Retaining the structural integrity of disulfide bonds in diphtheria toxoid carrier protein is crucial for the effectiveness of glycoconjugate vaccine candidates†

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The introduction of glycoconjugate vaccines marks an important point in the fight against various infectious diseases. The covalent conjugation of relevant polysaccharide antigens to immunogenic carrier proteins enables the induction of a long-lasting and robust IgG antibody response, which is not observed for pure polysaccharide vaccines. Although there has been remarkable progress in the development of glycoconjugate vaccines, many crucial parameters remain poorly understood. In particular, the influence of the conjugation site and strategy on the immunogenic properties of the final glycoconjugate vaccine is the focus of intense research. Here, we present a comparison of two cysteine selective conjugation strategies, elucidating the impact of both modifications on the structural integrity of the carrier protein, as well as on the immunogenic properties of the resulting glycoconjugate vaccine candidates. Our work suggests that conjugation chemistries impairing structurally relevant elements of the protein carrier, such as disulfide bonds, can have a dramatic effect on protein immunogenicity.

Introduction

Vaccines are considered as one of the most cost-effective interventions to prevent morbidity and mortality from infectious diseases.† Among the different approaches to vaccine design, glycoconjugate vaccines have been proven efficacious and cost-effective in the prevention of Haemophilus influenzae type b (Hib), Streptococcus pneumoniae (23 serotypes), Neisseria meningitidis (A, C, W135 and Y) and Salmonella typhi.‡ Conjugate vaccines are obtained by the covalent linkage of bacterial polysaccharides to immunogenic carrier proteins, and have been demonstrated to overcome the limitations frequently exhibited by unconjugated polysaccharide vaccines.§ The T-cell help provided by the protein epitopes of glycoconjugates imparts to the carbohydrates – which are per se T-cell independent antigens – the capacity to induce long-lasting and boostable IgG antibody production. Six proteins are currently used as carriers in licensed vaccines, including tetanus toxoid (TT), diphtheria toxoid (DT), Cross-Reactive Material 197 (CRM197), the outer membrane protein complex of Meningococcus B (OMPC), protein D from H. influenzae, and the recombinant exotoxin A of Pseudomonas aeruginosa.

Carbohydrates can be linked to proteins by using a variety of approaches. Amino acid residues that are most suitable for chemical linkage to sugars are those well-exposed onto the protein surface and whose side chains have reactive functional groups, such as primary amino groups of lysines and carboxylic groups of glutamic, or aspartic acid residues.¶• Generally, linking of a polysaccharide (PS) to a carrier protein results invariably in a random display of the carbohydrate on the protein surface, although some selectivity homogeneity can be obtained by modulating the carbohydrate to protein stoichiometry. A variety of factors that are associated with the conjugation methodology (e.g. conjugation chemistry, multiple attachment versus single-point attachment of carbohydrates, presence/absence of linkers) have an impact on the chemical and biological properties of different glycoconjugate...
constructs. This poses a serious limitation in the comparison of different glycoconjugates and in understanding their mechanism of action. Therefore, there is a need for site-selective glycoconjugation methods and effort has been recently devoted to the preparation of glycoconjugates with defined attachment. High regioselectivity has been achieved by targeting cysteine, cysteine disulfide bridges or tyrosines. Alternatively, amino acid tags for enzyme mediated conjugation or incorporation of unnatural amino acids have been exploited. The development of site-selective conjugation strategies on naturally occurring amino acids, such as cysteine, is appealing for the simplicity of these approaches, as more complicated and time-consuming steps like sequence engineering become unnecessary. Selectivity of glycoconjugation is achieved by targeting either amino acids with sufficiently high reactivity during chemical reactions, or amino acid patterns within the protein structure that allow selective reactivities. In this context, disulfide bridges are an optimal target for site-selective reactions, as they are usually present in limited numbers and, upon reduction, show nucleophilic properties that can be used for chemical reactions. Disulfide modifications have been successfully used for incorporation of small molecule payloads into antibodies and have attracted specific attention for the development of glycoconjugate vaccines, because of the selectivity that can be achieved on these groups on the carrier protein CRM197.

