antigen uptake

In another context of this thesis we have investigated the immune cells recruitment and the processing and re-exposure of glycoconjugate antigens in order to get information about the mechanism of action of this class of vaccines. For this purpose, CRM-MenC has been used as molecular model, and particularly it has been double labeled in order to track both protein and carbohydrate moiety during glycoconjugate processing.

Data obtained with cell recruitment experiments shows that professional antigen presenting cells (APC) like macrophages and B cells rapidly take up the labeled glycoconjugate. Uptake by macrophages is fast, non-specific and antigen will be processed and presented on MHC-II molecules to T cells. In contrast, antigen uptake by B cells is slower and only a minor cell fraction stains positive for the glycoconjugate. Also some cells of the innate arm of the immune system like eosinophils or neutrophils can bind antigen on their surface and act as antigen transporters. Yet, the major contribution to antigen uptake in our system comes from macrophages.

Intraperitoneally treatment with double labeled glycoconjugate has induced immune cells activation in mice as expected for a general immunity mechanism consequent to the antigen. Macrophages occupy a position of central importance in the immune response, since they are key effectors in both the innate and adaptive systems. They play a role in the inflammatory response during the early stages of host innate defense, phagocytosing foreign entities and secreting chemotactic factors and cytokines, after they function as accessory cells to the lymphocytes of the adaptive immune response, secreting pro-inflammatory cytokines and also processing and presenting antigen to T cells. Macrophages act also as effectors in the humoral immune response and in cell-mediated immunity.[107]

Also with confocal microscopy experiments we were able to distinguish two different types of cells, those with higher amount of antigen could be still referred to macrophages due to their prolonged

role on the antigen uptake; in these cells the antigens co-localized with Lamp-1, a lysosome-associated membrane proteins demonstrated to be present on the phagosomal membrane.[108]

Instead in the cells with lower antigen content we identified some region where both the peptide and the carbohydrate moieties are co-localized with the MHCII. Likely these are B cells where antigens are loaded on the MHCII for further interaction with T cells. Similar results have been obtained also by other research teams.[109]

The mechanism of action of glycoconjugate vaccines on the immune system, in particular about the function of the sugar moiety during antigen presentation in association with MHCII complex and subsequent interaction with T cell receptor, is still unclear. Knowing that CPSs are T independent antigens[13] and that most of the carbohydrate antigens are not able to bind MCHII after APC processing[110], the elicitation of T cell help by glycoconjugates has been attributed to the peptide moiety derived from protein processing.

Recently a different mechanism has been proposed by Kasper et al. In their model there are T-cell populations that recognize carbohydrate epitopes derived by APC processing of conjugate vaccines. When presented in association with MHCII, these epitopes recruit T-cell help for the induction of specific anti carbohydrate antibodies.[111]

This attractive model has been derived with GBS type III glycoconjugates and needs further investigations to understand if it is a general mechanism. Our results show that the carbohydrate portion of MenC glycoconjugate is exposed on the surface of B-cells in association with a peptide moiety and the MHCII; we cannot exclude that the carbohydrate further interacts with specific T-cell receptors, however additional experiments, for example isolation of a MenC specific T cell clone, are needed in order to confirm this.

3.5 Suggestions for the design of future glycoconjugate vaccines

Based on the data obtained in this work, the commonly used carriers protein (CRM197, DT and TT) appear suitable to develop also new glycoconjugate vaccines. In fact their clinical development will take advantages in using well known carriers with a well demonstrated history of safety and efficacy.

However if a new carrier would be needed, in order for example to reduce the amount of the same protein administered in human vaccines, our work identified other potential candidates able to work as carrier with a performance comparable to CRM197. Our method of investigation could also be used to select additional proteins as new carriers.

In the design of new glycoconjugate vaccines particular attention should be devoted to answer the question whether there is an optimal chain length to maximize the immune response in animal

models, and later on in humans. This information will be essential also to asses the feasibility of the synthetic approach for vaccine manufacturing, considering that the production of saccharides with short chain length will be easier to scale up.

Since to a selected saccharide chain length, probably corresponds an optimal loading onto the carrier protein, this need to be defined by comparing in animal model the immunogenicity of conjugates at different glycosylation degree and fixed oligosaccharide chain length.

 In order to obtain vaccines that can be deeply characterized, it is recommended the use of conjugation chemistries which involve the reducing end terminus to couple the sugar moiety to the protein. This will results in conjugates with a well defined radial structure, without cross-linking and easier to characterize.

Our data on different linker molecules suggest that the presence of an aromatic structure might jeopardize immune response against the sugar antigen.

Finally additional studies to better understand the mechanism of action of glycoconjugates vaccines will inform the development of future glycoconjugate vaccines.

This PhD work contributes to obtain more information on the properties that determine the optimal immunogenicity of the vaccine and leads to improve the design of glycoconjugate vaccines.

4   Materials and Methods

4.1   Reagents

Laminarin was obtained from Sigma Aldrich.

Meningococcal oligosaccharides avDP 15 serotypes ACWY were provided internally by the Manufacturing Department of Novartis V&D, Siena, Italy.[22] [23]

Synthetic *β-glucan* oligosaccharides were produced internally by Novartis V&D Research Center, Siena, Italy.

Succinimidyl4 p-maleimidophenyl butyrate (SMPB Thermo).

Bis succinimidyl penta ethylene glycol (Bis(NHS)PEG 5,Thermo).

