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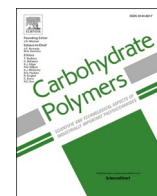
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Elucidating the role of *N*-acetylglucosamine in Group A Carbohydrate for the development of an effective glycoconjugate vaccine against Group A *Streptococcus*

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ABSTRACT

Group A Carbohydrate (GAC), conjugated to an appropriate carrier protein, has been proposed as an attractive vaccine candidate against Group A *Streptococcus* infections. Native GAC consists of a polyrhannose (polyRha) backbone with *N*-acetylglucosamine (GlcNAc) at every second rhamnose residue. Both native GAC and the polyRha backbone have been proposed as vaccine components. Here, chemical synthesis and glycoengineering were used to generate a panel of different length GAC and polyrhannose fragments. Biochemical analyses were performed confirming that the epitope motif of GAC is composed of GlcNAc in the context of the polyrhannose backbone. Conjugates from GAC isolated and purified from a bacterial strain and polyRha genetically expressed in *E. coli* and with similar molecular size to GAC were compared in different animal models. The GAC conjugate elicited higher anti-GAC IgG levels with stronger binding capacity to Group A *Streptococcus* strains than the polyRha one, both in mice and in rabbits.

This work contributes to the development of a vaccine against Group A *Streptococcus* suggesting GAC as preferable saccharide antigen to include in the vaccine.

1. Introduction

Streptococcus pyogenes, commonly known as Group A *Streptococcus* (Strep A), is a Gram-positive pathogenic bacterium accounting for a variety of infectious diseases, from superficial infections such as pharyngitis, skin infections and cellulitis to severe invasive diseases including puerperal sepsis, necrotizing fasciitis and streptococcal toxic shock syndrome, with a high frequency of serious sequelae, in particular

acute rheumatic fever, rheumatic heart disease, and acute glomerulonephritis (Ralph & Carapetis, 2013). Rheumatic heart disease, a chronic inflammatory heart valve condition, represents the main global burden of Strep A, responsible alone for 319,000 deaths, over 33 million cases and 10 million disability-adjusted life-years (DALYs) in 2015 (Watkins et al., 2017). Currently, there is no commercial vaccine against Strep A (Vekemans et al., 2019) and the only strategy to treat Strep A infections is antibiotic therapy (Dooling, Shapiro, Van Beneden, Hersh, & Hicks,

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2014) that can ultimately favor increase of resistance of other bacteria colonizing humans. In the recent years, the global challenge of increasing antimicrobial resistance has highlighted the need for alternative effective prevention strategies for Strep A too (Aslam et al., 2018; Baker, Payne, Rappuoli, & De Gregorio, 2018; Kennedy & Read, 2018; Micoli, Bagnoli, Rappuoli, & Serruto, 2021; Rappuoli, Santoni, & Mantovani, 2019).

Different vaccines are in development, based on various virulence factors exposed on the cell wall or secreted by Strep A, the most advanced being M protein based (Aranha et al., 2021; Dale, Penfound, Chiang, & Walton, 2011; Postol et al., 2013; Sekuloski et al., 2018; Vekemans et al., 2019). Additional strategies are in development and use more conserved antigens. Among these, Group A carbohydrate (GAC) and oligosaccharide (OS) fragments thereof have been proposed (Di Benedetto et al., 2020; Kabanova et al., 2010). GAC is constituted of a trisaccharide repeating unit with the main chain formed by alternate α -1,2- and α -1,3-linked L-rhamnose (Rha) residues and a β -linked N-acetyl-D-glucosamine (GlcNAc) residue as the side chain, attached to the 3-O position of the latter Rha residue (Fig. 1(a)). Human anti-GAC antibodies successfully promoted opsonophagocytic killing of several M protein-carrying Strep A serotypes (Salvadori, Blake, McCarty, Tai, & Zabriskie, 1995). Moreover, by immunising mice with GAC conjugated to tetanus toxoid (TT) or CRM (Cross-Reactive-Material-197 (CRM197), a mutant version of the diphtheria toxin) carrier proteins, protection was conferred against challenge with live Strep A (Kabanova et al., 2010; Sabharwal et al., 2006). It has been reported that colonization of Strep A in human throats is inversely correlated with the levels of anti-GAC antibodies (Sabharwal et al., 2006) that peak at around age 17 in correspondence to the reduced incidence of Strep A infection (Zimmerman, Auernheimer, & Taranta, 1971).

Although the main driver of Strep A autoimmune responses is the M protein (Faé et al., 2005), the association of GlcNAc side chain of GAC with the generation of cross-reactive antibodies relevant to immunopathogenesis of rheumatic diseases is unclear (Dudding & Ayoub, 1968; Goldstein, Rebeyrotte, Parlebas, & Halpern, 1968; Kirvan, Swedo, Heuser, & Cunningham, 2003; Kirvan, Swedo, Snider, & Cunningham, 2006; Malkiel, Liao, Cunningham, & Diamond, 2000; Shikhman, Greenspan, & Cunningham, 1993). Therefore, GAC polyrhamnose (polyRha) backbone lacking the GlcNAc side chain (Fig. 1(b)) has been proposed as an alternative antigen (Carapetis, Steer, Mulholland, & Weber, 2005; Gao, Uchiyama, et al., 2021). The ability of polyRha to induce phagocytic clearance of different *emm* types Strep A strains has been shown and active immunization suggest contribution to protection (van Sorge et al., 2014).

The aim of this study was to better elucidate the role of GlcNAc and polyRha on the immune response induced by GAC. Synthetic oligosaccharides (Geert Volbeda et al., 2017; Pitirollo et al., 2020) were prepared and used in antibodies binding studies. Over the last years, organic synthesis has been proposed for preparation of bacterial oligosaccharides, providing well defined structures easy to characterize, with absence of bacterial contaminants, and useful to correlate the immune response elicited by corresponding glycoconjugates with the oligosaccharide chemical structure (Micoli et al., 2019; van der Put et al., 2016;

Verez-Bencomo et al., 2004). Beyond vaccine manufacturing, synthetic oligosaccharides have become very advantageous for antigen identification and structure-guided vaccine design (Stefanetti, MacLennan, & Micoli, 2022). Longer polyRha chains were isolated from an *E. coli* strain genetically modified to express the GAC backbone linked to lipopolysaccharide (LPS) lipid A-core. For the first time, GAC and polyRha conjugates were compared in head-to-head immunogenicity studies in different animal models. This work supports the identification of optimal antigens to be included in a vaccine against Strep A.

2. Materials and methods

2.1. Materials

The following chemicals were used in this study: sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium cyanoborohydride (NaBH_3CN), sodium periodate (NaIO_4), sodium sulfite (Na_2SO_3), sodium borohydride (NaBH_4), deoxycholate (DOC), hydrochloric acid (HCl), sodium chloride (NaCl), acetic acid glacial (AcOH), dimethyl sulfoxide (DMSO), triethylamine (TEA), glycine, 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) (Merck, Darmstadt, Germany), boric acid solution, phosphate buffered saline tablets (PBS) (Honeywell Fluka, Charlotte, NC, USA), dithiothreitol (DTT) (Invitrogen, Waltham, MA, USA).

2.2. GAC preparation

GAC was extracted from a M protein-mutant strain (GAS51 Δ M1) generated from the wild-type strain HRO-K-51 kindly provided by the University of Rostock. The strain was grown in complete medium (1.8 g/L Na_2HPO_4 ; 14.8 g/L Yeast Extract; 10 g/L Dextrose Monohydrate).

GAC was chemically extracted from bacterial culture through nitrite/glacial acetic acid treatment (Pancholi & Fischetti, 1988) and purified as previously described (Kabanova et al., 2010). Purified GAC contained no hyaluronic acid, <4 % protein and <1 % DNA impurities (w/w with respect to GAC). Average molecular size of 7.0 kDa was estimated by HPLC-SEC analysis (TSK gel G3000 PWXL column) using dextrans (5, 25, 50, 80, 150 kDa) as standards (Merck, Darmstadt, Germany), corresponding to an average of 14 repeating units per chain. Sugar composition was confirmed by HPAEC-PAD and $^1\text{H-NMR}$ analyses.

2.3. PolyRha preparation

PolyRha was produced by engineering the *E. coli* K12 strain MG1655. Briefly, the polyRha biosynthetic locus (genes *gacB-gacG*) was amplified by PCR using GAS51 Δ M1 gDNA as template and the following primers: GTCGACTCCAACATGGCAAGAAGCCT (Forward) and GGATCCGTTG TGCCGTGGAGCTTACT (Reverse). The amplicon was then cloned in the pACYC184 vector using the *SalI* and *BamHI* restriction sites and the resulting plasmid was transformed in *E. coli* MG1655 (Supplementary Fig. 1). With this approach, biosynthesis of polyRha occurs constitutively and the polysaccharide is ligated to lipid A core region of *E. coli*

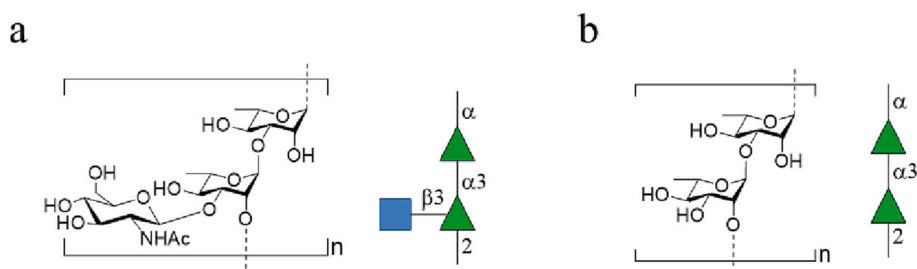


Fig. 1. Group A Carbohydrate (GAC) (a) and polyRha (b) repeating units.

