

Expert Opinion

1. Introduction
2. Glycoconjugate vaccines
3. Conjugate vaccines against non-infectious diseases
4. Expert opinion

informa
healthcare

The design of semi-synthetic and synthetic glycoconjugate vaccines

Paolo Costantino, Rino Rappuoli[†] & Francesco Berti

Novartis Vaccines, Via Fiorentina 1, Siena, Italy

Introduction: Glycoconjugate vaccines are among the safest and most efficacious vaccines developed during the last 30 years. They are a potent tool for prevention of life-threatening bacterial infectious diseases like meningitis and pneumonia. The concept of hapten-carrier conjugation is now being extended to other disease areas.

Areas covered: This is an overview of the history and current status of glycoconjugate vaccines. The authors discuss the approaches for their preparation and quality control as well as those variables which might affect their product profile. The authors also look at the potential to develop fully synthetic conjugate vaccines based on the progress of organic chemistry. Additionally, new applications of conjugate vaccines technology in the field of non-infectious diseases are discussed. Through this review, the reader will have an insight regarding the issues and complexities involved in the preparation and characterization of conjugate vaccines, the variables that might affect their immunogenicity and the potential for future applications.

Expert opinion: The immunogenicity of weak T-independent antigens can be increased in quantity and quality by conjugation to protein carriers, which provide T-cell help. Glycoconjugate vaccines are among the safest and most efficacious vaccines developed so far. Various conjugation procedures and carrier proteins can be used. Many variables impact on the immunogenicity of conjugate vaccines and a tight control through physicochemical tests is important to ensure manufacturing and clinical consistency. New and challenging targets for conjugate vaccines are represented by cancer and other non-infectious diseases.

Keywords: Alzheimer's disease, cancer, conjugate vaccines, drug addiction, oligosaccharides, polysaccharides

Expert Opin. Drug Discov. (2011) 6(10):1045-1066

1. Introduction

Conjugate vaccines are composed of a weak antigen (hapten, containing only a B-cell epitope, but lacking T-cell epitopes), generally a microbial poly- or oligosaccharide but also a low molecular mass compound, covalently linked to a protein as a source of T-cell epitopes (carrier). The hapten-carrier concept was known since 1929 when Avery and Goebel demonstrated that simple derivatives of glucose and galactose, otherwise non-immunogenic, when conjugated to proteins were able to induce specific antibodies in rabbits [1]. During the same period, the importance of surface polysaccharides in bacterial virulence and the protective role of antibodies against pneumococcal polysaccharides were investigated [2]. The first polysaccharide vaccines against selected pneumococcal serotypes were ready in 1945 [3]. Their development, however, was hindered by the advent of antibiotics which made physicians think that the problems associated with bacterial diseases were solved. Only later on when antibiotic-resistant strains developed was the research on antibacterial polysaccharide vaccines resumed and their efficacy demonstrated in large clinical trials [4-7]. This led to the first anti-meningococcal and anti-pneumococcal polysaccharide vaccines being licensed in the seventies. Polysaccharide vaccines, however, did not completely solve the problem of bacterial

Article highlights.

- Glycoconjugate vaccines are among the safest and most efficacious vaccines developed during the last 30 years.
- Carbohydrate–protein conjugate vaccines can be prepared with various methods, which can result in products with different molecular definitions.
- Conjugate vaccines can be adequately controlled by physicochemical tests; prediction of human immunogenicity based on animal models might not be reliable.
- Variables such as saccharide chain length and loading degree of carbohydrate onto the protein are interconnected.
- The nature of the protein carrier can influence the immunogenicity of conjugate vaccines. However, a direct comparison of the different proteins for their ability to function as carrier in the existing conjugate vaccines is confounded by other variables such as conjugation chemistry, saccharide chain length, adjuvant, formulation technology and concomitant vaccination with other antigens.
- Well defined homogeneous structures with high purity can be obtained by carbohydrate synthetic technologies and used to develop new conjugate vaccines.
- The concept of conjugate vaccines is being extended now to the evaluation of vaccines against non-infectious diseases.