Succinimidyl4 N-maleimidomethyl cyclohexane-1-carboxylate (SMCC Thermo).

Alexa Fluor® 488 (A488, Invitrogen).

Alexa Fluor® 647 (A647, Invitrogen).

Protein CRM197 was produced internally by Novartis V&D, Siena, Italy.

TT and DT were produced by Novartis V&D, Marburg, Germany.

Protein studied as new carrier candidates were produced internally by Novartis V&D Research Center, Siena, Italy.

4.2   Analytical methods

4.2.1   *Sodium Dodecyl Sulfate- Polyacrilamide gel electrophoresis (SDS-Page)*

SDS-Page has been performed on pre-casted polyacrylamide gels (NuPAGE®Invitrogen) that can be used with different polyacrylamide concentration, from 3-8% gradient to 7%. The electrophoretic runs have been performed in Tris-Acetate SDS running buffer (NuPAGE®Invitrogen) loading 2.5-5μg of protein each sample, using the electrophoretic chamber with a voltage of 150V for about 40 minutes. Samples were prepared by adding 3 μl of NuPAGE® LDS sample buffer. After electrophoretic runnig, the gel has been washed in H_2O for 3 times and then with dye comassie.

4.2.2 Size Exclusion High Performance Liquid Chromatography (SEC-HPLC)

SEC-HPLC has been performed on UltiMate® 3000 HPLC system (Dionex part of Thermo Fisher Scientific) equipped with a PDA, RF2000 Fluorescence Detector and RI-101 Shodex Detector. Chromatography has been performed in 0.1M $NaSO_4$ 0.1M NaPi 5% CH_3CN pH 7.2 on TSK 3000-4000 Gel SW (Tosoh Bioscience) analytical column at flow rate of 0.5 ml/min, with 50 µl of injection volume loading 15-25 µg of sample in protein content. The resulting chromatographic data processed using Chromeleon® 6.7 software.

4.2.3 Colorimetric analyses

Protein content on the conjugates has been determined by MicroBCA (Thermo) colorimetric assay.[112]

Amino groups were determined by colorimetric assay.[113]

Active ester groups introduced in the oligosaccharides were determined by analysis of released N-hydroxy-succinimido groups as previously published.[114]

Sulphydryl groups (SH) groups content on oligosaccharides have been determined by colorimetric assay.[115]

4.2.4 Spectrophotometric analysis

The label degree (DOL) of oligosaccharides labelled with fluorescent dyes has been determined by spectrophotometer. DOL calculation has been done by measuring the absorbance of the sample at the λ_{max} (Amax) of the dye and applying the Lambert-Beer law to calculate the number of dye molecule per molecule of sample knowing the sample concentration.

DOL= (Amax * MW)/[sample] * ϵ_{dey}

Where MW=molecular weight of the sample, ϵ_{dey}= extinction coefficient of the dye at its absorbance maximum, sample concentration was in mg/ml.

Dye	λ_{max}	Em	ϵ_{dey}	CF_{280}
Alexa Fluor® 488	495	519	71.000	0.11
Alexa Fluor® 647	650	665	239.000	0.03

Em= fluorescence excitation and emission maxima in nm; CF_{280}= correction factor at 280nm (A_{280} free dye/A_{max} free dye)

4.2.5 NMR analyses

^{1}H and ^{31}P NMR experiments were recorded at 25 $\pm$ 0.1°C on Bruker Avance III 400 MHz spectrometer, equipped with a high precision temperature controller, and using 5-mm broadband probe (Bruker). All the samples were dissolved in 0.75 mL of deuterium oxide (D$_2$O, 99.9% atom D, Aldrich) and inserted in 5-mm NMR tube (Wilmad). For data acquisition and processing, TopSpin version 2.6 software (Bruker) was used.

^{1}H NMR spectra were collected at 400 MHz over a 10 ppm spectral width, accumulating approximately 128 scans. The transmitter was set at the HDO frequency which was used as the reference signal (4.79 ppm). ^{31}P NMR spectra were recorded at 161.9 MHz over 20 ppm spectral width, accumulating approximately 1k of scans. 75% phosphoric acid in deuterium oxide was used as an external standard (0 ppm). All the NMR spectra were obtained in quantitative manner using a total recycle time to ensure a full recovery of each signal (5 x Longitudinal Relaxation Time T$_1$).

The avDP of MenA oligosaccharides were calculated by the integration of ^{31}P NMR peaks and expressed as [(P$_{de}$/P$_{me}$) +1], where P$_{de}$ is molar concentration of the phosphodiester in chain groups and P$_{me}$ the molar concentration of phosphomonoester end groups.

4.2.6 Profiling by use of ion chromatography

The chain length distribution of MenA oligosaccharides preparation has been controlled by profiling analysis. Analysis of the hydrolyzed products has been performed by HPAEC-PAD with a Dionex ICS3000 system, using a CarboPac PA200 column and a NaNO$_3$ gradient in 100mM NaOH. The resulting chromatographic data have been processed using Chromeleon® 6.8 software.

4.2.7 MALDI analysis

MALDI-TOF mass spectra linker-derivatized CRM$_{197}$ was recorded by UltraFlex III MALDI-TOF/TOF instrument (Bruker Daltonics) in linear mode and with positive ion detection. The samples for analysis were prepared by mixing 2.5 μL of product and 2.5 μL of Super DHB matrix. 2.5 μL of each mixture were deposited on samples plate, dried at room temperature for 10 min and subjected to the spectrometer.

