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Preclinical studies on new proteins as carrier for glycoconjugate vaccines

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ABSTRACT

Glycoconjugate vaccines are made of carbohydrate antigens covalently bound to a carrier protein to enhance their immunogenicity. Among the different carrier proteins tested in preclinical and clinical studies, five have been used so far for licensed vaccines: Diphtheria and Tetanus toxoids, the non-toxic mutant of diphtheria toxin CRM_{197}, the outer membrane protein complex of *Neisseria meningitidis* serogroup B and the Protein D derived from non-typeable *Haemophilus influenzae*. Availability of novel carriers might help to overcome immune interference in multi-valent vaccines containing several polysaccharide-conjugate antigens, and also to develop vaccines which target both protein as well saccharide epitopes of the same pathogen. Accordingly we have conducted a study to identify new potential carrier proteins. Twenty-eight proteins, derived from different bacteria, were conjugated to the model polysaccharide Laminarin and tested in mice for their ability in inducing antibodies against the carbohydrate antigen and eight of them were subsequently tested as carrier for serogroup meningococcal C oligosaccharides. Four out of these eight were able to elicit in mice satisfactory anti meningococcal serogroup C titers. Based on immunological evaluation, the *Streptococcus pneumoniae* protein spr96/2021 was successfully evaluated as carrier for serogroups A, C, W, Y and X meningococcal capsular saccharides.

1. Introduction

Glycoconjugate vaccines are safe and efficacious tools to prevent infection and mortality against different encapsulated bacterial pathogens [1–3].

In these type of vaccines carbohydrate antigens are covalently bound to a carrier protein which provides T-cell epitopes that confer a T dependent character to the saccharide moiety.

Five carrier proteins are currently included in licensed glycoconjugate vaccines [4–7]. Diphtheria (DT) and Tetanus toxoids (TT), derived 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 [8]. CRM_{197}, a non-toxic mutant of diphtheria toxin [9], has been extensively used as carrier for licensed conjugate vaccines against *Haemophilus influenzae* type b (Hib), *Streptococcus pneumoniae*, *Neisseria meningitidis*) and for other vaccines in clinical development. The outer membrane protein complex (OMPC) of *N. meningitidis* serogroup B has been used as carrier for the Hib conjugate vaccine developed by Merck [10]. Protein D, derived from non-typeable *H. influenzae* (NTHi), has been used as carrier for most of the polysaccharides included by GSK into the multivalent pneumococcal conjugate vaccine recently licensed [11,12].

Multivalent conjugates administration and vaccines co-administration can be associated with immune interference such as carrier-specific enhancement of T-cell help, carrier-induced-epitopic suppression (CIES) or bystander interference. Carrier priming and suppression effect were investigated in both animal and human studies and different behaviors between the commonly used carriers were reported [13–15]. Considering these aspects, novel carriers might help in avoiding or reducing the impact of the above mentioned issues and enable the further development of pediatric schedules and combinations [16].

Other proteins are increasingly used as carrier and some of them have also been tested in clinical trials. The recombinant non-toxic form of *Pseudomonas aeruginosa* exotoxin A (*r*EPA) has been used as carrier for *Shigella* O-antigens [17] and *Staphylococcus aureus* type 5 and 8 capsular polysaccahrides as well as for *Salmonella Typhi* Vi antigen [18–20]. *r*EPA is also used as a carrier for glycoconjugate vaccines directly synthesized in *Escherichia coli* against *Shigella dysenteriae* type 1 [21]. A rationally designed recombinant protein containing strings of promiscuous human CD4+ T-cell epitopes derived from various pathogens including

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tetanus, influenza virus, *Plasmodium falciparum* and hepatitis B virus, proved to be a very good carrier for Hib and meningococcal oligosaccharides [22–24]. Endeavors to develop carrier proteins which might also provide protection against a target pathogen, have recently broadened to include candidates from diverse vaccine categories [25–30].

Taking advantage from the availability of several recombinant proteins originated from a variety of reverse vaccinology projects undertaken in our Research Center, we have conducted a study to identify new carrier proteins, derived from different bacteria like extraintestinal pathogenic *E. coli* (ExPEC), *N. meningitidis* serogroup B (Men B), group A *Streptococcus* (GAS), group B *Streptococcus* (GBS) and *S. pneumoniae* (sp).

The strategy was based on two steps: (a) immunogenicity screening in mice of several recombinant proteins conjugated to a model saccharide in comparison to CRM_{197} as benchmark carrier protein; (b) testing selected carriers conjugated to meningococcal carbohydrate antigens. Here we describe the results of this study. Laminarin (Lam) was the model saccharide used for the first immunogenicity screening, a neutral sugar antigen studied as vaccine candidate against infections induced by *Candida albicans* [31].

2. Materials and methods

2.1. Reagents

Laminarin was obtained from Sigma Aldrich (L9634).

Serogroup A, C, W, Y meningococcal oligosaccharides were provided by the Manufacturing Department (GSK, Siena, Italy).