This box summarizes key points contained in the article.

diseases caused by encapsulated microorganisms because of their scarcely detectable immunogenicity in children below 2 years of age [4,5,8]. The concept of Avery and Goebel was taken up again and the first protein–polysaccharide conjugate vaccines to be developed were those against *Haemophilus influenzae* type b (Hib) [9–12], which were licensed between 1987 and 1990. The research on glycoconjugate vaccines has been directed also against other pathogens such as *Neisseriae meningitidis*, *Streptococcus pneumoniae* and group B *Streptococcus* [13–22]. A list of currently licensed glycoconjugate vaccines is shown in Table 1. The concept of conjugate vaccines has been largely associated with poly- or oligosaccharide–protein conjugates. Many bacterial pathogens, Gram-positive as well as Gram-negative, possess a polysaccharide coat called ‘capsule’. Surface polysaccharides have several functions: their hydrophilic character protects microorganisms from desiccation, thus facilitating host-to-host transmission; in some cases, for example, the hyaluronic capsule of group A *Streptococcus*, their adhesive properties help the pathogen to invade the host; other capsular polysaccharides interfere with innate immunity preventing the activation of the alternate complement pathway; and sometimes, their chemical structure mimics molecules produced by human cells so that the pathogen is not recognized as foreign by our immune system [23]. Due to the semi-synthetic nature of carbohydrate–protein conjugate vaccines, multiple expertise (biochemistry, chemistry, immunology and medicine) have been involved in their development and characterization. As compared to the development of conventional vaccines, the

novelty brought by conjugated vaccines was represented by the chemical approach needed to covalently couple poly- or oligosaccharides to protein carriers. Different strategies to prepare conjugate vaccines have been developed resulting in different molecular models (Figure 1) [24]. Many questions have been addressed concerning what are considered to be the key parameters for optimal immunogenicity of conjugate vaccines. Among these, we find size of carbohydrate moiety, O-acetylation degree of the carbohydrate, degree of carbohydrate loading onto the carrier protein, nature and length of the linker between the carbohydrate and the protein moieties, and the nature of the carrier proteins. Progress in synthetic carbohydrate chemistry made possible the synthesis of one or more repeating units of several bacterial oligosaccharides, which, after conjugation to carrier proteins, have been studied in animal models to understand the role played by some of these variables. A number of papers have been published trying to give an answer at least to some of these questions [25–31] and to open the way to a rational design of conjugate vaccines. On the wave of the success of bacterial glycoconjugate vaccines, the same concept is being now applied to the development and evaluation of vaccines against fungal diseases [32], malaria [33] and non-infectious diseases such as cancer, Alzheimer, hypertension, autoimmunity and drug abuse [34–41]. Conjugate vaccines have the potential to become of significant importance for the control of a large class of diseases beyond bacterial infections and a better understanding of the variables that influence their immunogenicity is crucial. In this paper, by reviewing different aspects of glycoconjugate vaccines including their immunological mechanism, conjugation strategies, variables influencing their immunogenicity and analytical testing, we highlight the key elements to be considered for their design. In addition, we briefly discuss other potential targets that might be addressed by conjugating peptides and small organic molecules to protein carriers.

2. Glycoconjugate vaccines

2.1 Immunological mechanism of glycoconjugate vaccines

Bacterial polysaccharides are made by repeated units constituted of monosaccharides linked together by glycosidic linkages. One of the main features of polysaccharide vaccines is that they are scarcely immunogenic in children < 2 years of age. In addition, the antibody response to bacterial polysaccharides is poorly affected by adjuvants and IgM represents the major class of antibodies induced. Last but not least, the immune response induced by polysaccharides does not induce memory, and thus it is not boosted by subsequent immunizations. These characteristics are due to the fact that, unlike proteins that are T-cell-dependent (TD) antigens, polysaccharides are T-cell independent (TI) antigens in that they do not require T-cell activation for the induction of specific B-cell (antibody) responses; in fact, they directly interact with polysaccharide-specific B cells which differentiate then into plasma cells to produce antibodies.

Table 1. Glycoconjugate vaccines currently licensed in the US, EU or WHO.