4.2.8 Determination of total saccharide in the conjugates preparations

Total saccharide was determined by high performance anionic exchange chromatography-pulsed amperometric detection (HPAEC-PAD) using different sample pre-treatment and chromatographic conditions depending on the saccharide antigens.

4.2.9 Laminarin conjugates

Total saccharide content of laminarin conjugates was determined by HPAEC-PAD[72] using a CarboPac PA1 column (50mm×250mm) coupled to a CarboPac PA1 guard column and connected

to a Dionex ICS3000 system. Samples, diluted at 5 µg/mL of saccharide were treated with trifluoroacetic acid (TFA) at a final concentration of 2M, heated at 100 ◦C for 2 h in a closed screw-cap test tube, dried on a Speed Vac concentrator fitted with a refrigerated condensation trap (Savant SC110) under vacuum, dissolved in 0.5mL of distilled water and filtered 0.45µm before the analysis. The separation was performed with a flow rate of 1mL/min using isocratic elution of 40mM NaOH for 12 min, followed by a washing step with 500mM NaOH for 5min. The chromatography was monitored using the pulsed amperometric mode with a gold working electrode and an Ag/AgCl reference electrode, a quadruple-potential waveform for carbohydrates was applied. The chromatographic data were processed using Dionex Chromeleon™ software. Calibration curve, treated as samples, was set up with glucose (Fluka) in the range 0.5–10.0µg/mL.

4.2.9.1 Meningococcal A,C,W ,Y conjugates

In MenACWY conjugates total saccharide was determined by HPAEC-PAD[116] using different sample pre-treatment and chromatographic conditions for the different saccharides. MenY and MenW conjugates were hydrolyzed by incubation in trifluoroacetic acid (TFA) with a final concentration of 2 M at 100 °C for 2 h in a closed screw-cap test tube, then centrifuged to combine the condensate with the bulk liquid and dried on a Speed Vac concentrator fitted with a refrigerated condensation trap (Savant SC110) under vacuum. Samples have been first dissolved in distilled and degassed water and then filtered (0.45 µm). Analysis of the hydrolyzed products has been performed by HPAEC-PAD with a Dionex ICS3000 system fitted with DP, DC and AS modules, using a CarboPac PA1 column and a NaOH/Na acetate step elution. The resulting chromatographic data have been integrated and processed using Chromeleon® 6.8 software. Quantification has been performed with calibration curves, treated as samples, set up with galactose and glucose, for MenW and MenY respectively, in the range of 0.5–4.0 µg/ml. Saccharide quantification for MenC conjugate has been performed by incubation in trifluoroacetic acid (TFA) with a final concentration of 1 M at 90 °C for 2 h in a closed screw-cap test tube, then samples have been chilled and centrifuged to combine the condensate with the bulk liquid before neutralization with sodium hydroxide (NaOH) 4M. Analysis of the hydrolyzed products has been performed by HPAEC-PAD with a Dionex ICS3000 system fitted with DP, DC and AS modules, using a CarboPac PA1 column and a NaOH/Na acetate elution gradient. The resulting chromatographic data have been integrated and processed using Chromeleon® 6.8 software. Quantification has been performed with a calibration curve, treated as samples, set up with sialic acid in the range of 0.5–5.0 µg/ml. Saccharide quantification for MenA conjugate has been performed by incubation in TFA with a final concentration of 2 M at 100 °C for 2 h in a closed screw-cap test tube, then samples has been chilled and centrifuged to combine the condensate with the bulk liquid before to be neutralized with NaOH 2M. Analysis of the hydrolyzed products has been performed by HPAEC-PAD with a Dionex ICS3000 system fitted with DP, DC and AS modules, using a CarboPac PA1 column and a

NaOH/Na acetate elution gradient. The resulting chromatographic data have been integrated and processed using Chromeleon® 6.8 software. Quantification has been performed with a calibration curve, treated as samples, set up with MenA polysaccharide in the range of 0.5–8.0 µg/ml.

4.2.10 Determination of unconjugated (free) saccharide content in glycoconjugates preparations

Unconjugated saccharide content (free saccharide %) was determined after separation of the free from the conjugated saccharide as follows. Conjugate vaccines (500 µl at saccharide concentration of 300µg/mL) have been applied to Microcon-30 filters previously washed with water, centrifuged at 10 000×g for 15 min at r.t. and washed twice with 500µl of water followed by centrifugation. Saccharide quantification on filtrates has been then performed as described above.

4.3   Preparation of oligosaccharides

4.3.1   Introduction of primary amino groups at the reducing end of laminarin oligosaccharide by reductive amination

Laminarin has been treated by reductive amination to introduce a NH_2 group on the reducing end of the sugar using these conditions: 2 mg/ml of polysaccharides were subjected to reductive amination by incubation for 5 days at 50°C in 300 mg/ml ammonium acetate (Sigma-Aldrich) and 0.2 M sodium cyanoborohydride pH 7.5 (Sigma-Aldrich). Aminated laminarin was purified from the reaction mixture by diafiltration on regenerated cellulose membrane (cut-off 1kDa; Millipore) and the amount of primary amino groups introduced was determined as reported in paragraph 3.2.