LPS molecules by the O-Antigen ligase WaaL. The engineered strain was grown in HTMC medium (15 g/L Glycerol, 30 g/L Yeast extract, 0.5 g/L MgSO₄, 5 g/L KH₂PO₄, 20 g/L K₂HPO₄), at 30 °C for 16 h. Then, polyRha was extracted by acetic acid hydrolysis (Micoli, Giannelli, & Di Benedetto, 2021), breaking the labile linkage between KDO and the lipid A. PolyRha was purified by a first step of Amicon Ultra (Merck, Darmstadt, Germany) 10 kDa cut-off against water (2000 ×g; 20 °C; 5 washes of 30 min) to remove low molecular weight impurities (including core molecules without polyRha repeats) and through a second step of anionic-exchange chromatography using Q-Sepharose FF resin (AKTA system, Cytiva) to remove charged impurities as proteins and nucleic acids. The material has been loaded in sodium phosphate buffer (NaPi) 20 mM at pH 7.2 and eluted in NaPi 20 mM pH 7.2 and NaCl 200 mM. A final buffer exchange against water was performed with Amicon 10 kDa cut-off (Merck, Darmstadt, Germany) against water (1000 ×g; 20 °C). Purified polyRha was characterised by <2 % protein and <1 % DNA impurities (w/w with respect to polyRha), with average molecular size of 7.0 kDa as estimated by HPLC-SEC analysis (TSK gel G3000 PWXL column) using dextrans (5, 25, 50, 80, 150 kDa) as standards (Merck, Darmstadt, Germany). Analyses by HPAEC-PAD and ¹H-NMR confirmed presence of only Rha, with no GlcNAc residues.

2.4. Synthesis of oligosaccharides

See Supplementary Material and Methods.

2.5. Preparation of glycoconjugates

2.5.1. Oligosaccharides conjugation to CRM with SIDEA linker

TetraRha 1, hexaRha 2 and GAC-dimer 3 (10 mg/mL in water) were added to 300 μL of DMSO. TEA (5 eq) and SIDEA (12 eq) were added. The reactions were stirred for 1 h after which the derivatized oligosaccharides 1a, 2a and 3a were purified by prepacked single-use PD MidiTrap G-10. Reaction mixtures were loaded in DMSO/H₂O 1:1 and eluted with water, according to manufacturer instructions. Eluted OS-SIDEA were dried in SpeedVac Concentrator, then conjugated to CRM in NaPi 100 mM pH 7.2 working with a CRM concentration of 5 mg/mL and a ratio OS/CRM of 2:1 in weight. Conjugation occurred overnight at room temperature. Reaction mixtures were purified by HiPrepTM 16/60 Sephacryl S-100 HR column. Samples were diluted to 1 mL with PBS, loaded and eluted in PBS with a flow rate of 0.5 mL/min. Pooled fractions corresponding to pure conjugates were concentrated by Vivaspinn 10 k.

2.5.2. GAC and polyRha conjugation to CRM

GAC was conjugated to CRM as previously described (Di Benedetto et al., 2020).

PolyRha was activated with CDAP according to the following procedure (Lees, Barr, & Gebretsaie, 2020; Lees, Nelson, & Mond, 1996; Lees & Zhou, 2021; Shafer et al., 2000): CDAP at 100 mg/mL in acetonitrile was rapidly added to a solution of polyRha 9 mg/mL in NaCl 2 M in a 1.5:1 w/w ratio CDAP/polyRha. Immediately, NaOH 0.3 M was added to reach pH 9. After 3 min, CRM was added to the solution in a 1:1 w/w ratio with respect to polyRha with final concentration of 5 mg/mL. The reaction was mixed for 2 h at room temperature maintaining the pH at 9 by adding NaOH 0.3 M. At the end, glycine 2 M at pH 9 was added in a w/w ratio of 7.5:1 glycine/polyRha in order to quench the reaction. The solution was kept in mild agitation over night at room temperature. The conjugate was purified by Amicon Ultra (Merck, Darmstadt, Germany) 30 kDa cut-off against PBS (3500 ×g; 4 °C; 8 washes).

2.6. Conjugates characterization

Purified conjugates were characterised by micro BCA (Thermo Scientific, Waltham, MA, USA) and HPAEC-PAD (Pitirolo et al., 2020) for total protein and total Rha content respectively and to determine the PS

to protein ratios. Free polysaccharide was separated through DOC precipitation and quantified by HPAEC-PAD (Lei, Lamb, Heller, & Pietronbon, 2000). Conjugates formation was verified by SDS-PAGE and HPLC-SEC analyses, comparing the conjugates with unconjugated CRM (Di Benedetto et al., 2020).

2.7. Immunogenicity studies

GSK is committed to the Replacement, Reduction and Refinement of animal studies (3Rs). Non-animal models and alternative technologies are part of our strategy and employed where possible. When animals are required, application of robust study design principles and peer review minimises animal use, reduces harm and improves benefit in studies.

Mouse studies were performed at the Toscana Life Sciences Animal Facility (Siena, Italy) and in GSK Animal Facility (Siena, Italy), in compliance with the relevant guidelines (Italian D.Lgs. n. 26/14 and European directive 2010/63/UE) and the institutional policies of GSK. The animal protocols were approved by the Italian Ministry of Health (AEC project No. 201309, approval date 20/12/2013; and 399/2017-PR, approval date 11/05/2017). The rabbit immunogenicity study was conducted in Charles River Laboratories (France) facility, in accordance with the European Directive 63/2010.

Female, 5 weeks old CD1 mice (8 per group) were vaccinated intraperitoneally (IP) with 200 μL of formulated antigens at study day 0 and 28, according to the scheme previously used with GAC conjugates (Di Benedetto et al., 2020). Approximately 100 μL bleeds (50 μL serum) were collected at day -1 (pooled sera) and at day 27 (individual sera), with final bleed at day 42.

Female New Zealand White rabbits Crl:KBL(NZW) (6 per group) were vaccinated intramuscularly (IM) with 500 μL of formulated antigens at study day 0, 21 and 35, as previously done with GAC conjugates (Kabanova et al., 2010). Sera were collected on study days 20 and 34. Maximum volume of blood was sampled according to ethic's recommendations. At bleed out at day 49, maximum obtainable volume of blood was collected from each rabbit and rabbits were euthanized.

Conjugates were formulated on Alhydrogel (0.7–2 mg/mL Al³⁺) and their adsorption (>90 %) was evaluated by analyzing formulation supernatants via SDS-PAGE with silver staining detection following manufacturer's instruction (SilverQuest Silver Staining kit, ThermoFisher Scientific, Waltham, MA, USA), after Alhydrogel removal by two sequential centrifugations (18,000 rcf, 15 min, 4 °C).

2.8. Sera analyses

Mouse sera were analysed for anti-GAC and anti-polyRha total IgG by enzyme-linked immunosorbent assay (ELISA), as previously described (Di Benedetto et al., 2020), using GAC-HSA (at the protein concentration of 1 μg/mL in carbonate buffer pH 9.6) and polyRha-HSA (at the protein concentration of 1 μg/mL in carbonate buffer pH 9.6) as coating antigens.

Rabbit sera were analysed for anti-GAC (Palmieri et al., 2022) and anti-polyRha total IgG by Luminex, using same conditions for both assays. GAC was coupled to MagPlex microspheres with Streptavidin after biotinylation while polyRha-HSA was coupled to MagPlex microspheres (20 μg of HSA in 1 × 10⁶ microspheres/mL) through the protein moiety via carbodiimide chemistry, with the protocol described in (Keeley et al., 2022) for Strep A recombinant proteins.

Mouse and rabbit sera were also analysed for their ability to bind different Strep A strains (provided by prof. Nikki Moreland, University of Auckland) by FACS. Conditions reported in (Palmieri et al., 2022) were used.

2.9. cELISA

The competitive ELISA (cELISA) was performed as previously described (Pitirolo et al., 2020) using round bottom ELISA plates

(NUNC, Maxisorp) coated overnight with 1 $\mu\text{g}/\text{mL}$ of GAC-HSA conjugate or triRha-BSA conjugate in PBS at 4 $^{\circ}\text{C}$, and the following day blocked with PBS-Milk 5 % for 1 h 25 $^{\circ}\text{C}$. Competitive ELISA was setup using as primary antibody polyclonal mouse sera raised against conjugated GAC or triRha. The antigen-specific polyclonal sera diluted at 1:100 (for anti-Rha serum) or 1:6000 (for anti-GAC serum) were spiked (ratio 1:1) with serial dilutions of samples, starting from a concentration of antigen ranging from 5 to 500 $\mu\text{g}/\text{mL}$. The mixtures prepared in PBS-Tween 0.05 % and BSA 1 % were incubated for 2 h at 25 $^{\circ}\text{C}$ on the coated ELISA plates, with samples and coating competing for the binding to the polyclonal serum. The stronger is the binding between the serum and the antigens present in the sample solution, the less will be the binding to the coated antigen, hence, the lowest signal will be obtained and vice versa. Plates were then washed three times with 300 μL of washing buffer (PBS 0.05 % Tween-20) and following steps were performed as standard ELISA.

2.10. STD-NMR

NMR experiments were carried out on a Bruker 600 MHz NMR instrument equipped with a TBI cooled probe at controlled temperature (± 0.1 K). Data acquisition and processing were performed using TOPSPIN 3.1 software. Proton-Carbon Saccharides resonances were assigned collecting both ^1D (^1H and ^{13}C) and 2D (COSY, HSQC, HMBC, NOESY) experiments, using standard pulse sequences. STD-NMR experiments were acquired with 96 scans over 96 accumulations and spectral width of 8000 Hz (12 ppm) at 298 K; a saturation transfer of 2/4 s. was applied to enhance the saturation transfer effect, irradiating at a frequency of 8.0 and -2.0 ppm (4000 and -1000 Hz respectively). Spectra acquired irradiating at a frequency of 8.0 ppm provide better signal/noise ration. To avoid pitfalls in the interpretation of STD-NMR spectra, a negative control spectrum was always recorded in absence of mAb (ligand and buffer only at the same molar concentration and pH of the protein-saccharide sample). The STD negative controls were always subtracted to the relative protein-saccharide STD spectrum, obtaining the STDD experiments.