Men X polysaccharide was produced internally by Research Center (GSK, Siena, Italy).

CRM_{197} was provided by the Manufacturing Department (GSK, Siena, Italy).

Proteins tested as new carrier candidates were produced internally by Research Center (GSK, Siena, Italy).

Men B 961c (NadA or GNA1994), GNA2132-1030 (NHBA-GNA1030) and GNA2091-fHbp (GNA2091–GNA1870) proteins were provided by the Manufacturing Department (GSK, Siena, Italy).

2.2. Preparation of protein carriers

The twenty eight recombinant proteins tested as potential new carrier candidates were expressed in *E. coli*, twenty five of them were expressed as His-tag fusion proteins and purified by immobilized metal affinity chromatography. Details of cloning, expression and purification strategy are reported in SI. All the proteins tested are reported in Table 1 SI with bacteria origin, protein name and alternative name reported in the bracket. Sp proteins were identified as spr when derived from R6 genome or D39 (spr1418 and spr1712), non spr are derived from TIGR4 genome or INV104 genome (PitB).

2.3. Preparation of conjugates

Lam has been treated by reductive amination using the following conditions: 2 mg/ml of polysaccharides were incubated for 5 days at 50 °C in 300 mg/ml ammonium acetate (Sigma–Aldrich) and 0.2 M sodium cyanoborohydride ($NaBH_3CN$) (Sigma–Aldrich) pH 7.5. Aminated Lam was purified by diafiltration on regenerated cellulose membrane (cut-off 1 kDa; Millipore) and the number of primary amino groups introduced were determined by colorimetric assay [32].

The amino-oligosaccharides were vacuum dried, solubilized in 1:9 H_2O:DMSO solution to a final amino group concentration of 40 μmol/ml, and reacted with a 12-fold molar excess of adipic acid bis(N-hydroxysuccinimmide) (SIDEA), in presence of 5-fold molar excess triethylamine as compared with amino groups [33]. The reaction was kept under gentle stirring at room temperature for 2 h. The activated oligosaccharides were obtained by precipitation with 4 volumes of acetone and dried under vacuum. The content of N-hydroxysuccinimide ester groups introduced was determined [34].

Lam conjugates have been prepared in 10–100 mM NaH_2PO_4 pH 7 or in phosphate buffer saline pH 7 (PBS, 150 mM NaCl, 10 mM Na_2HPO_4, 2 mM KCl, 2 mM KH_2PO_4) depending on the protein buffer, using an active ester (AE):protein molar ratio of 30:1, and a protein concentration in the range of 0.4–6 mg/ml. Reactions were carried out overnight at room temperature with gentle stirring.

The conjugates have been purified by affinity chromatography with His MultiTrap 96-Well Filter Plates (GE Healthcare) using the His tag portion on the recombinant protein to bind the conjugate to the resin. Conjugates have been loaded on the resin (250 μg of protein per well in a volume of 200 μl in the same buffer of conjugation) and incubated for 30 min after gently mixing on the well. After, the flow through have been taken by using the vacuum air and two washes 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 0.5 M NaCl, 0.5 M NaH_2PO_4, 0.5 M Imidazole pH 6.3, incubating for 15 min and collecting the flow through; the elution has been repeated two times. Eluted conjugates have been dialyzed with 6–8 kDa membrane (Spectra/Por 1, diameter 6.4 mm) against 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. Non His-tag protein conjugates were purified by Ultrafiltration with Vivaspin 10 K (Sartorius).

SIDEA-activated Men A, C, W, and Y oligosaccharides and their CRM_{197} conjugates were obtained from GSK Vaccine Manufacturing, produced as previously described [35]. Spr96/2021–Men A, C, W, Y conjugations were carried out in 10–100 mM NaH_2PO_4 pH 7 or in PBS pH 7 depending on the protein buffer, at a protein concentration of 3–3.5 mg/ml and a saccharide to protein molar ratio of 12:1. Reactions were let to proceed overnight at room temperature with gentle stirring. Purification has been done by gel filtration or by ultrafiltration as reported above. The same conditions were applied to obtain the conjugates between Men C activated oligosaccharides and the proteins Upec-5211, Orf3526, GNA2091-fHbp, RrgB I-II-III, spr907 and spr1418.

Men X polysaccharide was size reduced and activated for the conjugation as reported before [36]. Conjugation of spr96/2021 was performed at a protein concentration of 10 mg/ml with an oxidized polysaccharide to protein ratio 4:1 (w:w) and a saccharide to $NaBH_3CN$ ratio of 1:1 (w:w), at 37 °C for 48 h. The conjugate was purified by ammonium sulfate precipitation (500 g/l) and the pellet was dissolved in 10 mM NaH_2PO_4 at pH 7.2. CRM_{197}–Men X conjugate was prepared as previously published [36].

Conjugates were characterized by micro BCA for total protein content [37] and by HPAEC-PAD analysis (SI) for total saccharide content.