4.3.2   Derivatization of the laminarin amino oligosaccharides to active esters

The aminated oligosaccharides were vacuum dried, solubilized in 1:9 H_2O:DMSO solution with a final $[NH_2]=40$µmol/ml, and then reacted with the succinimido diester of adipic acid (SIDEA) in a 12-fold molar excess as compared with primary amino groups in the presence of 5-fold molar excess triethylamine as compared with primary amino groups.[72 117]

The reaction was kept under gentle stirring at room temperature for 2 h. The activated oligosaccharides were then separated from the reagents by precipitation with 1:4 of acetone, followed by washing of the precipitate with 1:4 acetone and dried under vacuum.

Content of N-hydroxysuccinimide ester groups introduced in the activated oligosaccharide was determined as reported in paragraph 3.1.

4.3.3 Introduction of primary amino groups or cystamine at the reducing end of meningococcal serogroup A oligosaccharides by reductive amination

MenA has been treated by reductive amination to introduce a NH_2 group on the reducing end of the sugar using these conditions: 4 mg/ml of polysaccharides were subjected to reductive amination by incubation for 5 days at 37°C in 300 mg/ml ammonium acetate (Sigma-Aldrich) and 0.2 M sodium cyanoborohydride (Sigma-Aldrich) at pH 6.5-7.

Reductive amination with cystamine has been performed using these conditions: 4 mg/ml of polysaccharides were subjected to amination with cystamine (cyst) by incubation for 5 days at 37°C in 1:23 mol/mol MenA/cyst and 1:40 mol/mol MenA/sodium cyanoborohydride at pH 6.5-7.

In both cases the modified MenA oligosaccharides, Oligo MenA-NH_2 and OligoMenA-Cystamine, have been purified by tangential flow filtration with 2kDa cut-off membrane to remove the excess of low molecular weight reagents and the retentate was collected. Amino group content has been determined by colorimetric assay as reported in 4.2.

4.3.4 Preparation of MenA oligosaccharides of different average degree of polymerization (avDP)

MenA CPS hydrolysis has been conducted in 50mM sodium acetate pH4.73 at saccharide concentration of 2.5mg/ml, 73°C for 30min-1h to obtain avDP 30/40, for 4-4.30h for avDP15 and for 7-7.30h to obtain avDP 6/10.

After hydrolysis the chain length selection has been performed applying different purification techniques. To select the avDP 30/40 after hydrolysis a tangential flow filtration has been done with 100kDa cut-off membrane (Sartorius) and the retentate containing the avDP saccharides higher than 30/40 have been eliminated. Instead the permeate has been treated with a second tangential flow filtration with 30kDa cut-off membrane that allow to eliminate in permeate the avDP saccharide lower than 30/40. The material in the 30kDa retentate contains the avDP 30/40 oligosaccharides of our interest.

To select the avDP6/10, the material has been treated with a tangential flow filtration with 2kDa cut-off membrane to eliminate in the retentate the saccharides at high avDP. The permeate has been further purified by anion exchange chromatography using a Q Sepharose resin (GEHealthcare): the sample has been charged on the column in 50mM NaCl and after a gradient to 500mM NaCl has been applied on 30 column volumes. After profiling analysis of the single fractions the pool containing the avDP oligosaccharides 6/10 has been collected. The oligosaccharides avDP 30/40 and 6/10 have been treated by reductive amination and activated with SIDEA as reported before.

4.3.5 Derivatization of OligoMenA-NH_2 with Succinimidyl diester of adipic acid (SIDEA)

This reaction as carried as described before for the laminarin oligosaccharides.

4.3.6 Derivatization of OligoMenA-NH₂ with Bis succinimidyl penta ethylene glycol (Bis(NHS)PEG5)

Bis(NHS)PEG5 (Thermo) activation has been performed on OligoMenA-NH₂ in 1:9 H₂O:DMSO solution with a final [NH₂]=40µmol/ml. Bis(NHS)PEG5 was used in the reaction at 1:9 mol:mol Bis(NHS)PEG5:NH2 groups and 4 equivalent of Et₃N.

The reaction was kept under gentle stirring at room temperature for 2 h. The activated oligosaccharides were then separated from the reagents by precipitation with 1:4 of acetone, followed by washing of the precipitate with 1:4 acetone and dried under vacuum.

Content of N-hydroxysuccinimide ester groups introduced in the activated oligosaccharide was determined as reported in paragraph 4.2.3.

4.3.7 Derivatization of OligoMenA-Cystamine with Succinimidyl4 p-maleimidophenyl butyrate (SMPB)

SMPB (Thermo) activation has been done on MenA oligosaccharides after amination with cystamine, after reduction with tris(2-carbossietyl)phosphine (TCEP, Pierce). Reduction has been done with 3 equivalent of TCEP, for 5h at a final [TCEP]=0.5M. After 5h sample has been purified by gel filtration using a G15 resin and H₂O as elution buffer. In the final sample SH groups content has been determined by colorimetric assay as described before. The linker reaction has been carried out with 1:3 mol:mol SH:SMPB in a solution 1:9 H₂O:DMSO at 10mg/ml of oligosaccharides concentration for 2h at R.T. After, acetone precipitation has been done and on lyophilized pellet active ester groups have been determined.

4.3.8 Derivatization of OligoMenA-Cystamine with Succinimidyl4 N-maleimidomethyl cyclohexane-1-carboxylate (SMCC)

SMCC (Thermo) activation has been done on MenA oligosaccharides after amination with cystamine, after reduction with TCEP. The linker reaction has been carried with 1:3 mol:mol SH:SMCC in a solution 1:9 H₂O:DMSO at 10mg/ml of oligosaccharides concentration for 2h at R.T. After, acetone precipitation has been done and on lyophilized pellet active ester groups have been determined.