Monoclonal antibody (anti-GAC from Abnova) was exchanged in the working buffer (Tris d-11 50 mM in D_2O at pH 7.2 ± 0.1) through 2 mL Zeba Spin desalting column pre-equilibrated with 3 cycles of working buffer. Oligomers were dissolved in the working buffer too. 200 μg of mAb were prepared in a final volume of 510 μL of buffer with 100 equivalents of GAC dimer. TetraRhamnose and *N*-acetyl glucosamine samples were prepared using the same Rha and the same GlcNAc molar concentration contained in GAC dimer, respectively, and mixed with 200 μg of mAb in 510 μL of buffer.

2.11. Statistics

Mann-Whitney two-tailed test was used to compare the immune response elicited by two different antigens at same time point. Statistical analysis was performed using GraphPad Prism 7.

3. Results

3.1. Synthetic approach to produce GAC fragments with well-defined structure

OligoRha structures of different chain length, tetraRha **1** and hexaRha **2**, were synthesized as recently reported (Pitirollo et al., 2020); longer oligoRha (octaRha **21**, decaRha **22**, tetradecaRha **23**, hexadecaRha **24**) were prepared by automated synthesis (Fig. 2) (Geert Volbeda et al., 2017).

GAC dimer **3** (Fig. 2) was prepared with a double glycosylation of tetraRha **18** and trichloroacetimidate **19**, as retrosynthetically shown in Fig. 3. The synthesis of donor **19** was recently reported (Del Bino et al., 2019). The tetraRha **18** was synthesized from disaccharides **13** and **15** that have a naphthyl protective group at the C-3-position of terminal Rha residues. The disaccharides were prepared from monosaccharides **10**, **11** and **12**, (Fig. 3). The synthesis of monosaccharides **11** and **12** has been described previously (Crotti et al., 2014).

The synthesis of monosaccharide donor **10** is shown in Fig. 4. L-rhamnose was peracetylated using iodine and acetic anhydride to give **4**, which was treated with allyl alcohol and $\text{BF}_3\text{Et}_2\text{O}$ to obtain **5** in 62 % yield. Deacetylation of compound **5** in MeONa/MeOH followed by introduction of an isopropylidene at the C-2- and 3-positions allowed to obtain **6** in 87 % yield. After benzylation of the C-4-position to obtain **7**, the cleavage of isopropylidene and the selective protection of C-3-position with a naphthylmethyl group using dibutyl tin oxide resulted in compound **8** in 58 % yield. Finally, acetylation of the C-2-position provided **9** and deallylation followed by activation as trichloroacetimidate gave donor **10** in 85 % yield.

As illustrated in Fig. 5, compound **10** reacted with acceptor **11**, containing the linker for future conjugation, in the presence of TMSOTf to obtain disaccharide **13** in 85 % yield. Disaccharide **13** was deacetylated with MeONa/MeOH leading to the formation of disaccharide acceptor **14**. In parallel, donor **10** and acceptor **12** were coupled to obtain disaccharide **15** in 72 % yield. Disaccharide **15** was deallylated and transformed into trichloroacetimidate **16** which reacted with disaccharide **14** to obtain tetrasaccharide **17** in 87 % yield. The simultaneous cleavage of the two Nap groups provided tetrasaccharide acceptor **18** in 78 % yield.

A double glycosylation was performed between tetrasaccharide

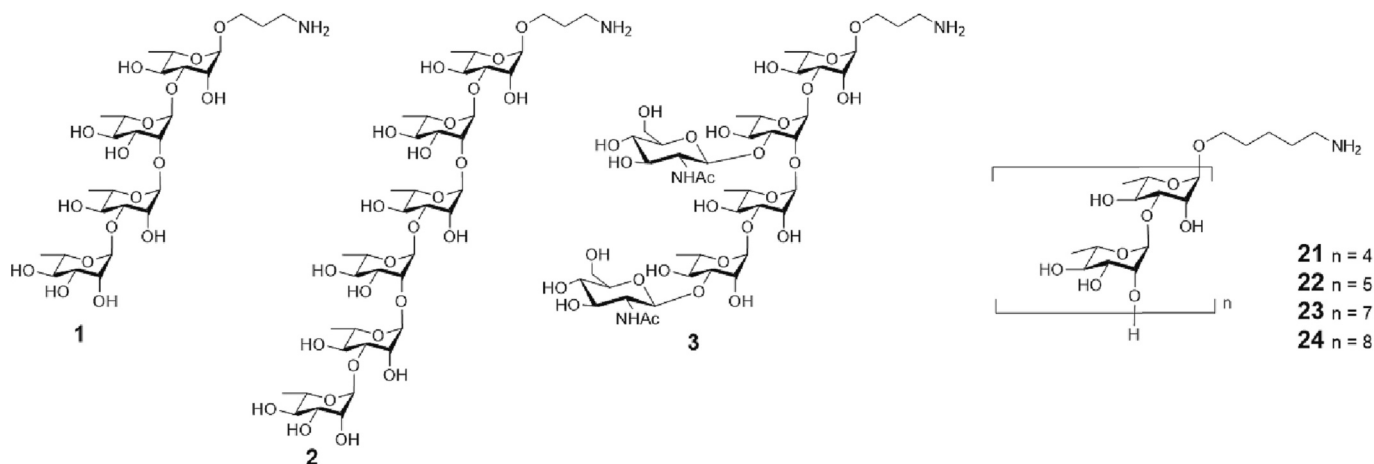


Fig. 2. Synthetic GAC oligosaccharides. TetraRha **1**, hexaRha **2**, GAC dimer **3**, octaRha **21**, decaRha **22**, tetradecaRha **23**, hexadecaRha **24**.

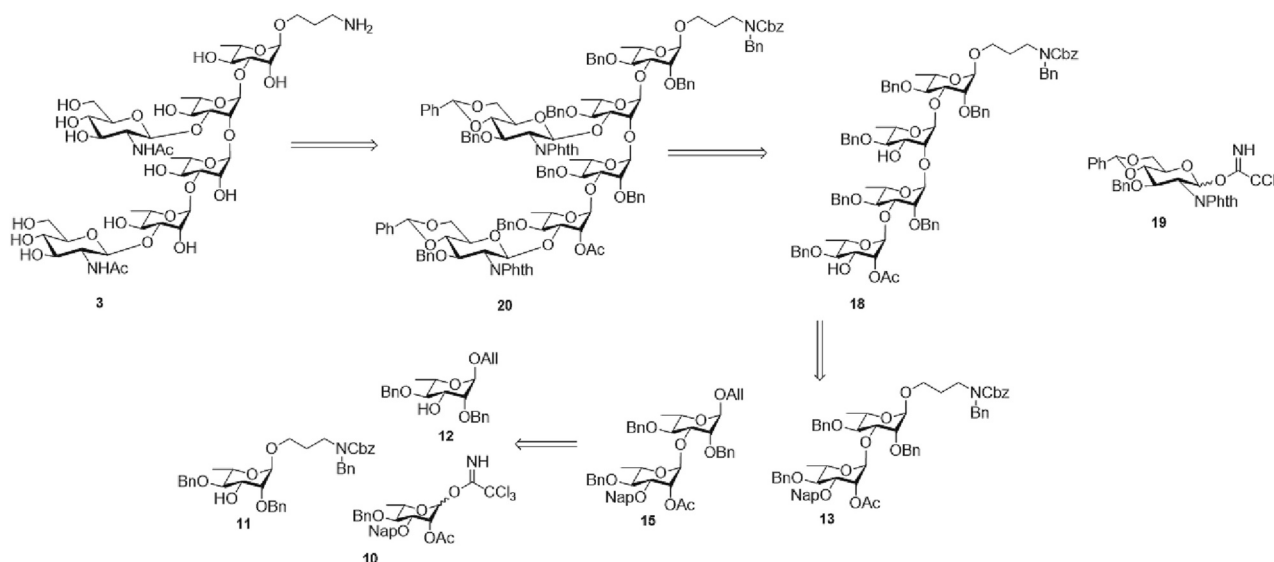


Fig. 3. Retrosynthesis of GAC dimer 3.

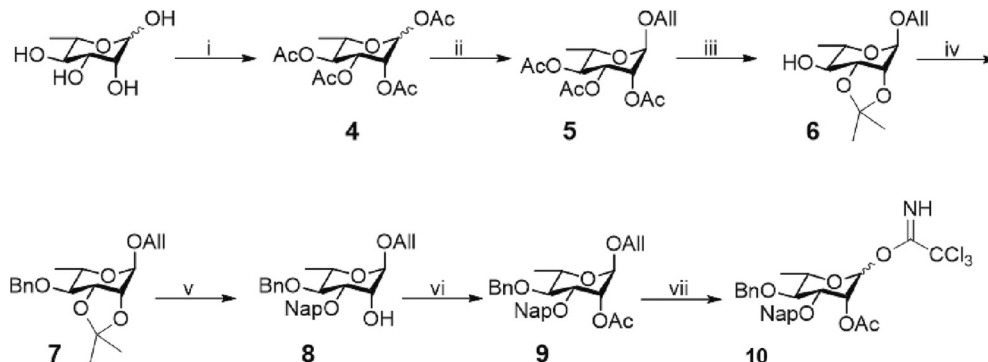


Fig. 4. Synthesis of building blocks 10. Reagents and conditions: i) I_2 , Ac_2O , quantitative yield; ii) CH_2CHCH_2OH , BF_3Et_2O , DCM, $0\text{ }^\circ\text{C} \rightarrow \text{rt.}$, 62 % yield; iii) a) $MeONa/MeOH$; b) $(CH_3O)_2C(CH_3)_2/DCM$ 4:1, $p\text{-TsOH}$, 87 % yield; iv) NaH , $BnBr$, DMF , 95 % yield; v) a) TFA 50 %, DCM; b) Bu_2SnO , toluene; $NapBr$, TBAI, $45\text{ }^\circ\text{C}$, 58 % yield; vi) Py , Ac_2O , 98 % yield; vii) a) Ir cat, THF , H_2 ; I_2 , H_2O ; b) CCl_3CN , DBU , DCM, 85 % yield.

acceptor **18** and donor **19** using TMSOTf as a promoter to give the fully protected GAC dimer **20** in 47 % yield. The cleavage of the two phtalimido groups followed by *N*-acetylation, treatment with $NaOMe$ in $MeOH$, and hydrogenolysis of the remaining protecting groups allowed to obtain the target GAC dimer **3** in 40 % yield (Fig. 5).

