

# Development of a glycoconjugate vaccine to prevent meningitis in Africa caused by meningococcal serogroup X

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*Neisseria meningitidis* is a major cause of bacterial meningitis worldwide, especially in the African meningitis belt, and has a high associated mortality. The meningococcal serogroups A, W, and X have been responsible for epidemics and almost all cases of meningococcal meningitis in the meningitis belt over the past 12 y. Currently no vaccine is available against meningococcal X (MenX). Because the development of a new vaccine through to licensure takes many years, this leaves Africa vulnerable to new epidemics of MenX meningitis at a time when the epidemiology of meningococcal meningitis on the continent is changing rapidly, following the recent introduction of a glycoconjugate vaccine against serogroup A. Here, we report the development of candidate glycoconjugate vaccines against MenX and preclinical data from their use in animal studies. Following optimization of growth conditions of our seed MenX strain for polysaccharide (PS) production, a scalable purification process was developed yielding high amounts of pure MenX PS. Different glycoconjugates were synthesized by coupling MenX oligosaccharides of varying chain length to CRM<sub>197</sub> as carrier protein. Analytical methods were developed for in-process control and determination of purity and consistency of the vaccines. All conjugates induced high anti-MenX PS IgG titers in mice. Antibodies were strongly bactericidal against African MenX isolates. These findings support the further development of glycoconjugate vaccines against MenX and their assessment in clinical trials to produce a vaccine against the one cause of epidemic meningococcal meningitis that currently cannot be prevented by available vaccines.

A major cause of bacterial meningitis worldwide, *Neisseria meningitidis* has significant associated mortality (1). Among the 13 distinct meningococcal serogroups, which are classified on the structure of their capsular polysaccharide (PS), serogroups A, B, C, Y, W, and X most commonly cause invasive disease, including meningitis and septicemia, in humans. The highest incidence of meningococcal meningitis occurs in the meningitis belt of sub-Saharan Africa, extending from Senegal to Ethiopia.

Since records began, meningococcal serogroup A (MenA) has been the dominant cause of epidemics of meningococcal meningitis in this region (2), but MenW (3) and MenX (4–6) have also been responsible for epidemics. From 2010 to 2012, MenX was responsible for annual meningitis outbreaks in Burkina Faso. In 2011, MenX accounted for 59% of confirmed cases of meningococcal meningitis in this country (7). Higher case fatality rates have been reported for meningitis caused by MenX compared with MenA (4, 6), and children aged 1–9 y constitute the most affected age group (4, 8).

In 2010, a MenA conjugate vaccine (MenAfriVac) was rolled out in a mass vaccination program in Burkina Faso, Mali, and

Niger (9). Early reports indicate that this has been highly effective at reducing cases of MenA meningitis. Removal of serogroup A strains from circulating among the population may confer an advantage to MenX, previously less able to compete with the more virulent serogroup A (10, 11). Capsule replacement of carried meningococci did not occur following the implementation of serogroup C conjugate vaccines in the United Kingdom (12). However, the conditions in the meningitis belt are very different from those in industrialized nations and a recent study of carriage before and after the introduction of the MenA conjugate vaccine in Burkina Faso found significantly higher levels of MenX carriage following the introduction of the vaccine (13).

MenW PS vaccine is used for outbreak control of meningitis caused by MenW in the meningitis belt and a change in the epidemiology of meningitis due to MenW could necessitate its increased demand. Polyvalent vaccines, including MenW glycoconjugate, are currently produced and used in developed countries and could potentially be mobilized for use in Africa. In contrast, although the need for a vaccine against serogroup X *Neisseria meningitidis* has been recognized for many years (4, 5, 14, 15), none is currently available.

Given the success of other meningococcal glycoconjugate vaccines (16), the MenX PS antigen is a logical target for vaccine design. Plain PS could facilitate epidemic control, whereas conjugation to a carrier protein would provide enhanced immunogenicity,

## Significance

Meningococcal serogroup X has recently emerged as a cause of meningitis outbreaks with epidemic potential in sub-Saharan Africa. Novel conjugation technologies, compatible with a reproducible production process, have been successfully used to develop immunogenic polysaccharide conjugate vaccine candidates that are likely to protect against meningococcal X disease. The timely development of an anti-meningococcal X conjugate vaccine appears a logical next step in the broadest control of meningococcal disease and requires commitment now.