4.3.9 Derivatization of serogroup C menigococcal oligosaccharide with fluorescent labels

To obtain a glycoconjugate selectively labelled on the protein and on the saccharide moiety, MenC oligosaccharides avDP15 have been labelled with Alexa488 (A488, Invitrogen). At first MenC was aminated by reductive amination using the same conditions described for MenA. After, the hydrazide groups on A488 have reacted with the carboxylic groups on the sialic acid of MenC sugar: 10% mol of A488 respect to mol of MenC-COOH were put in reaction with 50% mol of EDC (Sigma) in Mes buffer (2-(N-morpholino) ethanesulfonic acid) 30mM pH 5.6 for 3h in gentle stirring. After 3h

the reaction has been dialyzed on 3.5kDa cut-off membrane against H_2O. After DOL determination (DOL=0.484), MenC/A488 has been activated with SIDEA as reported before and active ester group determination has been done on the lyophilized sample. All reactions have been done in the dark as required by the dye usage instructions.

4.4   Conjugates preparation and purification

4.4.1   Conjugates with new carrier candidate proteins

Laminarin conjugations have been carried out in 10-100mM NaH_2PO_4 pH 7 or in phosphate buffer saline pH7 (PBS, 150mM NaCl, 10mM Na_2HPO_4, 2mM KCl, 2mM KH_2PO_4) depending on the protein buffer, using an active ester(AE):protein ratio mol:mol of 30:1. The reaction has been carried over night (o.n.) at RT with gentle stirring.

The conjugates obtained have been purified from the unreacted sugar by affinity chromatography with His MultiTrap 96-Well Filter Plates (GEHealthcare) using the His tag portion on the recombinant protein to bind the conjugate to the resin. Conjugates have been charged on the resin 250µg per well on volume of 200µl in the same buffer of conjugation and incubate for 15min after mixing on the well, other 15min of incubation has been done. After, the flow through have been taken by using the vacuum air and two wash of the resin have been done with 200 µl of PBS buffer pH 7.2. Conjugates have been eluted from the resin by adding 150 µl of elution buffer 0.5M NaCl, 0.5M NaH_2PO_4, 0.5M Imidazole pH 6.3, incubating for 15min and collecting the flow through; the elution has been repeated two times. Eluted conjugates have been dialyzed with 3-8kDa membrane vs PBS buffer pH 7.2 to eliminate the elution buffer, dialysis have been carried out one day at 4°C changing the buffer for four times.

If the protein used in the conjugate was expressed without the His tag, conjugates have been purified by size exclusion chromatography or by ultrafiltration with Vivaspin system (Sartorius) using a 30kDa cut-off membrane.

MenC conjugations reaction have been carried with an AE:protein mol:mol of 12:1, in 10-100mM NaH_2PO_4 pH 7 or in PBS pH 7 depending on the protein buffer, and incubated o.n. at RT with gentle stirring. Purification has been done or by His MultiTrap 96-Well Filter Plates (GEHealthcare) or by gel filtration or by ultrafiltration as reported above.

PNEU20 conjugations with MenAWY oligosaccharides have been done using the same conditions described above for MenC.

4.4.2 Conjugates preparation with CRM197, TT and DT as carrier proteins

Protein CRM197 was produced internally by Novartis V&D, Siena, Italy. TT and DT were produced by Novartis V&D, Marburg, Germany. For TT protein, the monomer portion has been isolated by gel filtration using a Sephacryl S300 resin and 0.15M NaCl, 0.01M NaPi pH7.2 as running buffer and collecting the fractions at lower molecular weight.

Meningococcal ACWY oligosaccharides avDP15 after SIDEA activation have been conjugated to the three different carrier with an AE:protein ratio mol:mol of 12:1, in 100mM NaH_2PO_4 pH 7 and incubated o.n. at RT in gentle stirring. CRM197, TT and DT conjugates have been purified by $(NH_4)_2SO_4$ precipitation to remove the unreacted oligosaccharides. Precipitation has been carried out adding 500g/l of solid ammonium sulphate to the conjugate solution; the suspension has been incubated on ice for 15 min and then centrifuged at 4°C for 30 min. After centrifugation, the pellet has been dissolved in 0.01M NaH_2PO_4 solution pH 7 and the treatment has been repeated for 3 times. Then, the pellet has been washed 3 times with an ammonium sulphate solution 500g/l. Finally pellets have been dissolved in 0.01M NaH_2PO_4 pH 7.

4.4.3 Conjugates with different saccharide chain length and different glycosylation degree

Activated oligosaccharides at different avDP (6/10, 15, 30/40) have been conjugated with CRM197 after SIDEA activation and using different reaction conditions conjugates with different glycosylation degree have been obtained.

Conjugation conditions for avDP6-11 oligosaccharides: two different conjugates have been prepared (M13 13:1 prot 40mg/ml, M15 13:1 prot 40mg/ml) with AE:protein ratio mol:mol of 13:1, in 100mM NaH_2PO_4 pH 7 and incubated o.n. at RT in gentle stirring. Conjugates have been purified by gel filtration using a Sephacryl S300 resin and 0.01M NaCl, 0.01M NaPi pH 7.2 as running buffer to eliminate the unreacted sugar.