3.2. Characterising the epitopic specificity of anti-GAC antibodies

3.2.1. Competitive ELISA experiments

OligoRha of different length (from 2 to 8 repeating units) were tested in competitive enzyme-linked immunosorbent assay (cELISA) to assess their ability to compete with GAC-HSA (used as plate coating antigen), in the binding to polyclonal anti-GAC antibodies, in comparison to native GAC isolated from wild type bacteria and GAC-CRM conjugate (Fig. 6(a)). The oligoRha structures, regardless of their size, did not compete with GAC-HSA for binding with antibodies generated against GAC, as opposed to native GAC and GAC dimer that showed similar levels of competition. As expected, conjugation of GAC to CRM increased the binding +20 times compared to unconjugated GAC. These results suggest that the GlcNAc residues are part of the dominant epitope in the GAC structure.

A second experiment was performed using a triRha-BSA conjugate as coating antigen and sera generated from mice immunized with a triRha-CRM conjugate (Pitirollo et al., 2020) (Fig. 6(b)). In this case, all

oligoRha structures competed for binding, longer structures showing stronger binding. The GAC-CRM conjugate proved to be a poor competitive binder, suggesting that the GlcNAc residue can mask the polyRha structure for Rha-specific antibodies.

Interestingly we also verified that, free GlcNAc, GlcNAc conjugated to HSA or GlcNAc present in the structure of unrelated GBS type III OS did not compete well with GAC-HSA coating antigen for binding to anti-GAC antibodies, in contrast to the GAC dimer (Fig. 6(c)).

All these data suggest that neither GlcNAc nor oligoRha alone are sufficient to bind anti-GAC antibodies. This is in line with an epitope containing both GlcNAc and Rha residues (Michon et al., 2005).

3.2.2. Saturation Transfer Difference (STD)-NMR experiments

Saturation Transfer Difference (STD)-NMR was undertaken to map the epitope recognized by an anti-GAC monoclonal antibody (mAb) comparing the recognition of polyRha fragments with and without GlcNAc residues. Initially, the GAC dimer **3** was tested (Fig. 7(a)). In general, all the spins systems received saturation. The highest transfer of saturation was found for the methyl protons of the GlcNAc residue C, which was normalized to 100 %, and those of GlcNAc residue C' which received a relative saturation of 85 %. This highlighted the NAC moieties as major anchoring points for the binding.

The protons H2, H4, H5 and H6 of the GlcNAc-C moiety also appeared to engage in interaction with the mAb, with a relative intensity

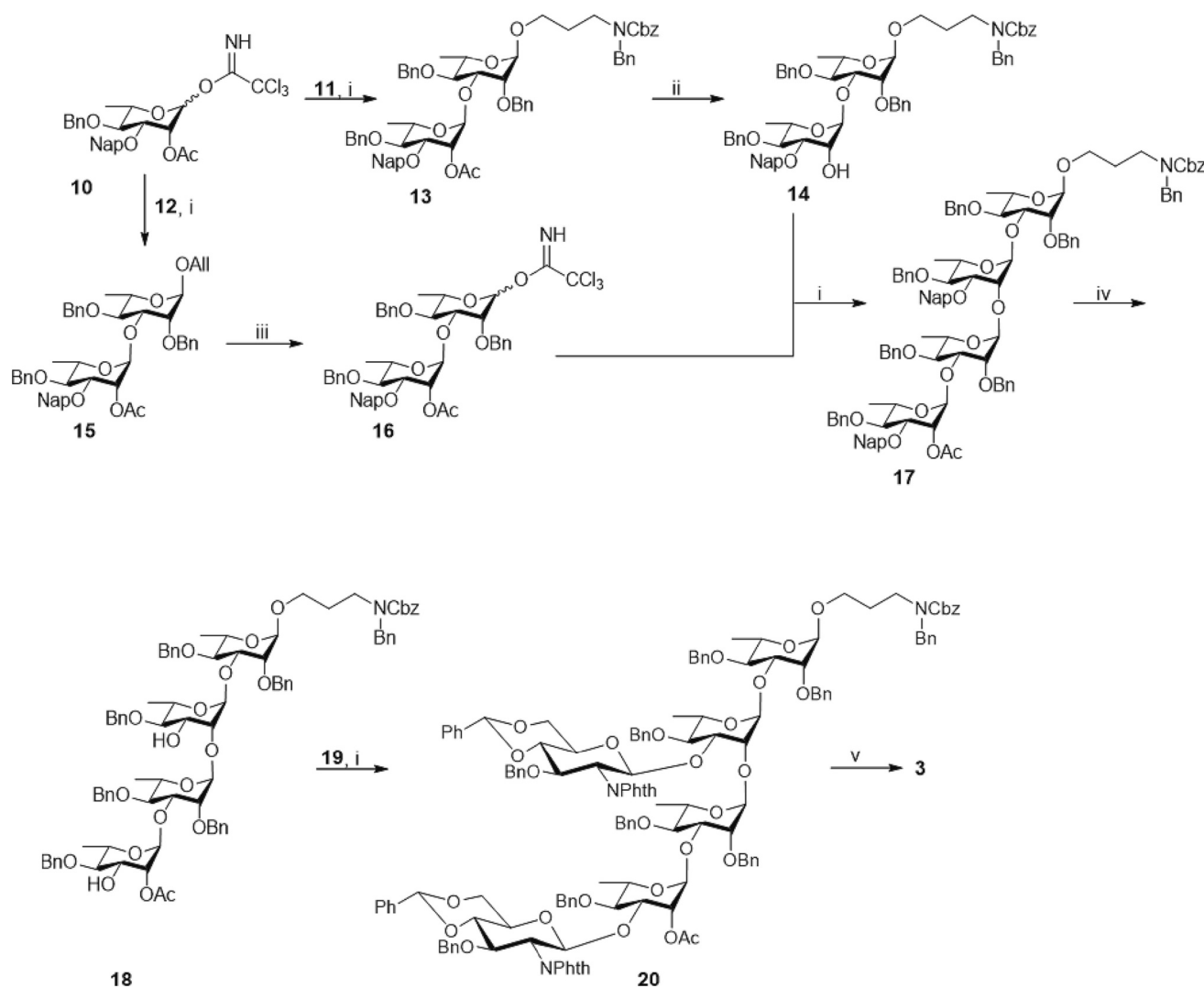


Fig. 5. Synthesis of GAC dimer **3**. Reagents and conditions: i) TMSOTf, DCM, 0 °C, 72 %–87 % yield; ii) NaOMe/MeOH, quantitative yield; iii) a) Ir cat, THF, H₂; I₂, H₂O; b) CCl₃CN, DBU, DCM, 85 % yield; iv) DDQ, DCM/MeOH 4:1, 78 % yield; v) a) ethylenediamine, EtOH; Py, Ac₂O; b) NaOMe/MeOH; c) H₂O/EtOH 1:1, Pd/C, H₂ (3 bar), 40 % yield.

of 43 %, 47 %, 47 % and 48 %, respectively.

Of the ring protons, H2 of Rha A received the higher transfer of saturation (69 %), followed by H2 of Rha B and B', with a relative intensity of 65 % and 58 %, respectively. Considering that H2 of Rha A' showed a lower percentage of saturation (57 %), it can be deduced that the two consecutive Rha RUs are the primary determinant of recognition.

The H3 protons of the Rha B, B' and A showed a relative saturation of 45 %, 60 % and 45 %, respectively, while the H4 protons of B and B' have a relative saturation of 45 % and 49 %, respectively, which supported a slighter preference of transfer of saturation towards the Rha B' instead of B.

We also verified the ability of the mAb to bind tetraRha **1**, corresponding to the dimer lacking the GlcNAc residues. The protons H2, H4 and H6 were most involved in binding, receiving between 60 and 80 % saturation transfer, similar to its GAC dimer congener (Fig. 7(b)).

The maximum relative intensity in the STD-NMR spectrum was found for H2 of Rha A (100 %), followed by H2 from Rha B and B' (70 %). This indicates that Rha A-H2 is positioned in closer proximity to the protein upon complex formation than the H2 protons of Rha B and Rha B'. The H2 of the non-reducing Rha A' showed the weakest saturation (65 %) of the H2 protons, suggesting that this moiety is most distant from the binding pocket upon complex formation. A strong saturated

resonance was observed for H3 of Rha A (86 %), while H4 of the same Rha showed a weaker saturation (64 %). The saturation of H2, H3 and H4 from the Rha A suggests a preferential recognition of the (1 → 3)-α-L-Rhap(1 → 2) epitope, while the reducing end (1 → 3)-α-L-Rhap(1 → spacer) motif seems less involved in binding. The well resolved methyl signals between 1.20 and 1.00 ppm showed the lowest relative saturation, suggesting that these positions are less important for binding.

To further probe which of the two structures, the GAC dimer or the tetraRha, preferentially binds the mAb, an STD-NMR experiment was performed using the two glycans in competition with the mAb (Fig. 7(c)).