Indication and structures of capsular polysaccharides	Type of conjugate	Manufacturer
Haemophilus influenzae type b →3)-β-D-Ribf-(1→1)-D- Ribitol-(5→OPO ₃ ⁻ →	PRP-TT PRP-OMPC PRP-CRM ₁₉₇ Hib-CRM ₁₉₇	Sanofi-Pasteur Merck Pfizer Novartis Vaccines
Neisseria meningitidis serogroups A →6)-α-D-ManpNAC(3/4OAc)-(1→OPO ₃ ⁻ → C →9)-α-D-Neup5Ac(7/8OAc)-(2→ W ₁₃₅ →6)-α-D-Galp-(1→4)-α-D-Neup5Ac(9OAc)-(2→ Y →6)-α-D-Glcp-(1→4)-α-D-Neup5Ac(9OAc)-(2→	MenA-TT MenC-CRM ₁₉₇ MenC-TT MenACWY-DT MenACWY-CRM ₁₉₇	Serum Institute India Pfizer Novartis Vaccines Baxter Sanofi-Pasteur Novartis Vaccines
Haemophilus influenzae type b/Neisseria meningitidis group C	MenC/Hib-TT	GSK
Streptococcus pneumoniae serotypes 1 →3)-D-AAT-α-Galp-(1→4)-α-D-GalpA(2/3OAc)-(1→3)-α-D-GalpA-(1→ 3 →3)-β-D-GlcA-(1→4)-β-D-Glcp-(1→ 4 →3)-β-D-ManpNAC-(1→3)-L-FucpNAC-(1→3)-α-D-GalpNAC-(1→4)-α-D-Galp2,3(S)Py-(1→ 5 →4)-β-D-Glcp-(1→4)-[α-L-PnepNAC-(1→2)-β-D-GlcpA-(1→3)]-α-L-FucpNAC-(1→3)-β-D-Sugp-(1→ 6A →2)-α-D-Galp-(1→3)-α-D-Glcp-(1→3)-α-L-Rhap-(1→3)-D-Ribitol-(5→ OPO ₃ ⁻ → 6B →2)-α-D-Galp-(1→3)-α-D-Glcp-(1→3)-α-L-Rhap-(1→4)-D-Ribitol-(5→ OPO ₃ ⁻ → 7F →4)-β-D-Glcp-(1→3)-[α-D-GlcpNAC-(1→2)-α-L-Rhap-(1→4)]-β-D-GalpNAC-(1→6)-[β-D-Galp-(1→2)]-α-D-Galp-(1→3)-β-L-Rhap(2OAc)-(1→ 9V →4)-α-D-GlcpA(2/3OAc)-(1→3)-α-D-Galp-(1→3)-β-D-ManpNAC(4/6OAc)-(1→4)-β-D-Glcp-(1→4)-α-D-Glcp-(1→ 14 →4)-β-D-Glcp-(1→6)-[β-D-Galp-(1→4)]-β-D-GlcpNAC-(1→3)-β-D-Galp-(1→ 18C →4)-β-D-Glcp-(1→4)-[α-D-Glcp(6OAc) (1→2)]-[Gro-(1→P→3)]-β-D-Galp-(1→4)-α-D-Glcp-(1→3)-β-L-Rhap-(1→ 19A →4)-β-D-ManpNAC-(1→4)-α-D-Glcp-(1→3)-α-L-Rhap-(1→ OPO ₃ ⁻ → 19F →4)-β-D-ManpNAC-(1→4)-α-D-Glcp-(1→2)-α-L-Rhap-(1→ OPO ₃ ⁻ → 23F →4)-β-D-Glcp-(1→4)-[α-L-Rhap-(1→2)]-[Gro-(2→ OPO ₃ ⁻ →3)]-β-D-Galp-(1→4)-β-L-Rhap-(1→	7 valent-CRM ₁₉₇ (4, 6B, 9V, 14, 18C, 19F, 23F) 13 valent-CRM ₁₉₇ (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) 10 valent-DT/TT Protein D (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F)	Pfizer Pfizer GSK

CRM₁₉₇: Non-toxic mutant of diphtheria toxin; DT: Diphtheria toxoid; Gro: Glycerol residue; OMPC: *Neisseria meningitidis* B outer membrane protein complex; Protein D: Hib related protein; PRP: Poly-ribosyl-ribitol phosphate; TT: Tetanus toxoid.