CRM197 is an enzymatically inactive and nontoxic form of diphtheria toxin, which has been detoxified through a single G52E mutation. CRM197 is a well-characterized protein, whose X-ray structure has been elucidated. It is synthesized as a single-chain holoprotein, which comprises two domains, fragment A (catalytic domain) and fragment B (transmembrane domain). Two disulfide bridges are present in the intact holoprotein: one bridge joins C186 to C201, linking fragment A to fragment B, while a second bridge joins C461 to C471 within fragment B. Polysaccharide conjugates of CRM197 are components of vaccine formulations protecting against important bacterial pathogens including Streptococcus pneumoniae (Prevnar), Haemophilus influenzae type b (HibTITER, Vaxem-Hib) and Neisseria meningitidis serogroup A, C, Y and W-135 (e.g. Menveo and Menjugate). Recently, disulfide re-bridging with acetone was developed for site-selective conjugation of Salmonella O-antigen to CRM197 and improved immunogenicity of the protein as an antigen was observed after grafting one of the two disulfide bonds with oxetane.

In this study, we investigated the scope and limitations of two different site-selective modification methods on the disulfide functionalities of the carrier protein CRM197. By choosing two opposing strategies, we sought to elaborate the importance of retaining the structural integrity of a disulfide functionality through acetone re-bridging or, in contrast, the effect of opening and converting a disulfide bridge into two dehydroalanine residues, on the properties of a resulting glycoconjugate vaccine candidate. In depth structural characterization of the modified CRM197 was carried out to unravel differences between the two methods, and the X-ray crystallographic structure of acetone modified CRM197 was successfully resolved. Capsular polysaccharides from group B Streptococcus (GBS) serotypes Ia and III, as well as capsular polysaccharide from S. pneumoniae (Sp) serotype 14, were chosen as model antigens and the generation of immune responses after administration of these vaccine candidates was evaluated.

Results
Selective glycoconjugation at C186–201 of CRM197
From the two disulfide bridges present in the carrier protein CRM197, the C461–C471 bond appeared to be buried inside the protein, while the C186–201 is surface-exposed. We have already reported that selective modification of the latter disulfide bridge can be achieved by partial reduction of CRM197 to release C186 and C201 in the presence of tris(2-carboxyethyl) phosphine (TCEP). We envisaged to modify the C186–C201 bond using two different strategies for the subsequent two-step conjugation of large polysaccharides (Scheme 1). First, re-bridging of C186–C201 with 1,3-dichloroacetone (DCA) would allow insertion of a ketone handle, which can be further modified with a bifunctional linker bearing an aminoxy to form an aminooxy derivate, and an azide group useful for the conjugation of polysaccharides derivatized with azlky functionalities via azido-alkyne Huisgen (3 + 2) cycloaddition (click chemistry). This method enables incorporation of a single carbohydrate moiety per protein. Also, preliminary experiments with sugar modified with aminooxy linkers proved the direct conjugation challenging, due to the size of GBS polysaccharides (MW ~150–200 kDa).

The multivalent presentation of sugar antigens on a protein carrier is known to be an important factor for the efficient generation of an immunogenic response. To enable selective conjugation of a higher number of sugar molecules on the protein, a second strategy was developed, relying on the opening of C186–C201 and subsequent conversion of the free cysteine residue into dehydroalanine (Dha). In this approach, further conjugation can be achieved through thiol Michael addition on the introduced Dha groups of a bifunctional thiol linker bearing an azido moiety for the subsequent conjugation reaction to alkyn derivatized polysaccharides by click chemistry (Scheme 1). These two methods appeared very attractive to be compared in terms of the construction of site-selective glycoconjugates and immunogenicity of the resulting biomolecules, since the first strategy retains the covalent connection between cysteine residues C186 and C201, whereas the second strategy results in the opening of this disulfide bridge.

For the generation of CRM197-DCA, after selective reduction of C186–C201 in the presence of tris(2-carboxyethyl) phosphine (TCEP, 12 equiv.) at pH 7.5 for three hours, the protein was incubated with 1,3-dichloroacetone (10 equiv.) for 3.5 hours yielding the modified CRM197. Size exclusion chromatography allowed the removal of small molecules and exchange of buffer to 100 mM sodium phosphate at pH 6.3 for acid catalyzed reaction with the aminooxy linker (Scheme 1). Mass spectrometry revealed virtually complete derivatization of the starting

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material (Fig. 1). The impact of the installation of the DCA moiety into CRM197 on its structure was also studied by CD (Fig. 1B) and dynamic light scattering (DLS) analysis (Fig. S4, ESI†) and compared with the native protein. We found that CD and DLS spectra of CRM197-DCA were nearly identical to those of CRM197, which indicates that the secondary structure was preserved upon the chemical stapling. The CRM197-DCA was used for condensation with an excess of aminoxy-PEG5-azido linker (600 equiv.) for quantitative insertion of an azido moiety for further polysaccharide conjugation (Scheme 1), as confirmed by LC-MS analysis (Fig. S1, ESI†).