The highest signal recovery was found for the NAc moieties of GAC dimer (100 %). Moreover, a remarkable differential transfer of saturation was observed for the non-overlapping signals of both inhibitors: the H2 of Rha B and B' of the GAC dimer and the proton Rha A H2 of the tetraRha. These three protons were diagnostic to track changes in saturation under competitive conditions. In particular, the H2 of Rha B and B' had a relative saturation of 73 % and 48 %, respectively, under the competition conditions, which is comparable to the initial percentage of 65 % and 58 % observed in the direct binding of the GAC dimer. On the other hand, the H2 of Rha A of the tetraRha showed a depletion of saturation from an intensity of 100 % to 36 %, in the presence of the GAC dimer competitor.

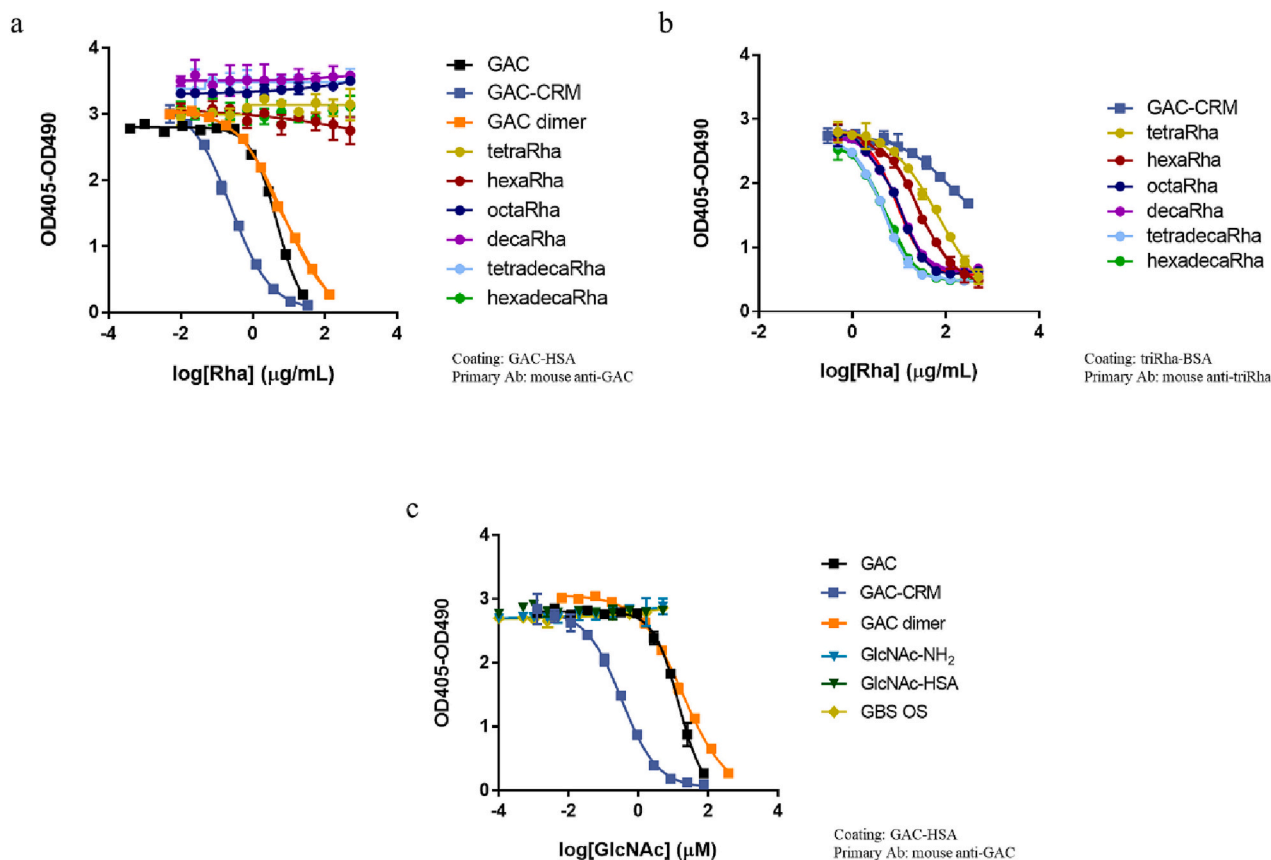


Fig. 6. cELISA experiments. Oligosaccharides of different chain length (tetraRha 1, hexaRha 2, octaRha 21, decaRha 22, tetradecaRha 23, hexadecaRha 24) compared to GAC dimer 3, native GAC and native GAC-CRM conjugate in the ability (a) to compete with GAC-HSA conjugate for anti-GAC antibodies binding; (b) to compete with triRha-BSA conjugate for anti-Rha antibodies binding. (c) Native GAC, native GAC-CRM conjugate, GAC dimer 3, free GlcNAc, GlcNAc conjugated to HSA or GlcNAc present in the structure of unrelated GBS type III OS compared in the ability to compete with GAC-HSA conjugate for anti-GAC antibodies binding.

Furthermore, STD-NMR of a stand-alone GlcNAc was performed with the mAb showing no transfer of saturation (Supplementary Fig. 2). Overall, these data indicate that the GlcNAc is not per se a relevant epitope, unless presented in the context of the Rha residues, and the affinity of the GAC dimer is higher than that of tetraRha.

To reveal the structural features of both the GAC dimer and tetraRha, models were built using the carbohydrate builder GLYCAM force fields (<http://glycam.org>). Analysis of the GAC dimer dihedral angles are summarized in Fig. 8(a) and revealed that all Φ torsion angles are in agreement with the *exo*-anomeric effect and show a + *gauche* conformation, of 54.1°, 58.9° and 55.9° respectively for the α -(1 → 3) A'-B', α -(1 → 2) B'-A, and α -(1 → 3) A-B linkages. The same *exo*-anomeric periplanar conformation was found around the Ψ torsion angles of these glycosidic bonds, being 1.1°, 3.1° and 1.5°, respectively. The β -(1 → 3) Φ torsion angles between B'-C of 54.7° is indicative of a + *gauche* conformation which allows for a stabilizing *exo*-anomeric effect and is similar to the B-C' dihedral angle of 56.8°. On the other hand, the Ψ torsion angles of the same bonds are periplanar with dihedral angles of -4.5° and -3.5° for the B'-C and B-C' glycosidic bonds respectively. To validate these structures, the NOESY spectra for both fragments were acquired, the analysis of which unequivocally revealed key inter-residue cross-peaks, namely i) RhaAH1-RhaB'H2, ii) RhaB'H1-RhaA'H3 and iii) RhaBH1-RhaAH3 NOE's for RhaAH1 confirmed the conformation around both glycosidic linkages. Notably, the former torsion angles where in agreement with those of the tetraRha (A'-B': $\Phi/\Psi = 55.1^\circ/-2.1^\circ$; B'-A: $\Phi/\Psi = 57.0^\circ/5.8^\circ$; A-B: $\Phi/\Psi = 53.0^\circ/1.1^\circ$) suggesting that the helical shape is promoted by the core Rha backbone. The lack of trajectories prevent a more insightful understanding or the role GlcNAc branching units in the dynamic behavior of the hapten.

The alternated α -(1 → 2) and α -(1 → 3) Rha residues promote the formation of a compact left-handed helix with eight residues per turn with a diameter of 7.58 Å and a pitch of 11.71 Å. The β -(1 → 3) GlcNAc units in the native GAC are exposed on the exterior face of the helix and these, therefore, have relatively large surface area that is accessible for solvent (400 Å²). In contrast, the folded rhamnan backbone has its alpha face oriented towards the interior leaflet of the helix while their intercalated decoration results in a solve-accessible surface area approximately 10-fold lower than the GlcNAc as shown in Fig. 8(b).

3.3. GAC and polyRha conjugates: synthesis and immunogenicity in animals

TetraRha 1, hexaRha 2 and GAC dimer 3 were conjugated to CRM carrier protein, through adipic acid bis-(N-hydroxysuccinimide) ester (SIDEA) chemistry (Supplementary Fig. 3(a)). The derivatization reactions of 1, 2 and 3 with SIDEA were performed using a large excess of linker to favor the formation of monofunctionalized OS-SIDEA derivatives 1a, 2a and 3a. Conjugation to CRM was confirmed by SDS-PAGE analysis (Supplementary Fig. 3(b)) and all resulting conjugates were characterized by a molar ratio of oligosaccharides to CRM close to 5, as calculated by micro BCA and HPAEC-PAD analyses.

The conjugates produced were tested in mice in comparison to a native GAC-CRM conjugate (Di Benedetto et al., 2020). Differently from GAC-CRM, all oligosaccharide conjugates, including the GAC dimer conjugate, induced very low anti-GAC IgG responses under the conditions tested (Supplementary Fig. 4(a)) and therefore did not allow for the comparison of the GAC and polyRha structures in terms of immunogenicity. Also FACS analysis of Strep A bacterial cells stained using the