2.4. Mice immunization

Groups of 6–8 mice (BALB/c or CD1) were immunized subcutaneously on days 1, 14 and 28; bleedings were performed on day 0 (pre immune), day 27 (post 2) and day 42 (post 3).

All vaccines were administered on saccharide base in a volume of 200 μl. Lam conjugates were tested at 5 μg dose, meningococcal conjugates at 2 μg for Men A and 1 μg for Men C, W, Y, X.

Lam and Men A, C, W, Y conjugates were tested without any adjuvant while Men X conjugates were formulated with $AlPO_4$ at 0.12 mg Al^{3+}/dose.

Control groups received PBS alone or PBS/adjuvant.

Animal experimental guidelines set forth by the Animal Welfare Body of the Animal Resource Centre of our Company have been followed for conducting all animal studies.

2.5. Analysis of antibody response by ELISA

2.5.1. Antibody response induced by the glycoconjugates against the homologous polysaccharide

The antibody response induced by the glycoconjugates against the homologous polysaccharide has been measured by ELISA. Plates have been coated with the different meningococcal polysaccharides by adding 100 μl/well of a 5 μg/ml polysaccharide solution in PBS buffer at pH 8.2 followed by incubation overnight at 4 °C. Lam coating has been done at 50 μg/ml of polysaccharide solution in 0.05 M Na_2CO_3–$NaHCO_3$ buffer at pH 9.6. Coating solutions were removed from the plates by washing three times with PBS buffer with 0.05% v/v of Tween 20 (Sigma) (TPBS). A blocking step has been then performed by adding 100 μl/well of BSA solution at 3% w/v in TPBS and incubating the plates 1 h at 37 °C. Blocking solution has been removed from the plates by washing three times with TPBS. 200 μl/well of pre-diluted serum (1:25 for pre immune (negative control), 1:100–1:200 for a reference serum and from 1:50 to 1:500 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 two-fold serial dilutions along each column were then performed by transferring from well to well 100 μl of sera solutions. After primary antibody dilution, plates have been incubated for 2 h at 37 °C. After three washes with TPBS, 100 μl/well TPBS solutions of secondary antibody alkaline phosphates conjugates (anti mouse IgG 1:10,000, Sigma–Aldrich) were added and the plates incubated 1 h at 37 °C. After three more washes with TPBS, 100 μl/well of a 1 mg/ml of p-NPP (Sigma) in a 0.5 M diethanolammine buffer pH 9.6 was added. After 30 min of incubation at room temperature, plates were read at 405 nm using a Biorad plate reader. Raw data acquisition was 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 depending on the study. Each immunization group has been represented as the geometrical mean (GMT) with 95% CI of the single mouse titers. The statistical and graphical analysis has been done by GraphPad Prism 6.0 software.

2.5.2. Antibody response against the carrier proteins

The antibody response induced by the glycoconjugates against the protein has been measured by ELISA. Plates were coated with the different proteins at 2 μg/ml in PBS buffer at pH 7.2. Pools of sera were tested applying the same procedure described in Section 2.5.1.

2.5.3. Serum bactericidal assay (rSBA)

Functional antibodies induced by vaccine immunization were analyzed by measuring the complement-mediated lysis of *N. meningitidis* with an in vitro bactericidal assay [38].

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. 10^4 CFU/ml). The total volume in each well was 50 μl with 25 μl of serial two-fold dilutions of the test serum, 12.5 μl of bacteria at the working dilution and 12.5 μl of baby rabbit complement. The tested sera were pooled and heat-inactivated for 30 min 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). The microtiter plate was incubated for 1 h 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 titer 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 150–200% increase in CFU/ml, during the 60 min incubation time. The reference strains for each meningococcal serotype used were: F8238 (Men A); C11 (Men C); 240070 (Men W); 860800 (Men Y); Z9615 (Men X). Men A, C, W and Y strains are O-acetyl positive.

2.6. MHC II binding prediction

Investigation on MHC II high affinity binding peptides on the proteins has been done by IEDB Analysis Resource [39], as reported in SI.