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particularly from early infancy, by converting the PS into a T-cell-dependent antigen (17, 18). As recognized for other PS, conjugation to an appropriate carrier protein overcomes the limits of PS vaccines, such as poor efficacy in children less than 2 y, lack of immunological memory with poor booster responses, and relatively short duration of protection (19–21). Meningococcal conjugate vaccines are also able to overcome the immune hyporesponsiveness that is induced by PS vaccines (22, 23). Additionally, as documented for group C, meningococcal conjugate vaccines can reduce carriage of *N. meningitidis* in the nasopharynx, decreasing transmission (24), whereas PS vaccines have not been shown to provide substantial herd immunity (25). The impact of vaccination with the MenAfriVac conjugate vaccine in Burkina Faso on carriage and herd immunity has been recently reported (13).

The MenX PS was first characterized and defined as a distinct serogroup in the 1960s (26, 27) and was shown to be immunogenic in rabbits (28, 29). The structure of MenX PS consists of *N*-acetylglucosamine-4-phosphate residues held together by  $\alpha$ -(1-4) phosphodiester bonds without *O*-acetyl groups (28, 30–33).

Here, we describe the process of synthesis of MenX glycoconjugate vaccines, together with data from preliminary immunological evaluation in mice. MenX oligosaccharides (OS) of different length and three different conjugation chemistries were compared, using CRM<sub>197</sub>, a nontoxic mutant of diphtheria toxin (34), as carrier protein. When tested in mice, all MenX–CRM<sub>197</sub> conjugates resulted in high IgG antibody levels and serum bactericidal activity (SBA) titers, indicating the path ahead for the clinical development of a vaccine to prevent the remaining cause of epidemic meningococcal meningitis in Africa for which no vaccine is available.

## Results

**MenX PS Production.** To identify bacterial growth conditions for the optimal production and release of MenX PS into the supernatant, three different media were tested using the MenX strain 5967 (ST 750) (*SI Materials and Methods*). Modified Frantz medium resulted in the highest amount of PS expression (Table 1) and therefore was selected for higher scale (18 L) fermentation, which yielded 356  $\mu$ g/mL MenX PS. Proton NMR spectroscopy (<sup>1</sup>H NMR) was used to monitor PS expression (*SI Materials and Methods*).

The process for purifying MenX PS was built on the knowledge of equivalent processes for the quadrivalent meningococcal A, C, W-135, and Y glycoconjugate vaccine (Menveo) and for *Salmonella* Vi (35, 36). Applying our high-performance anion-exchange chromatography with pulsed amperometric detection method (37), able to quantify very low MenX PS concentrations ( $\geq 0.5$   $\mu$ g/mL) and, unlike other methods (32, 38), applicable to all process intermediates including fermentation broth, we demonstrated that our purification process provides high yields at each step (six steps in total), with an overall recovery of 56% when scaled to 16 L of clarified fermentation broth.

All purified MenX PS preparations contained low levels of contaminants [protein and nucleic acid <1% [weight to weight (wt/wt)] of PS, and endotoxin <5 EU/ $\mu$ g of PS]. The structural integrity of MenX PS was confirmed by NMR analysis (31–33) (*SI Materials and Methods*). Applying high-performance liquid

chromatography–size exclusion chromatography (HPLC-SEC), the PS eluted at a  $K_d$  of 0.34 from a TSK gel 6000PW connected in series with a TSK gel 5000PW column, without evidence of PS depolymerization or aggregation (*SI Materials and Methods*).

**MenX–CRM<sub>197</sub> Conjugates.** To identify a feasible and reproducible conjugation process, low-scale preparations were first performed using the native MenX PS. By applying a random activation of the phosphate groups along the saccharide chain with carbodiimide, complex products difficult to purify and characterize were obtained. MenX OS preparations with shorter chain lengths, obtained by mild acid hydrolysis (see *SI Materials and Methods* for details of preparation and characterization), were consequently used to prepare conjugates with three different coupling strategies (Fig. 1).