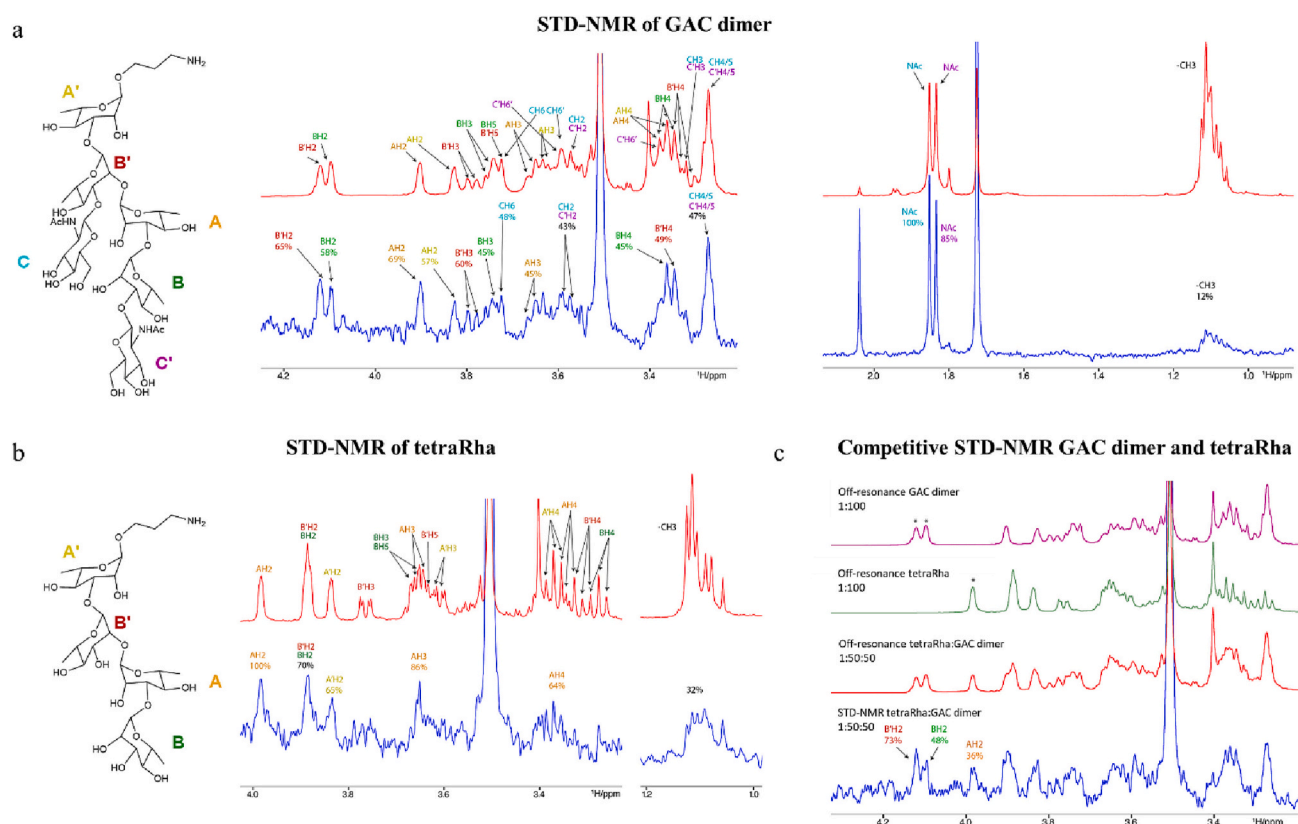


Fig. 7. STD-NMR of GAC dimer (a). ^1H NMR off-resonance spectrum (top red) of GAC dimer and STD-NMR spectrum (bottom blue) of the complex containing 6 μM of ligand and 600 μM of protein are shown. Individual peaks of the methyl moieties from the GlcNAc can be visualized in the region 1.9–1.8 ppm. Methyl moieties of the four Rha moieties are observed in the region between 1.2 and 1.0 ppm. The relative percentage of binding upon complex formation between GAC dimer and the mAb is shown above each resonance peak. All resonances were normalized to the methyl protons of GlcNAc (100 %). STD-NMR of tetraRha (b). At the left: spectrum on the top (red) is the ^1H NMR off-resonance spectrum of the tetraRha. Spectrum on the bottom (blue) represents the STD-NMR spectrum of the complex containing 6 μM of ligand and 600 μM of protein. At the right: methyl moieties region between 1.2 and 1.0 ppm are shown. The relative percentage of binding upon complex formation between the tetraRha and the mAb is shown above each resonance peak. All resonances were normalized to the proton H2 of the Rha A (100 %). Competitive STD-NMR between GAC dimer and tetraRha (c). Spectrum in purple represents the ^1H NMR off-resonance spectrum of GAC dimer. Spectrum in green represents the ^1H NMR off-resonance spectrum of tetraRha. Spectrum in red represents the ^1H NMR off-resonance spectrum of the equimolar mixture of tetraRha and GAC dimer both in 50 mol/mol excess over the mAb (3 μM of each ligand and 600 μM of protein). All resonances were normalized to the methyl protons of GlcNAc (100 %).

raised antibodies showed higher binding of the anti-native GAC antibodies in comparison to all anti-oligosaccharide antibodies (Supplementary Fig. 4(b)).

In order to produce longer polyRha structures for comparison with native GAC, we moved from chemical synthesis to biosynthesis. To this end, an *E. coli* strain was genetically engineered to express polyRha chains without GlcNAc side chain residues. The biosynthetic genes for polyRha expression were cloned from *S. pyogenes* and provided to *E. coli* using an episomal vector, as previously described (Shibata, Yamashita, Ozaki, Nakano, & Koga, 2002). PolyRha chains were transferred to the core region of *E. coli* LPS molecules and were isolated after acetic acid hydrolysis that breaks the labile linkage between KDO and the lipid A anchor (Micoli et al., 2013; Micoli, Giannelli, et al., 2021) (Fig. 9(c)). The resulting polyRha chains showed average molecular weight similar to native GAC as estimated by High Performance Liquid Chromatography–Size Exclusion Chromatography (HPLC-SEC) analysis (Fig. 9(a)). High-performance Anion Exchange Chromatography coupled with Pulsed Amperometric Detection (HPAEC-PAD) confirmed the expected composition for the polyRha chains (Fig. 9(b)).

Both polyRha and GAC were randomly conjugated to CRM (Fig. 9(c)), using two different approaches. GAC was oxidised with sodium periodate on GlcNAc residues to produce aldehydic groups for following reductive amination with lysines on CRM (Di Benedetto et al., 2020). It should be noted that oxidation of polyRha, impacting the Rha units in

the backbone, led to degradation of the sugar chain. For this reason, an alternative chemistry was selected for polyRha: it was randomly activated at the hydroxyl groups along the polysaccharide chain using the 1-cyano-4-dimethylaminopyridine tetrafluoroborate (CDAP) cyanating agent (Lees et al., 1996; Lees et al., 2020; Lees & Zhou, 2021; Shafer et al., 2000), followed by conjugation through a isourea linkage by reaction with lysine residues on the CRM carrier protein. Conjugates formation was confirmed by HPLC-SEC, revealing the absence of unreacted protein, and the conjugates showed a w/w saccharide to protein ratio of 0.12 (polyRha-CRM) and 0.51 (GAC-CRM) corresponding to a saccharide/CRM molar ratio of 1.0 and 4.3 respectively. Free saccharide was <2 % in both cases.

When tested in mice, polyRha-CRM induced significantly lower anti-GAC IgG response than GAC-CRM, both 4 weeks after the first and 2 weeks after the second injection (Fig. 10(a)). The results in mice were confirmed in rabbits: at all-time points GAC-CRM elicited significantly higher anti-GAC IgG levels than polyRha-CRM (Fig. 10(b)). Interestingly, in both animal models, higher anti-polyRha IgG responses were elicited by the polyRha conjugate than by the GAC conjugate (Fig. 10(c) and (d)).

Flow cytometry was used to evaluate the binding affinity of polyclonal mouse- and rabbit-derived sera to the surface of 5 wild-type Strep A strains of different M protein serotypes (M1, M12, M53 and M75). For all strains, the Strep A surface binding of the antisera raised against the

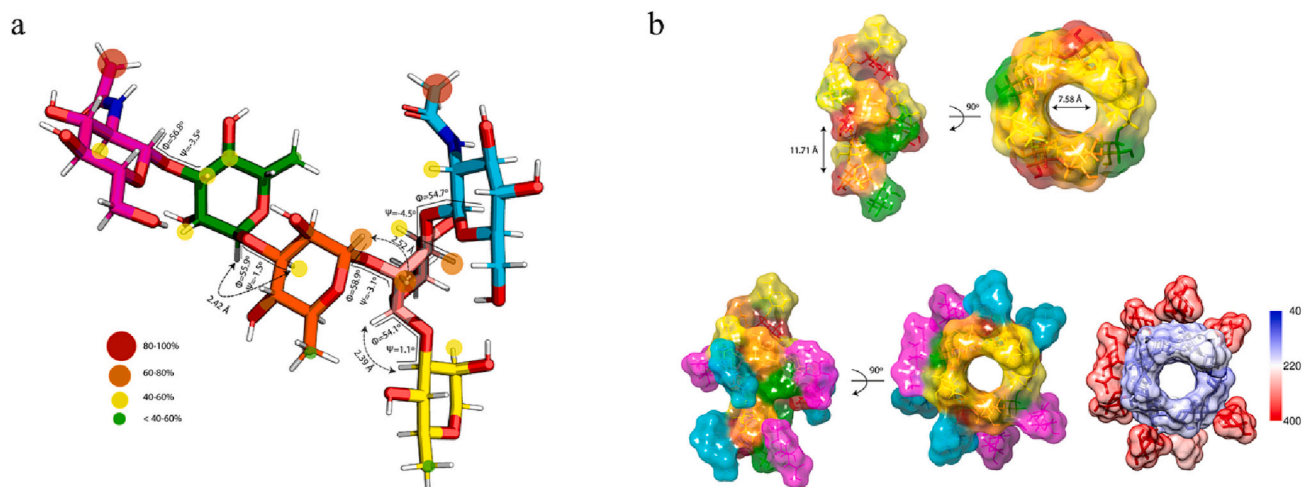


Fig. 8. Epitope mapping and conformational analysis of GAC dimer (a). Individual contributions from the different protons are depicted according to their ASTD (red: 80–100 %; orange: 60–80 %; yellow: 40–60 %; green: <40 %). All relative intensities were normalized to the methyl resonances of the GlcNAc (100 %). The triad composed by the AB'C is the determinant region of the GAC dimer receiving more saturation. Through space correlations observed by ^1H - ^1H -NOESY were confirmed between AH1-B'H2 (2.52 Å), B'H1-A'H3 (2.39 Å) and BH1-AH3 (2.42 Å). Torsion angles described for the free hexasaccharide determined according to the following definition $\Phi = \text{H1-C1-Ox-Cx}$ and $\Psi = \text{C1-Ox-Cx-Hx}$. Molecular surface representation of Rha core backbone its respective branched hapten (b). Upper panel show the molecular surface representation of a DP8 composed of alternating α -(1 \rightarrow 3) and α -(1 \rightarrow 2) rhamnans. This structure forms a left-handed helix with and internal diameter of 7.58 Å composed of for repeating units per turn with a pitch of 11.71 Å. Lower panel of the picture show the molecular representation of a native Strep A DP8 with its α -(1 \rightarrow 3) represented in purple and cyan. Solvent-accessible surface area (SASA) representation for each residue in Å² (blue: 40 Å²; White: 220 Å²; Red: 400 Å²).