3. Results

3.1. Screening of potential new carrier proteins using Lam as carbohydrate pilot model

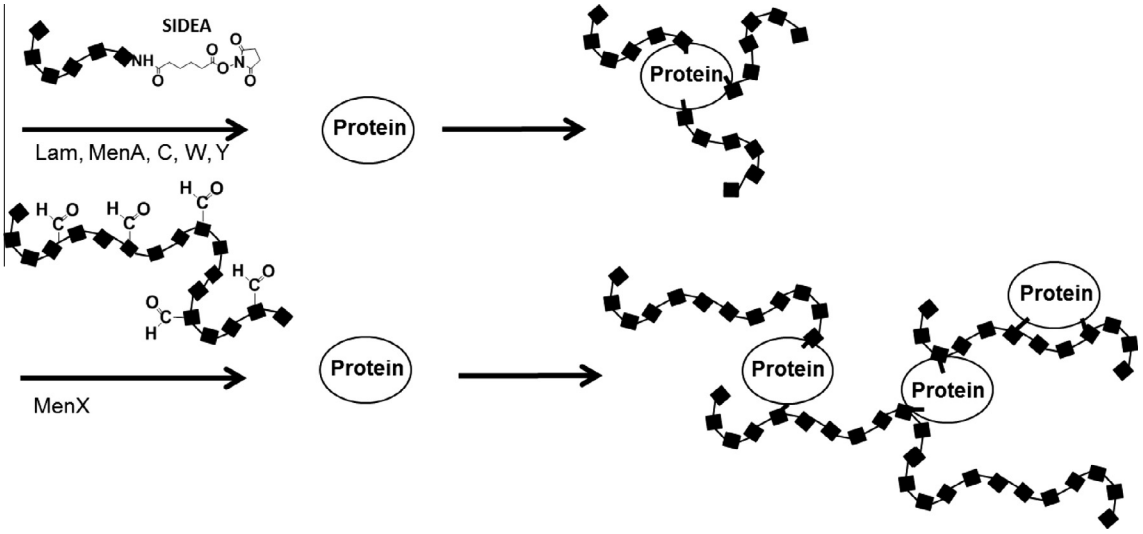
A panel of twenty-eight proteins derived from different pathogens and expressed recombinantly in *E. coli*, was conjugated to the neutral sugar Laminarin (Lam), a β-(1, 3) glucan with sporadic β-(1, 6) branches, that has been studied as vaccine candidate against *C. albicans* infections [31,40,41].

The proteins (Table 1 SI) were selected using the following criteria: not expected toxicity based on available information, solubility in physiological buffers at concentrations of 0.4–6 mg/ml, and presence of a sufficient number of lysines for conjugation. In addition we have in general preferred proteins with molecular weight between 40 and 100 kDa. Lam was derivatized, at its end reducing group, with the linker succinimido diester of adipic acid (SIDEA) in such a way to have a terminal succinimido ester group available to react with the lysine residues of the proteins. The activated oligosaccharide was then reacted with the different proteins using the same active ester/protein molar ratio (Scheme 1). A CRM_{197}–Lam conjugate was prepared to be used as a control in each immunogenicity study.

In sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–Page) the glycoconjugates appeared with a typical smear, due to the variable number of sugar chains bound to the protein and to the polydispersion of the sugar chain length; an example is reported in Fig. 1 SI.

The average glycosylation degree of the different glycoconjugates, calculated as saccharide to protein w/w ratio, varied from 1.7 to 0.1 w/w and an example is reported in Table 1. This diversity did not correlate with the proportion of lysines on each protein and might be due to their different reactivity depending on local environment.

The conjugates were tested in mice without using any adjuvant in order to highlight the intrinsic value of the carrier only. In each immunogenicity study a control group of mice was immunized with CRM_{197}–Lam. Only the conjugate with spr96/2021 induced an anti-Lam antibody titer significantly higher than the one induced by CRM_{197}–Lam ($p = 0.01$; Fig. 1). As determined by ELISA



Scheme 1. Different chemical approaches applied to obtain activated oligosaccharides or polysaccharide to be covalently coupled to the carrier protein. End terminal reducing chemistry was applied to Lam, Men A, C, W, Y and random oxidation chemistry to Men X polysaccharide activation.

Table 1
Lam and Men C glycoconjugates characterization by saccharide to protein ratio in term of weight/weight (w/w) and relative % of lysine residues on the carrier protein.

Carrier protein	Lam conjugates Saccharide/protein (w/w)	Men C conjugates Saccharide/protein (w/w)	% of lysines in the carrier molecule
Upec-5211	0.4	0.3	7.49
Orf3526	0.3	0.1	6.05
GNA2091-fHbp	0.4	0.5	6.91
RrgB I-II-III	0.1	0.1	9.38
Spr907	0.2	0.1	7.38
Spr96/2021	0.5	0.3	5.04
Spr1418	1.2	0.6	7.33
Spr1875	0.4	0.3	4.20
CRM_{197}	0.7	0.6	7.28

on pool of sera after the third injection, and similarly to CRM_{197}, spr96/2021 conjugate was able to induce also anti-carrier IgG, interesting information considering the potential of spr96/2021 as antigen against *S. pneumoniae* (Fig. 2 SI). In Fig. 3 SI we have reported the immunogenicity of all the Lam conjugates expressed as the difference between the median of each group and the median of the relative control group CRM_{197}–Lam. The small number of mice in each group and the variability in the immunological response, probably due to the absence of adjuvant in the formulations, limited the ability to highlight statistical significant differences between several carrier proteins and their control. Since spr96/2021 is a recombinant fusion of the two pneumococcal proteins spr0096 (LysM domain secreted protein) and spr2021 (secreted PcsB protein) [42–44], we addressed the question whether one of the two moieties could be responsible for this strong carrier effect and we tested in mice Lam conjugates with spr0096 and spr2021 respectively. Surprisingly no response was induced by the spr0096 conjugate and very low by the spr2021 one (Fig. 4 SI), indicating that some feature derived from the fusion of the two proteins is critical for the carrier activity.