Conjugation conditions for avDP15 oligosaccharides: two different conjugates have been prepared (M5 40:1, M10 13:1) with AE:protein ration mol:mol of 40:1-13:1, in 100mM NaH_2PO_4 pH 7 and incubated o.n. at RT in gentle stirring. Conjugates have been purified by by gel filtration using a Sephacryl S300 resin and 0.01M NaCl, 0.01M NaPi pH 7.2 as running buffer to eliminate the unreacted sugar.

Conjugation conditions for avDP30-40 oligosaccharides: tree different conjugates have been prepared (M8, M11, MT2) with AE:protein ration mol:mol of 25:1-13:1-8:1, in 100mM NaH_2PO_4 pH 7 and incubated o.n. at RT in gentle stirring. Conjugates have been purified by: M8 ultrafiltration with 100kDa cut-off membrane (0.1m^2 Hydrosart Sartorius) against 10 volumes of 10mM NaH_2PO_4 pH 7; MT2 by gel filtration using a Sephacryl S300 resin and 0.15M NaCl, 0.01M NaPi pH 7.2 as running buffer to eliminate the unreacted sugar and by hydrophobic resin Phenyl Sepharose (GEHealthcare)

using 3M NaCl as sample loading buffer and stripping with a gradient of H_2O to eliminate the unconjugate protein; M11 by gel filtration using a Sephacryl S300 resin and 0.01M NaCl, 0.01M NaPi pH 7.2 as running buffer to eliminate the unreacted sugar.

4.4.4   Conjugates with different linkers

MenA oligosaccharides avDP 15 have been activated as reported above with different linker molecules. Activated oligosaccharides have been conjugated with CRM197 using these conditions of reaction:

MenASIDEA: AE:protein ratio mol:mol 12:1, in 50-100mM NaH_2PO_4 pH 7 and incubated o.n. at RT in gentle stirring.

MenAPEG: AE:protein ratio mol:mol 13:1, in 50-100mM NaH_2PO_4 pH 7 and incubated o.n. at RT in gentle stirring.

MenASMPB: AE:protein ratio mol:mol 13:1, in 50-100mM NaH_2PO_4 pH 7 and incubated 48h at RT in gentle stirring.

MenASMCC: AE:protein ratio mol:mol 13:1, in 50-100mM NaH_2PO_4 pH 7 and incubated 48h at RT in gentle stirring.

With SMCC a second type of conjugate has been obtained activating the protein moiety with the linker molecule and after conjugating with the saccharide: CRM197 has been taken in reaction with 30% of mol of SMCC linker considering the total mol of lysine in the CRM197 (39 lysine present in CRM197), that are the NH_2 groups free to react. Reaction has been carried in 50mM NaH_2PO_4 pH 7 for 3h at RT. After, CRM197-SMCC has been purified in size exclusion chromatography using a G25 resin (GE Healthcare) and 10mM NaH_2PO_4 pH 7 with 10% of saccharose as running buffer. Purified CRM197-SMCC has been conjugated with MenA oligosaccharides after cystamine activation and reduction. Conjugation has been carried SH:SMCC ratio mol:mol of 10:1, in 50-100mM NaH_2PO_4 pH 7 and incubated 48h at RT in gentle stirring. Conjugate has been purified by size exclusion chromatography using a S300 resin (GEHealthcare) and 10mM NaH_2PO_4 10mM NaCl pH 7 with as running buffer.

4.4.5   Labeling of glycoconjugate

MenC oligosaccharide avDP15 labelled with A488 as reported and after SIDEA activation, have been conjugated with CRM197 with an AE:protein ratio mol:mol 13:1, in 50mM NaH_2PO_4 pH 7 and incubated o.n. at RT in gentle stirring. After conjugation a second labelling has been done selectively on the protein moiety with Alexa Fluor® 647 (A647, Invitrogen) using the active ester group on the dye to react with the free amino group on the protein after conjugation. A647 reaction with MenC/A488-CRM197 conjugate has been carried with CRM197:A647 ratio mol:mol 1:30 in

0.1M NaHCO$_3$ buffer pH8.2 for 6h at RT. After, the sample has been diluted 1:2 with 10mM NaH$_2$PO$_4$ and purified by (NH$_4$)$_2$SO$_4$ precipitation to remove the unreacted sugar. Precipitation has been carried out adding 500g/l of solid ammonium sulphate to the conjugate solution; the suspension has been incubated on ice for 15 min and then centrifuged at 4°C for 30 min. After centrifugation, the pellet has been dissolved in 0.01M NaH$_2$PO$_4$ solution pH 7 and the treatment has been repeated for 3 times. Then, the pellet has been washed 3 times with an ammonium sulphate solution 500g/l. Finally pellets have been dissolved in 0.01M NaH$_2$PO$_4$ pH 7. After purification by precipitation, the sample has been dialyzed to remove the unreacted dye, dialysis has been done on 3-8kDa cut-off membrane on 0.01M NaH$_2$PO$_4$ pH7 for 24h at 4°C, changing the buffer for 5 times.

4.5 Vaccines formulation and immunological studies

4.5.1 Preparation of glycoconjugates formulations

Antigens formulations have been done under sterile woods using sterile instrumentation and solutions.

All formulations have been done using PBS pH 7.2 as buffer where the vaccines have been diluted to obtain the require dosage of saccharide per mice in a total volume of 200µl.

Aluminium phosphate (AlPO$_4$) has been used as adjuvant and has been prepared as 2x solution to be mixed 1:1 to the total volume of PBS. Each dose contained 0.12 mg (expressed as Al^{3+}) of aluminium phosphate.