The second strategy was intended to open the disulfide bond C186-C201 in a selective manner and convert each of the two cysteine residues into the amino acid Dha. Similar to the previous approach, CRM197 was first selectively reduced at C186-C201, and then introduction of Dha was achieved by treatment with 500 equiv. of methyl 2,5-dibromopentanoate over a period of 5 hours at pH 11. Residual small molecules were removed by size exclusion chromatography with 100 mM sodium phosphate at pH 6.3 for elution. The reaction with methyl 2,5-dibromopentanoate works through a bisalkylation mechanism, followed by an elimination step. This second part

Scheme 1 Site-selective modification of CRM197 into CRM197-DCA or CRM197-DHA, (i) 12 equiv. TCEP, 3 h, r.t., (ii) 10 equiv. 1,3-Dichloroacetone, 3.5 h r.t., (iii) 500 equiv. Methyl 2,5-dibromopentanoate, 5 h, r.t., (iv) 600 equiv. aminoxy-PEG5-azide, 3 d, r.t., (v) TCEP, 100 equiv. bis(11-azidoundecyl)disulfide, 18 h, r.t.

Fig. 1 A) LC-MS spectra of native CRM197 and the modified CRM197-DCA and CRM197-DHA highlight complete conversion of native protein. (B) Secondary structure of CRM197, CRM197-DHA and CRM197-DCA (5 µM) determined by circular dichroism. CD spectra were obtained in phosphate buffer, pH 7.4 at 25 °C. All the values are mean values ± SEM from at least two independent experiments. (C) Estimated secondary structure content (%) from CD spectra using the BeStSel server.
was the critical step, as reactions at lower pH values were found to result in incomplete elimination products with the pentanoate molecule attached to the protein. A concentration of 5 mg mL⁻¹ protein was key to achieve a high level of modification. Higher concentrations resulted in protein precipitation during the reaction. The introduction of two Dha residues (Scheme 1) and complete conversion of the starting material was confirmed by LC-MS analysis (Fig. 1A and S2, ESI†). Despite the ring opening, a minimal level of protein aggregation was observed when freshly prepared samples were used for the subsequent modifications (Fig. S3, ESI†). The impact of the opening of the disulfide bridge and subsequent conversion of cysteine in Dha residues on the CRM₁₉₇ structure was evaluated by CD (Fig. 1B) and DLS (Fig. S4, ESI†) experiments using the native protein as the control. We found a few differences both in CD and DLS spectra corresponding probably to a less stable structure for CRM₁₉₇-DCA and the native form, further structural insights were obtained from DCA and the native form, further structural insights were obtained from

DCA, typical of the native form (Fig. S4, ESI†). The distances within C atoms of C186 and C201, angles and length bonds values are in agreement with the geometry of Cys–acetone–Cys bridges. Moreover, the flexibility of the loop accommodating the modified S–S bridge allows this acetone moiety to be solvent-exposed and potentially accessible to the conjugation of glycogen antigens. In contrast, no modification of the C461–C471 disulfide bond was found (Fig. 2C). Remarkably, our structural data expand what previously shown by entire mass analysis,²⁸ indicating the unambiguous presence of selective acetone insertion in the S–S bond C186–C201 of the CRM₁₉₇-DCA and showing no major impact of this modification on the tertiary structure of the protein.

Glycoconjugation with CRM₁₉₇-DCA and CRM₁₉₇-DHA

To compare the influence of the introduced protein modifications on the immunogenicity of resulting glycoconjugate constructs, GBS and Sp polysaccharide antigens were chosen for conjugation.