3.2. Evaluation of selected proteins as carrier for serogroup C meningococcal oligosaccharide

We then decided to test in mice the protein spr96/2021 as carrier for meningococcal serogroup C (Men C) oligosaccharides. We also included in the evaluation other seven proteins, among those initially tested in the Lam model, whose median of the anti Lam response was similar, intermediate or low when compared to their CRM_{197}–Lam control: Orf3526 and spr907, Upec-5211 and spr1418, GNA2091-fHbp [45], RrgB I-II-III, and spr1875. The conjugation to Men C oligosaccharides was carried out using the same reaction scheme described above for the Lam conjugates (Scheme 1). The activated oligosaccharides were reacted with the different proteins as described in Methods. The purified glycoconjugates had a saccharide to protein ratio ranging from 0.6 to 0.1 w/w (Table 1). Also in this case a CRM_{197}–Men C conjugate was prepared to be used as control.

The spr96/2012 conjugate induced anti Men C polysaccharide IgG titers comparable to those developed by CRM_{197} conjugate ($p > 0.05$). Interestingly also Upec-5211, Orf3526, and spr1875 conjugates response was comparable to the control ($p > 0.05$), while GNA2091-fHbp, RrgB I-II-III, spr907 and spr1418 conjugates were inferior to the control ($p < 0.01$) (Fig. 2).

3.3. Evaluation of spr96/2021 as carrier for serogroup A, W, Y, and X meningococcal saccharides

Based on the attractive behavior of spr96/2021 as carrier in both Lam and Men C models, we decided to further explore its potential with the meningococcal serogroups A, W, Y oligosaccharides (Men A, Men W, Men Y), which are currently part of licensed vaccines [46,47], and X polysaccharide (Men X), recently identified as a potential candidate for vaccine development [36]. The conjugation to the individual Men A, Men W and Men Y oligosaccharides was carried out essentially as described above for Men C. Similarly to what already described for CRM_{197}, the conjugation to Men X was achieved after size reduction of the polysaccharide to an average polymerization degree (avDP) of about 130 followed by random oxidation of the N-acetyl glucosamine phosphate primary hydroxyl groups and coupling of the resulting aldehyde groups to lysine ε-amine groups of the protein by reductive amination (Scheme 1) [36]. Purified conjugates were characterized by SDS–Page (Fig. 5 SI) and for their saccharide and protein content. The glycosylation degrees were in the range between 0.7 and 0.2 w/w (Table 2). The SDS–Page profile of spr96/2021–Men X is similar to the one reported for CRM_{197}–Men X [36] and is consistent with an high molecular weight conjugate and confirmed by the SEC-HPLC analysis reported in SI (Fig. 6 SI). This is not surprising considering that the random conjugation chemistry used for this conjugate can generate cross