To study the absorption of vaccines on AlPO$_4$, formulated vaccines were incubated 1h at R.T. measuring the pH and after centrifuged at 13000g, 4°C for 40 min. Protein dosage has been done as reported on the supernatant. The % of absorption has been calculated relatively to the formulation without adjuvant.

MenA vaccines have been administered to mice in 2µg per dose in saccharide content; MenCWY vaccines have been administered to mice in 1µg per dose in saccharide content; Laminarin vaccines have been administered to mice in 5µg per dose in saccharide content.

Mice have been immunized subcutaneously at day 1, 14 and 28. Bleedings were performed at day 0 (pre immune), day 28 (post 2) and day 42 (post 3). Control groups received PBS. Animal experimental guidelines set forth by the Novartis Animal Care Department were followed in the conduct of all animal studies.

4.5.2   Immunochemical evaluation of response

The antibody response induced by the glycoconjugates against the homologous polysaccharide and carrier protein has been measured by ELISA. Plates have been coated with the different meningococcal polysaccharides or carrier proteins by adding 100 µl/well of a 5 µg/ml polysaccharide solution in PBS buffer at pH 8.2 or 100 µl/well of a 1-2 µg/ml carrier protein solution in PBS buffer at pH 7.2, followed by incubation overnight (o.n.) at 4°C. Instead Laminarin coating has been done at 5 µg/ml of polysaccharide solution in 0.05M Na_2CO_3-$NaHCO_3$ buffer at pH 9.6. Coating solutions were removed from the plates by washing tree times per well with PBS buffer with 0.05% of Tween 20 (Sigma) (TPBS). A blocking step has been then performed by adding 100 µl of BSA solution at 3% in TPBS and incubating the plates 1 h at 37°C. Blocking solution has been removed from the plates by washing three times per well with TPBS. Two hundred µl of pre-diluted serum (1:25 for pre immune, 1:100-1:200 for a reference serum from 1:50 to 1:1000 for test sera) was added in the first well of each column of the plate, while on the other wells 100 µl of TPBS has been dispensed. Eight twofold serial dilutions along each column were then performed by transferring from well to well 100 µl of sera solutions. After primary Abs dilution, plates have been incubated for 2h at 37°C. After three washes with TPBS, 100 µl TPBS solutions of secondary antibody alkaline phosphates conjugates (anti mouse IgG 1:10000 or anti mouse IgG 1, 2a, 2b, 3 , Sigma-Aldrich, diluted 1:2000 or anti mouse IgM 1:5000) were added and the plates incubated 1h at 37°C. After three more washes with TPBS, 100 µl/well of a 1mg/ml of p-NPP (Sigma) in a 0.5 M di-ethanolammine buffer pH 9.6 was added. After 30min of incubation at room temperature, plates were read at 405nm using a Biorad plate reader. Raw data acquisition has been performed by Microplate Manager Software (Biorad). Sera titers were expressed as the reciprocal of sera dilution corresponding to a cut-off OD = 1 or to a cut-off OD=0.2. Each immunization group has been represented as the geometrical mean (GMT) of the single mouse titers. The statistical and graphical analysis has been done by GraphPad 5.0 software.

4.5.3   Competitive ELISA

The protocol described above was followed to prepare laminarin-coated plates. The plate was designed to contain (a) a blank column with TPBS alone, without serum and inhibitors, and (b) a column with serum alone, without inhibitors (b0); the other columns contained both, the serum and the inhibitors, which included also laminarin and the not correlated polysaccharide MenC as positive and negative controls, respectively. The different competitors (compounds **1**, **2**, **3**, **4,** and **6**) were prediluted to obtain the starting concentration of 4 mM and eight 10-fold dilutions were performed on the plate. Meningococcal C polysaccharide was used as negative control.

The competitors at different concentrations starting from 10 mg/mL concentration were mixed with an equal volume of a fixed dilution of antilaminarin immune serum, followed by 2 h incubation at

37C. After primary antibody incubation the general protocol described in the ELISA Analysis section was followed. All OD lectures were subtracted from the mean value of the blank column (b). The inhibition percentage was expressed as follows: % inhibition = $[(B0-ODx)/B0] \times 100$, where B0 is the mean values of the b0 column (serum without inhibitor) and ODx is the optical density corresponding to each inhibitor concentration. IC_{50} was defined as the inhibitor concentration resulting in 50% inhibition of the main reaction. Fitting of inhibition curves and calculation of IC_{50} values were performed on the Graphpad Prism software using the variable slope model (Graphpad Prism Inc.).