Structurally similar polysaccharides from two different bacteria (GBS serotypes types III and Ia and Sp serotype 14) were partially de-N-acetylated and reacted by reaction with dibenzocyclooctyne-N-hydroxysuccinimidyld (DBCO) ester to insert a handle suited for azide–alkyne cycloaddition, which we have shown to be particularly efficient with large polysaccharides.¹¹ To minimize the impact on the polysaccharide structure and preserve sugar epitopes, a limited number of repeating units were modified. The relative ratio of DBCO units per polysaccharide molecule was determined by NMR spectrometry considering the ratio of the peak intensity of the aromatic protons of the linker and the H-3-equatorial or H-3-axial of the sialic acid for GBS PS or the H-2 of the Glc residue Sp14 PS, respectively (Fig. S7, ESI†). Typically one out of 20 and 40 repeating units was modified, for GBS PS and Sp14, respectively. Azide moieties were incorporated into the protein using either a PEG or an alkyl linker (Fig. 3). Based on our previous experience with strain promoted click chemistry,¹²⁻¹⁷ it was found that while the alkyl or PEG chain is immunosilent, rigid aromatic systems like DBCO are not; however this type of linker does not shift the immune response away from the carbohydrate. Therefore, although not identical, we considered them not to impact the immunogenicity outcome.

Through the inserted DBCO moiety GBS types Ia and III PS and Sp type 14 were conjugated to CRM₁₉₇-DCA and CRM₁₉₇-DHA...
(Fig. 3) at 2:1 polysaccharide/protein w/w ratio and a protein concentration of 2 mg mL$^{-1}$. Larger amounts of sugar did not further improve the course of conjugation. After removal of the unconjugated polysaccharide, the level of sugar incorporation was estimated by HPAEC-PAD and the protein content was assessed by colorimetric assay (bicinchoninic acid assay). The characteristics of the synthesized site-selective conjugates are summarized in Table 1. The conversion of cysteine residue from
C186–C201 into Dha was already shown to be more impactful on the protein 3D structure as compared to the disulfide rebridging. To further assess that the structural integrity of CRM197-DCA was preserved upon glycoconjugation, CD spectra were recorded for CRM197-PSIa and CRM197-DCA-PSIa, using unmodified CRM197 and CRM197-DCA as controls. The CD spectra of the two conjugates (which exhibited a similar size in the DLS analysis) showed that both random and selective conjugation caused a slight shift of the minimum at 218 nm to higher molar ellipticity values, with respect to CRM197 and CRM197-DCA, which appeared almost overlapping (Fig. S8, ES†).

In vivo results

Immune responses induced by site-selective CRM197-DCA-PSIII and CRM197-DHA-PSIa conjugates were compared to the ones obtained with random conjugates where polysaccharide is attached randomly to lysines on the surface of CRM197. These
conjugates were prepared as previously reported\textsuperscript{33} and are considered viable candidates for clinical studies.\textsuperscript{34,35} In the immunization experiments, groups of ten mice received three doses of the prepared conjugates adjuvanted with alum hydroxide. Two weeks after the second and third vaccine doses, individual IgG titers were measured by ELISA using full-length PSIII or PSIa conjugated to Human Serum Albumin as the coating agent.

The immunogenicity of the CRM\textsubscript{197}-DCA-PSIa conjugate was 3 fold higher than that of the CRM\textsubscript{197}-DHA counterpart and statistically comparable to that of the random CRM\textsubscript{197}-PSIa conjugate, although a trend to be higher for the latter was observed. The functional activity of the elicited antibodies was estimated on pooled sera by OPKA, an assay that mimics \textit{in vivo} GBS killing by effector cells in the presence of complement and specific antibodies, and correlates with mouse protection.\textsuperscript{36} In agreement with the ELISA outcome, OPKA titers of the pooled sera after the second and third doses were comparable for CRM\textsubscript{197}-DCA-PSIa and CRM\textsubscript{197}-PSIa conjugates (Table 2), while they were lower for the CRM\textsubscript{197}-DHA-PSIa conjugate.

In addition, the anti-protein antibodies for CRM\textsubscript{197}-DCA were comparable to those for CRM\textsubscript{197} and more than 10-fold higher as compared to those for CRM\textsubscript{197}-DHA (Fig. 4B and ESI, Fig. S9\textsuperscript{†}), possibly as a result of protein stabilization.\textsuperscript{30} It is noteworthy that the protein dose for CRM\textsubscript{197}-DCA was 2 fold lower than that for the random conjugate.