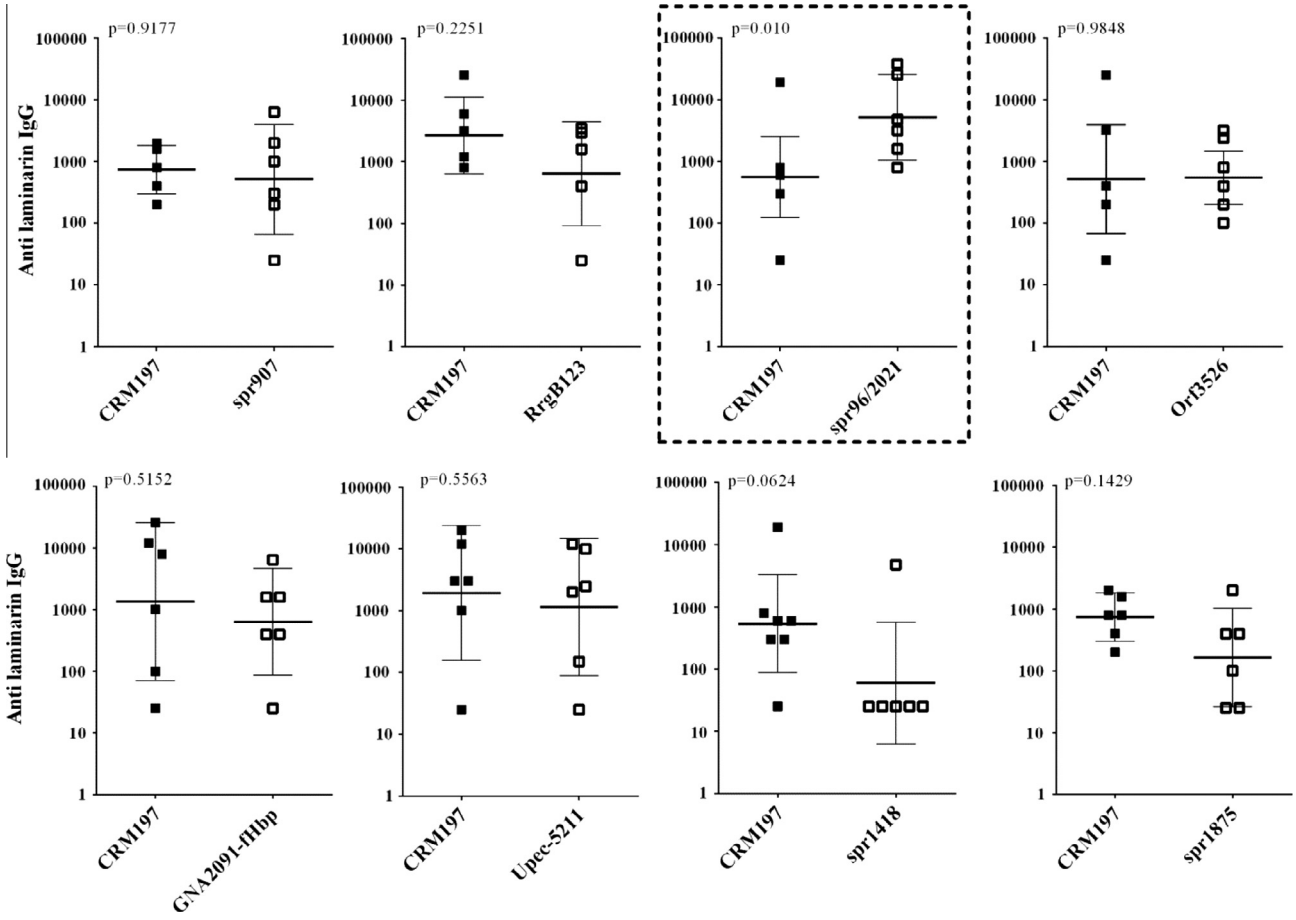


Fig. 1. Anti-Lam antibody levels induced by Lam conjugates with new candidate carrier proteins in comparison with CRM_{197}–Lam used as positive control. Each spot indicates a single mouse ELISA titer, the horizontal bar refers to the geometric mean of the group and the vertical bar shows the 95% CI. Non parametric Mann–Whitney test has been used for *p* value calculation.

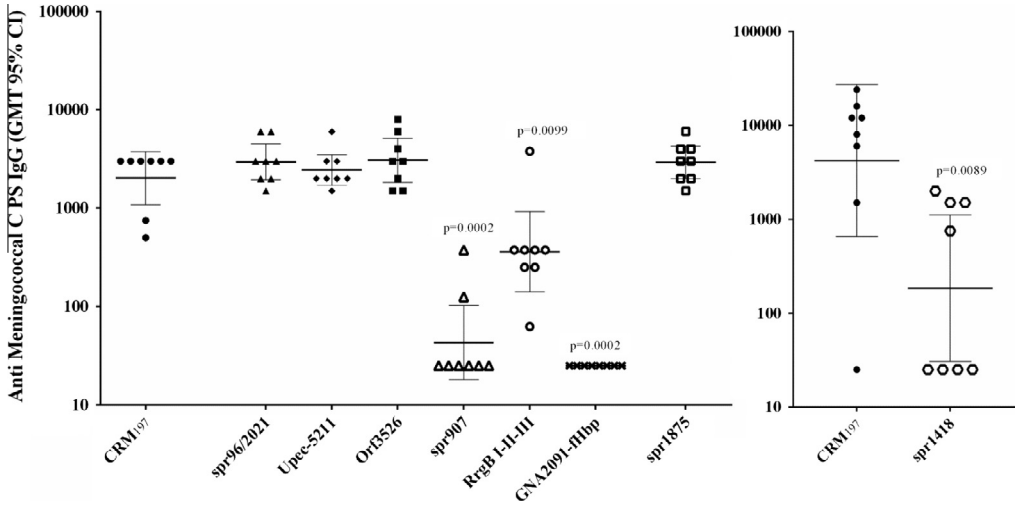


Fig. 2. Anti-Men C antibody levels induced by glycoconjugates with different carriers in comparison with the positive control CRM_{197}. Each dot indicates a single mouse ELISA titer, the horizontal bar is the geometric mean of the group and the vertical bar shows the statistical 95% CI. Non parametric Mann–Whitney test has been used for *p* value calculation.

linked products (Scheme1) and that Men X saccharide has been subject to a modest size reduction. Also in this case control CRM_{197}–Men A, W, Y and X conjugates were prepared.

When tested in mice, the spr96/2021–Men A, W,Y and X based conjugates, after three doses of vaccine, were able to induce a level of specific anti-Men IgGs substantially comparable with that of CRM_{197} conjugates ($p > 0.05$; $p = 0.2620$; Fig. 3).