MenX<sub>OX</sub>–CRM<sub>197</sub> (Fig. 1A) was prepared using MenX OS of average degree of polymerization (avDP) 80–100 (average  $M_r$ , 24–30 kDa). The primary hydroxyl groups of *N*-acetyl glucosamine phosphate repeating unit were randomly oxidized using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), to introduce aldehyde groups which were then directly coupled to Lys  $\epsilon$ -amine groups of CRM<sub>197</sub> by reductive amination. <sup>1</sup>H NMR was used to confirm the presence and estimate the concentration of functional aldehyde groups generated at position C<sub>6</sub> by oxidation (*SI Materials and Methods*). Although proton NMR signals were expected both for the aldehyde and the hydrate aldehyde forms, only the signal at 5.32 ppm representing the hydrate aldehyde form was detected, possibly because the conditions used to perform the experiment shifted the equilibrium toward this species. The saccharide oxidation was estimated by integration of the hydrate aldehyde peak ( $H_{6ha}$ ) in comparison with  $H_1$  peak (proton at position C<sub>1</sub>): one repeating unit every 15–25 units was found to be oxidized. As confirmed by HPLC-SEC analysis, no degradation or aggregation of MenX OS occurred due to oxidation.

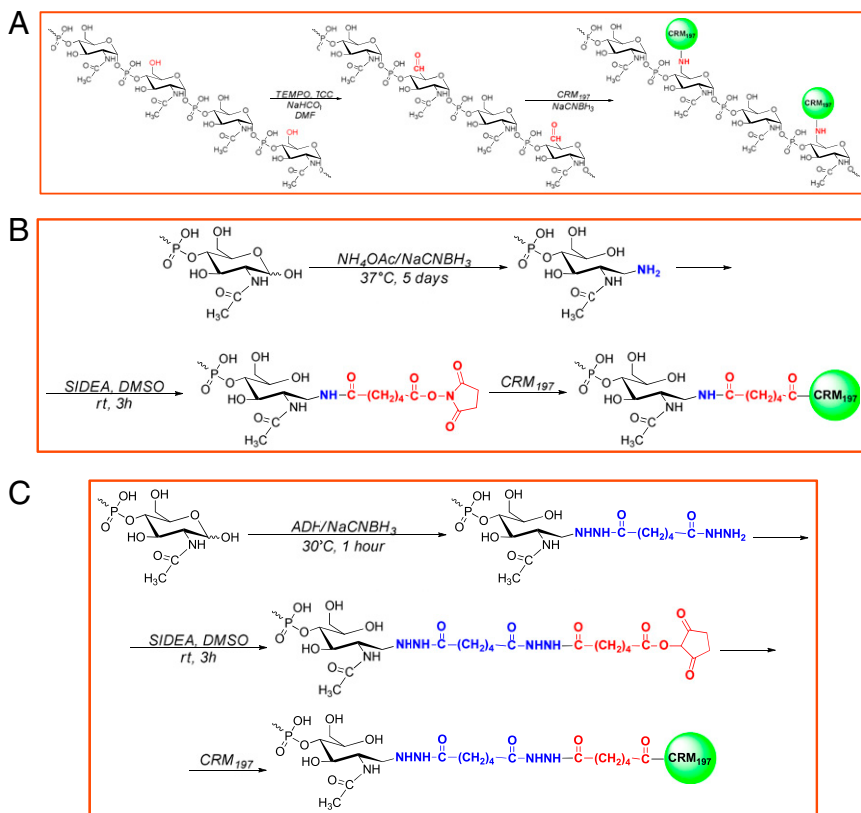
The other two coupling strategies were based on MenX OS of avDP 15–20 (average  $M_r$ , 4.5–6 kDa). In one case, the Menveo conjugation chemistry (35), consisting of reductive amination at the end of the sugar chain with ammonium acetate (NH<sub>4</sub>OAc), followed by reaction with adipic acid bis(*N*-hydroxysuccinimide) (SIDEA) and conjugation to CRM<sub>197</sub>, was applied (Fig. 1B). A similar chemistry was evaluated where the reductive amination was conducted using adipic acid dihydrazide (ADH) followed also in this case by derivatization with SIDEA, thus resulting in a longer linker (Fig. 1C) (39). The use of more reactive ADH molecule resulted in shorter reaction time of the first step (from 5 d to 1 h) and higher derivatization yield: 40% of MenX chains derivatized with NH<sub>4</sub>OAc, compared with 81% using ADH, as quantified by 2,4,6-trinitrobenzenesulfonic acid (TNBS) colorimetric method. In both cases, >80% of saccharide chains were derivatized with SIDEA in the following reaction step. For all these process intermediates, no changes of  $M_r$  distribution occurred, as verified by HPLC-SEC analysis.