Given the positive results obtained with the CRM\textsubscript{197}-DCA conjugate, we assessed whether strong immunogenicity could be elicited through this conjugation approach despite the type

| Glycoconjugate        | Glycosylation ratio (w/w) | Free saccharide (%) |
|-----------------------|---------------------------|--------------------|
| CRM\textsubscript{197}-PSIa | 2.5                      | 16                 |
| CRM\textsubscript{197}-DHA-PSIa | 1.1                  | <6                 |
| CRM\textsubscript{197}-DCA-PSIa | 4.8                  | 11.9               |
| CRM\textsubscript{197}-PSIII | 1.2                    | <2                 |
| CRM\textsubscript{197}-DCA-PSIII | 1.4                  | Nd                 |
| CRM\textsubscript{197}-PN14PS | 1.0                    | Nd                 |
| CRM\textsubscript{197}-DCA-PN14PS | 1.9                  | <3                 |

Table 2 OPKA titers measured for sera elicited against GBS PSIa

| Glycoconjugate | OPKA titers | | |
|----------------|-------------| | |
| | | | |
| CRM-PSIa\textsuperscript{a} | 120; 60 | 125; 75 |
| CRM-Dha-PSIa\textsuperscript{a} | <50 | <50 |
| CRM-PSIa\textsuperscript{b} | 63; 62 | 253; 179 |
| CRM-DCA-PSIa\textsuperscript{b} | 104; 51 | 265; 143 |

\textsuperscript{a} OPKA associated with ELISA shown in the Fig. 4A left panel. \textsuperscript{b} OPKA associated with ELISA shown in the Fig. 4A right panel.

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**Fig. 4** Enzyme-linked immunosorbent assay immunoglobulin G (IgG) titers anti GBS PSIa (A) and anti CRM\textsubscript{197} (B) in mouse serum samples collected after 2 and 3 vaccine doses, reported as arbitrary units (EU mL\textsuperscript{-1}); bars represent the geometric mean titers with 95% confidence intervals from 10 serum samples; *p < 0.05, 0.001 < p**** < 0.0001 (Kruskal–Wallis and Dunn multiple comparisons test). Enzyme-linked immunosorbent assay immunoglobulin G (IgG) titers anti GBS PSIII (C) and Sp PS14 (D) in mouse serum samples collected after 2 and 3 vaccine doses, reported as arbitrary units (EU mL\textsuperscript{-1}); bars represent the geometric mean titers with 95% confidence intervals from 10 serum samples.
of polysaccharide used. As shown in Fig. 4C, the immune response after two and three injections was comparable for CRM$_{197}$-PSIII and the site selective CRM$_{197}$-DCA-PSIII conjugate. The functional activity elicited by site selective CRM$_{197}$-DCA-PSIII (67 and 283 are average OPK titer of three different experiments from the pool of mouse sera obtained after two and three doses, respectively) was also comparable with the one obtained by immunizing mice with the reference random conjugate (143 and 154, respectively). Finally, a site-selective CRM$_{197}$-DCA conjugate of SpI4 PS, which is part of commercial pneumococcal vaccines, was prepared and tested in mice. Again, in vivo immunogenicity on 10 mice showed after two and three vaccine doses a level of elicited specific anti-SpI4 PS antibodies comparable to those for the respective random conjugate (Fig. 4D).

**Discussion**

The rational modification of the structure of peptides and proteins offers a wide range of opportunities for the modulation of their biological activity.

Glycoconjugates present in licensed vaccines are generally generated by classic lysine random conjugation of carbohydrates on the surface of carrier protein. These conjugates have often heterogeneous compositions, which cause batch-to-batch variability of structure and activity, often leading to an incomplete understanding of their mechanism of action. Site-selective conjugation is a powerful method to direct polysaccharide conjugation at predetermined sites of the protein and ensure higher batch-to-batch consistency in comparison to classic non-specific conjugation procedures, particularly when the protein is used with the dual role of antigen and carrier.

Stefanetti et al.$^a$ demonstrated that specific site-selective single or double attachment of glycan antigens to carrier protein CRM$_{197}$ is sufficient to induce high levels of anti-Salmonella typhimurium O-antigen IgG specific antibodies with serum bactericidal activity. Conjugation at the C186–C201 bond resulted in high anti-O-antigen bactericidal antibody titers.

Starting from this discovery, we generated two different vaccines with a defined conjugation point, by modifying the same C186–C201 disulfide bridge in CRM$_{197}$. In the first approach, a DCA graft was installed in CRM$_{197}$. The second strategy involved the conversion of cysteine residues in dehydroalanine. Both methods offer the possibility to attach through condensation reaction or thiol Michael addition bifunctional linkers for click reactions with carbohydrates. Particularly, here we incorporated azido moieties, ready for strain promoted click chemistry glycoconjugation with DBCO-derivatized polysaccharides from group B Streptococcus and S. pneumoniae.