GAC-CRM was significantly higher than the level of antibodies binding observed with antisera raised against polyRha-CRM conjugate. The difference was more pronounced with mice sera (Fig. 11(a)) than with rabbit sera (Fig. 11(b)).

4. Discussion & conclusion

No vaccines are currently available against Strep A (Vekemans et al., 2019), a leading cause of infectious disease burden worldwide, responsible for a broad spectrum of diseases with about 0.5 million annual deaths, particularly in young adults (Carapetis et al., 2005). GAC is universally conserved, essential for Strep A survival, abundant on the bacterial cell surface, and accessible to antibody binding regardless of the degree of encapsulation (Gao, Rodas Lima, & Nizet, 2021).

Both GAC and the polyRha backbone, lacking the GlcNAc side chains, conjugated to appropriate carrier proteins, have been proposed as key antigens for the development of a cross-protective vaccine against Strep A (Di Benedetto et al., 2020; Gao, Uchiyama, et al., 2021; Kabanova et al., 2010; van Sorge et al., 2014). Here, for the first time, the two sugars have been directly compared for their ability to induce an immune response in animal models. GAC induced higher anti-GAC IgG response than polyRha, both in mice and rabbits, showing stronger binding to a panel of Strep A bacterial strains. PolyRha was generated in *E. coli* to provide well-defined oligosaccharide structures, devoid of GlcNAc residues, with a similar length as native GAC. Indeed, our preliminary attempts to compare short synthetic oligomers (e.g., GAC dimer and tetra- and hexaRha) failed: both the oligoRha-CRM conjugates and the GAC dimer conjugate elicited much lower anti-GAC IgG responses in mice than native GAC conjugate. For the GAC dimer this result stands in contrast to what has previously been reported by (Kabanova et al., 2010), who showed that CRM conjugates of synthetic oligosaccharides and native GAC elicited comparable high levels of serum GAC-specific IgG. Also, (Auzanneau, Borrelli, & Pinto, 2013) reported that a similar immune response can be elicited by a GAC dimer and native GAC conjugated to TT. This different outcome could be due to the lower GAC doses we injected and the different immunization schedule, which here was based on only two injections, to allow for better differentiation between the constructs. In addition, the synthetic conjugate used in this

work had a lower glycosylation degree in comparison to our previous report (Kabanova et al., 2010). The critical role for GAC chain length in inducing protective antibodies against Strep A has recently also been confirmed by (Wang et al., 2020).

However, the synthetic oligosaccharides with the well-defined structures were instrumental to shed further light on the dominant GAC epitope. A synthetic strategy to prepare a GAC trisaccharide and a GAC hexasaccharide (GAC dimer) was reported by (Marino-Albernas, Harris, Varma, & Pinto, 1993). An efficient and convergent synthesis of longer GAC oligosaccharides was achieved a few years later by the same group, following a strategy that limits the number of protection and deprotection steps, to deliver an allyl glycoside for conjugation to a carrier protein. The oligomers were conjugated to BSA (bovin serum albumin) and Ovalbumin carrier proteins using N-acryloylated or diethyl squarate linkers (Auzanneau & Pinto, 1996). Here, the strategy for the synthesis of the GAC dimer was further simplified by constructing the polyRha backbone first and inserting the two GlcNAc residues simultaneously.

The prepared synthetic oligosaccharides were instrumental to further clarify the role of GlcNAc and the polyRha backbone in binding with antibodies. Competitive ELISA and STD-NMR experiments confirmed that the epitope motif of GAC is composed of a GlcNAc in the context of the polyrhamnose backbone. Indeed, in cELISA, the polyRha backbone, irrespectively of its chain length, and stand-alone GlcNAc or GlcNAc in the context of other OS structures, were not able to compete with GAC for binding to anti-GAC antibodies. Importantly, the GlcNAc residues were shown to be able to mask the polyRha backbone for Rha-specific antibodies. These results are in agreement with our structural studies, indicating that the alternating α -(1 \rightarrow 2) and α -(1 \rightarrow 3) rhamnans promote the formation of a compact left-handed helix, decorated with β -(1 \rightarrow 3) GlcNAc units that are exposed to the exterior of the helix. STD-NMR experiments confirmed that the GlcNAc side chain is strongly involved in the binding of GAC specific monoclonal antibodies. This is in agreement with previous studies indicating that the GAC epitope consists of the entire branched trisaccharide repeating unit (Johnson & Pinto, 2002; Michon et al., 2005; Pitner et al., 2000; Stuike-Prill & Pinto, 1995). In addition, the comparison of the polyRha structure, with and without the GlcNAc residues, and the GlcNAc monomer,

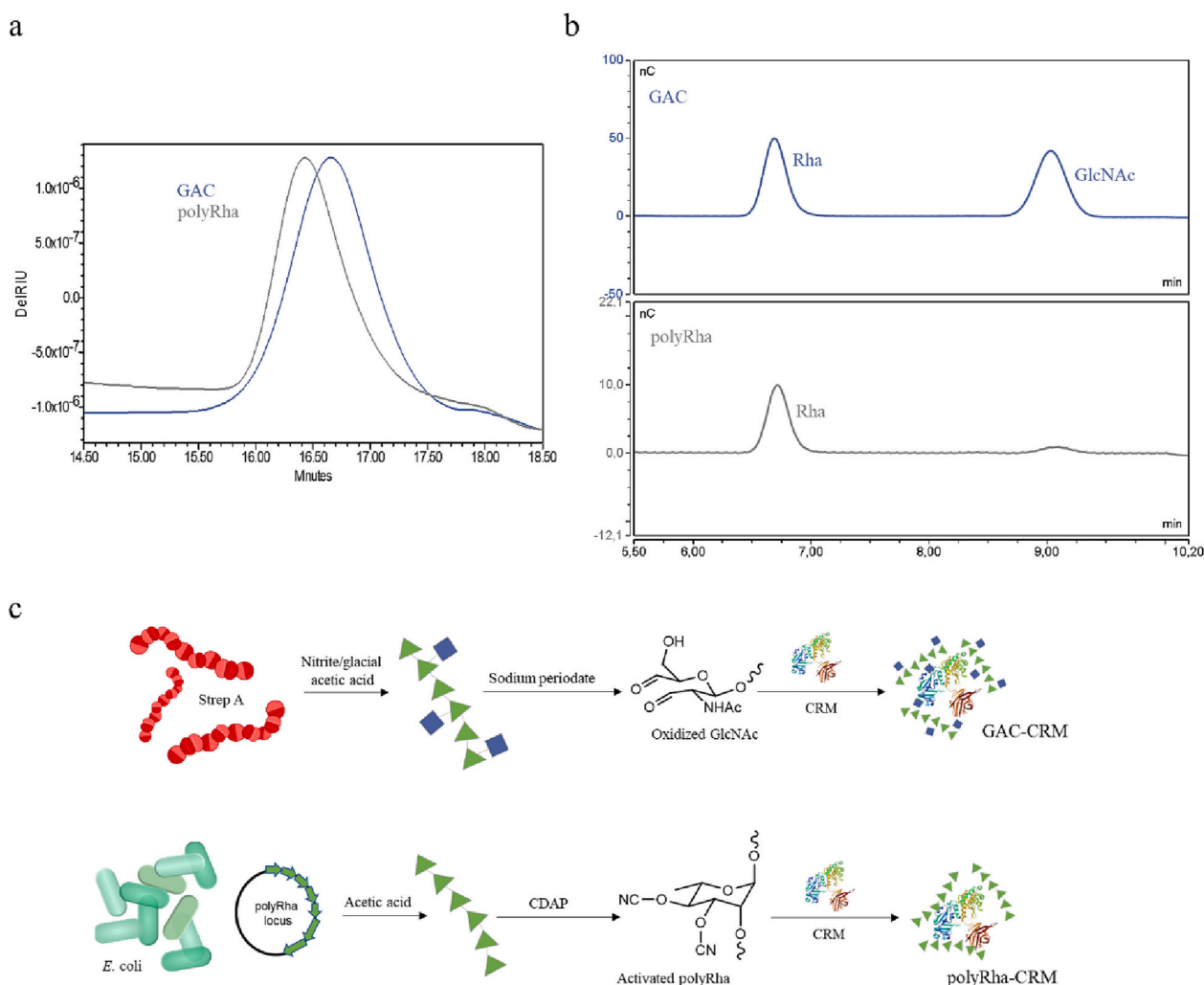


Fig. 9. HPLC-SEC profiles (a) (refractive index detection) of GAC (blue line) and polyRha (grey line), 80 μ L of sample injected on a TSK gel G3000 PWXL column; 0.1 M NaCl 0.1 M NaH_2PO_4 5 % CH_3CN pH 7.2 at 0.5 mL/min. V_{tot} 23.326 min, V_0 10.663 min. HPAEC-PAD profiles (b) of GAC (blue line) and polyRha (grey line) obtained after hydrolysis with TFA 4 M for 2 h and 30 min and eluted in CarboPac PA1 column with 18 mM NaOH for 20 min, followed by 100 mM AcONa in 28 mM NaOH regeneration step and re-equilibration, at a flow rate of 1.0 mL/min. Strategies used for producing GAC-CRM and polyRha-CRM conjugates (c). GAC was chemically extracted from bacterial culture through nitrite/glacial acetic acid treatment and conjugated to CRM via reductive amination between the aldehyde groups randomly generated through its oxidation and lysines of the carrier protein; polyRha was produced by engineering an *E. coli* strain. Then, polyRha was extracted by acetic acid hydrolysis and conjugated to CRM through random activation of hydroxyl groups along the polysaccharide chain using the cyanilating agent CDAP, followed by isourea linkage with lysines on the carrier protein.

unambiguously showed that the involvement of the GlcNAc residues in binding occurs only in the context of the polyRha structure.