After verifying conjugate formation by SDS/PAGE and HPLC-SEC (Fig. 2), the three conjugation reaction mixtures were purified by preparative size exclusion column (Sephacryl S300 column). The fractions not overlapping with those corresponding to free CRM<sub>197</sub> and free MenX OS were collected. Table 2 summarizes the characterization data of the purified conjugates tested in mice. The conjugates obtained by end-groups activation showed comparable OS to protein ratios, higher than that obtained for the random conjugate. All of the conjugates were of higher  $M_r$  than free CRM<sub>197</sub> and free OS, as shown by the corresponding  $K_d$  values.

**Immunogenicity of Conjugates in Mice.** To assess the ability of conjugates to induce anti-MenX antibodies, female BALB/c (5–6 wk old) mice were immunized s.c. three times at 2-wk intervals with the vaccine candidates plus adjuvant. All MenX–CRM<sub>197</sub> conjugates were immunogenic, eliciting significantly higher anti-

**Table 1. MenX 5967 (ST-750) strain growth in different media and relative PS production**

Growth medium	OD, 600 nm	Saccharide,	
		$\mu$ g/mL	$\mu$ g Saccharide/OD
Modified Catlin v.6	2	22.55	13.3
MCDM1	6	42.73	7.1
Modified Frantz	2.8	62.6	22.4

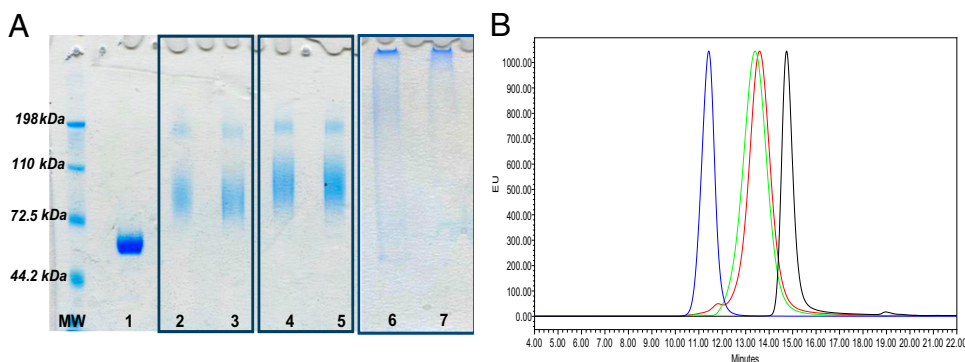


**Fig. 1.** Reaction scheme for the synthesis of MenX<sub>Ox</sub>-CRM<sub>197</sub> conjugate (A), MenX-NH<sub>2</sub>-SIDEA-CRM<sub>197</sub> conjugate (B), and MenX-ADH-SIDEA-CRM<sub>197</sub> conjugate (C).

MenX PS IgG ELISA units than the control group, which received PBS plus adjuvant ( $P$  value < 0.0001) (Fig. 3A). Mice immunized with unconjugated MenX PS or with the physical mixture MenX PS plus CRM<sub>197</sub> did not show anti-IgG response to MenX PS. MenX<sub>Ox</sub>-CRM<sub>197</sub> and MenX-ADH-SIDEA-CRM<sub>197</sub> conjugates, administered at a 1- $\mu$ g saccharide dose, induced peak IgG antibody levels after two injections. In contrast, the MenX-NH<sub>2</sub>-SIDEA-CRM<sub>197</sub> conjugate resulted in IgG antibody levels comparable to the other two conjugates only after the third dose, with a statistically significant booster effect compared with the level after the second dose ( $P$  value = 0.0002). By immunizing mice with a lower dose of conjugate (0.1  $\mu$ g of saccharide of MenX-ADH-SIDEA-CRM<sub>197</sub>), lower IgG titers were obtained after the second dose, with a booster effect now observed after the third dose ( $P$  value = 0.002), that was capable of yielding comparable ELISA titers to the values obtained when immunizing with 1  $\mu$ g of conjugate. Anti-MenX PS IgM ELISA units were low for all of the conjugates, as

expected for conjugate vaccines and likely due to effective isotype switching from IgM to IgG (40, 41).