Native MenA CPS and oligosaccharides were provided by Novartis Vaccines and Diagnostics, Siena, Italy. Polyclonal serum derived from immunization of mice with a native meningococcal A oligosaccharide-protein conjugate was provided by Preclinical Serology Laboratory in Novartis Vaccines and Diagnostics, Siena, Italy. 96-Well Maxisorp plates (Nunc, Thermo Fisher scientific) were coated overnight at $+4 \circ$C with Meningococcal A native capsular polysaccharide (MenA CPS), 5 µg/well in Phosphate Saline Buffer (PBS) pH 8.2. After coating, the plates were washed three times with 300 µl per well of PBS with 0,05% Tween 20 (TPBS) at pH 7.4. Plates were blocked with 3% bovine serum albumin (Fraction V, Sigma–Aldrich) in TPBS for 1 h at 37°C and then washed again. 50 µl of polyclonal immune mouse sera pre-diluted in TPBS were put on the plate and mixed with 50 µl of inhibitor previously diluted with tenfold serial dilution on another plate. On the column without inhibitor 50 µl of immune mouse sera pre-diluted in TPBS were mixed with 50 µl of TPBS. Polyclonal serum obtained from native Meningococcal A immunization was used at the final dilution of 1:1600. After 2h of incubation at 37°C and washing with TPBS, 100 µl/well of 1:10,000 of anti-mouse IgG alkaline phosphatase conjugated (Sigma–Aldrich) were added and plates were incubated for 1 h at 37°C. After, plates were developed for 30 min at room temperature with 100 µl/well of 1 mg/ml p-nitrophenyl phosphate disodium (Sigma–Aldrich) in 1 M diethanolamine (pH 9.8) and red at 405 nm with a microplate spectrophotometer (Biorad). The plate was designed to contain: a) blank column with TPBS alone, without serum and inhibitors, b) column with serum alone, without inhibitors (b0); the other columns contained both, the serum and the inhibitors which included also MenA CPS, native MenA oligosaccharides and the not correlated Laminarin polysaccharide as positive and negative controls respectively.

The different competitors (compounds **1** = monosaccaride, **2** = disaccharide, **3** = trisaccharide and native*av*DP3 = native MenA oligosaccharide average polymerization degree 3) were pre-diluted to obtain the starting concentration of 0.5 mM and ten fold dilutions were performed eight times on the plate. Native MenA CPS and *av*DP15 oligosaccharide used as positive control were pre-diluted to obtain a starting concentration of 0.05mM, instead Laminarin of 1mM and was used as negative control. All OD lectures were subtracted of the mean value of the blank column (b). The inhibition

percentage was expressed as follows: % inhibition= [(B0 - ODx)/B0] x 100. Where B0 is the mean values of the b0 column (serum without inhibitor) and ODx is the optical density corresponding to each inhibitor concentration. IC50 was defined as the inhibitor concentration resulting in 50% inhibition of the main reaction. Fitting of inhibition curves and calculation of IC_{50} values was performed on the Graphpad Prism software using variable slope model (Graphpad Prism Inc.).

4.5.4 Serum bactericidal assay (rSBA)

Functional antibodies induced by vaccine immunization were analysed by measuring the complement-mediated lysis of *Neisseria meningitidis* with an in vitro bactericidal assay.[118] A commercial lot of baby rabbit complement was used as source of active complement. Briefly, N. meningitidis strains were grown overnight on chocolate agar plates at 37°C in 5% CO_2. Colonies were inoculated in Mueller-Hinton broth, containing 0.25% glucose to reach an OD600 of 0.05-0.08 and incubated at 37°C with shaking. When bacterial suspensions reached OD600 of 0.25-0.27, bacteria were diluted in the assay buffer (Gey's balanced salt solution with 1% BSA) at the working dilution (ca. 104 CFU/mL). The total volume in each well was 50µL with 25µL of serial two-fold dilutions of the test serum, 12.5µL of bacteria at the working dilution and 12.5µL of baby rabbit complement. All sera to test were heat-inactivated for 30 minutes at 56°C. Negative controls included bacteria incubated, separately, with the complement serum without the test serum and with test sera and the heat-inactivated complement. Immediately after the addition of the baby rabbit complement, negative controls were plated on Mueller-Hinton agar plates, using the tilt method (time 0). Plate were incubated for 1 hour at 37°C, then each sample was spotted in duplicate on Mueller-Hinton agar plates while the controls were plated using the tilt method (time 1). Agar plates were incubated overnight at 37°C and the colonies corresponding to time 0 and time 1 (surviving bacteria) were counted. The serum bactericidal titre was defined as the serum dilution resulting in 50% decrease in colony forming units (CFU) per mL, after 60 min incubation of bacteria in the reaction mixture, compared to control CFU per mL at time 0. Typically, bacteria incubated without the test serum in the presence of complement (negative control) showed a 150 to 200% increase in CFU/mL, during the 60 min incubation time. The reference strains for each meningococcal serotype used were: F8238 (MenA); C11 (MenC); 240070 (MenW135); 860800 (MenY).

4.5.5 In vivo and ex vivo cell uptake experiments

Balb/c mice were treated IP with double labeled CRM-MenC antigen containing an equivalent of 1 mcg of MenC/ mouse in a volume of 200 µl. Peritoneal wash samples were collected at time 0, 30 min, 6 h and 24 h post treatment. Cells were harvested by centrifugation at 1300 rpm, washed 2 times with PBS and then fixed with 2% of formaldehyde solution for 20 min at RT. Cells were then washed and stained in 2% rabbit serum with the following antibody cocktail: CD19-PE; MHCII-A700; F4/80-Pacific Blue; Gr1-PerCP Cy5.5; CD11c-APC e700 CD11b PECy7. Samples were acquired

using a FACS LSRII (BectonDickinson, special order system) and analyzed using BD-DIVA software (BectonDickinson).

For confocal microscopy analysis, fixed cells collected from peritoneal wash samples were permeabilized with PBS, 0.1% saponin, 1% BSA (permeabilization buffer, PB) fro 30 minutes. Then cells were stained with anti MHCII or with anti Lamp1 antibodies, followed by secondary AF568 conjugated anti mouse IgG antibodies. Cells were washed twice with PB and then resuspended in 100 ul of PBS and centrifuged onto a slide by cytospin (300 rpm, 4 min). Samples were treated with ProLong antifade medium and analyzed by laser scanning microscopy (LSM700, Zeiss).

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