The functionality of the antibodies elicited by the spr96/2021 glycoconjugates against each meningococcal bacterial strain was comparable with that induced by CRM_{197} conjugates, as

Table 2
Men A, W, Y and X glycoconjugates with spr96/2021 and CRM_{197}, characterization by saccharide to protein ratio in term of weight/weight (w/w).

Spr96/2021 conjugates	Saccharide/ protein (w/w)	CRM_{197} conjugates	Saccharide/ protein (w/w)
Men A	0.3	Men A	0.4
Men W	0.7	Men W	0.7
Men Y	0.4	Men Y	0.6
Men X	0.2	Men X	0.3

Table 3
rSBA titers induced by mice iper-immune serum obtained after immunization with CRM_{197}–Men A, C, W, Y and X and spr96/2021–Men A, C, W, Y and X glycoconjugate vaccines. Men A, C, W, Y sera were tested after two doses of vaccines instead Men X after three doses.

Rabbit complement serum bactericidal activity		
Conjugates	CRM_{197}	Spr96/2021
Men A	2048	4096
Men C	1024	2048
Men W	128	512
Men Y	2048	1024
Men X	256	1024

determined by rabbit complement mediated bactericidal assay (rSBA) (Table 3).

3.4. MHC II binding peptides prediction

Following the indication reported on IEDB, we have chosen 1.5 percentile rank as cut-off for a comparative analysis between the different proteins based on the number of MHC II high affinity peptides. This value allows us to take in consideration only the peptides with very high affinity and obtain a useful number of peptides to compare all the proteins tested. This rank corresponds also to the first 20 peptides with high affinity obtained for the amino acidic sequence of CRM_{197} that is the protein used as benchmark in our studies. Based on this investigation we obtained, among all the proteins tested, a variable number of high affinity binder peptides, from 0 of NarE, SAG0646 and spr1287 to 68 of SPyAD. We did not find any correlation between the carrier functionality and these results.

4. Discussion

Few carrier proteins have been tested so far for clinical evaluation of conjugate vaccines and only five of them (DT, TT, CRM_{197}, OMPC and NTHi protein D) are currently used for licensed vaccines against bacterial infections. The increased attention to multivalent combination vaccines and the possible CIES or bystander interference affecting polysaccharide conjugate antigens drives through the need of alternative carrier proteins, which could also be useful to target multiple and different virulence factors (protective epitopes) of pathogens. Accordingly we have conducted a screening of several bacterial proteins to investigate their potential as carrier for glycoconjugate vaccines and identify possible candidates for further development. In a first screening step, twenty eight proteins were conjugated to Lam using the end-groups chemistry illustrated in Scheme 1.

The protein spr96/2021 emerged as a promising carrier inducing titers significantly higher than CRM_{197}–Lam (Fig. 1). Since Lam is a non-charged saccharide we thought interesting to extend the study using Men C oligosaccharides which have a negatively charged repeating unit. Accordingly spr96/2021 and also some other proteins of those initially tested in the Lam model and showing similar, intermediate or low median anti Lam response as compared to their CRM_{197}–Lam control, were conjugated to Men C oligosaccharides and tested in mice. Spr96/2021–Men C, induced anti-Men C CPS IgG titers comparable to those developed by CRM_{197}–Men C, confirming the results obtained in the Lam model. Subsequently the potential of spr96/2021 to work as carrier for carbohydrate antigens was confirmed with Men A, Men W, Men Y and Men X. The corresponding conjugates induced in mice anti-meningococcal IgGs and rSBA titers comparable to those developed by glycoconjugates of CRM_{197} which is a well-known carrier and successfully used in several licensed glycoconjugate vaccines like *Menjugate*™, *MENVEO*™ and *Prevenar* (Wyeth).

The response induced by the Men C conjugates with the other selected proteins did not always follow the same trend of the initial screening with Lam. In fact while Upec-5211, Orf3526 and spr1875 conjugates induced anti-Men C CPS IgG titers comparable to CRM_{197}–Men C, spr907, GNA2091-fHbp, RrgB I-II-III conjugates were significantly less immunogenic.

The role of the carrier in glycoconjugate vaccines is to provide T-cell epitopes which bind to the class II MHC and subsequently, on the surface of the antigen presenting cells, interact with the CD4+ T cell receptor to trigger signals which induce B-cell differentiation in carbohydrate-specific plasma and memory cells. Many factors are important in determining the ability of a protein to work as carrier. As well as the number of MHC II high affinity binding peptides, other factors like the glycoconjugate processing inside the antigen presenting cells, the strength of the interaction