SBA assays were performed with pooled sera collected 2 wk after the second and the third immunization against the same strain used for MenX PS production, and indicated that antibodies induced by immunization with the MenX-CRM<sub>197</sub> conjugates were functional (Table 3). Fourteen days after the second dose, the mean bactericidal titer was higher with MenX-ADH-SIDEA-CRM<sub>197</sub> and MenX<sub>Ox</sub>-CRM<sub>197</sub> than MenX-NH<sub>2</sub>-SIDEA-CRM<sub>197</sub>, corresponding to IgG titers. Fourteen days after the third dose, the SBA titers were boosted for all conjugates, unlike IgG titers for MenX<sub>Ox</sub>-CRM<sub>197</sub> and MenX-ADH-SIDEA-CRM<sub>197</sub>. A possible explanation for this lack of correlation between ELISA and SBA titers is that ELISA detects both high- and low-avidity antibodies (42), whereas higher-avidity antibodies elicit more complement-mediated SBA (43, 44). To test this hypothesis, anti-MenX antibodies titers were measured by a modified ELISA, which uses a chaotropic salt to select higher avidity IgG antibodies. The results more closely matched the SBA data (Fig. 3B and Table 3).



**Fig. 2.** (A) SDS/PAGE analysis of CRM<sub>197</sub> (lane 1); MenX-NH<sub>2</sub>-SIDEA-CRM<sub>197</sub>, MenX-ADH-SIDEA-CRM<sub>197</sub>, and MenX<sub>Ox</sub>-CRM<sub>197</sub> conjugation mixtures (2, 4, and 6, respectively); corresponding purified conjugates (3, 5, 7); 5  $\mu$ g of protein loaded per each sample. (B) HPLC-SEC profiles (fluorescence emission) of purified MenX<sub>Ox</sub>-CRM<sub>197</sub> (blue), MenX-NH<sub>2</sub>-SIDEA-CRM<sub>197</sub> (red), and MenX-ADH-SIDEA-CRM<sub>197</sub> (green) in comparison with free CRM<sub>197</sub> (black). Chromatograms were normalized with respect to y axis.

**Table 2. Characterization of the MenX–CRM<sub>197</sub> conjugates tested in mice**

Conjugate	Conjugation chemistry	avDP MenX OS	MenX/CRM <sub>197</sub> , wt/wt	MenX/CRM <sub>197</sub> , mol/mol	K <sub>d</sub>	Endotoxin, EU/μg
MenX <sub>OX</sub> –CRM <sub>197</sub>	Random	80	0.69	1.7	0.07	1.6
MenX–ADH–SIDEA–CRM <sub>197</sub>	Selective with longer spacer	17	0.36	4.1	0.22	5.3
MenX–NH <sub>2</sub> –SIDEA–CRM <sub>197</sub>	Selective	17	0.33	3.7	0.24	0.8

K<sub>d</sub> values were of 0.33 for unconjugated CRM<sub>197</sub> and of 0.24 and 0.40 for MenX OS of average degree of polymerization (avDP) 80 and 15, respectively.

Anti-MenX PS IgG ELISA units, measured using this high-avidity ELISA, were low for all conjugates both after the second and third dose compared with units by standard ELISA. Now a statistically significant booster effect could be detected after the third dose for all conjugates ( $P$  from 0.0006 to <0.0001).

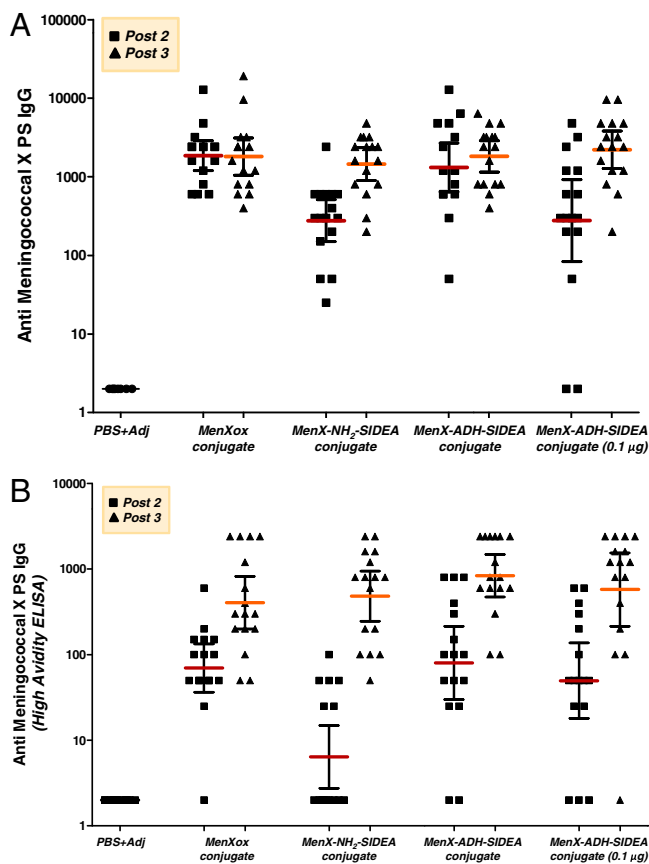
Pooled sera collected 2 wk after the third immunization were evaluated for functional activity against two additional MenX strains, BF7/07 and Kenya 1/06, respectively isolated in Burkina Faso (2007) and Kenya (2006), and provided by the World Health Organization Collaborating Centre for Reference and Research on Meningococci (Oslo, Norway). High SBA titers were found against each strain for all conjugates (Table 3).