In vivo data highlighted that CRM$_{197}$-DCA conjugates elicited an immune response comparable to the reference random CRM$_{197}$-GBS PSlAs and PSIII conjugates, which are vaccine candidates under clinical development, while CRM$_{197}$-DHA resulted in poor immunogenicity. Moreover, antibodies elicited by CRM$_{197}$-DCA-PSIa and PSIII are functional with an OPK titer comparable to the reference vaccines. Also, CRM$_{197}$-DCA conjugation of S. pneumoniae equally provided a strongly immunogenic conjugate. This observation converges with the work done by Martinez-Saez et al.$^{28}$ according to which oxetane graft installation on protein through the regioselective disulfide stapling of the protein carrier CRM$_{197}$ enables stabilization of folded structures and results in an enhanced bioactivity, e.g. a significant increase in its immunogenicity in vivo.

It has been reported that the 3D structure of CRM$_{197}$ is more altered by random conjugation of glycans in comparison to the one of formaldehyde treated proteins, including DT or CRM$_{197}$ itself.$^{29}$ The loss of the CRM$_{197}$ tertiary structure with potential detrimental impact on certain conformational epitopes could partially explain the lower propensity of this protein to be subjected to immune interference in the presence of pre-existing anti-protein antibodies.$^{30,31}$ Of note, the modification induced by glycoconjugation might appear more evident in CRM$_{197}$ with respect to formaldehyde treated proteins because the marked unfolding caused by chemical detoxication might result in negligible further structural impact caused by glycan coupling.

Our data complement this information and suggest that slight modifications of the protein tertiary structure, such as the ones induced by a random reaction of surface exposed lysine residues, are compatible with a strong anti-carbohydrate immune response.

Among the two cysteine directed chemistries herein tested, disulfide re-bridging of CRM$_{197}$ with DCA is shown to aid preservation of the 3D structure of the protein, as demonstrated by combined CD and DLS and X-ray experiments.

Importantly, the crystal structure of CRM$_{197}$-DCA presented herein clearly shows the selective presence of one disulfide bridge at C185–C201, while the second disulfide bridge (C461–C471) remains untouched, and preservation of the 3D protein structure compared to the native form. Minimal alterations of the 3D structure caused by glycoconjugation and detectable by CD did not impair its carrier properties, resulting in a robust anti-carbohydrate response.

Conversely, a modification based on ring opening (CRM$_{197}$-DHA) resulted in structural changes that strongly impacted the immunogenicity of the conjugated glycan.

Overall this study underpins that selective protein modifications based on bridging of disulfide bonds are optimal to preserve the structural integrity and stability, and consequently the immunogenicity of the glycoconjugates.

**Conclusions**

This work underscores the impact of protein modification on the stability and immunogenicity of glycoconjugates and highlights disulfide stapling as an effective strategy for selective protein conjugation, widely applicable to different polysaccharides. In addition, it opens the path for the use of highly selective chemical methods for the preparation of glyco-conjugate vaccines with advantages in terms of consistency of production and characterization, and a better understanding of their immunological mechanism of action.
Ethical statement

Animal experiments were performed in accordance with the regulations of the Directive 2010/63/EU and GSK ethical guidelines, under the approval of the Italian Ministry of Health (Italian Legislative Decree no. 26/2014). All mice were housed under specific pathogen-free conditions at the GSK Vaccines Animal Resource Centre in compliance with the relevant guidelines.

Data availability

All relevant data are reported as ESI.†

Author contributions

FC, AK, GJLB and RA conceived the study; FC, AK, MCM, DV, LDi, EB, BB, FA, and LDB performed experimental work; FC, AK, MCM, IM, MRR, GJLB and RA analyzed results; FC, AK, GJLB and RA wrote the manuscript; all revised the manuscript.

Conflicts of interest

FC, DV, LDi, DO, EB, BB, FA, LDB, IM, MRR, and RA are employees of the GSK group of companies. AK was hosted in a secondment in GSK during her PhD programme. Prevnar and HibTITER are trademarks from Pfizer; Vaxem-Hib, Menveo and Menjugate are trademarks from GSK.

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