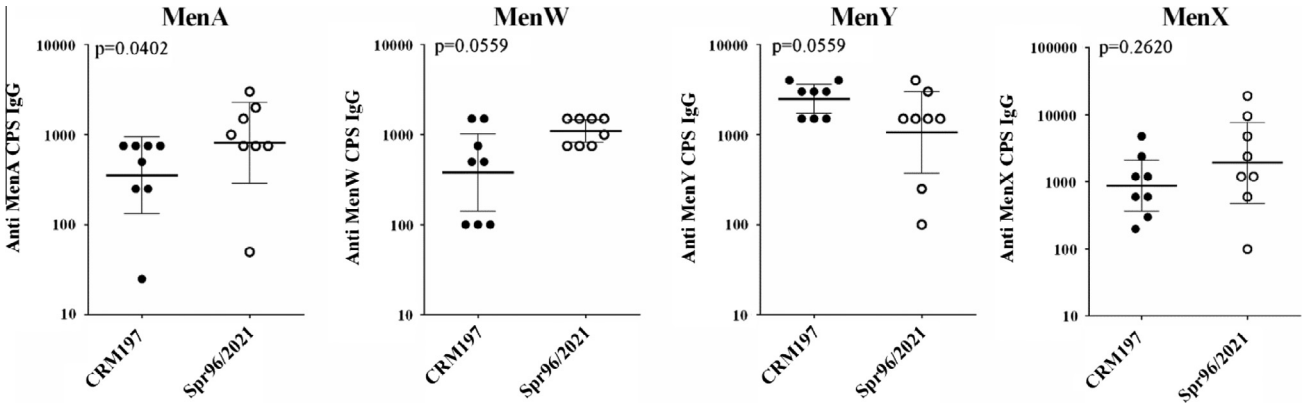


Fig. 3. Anti-Men A, W, Y, X antibody levels induced by spr96/2021–Men A, W, Y, X glycoconjugates in comparison with the positive control CRM_{197}–Men A, W, Y, X conjugates. Each dot indicates a single mouse ELISA titer, the horizontal bar is the geometric mean of the group and the vertical bar shows the statistical 95% CI. Non parametric Mann–Whitney test has been used for *p* value calculation.

with the T-cell receptor, the chemical glycosylation procedure, the extent and localization of glycosylation, might affect the integrity of the key T-cell epitopes, therefore determining the immunogenicity of the conjugate.

By applying the MHC class II prediction algorithms IEDB Analysis Resource (IEDB-AR) [39] we did not find any correlation between the number of high affinity MHC II binders in the carriers and the immunogenicity of their corresponding Lam conjugates. Similarly we did not find a correlation between immunogenicity of the Lam conjugates and their glycosylation degree as well as the carrier molecular weight.

In terms of chemical procedure we should consider that the chemistry used in this work is based on the conjugation to the lysine residues of the proteins. This process might have selected preferentially the protein whose highest affinity T-cell epitopes do not contain lysines in critical position. Moreover for a given carrier the glycosylation profile obtained with a neutral oligosaccharide might differ from that of charged one. It would be interesting to test conjugation chemistries that involve different amino acid residues and to analyze the glycosylation profiles of carriers conjugated to charged and non-charged saccharides, as well as the anti-carrier antibodies induced by the different conjugates.

Interestingly when the non-fusion proteins spr0096 or spr2021 were tested as Lam conjugates, no response or significantly lower than CRM_{197} response was induced respectively by spr0096 and spr2021. This behavior could be due to several factors including the different molecular weights (18.7 kDa for spr0096 and 38.9 kDa for spr2021) and the number and affinity of MHC class II binding peptides. Moreover the glycosylation profile of the fusion protein could be different for the fusion and single proteins, leading to a different impact on the preservation of T-cell epitopes and/or the conjugates processing inside the antigen presenting cells; we are currently investigating this aspect.

In conclusion, by screening a large number of recombinant proteins and focusing on two types of saccharide structures, the β-glucan based non charged Laminarin and the polyanionic meningococcal oligosaccharides, we have identified, based on immunological data, a panel of proteins that deserve further attention. In particular spr96/2021 is a new potential protein carrier for carbohydrate haptens which might also offer pathogen-protective effect. We are accumulating evidences that spr96/2021 can be successfully used as carrier for pneumococcal derived carbohydrates and in meningococcal multivalent formulations, this aspect has not been addressed in this paper and will be part of a comprehensive preclinical investigation. For a new vaccine, including conjugate vaccines, time to market is variable. While in the 1980s it was determined more by the discovery phase, impressive technological revolutions made the discovery phase significantly shorter, but at the same time the development path which includes process development, clinical and regulatory development, and GMP manufacturing is longer and fully justified by increased cGMP and good clinical practice standards, making the overall time to market still in the range of 10–15 years [48].

Conflict of interests

All authors are employees of the GSK group of companies.

Tontini M., Romano M. R., Proietti D., Micoli F., Balocchi C., Masignani V., Berti F. and Costantino P. were listed as an inventor on patents owned by the GSK group of companies.

Author contributions

TM, RMR, MF, BF and CP were involved in the conception and design of the study. TM, PD, BE, MF, BC and SL acquired the data. TM, RMR, MF, BF, VM and CP analyzed and interpreted the results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

Trademark statement

MENVEO and Menjugate are trademarks of the GSK group of companies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.06.039.

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