## Discussion

Despite the widespread implementation of a MenA–tetanus toxoid conjugate vaccine in the African meningitis belt and the availability of PS and conjugate MenW vaccines, this area is vulnerable to increased incidence of meningitis caused by MenX for which no vaccine is available (45). Because the development of a new vaccine through to licensure takes many years, this leaves Africa vulnerable to new epidemics of MenX meningitis at a time when the epidemiology of meningococcal meningitis on the continent is changing rapidly (13). Glycoconjugates are among the safest and most efficacious vaccines developed during the last 30 y (46). Following the success of serogroup C meningococcal conjugate vaccines (16), which widespread vaccination confirmed to be immunogenic, inducing immunological memory, reducing colonization, and providing herd immunity, tetravalent conjugate vaccines covering serogroup A, C, W135, and Y (47–49) have been developed to broaden protection against meningococcus. The timely development of an anti-MenX conjugate vaccine appears a logical next step in the broadest control of meningococcal disease and requires commitment now.

Using the process developed in this study, MenX PS was purified at high yields with good final product quality. The process is scalable and suitable for good manufacturing practice production. Approximately 200 mg of purified MenX PS was obtained from 1 L of fermentation broth, a productivity much higher than previously reported with MenX isolates (9.6–20 mg/L) (31, 50, 51). Studies on MenX PS and derived OS indicated that they are stable in aqueous solution (31, 33), providing the opportunity to develop a fully liquid vaccine formulation with clear advantages in terms of cost and practical utilization compared with freeze-dried formulations that require reconstitution with water.

Conjugation of native MenX PS, necessarily through the use of random chemistries, yielded heterogeneous products that were difficult to purify and characterize. As reported for other conjugate vaccines, cross-linked chemistries can be affected by low batch-to-batch consistency (52, 53). The use of shorter saccharides has allowed the production of more defined and less cross-linked conjugates, with advantages in relation to reproducibility and vaccine characterization. Parameters, including conjugation chemistry (random against selective carbohydrate modification), linker used for coupling and saccharide chain length, can have an impact on the immunogenicity of the corresponding conjugate vaccines (53–56). The effect of varying such parameters is antigen dependent and was investigated in the current study to optimize the design of a vaccine against MenX. Three different conjugate vaccines were synthesized using MenX OS of avDP 15–20 or 80–100 and CRM<sub>197</sub> (34) as carrier protein. The chemistry developed for Menveo was adapted to the shorter OS (35). However, working with MenX PS, the first step of reductive amination (Fig. 1B) resulted in low activation yields. The use of ADH as a more reactive linker (Fig. 1C) (39) increased the yield of this first step and reduced the reaction time. Working with longer OS, a completely different chemistry was required (Fig. 1A). Random oxidation at position C<sub>6</sub> of *N*-acetyl glucosamine phosphate repeating unit resulted in multiple activation sites along the saccharide chain instead of just at the terminal reducing end. This process was characterized by one step less



**Fig. 3.** (A) Antibody response to meningococcal group X PS induced in female BALB/c mice (5–6 wk old) by MenX–CRM<sub>197</sub> conjugate vaccines at 1-μg dose. MenX–ADH–SIDEA–CRM<sub>197</sub> was also tested at 0.1-μg dose. Anti-MenX IgG ELISA units measured by standard ELISA in sera collected 2 wk after the second and third immunizations. Individual animals are represented by each point; the horizontal bars indicate geometric mean and 95% confidence intervals. (B) Anti-MenX IgG ELISA units measured by high-avidity ELISA using 0.1 M ammonium thiocyanate as chaotropic agent.