To conclude, our results support the use of GAC for a glycoconjugate vaccine against Strep A and confirm GlcNAc recognition by anti-GAC antibodies only in the context of the entire polysaccharide structure. Also, our work shows how synthetic oligosaccharides can be used as powerful tools to facilitate glycoepitopes identification for vaccine design.

CRedit authorship contribution statement

Roberta Di Benedetto, Gianmarco Gasperini, Luigi Lay, Roberto Adamo, Francesca Micoli: **conceptualization**; Olimpia Pitirollo, Roberta Di Benedetto, Pedro Henriques, Gianmarco Gasperini, Francesca Mancini, Martina Carducci, Luisa Massai, Anne Geert Volbeda: **data curation**; Olimpia Pitirollo, Roberta Di Benedetto, Pedro Henriques, Gianmarco Gasperini, Francesca Mancini, Martina Carducci, Luisa Massai, Omar Rossi, Jeroen D C Codée, Francesco Berlanda Scorza, Danilo Gomes Moriel, Francesca Necchi, Luigi Lay, Roberto Adamo,

Francesca Micoli: **formal analysis**; Olimpia Pitirollo, Roberta Di Benedetto, Francesca Mancini, Martina Carducci, Luisa Massai, Omar Rossi, Jeroen D C Codée, Francesca Necchi, Luigi Lay, Francesca Micoli: **methodology**; Olimpia Pitirollo, Roberta Di Benedetto, Roberto Adamo, Francesca Micoli: **roles/writing - original draft**; Olimpia Pitirollo, Roberta Di Benedetto, Pedro Henriques, Gianmarco Gasperini, Francesca Mancini, Martina Carducci, Luisa Massai, Omar Rossi, Anne Geert Volbeda, Jeroen D C Codée, Francesco Berlanda Scorza, Danilo Gomes Moriel, Francesca Necchi, Luigi Lay, Roberto Adamo, Francesca Micoli: **writing - review & editing**.

Declaration of competing interest

This work was undertaken at the request of and was sponsored by GlaxoSmithKline Biologicals SA. GVGH is an affiliate of GlaxoSmithKline Biologicals SA.

Roberta Di Benedetto, Gianmarco Gasperini, Francesca Mancini, Martina Carducci, Luisa Massai, Omar Rossi, Francesco Berlanda Scorza, Danilo Gomes Moriel, Francesca Necchi, Roberto Adamo and Francesca

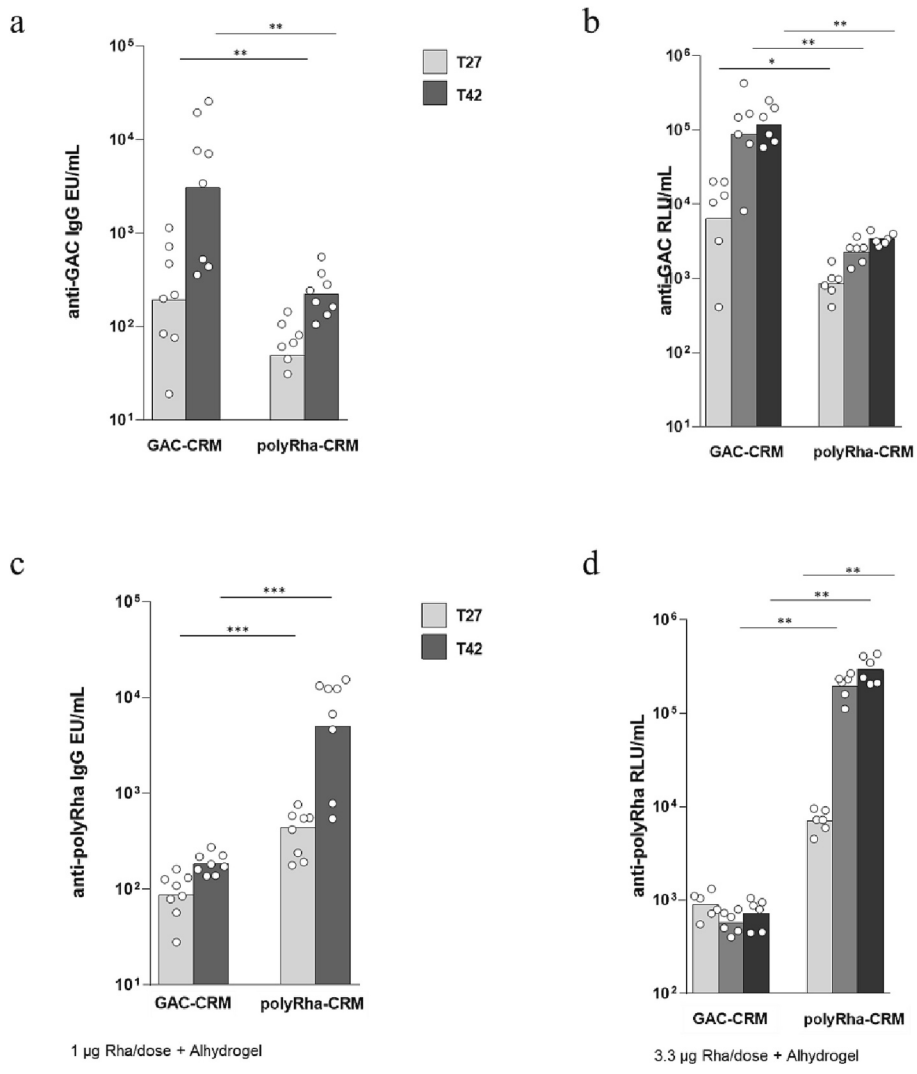


Fig. 10. Immunogenicity of polyRha and GAC conjugates. (a, c) CD1 mice were immunized intraperitoneally (IP) at day 0 and 28 with 1.0 µg (Rha based)/dose formulated with 2 mg/mL Alhydrogel. (b, d) New Zealand white rabbits were immunized intramuscularly (IM) at day 0, 21 and 35 with 3.3 µg (Rha based)/dose formulated with 0.7 mg/mL Alhydrogel. Summary graphs of anti-GAC (a, b) and anti-polyRha (c, d) specific IgG geometric mean units (bars) and individual antibody levels (dots) are reported. Mann-Whitney two-tailed test was performed to compare the response induced by the two immunization groups.

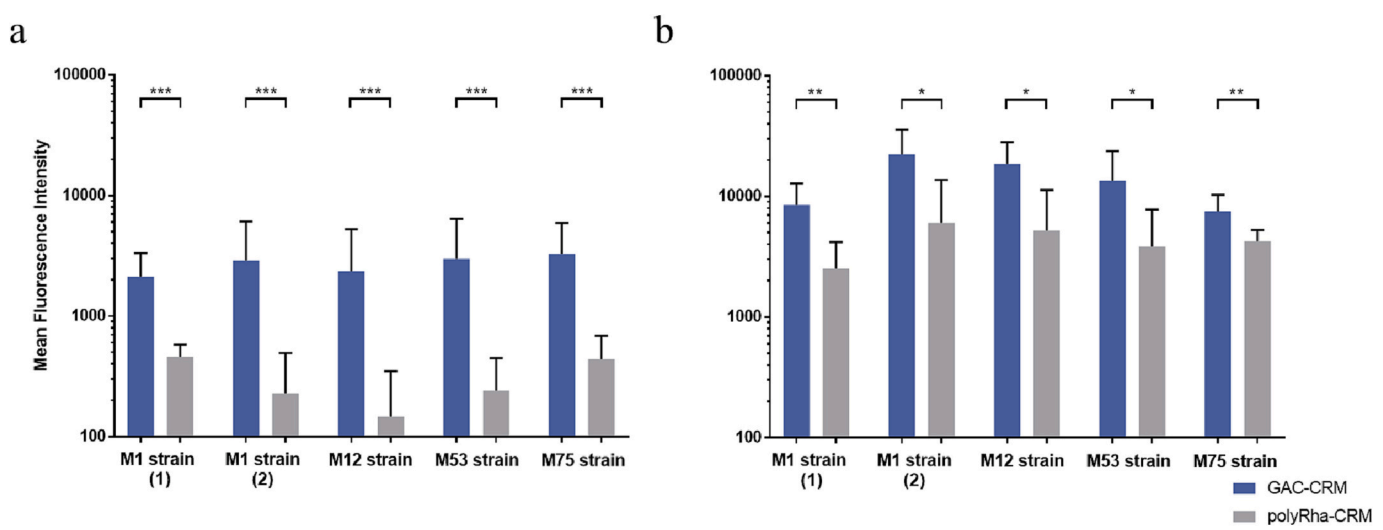


Fig. 11. Individual mice sera at day 42 (a) and individual rabbit sera at day 49 (b) were tested in Flow cytometry (FACS) to evaluate their ability to bind to Strep A bacterial cells of different M protein serotypes. Following incubation of bacteria with the different sera, anti-mouse conjugated to AF647 and anti-rabbit conjugated to PE secondary antibodies were used for detection. The mean fluorescence intensity (MFI) of 10,000 acquired events, measured for each serum, is reported as compared to pre-immune sera. Results obtained with sera diluted 1:500 are reported.

Micoli are employees of the GSK group of companies. Roberta Di Benedetto, Omar Rossi, Francesco Berlanda Scorza and Danilo Gomes Moriel report shares in the GSK group of companies. Outside the submitted work, Francesco Berlanda Scorza and Danilo Gomes Moriel reports funding from CARB-X.

Olimpia Pitirollo and Pedro Henriques participated in PhD programs at GSK at the time of the experimental work.

Data availability

The authors declare that data are contained within the article and in Supplementary Materials.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.carbpol.2023.120736>.

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