**Table 3. Serum bactericidal activity of pooled mouse sera against MenX strains and anti-MenX IgG GMT ELISA units 95% confidence interval 14 d after the second (post 2) and third (post 3) injection of MenX-CRM<sub>197</sub> conjugate vaccines and control**

Group	SBA titers				Standard ELISA		High-avidity ELISA	
	Pooled sera post 2		Pooled sera post 3		Sera post 2	Sera post 3	Sera post 2	Sera post 3
	5967 (ST 750)	5967 (ST 750)	BF7/07	Kenya 1/06				
PBS + adjuvant	<16	<4	<4	<4	2	2	2	2
MenX <sub>OX</sub> -CRM <sub>197</sub>	1,024	4,096	4,096	2,048	1,857	1,810	69.8	404.6
MenX-NH <sub>2</sub> -SIDEA-CRM <sub>197</sub>	128	4,096	4,096	2,048	276	1,447	6.4	482.7
MenX-ADH-SIDEA-CRM <sub>197</sub>	1,024	4,096	4,096	2,048	1,313	1,816	80.1	836.1
MenX-ADH-SIDEA-CRM <sub>197</sub> (0.1 μg)	512	4,096	2,048	2,048	277.5	2,208	49.6	576.8

Female BALB/c (5–6 wk old) mice were immunized three times at 2-wk intervals with MenX-CRM<sub>197</sub> conjugate vaccines at 1-μg dose. MenX-ADH-SIDEA-CRM<sub>197</sub> was also tested at 0.1-μg dose. All vaccines were tested with aluminum phosphate as adjuvant. Bactericidal titers are defined as the reciprocal serum dilution that gives a 50% decrease of colony-forming units after 60-min incubation in the reaction mixture, compared with the mean number of colony-forming units in the control reactions at time 0.

compared with the other two methods. We estimated that a derivatization density of one repeating unit every 15–25 unit and the use of higher avDP OS should preserve epitopes of the native saccharide for interaction with the B cells.

All conjugates gave high anti-MenX IgG titers in mice (Fig. 3A) and induced functional antibodies when tested against African meningococcal X isolates (Table 3). High-avidity IgG antibody titers closely matched SBA titers, which is a good surrogate for meningococcal vaccine efficacy (42, 57, 58) (Table 3). Comparing MenX<sub>OX</sub>-CRM<sub>197</sub> and MenX-ADH-SIDEA-CRM<sub>197</sub>, saccharide chain length and conjugation chemistry do not seem to affect immunogenicity significantly. However, MenX-ADH-SIDEA-CRM<sub>197</sub>, which contains a longer spacer compared with MenX-NH<sub>2</sub>-SIDEA-CRM<sub>197</sub>, resulted in higher antibody and SBA titers after two doses, indicating an effect from the length or the nature of the spacer on the antibody response.

MenX has recently emerged as a cause of meningitis outbreaks with epidemic potential in sub-Saharan Africa, where MenA and MenW have previously been the dominant causes of meningococcal disease (2, 45). Although it is uncertain how MenX will contribute to the problem of meningitis in Africa in the future, preparedness would be prudent in the face of changing epidemiology surrounding the introduction of the serogroup A conjugate vaccine. A trivalent conjugate vaccine covering MenX, MenA, and MenW would be particularly useful for prevention of meningococcal meningitis in the African meningitis belt. Such a vaccine could potentially have protected against over 90% of invasive meningococcal meningitis cases occurring over the past 10 y in this area. A major challenge to the development of a vaccine against MenX is the lack of commercial incentive, because cases of MenX meningitis are rare outside of Africa. Therefore, to progress new vaccine initiatives requires a public health demand from the population, health care professionals and ministries of health in the affected countries, together with foresight and support from global health policy makers and major funders of global health research. This successfully occurred with the formation of the Meningitis Vaccine Initiative, which was responsible for the development of the MenA conjugate vaccine.

This study has demonstrated that conjugation technologies, compatible with a reproducible production process, can be successfully used to develop immunogenic PS conjugate vaccine candidates that are likely to protect against MenX disease. This forms a basis for further vaccine development to identify the best candidate and formulations to proceed to clinical trials and indicates the next steps needed to develop a vaccine to protect against this important cause of epidemic meningitis in Africa.

## Materials and Methods

*SI Materials and Methods* feature additional information to the section provided here.

**MenX PS Production.** *Neisseria meningitidis* X 5967 strain (ST 750) was obtained from the Swiss Tropical Public Health Institute. It was originally isolated during a diagnostic procedure from the cerebrospinal fluid of a meningitis patient in Germany.

MenX PS was purified adapting methods previously developed for the purification of meningococcal and Vi PS (35, 36). A standard purification is described in *SI Materials and Methods* together with analytical methods to assess its purity and full characterization.

**MenX-CRM<sub>197</sub> Conjugates. Random oxidation along the MenX chain and conjugation with CRM<sub>197</sub> (Fig. 1A).** MenX OS (avDP 80–100), obtained by mild acid hydrolysis as described in *SI Materials and Methods*, was loaded in water onto a Sepharose SP column (~4–5 mg of OS loaded per 1 mL of resin at 75 cm/h) preequilibrated with tetrabutyl ammonium bromide (TBABr) to introduce tetrabutyl ammonium as MenX counterion, allowing the subsequent dissolution of the saccharide chains in dimethylformamide (DMF). To MenX OS at 2.5 mg/mL in DMF, TEMPO (0.06 eq respect to MenX repeating units), NaHCO<sub>3</sub> (9 eq respect to MenX repeating units), and trichloroisocyanuric acid (TCC) (2 eq respect to MenX repeating units) were added, and the suspension was incubated at 0 °C overnight with stirring. The reaction mixture was centrifuged (8,000 × g at 20 °C for 15 min), and the supernatant, containing the oxidized MenX, purified by precipitation, adding 1 M NaCl at a final concentration of 200 mM and acetone to 90% (vol/vol) in the final solution. The pellet was dissolved in water and desalted against water on a SEC Sephadex G-15 column (~0.3 mg of OS loaded per 1 mL of resin at 30 cm/h).

For protein conjugation, CRM<sub>197</sub> and NaH<sub>2</sub>PO<sub>4</sub> buffer, pH 7.2, were added to oxidized MenX with a resulting protein concentration of 10 mg/mL, final buffer capacity of 10 mM, and a wt/wt ratio of saccharide to CRM<sub>197</sub> of 4:1. NaBH<sub>3</sub>CN was added 1:1 in weight to the OS. The reaction was slowly stirred at 37 °C for 72 h. The conjugate was designated as MenX<sub>OX</sub>-CRM<sub>197</sub>.

**Derivatization of the reducing end with NH<sub>2</sub>OAC or ADH by reductive amination, followed by reaction of with SIDEA and conjugation with CRM<sub>197</sub> (Fig. 1 B and C).** The procedure developed for meningococcal serogroup A, C, W135, and Y OS was adapted to MenX OS (avDP 15–20) (35), as detailed in *SI Materials and Methods*.

**Purification and characterization of MenX-CRM<sub>197</sub> conjugates.** After verifying conjugate formation by SDS/PAGE and HPLC-SEC analysis, all of the conjugates were purified by size exclusion chromatography on a 1.6 × 90-cm SEC Sephacryl S-300 HR column eluted at 0.5 mL/min in 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.2. Purified material was 0.2-μm filtered and stored at –20 °C. Methods used for MenX-CRM<sub>197</sub> conjugates characterization are detailed in *SI Materials and Methods*.

**Immunization Protocol and Serum Analysis.** All animal protocols were approved by the local animal ethical committee and by the Italian Ministry of Health in accordance with Italian law. Groups of 16 female BALB/c mice (5–6 wk old) were immunized on days 1, 14, and 28 with 0.1 or 1 μg of MenX conjugated saccharide antigen formulated in PBS buffer, pH 7.2, with aluminum phosphate as adjuvant (0.12 mg of Al<sup>3+</sup>-based content). All immunizations

were performed by administering 200  $\mu$ L of vaccine dilution via the s.c. route. Adjuvant alone was used for the negative control group. Sera were collected at days 0 (preimmune serum), 27 (post 2 serum), and 42 (post 3 serum). Specific anti-PS antibody titers and functional antibodies were estimated 2 wk after the second and the third immunization by IgG ELISA and SBA assay.

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