However, differentiation into memory B cells does not occur and, moreover, a pre-existing memory B-cell pool can be depleted by immunization with unconjugated polysaccharide, with risk of hypo-responsiveness on subsequent immunizations.

Proteins and peptides are instead TD antigens as they stimulate helper T lymphocytes to elicit an immune response leading to specific antibody response. Unlike TI antigens, TD antigens are immunogenic early in infancy, the immune response induced can be boosted, enhanced by adjuvants, and characterized by an antibody class switch and production of antigen-specific IgG.

Following interaction with antigen-presenting cells such as dendritic cells, macrophages and B cells, TD antigens are internalized and processed into small peptides which are then re-exposed and presented to T lymphocytes in association with the MHC class II molecules. Interaction with T cells induces B cells to differentiate into plasma cells and memory B cells. TD antigens induce an immune response that is long lasting; antibodies are of high affinity and with prevalence of IgG.

Chemical conjugation of polysaccharides to protein carriers allows processing of the protein carrier by polysaccharide-specific B cells and presentation, on their surface, of the resulting

peptides or glycopeptides in association with MHC class II. Further interaction with carrier-specific T cells then induces polysaccharide-specific B-cell differentiation (Figure 2). As a consequence, a conjugate vaccine induces a TD response already early in life which leads to immunological memory and boosting of the response by further doses of the vaccine.

The key aspects of the immune response to polysaccharide and glycoconjugate antigens have been reviewed by many authors [42-46].

2.2 Chemistry of glycoconjugate vaccines

In glycoconjugate vaccines, a covalent linkage between the carbohydrate moiety and the carrier protein has to be installed (Figure 1). Two main approaches have been traditionally used for glycoconjugate vaccines preparation: one based on random chemical activation of hydroxyl or carboxyl groups present along the chain of the native or slightly size reduced polysaccharide, followed by conjugation; the other based on oligosaccharides coupled to the protein through activation of their terminal groups. Progress in synthetic carbohydrate chemistry is making increasingly attractive the synthetic approach

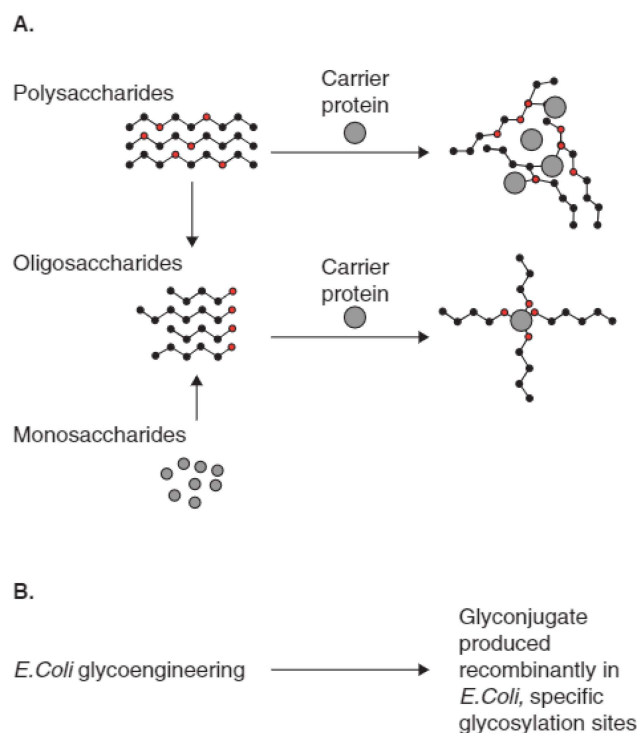


Figure 1. Schematic representation of A) different poly-/oligosaccharide-protein chemical couplings and B) Different way to make glycoconjugate vaccines.

Adapted from [24].

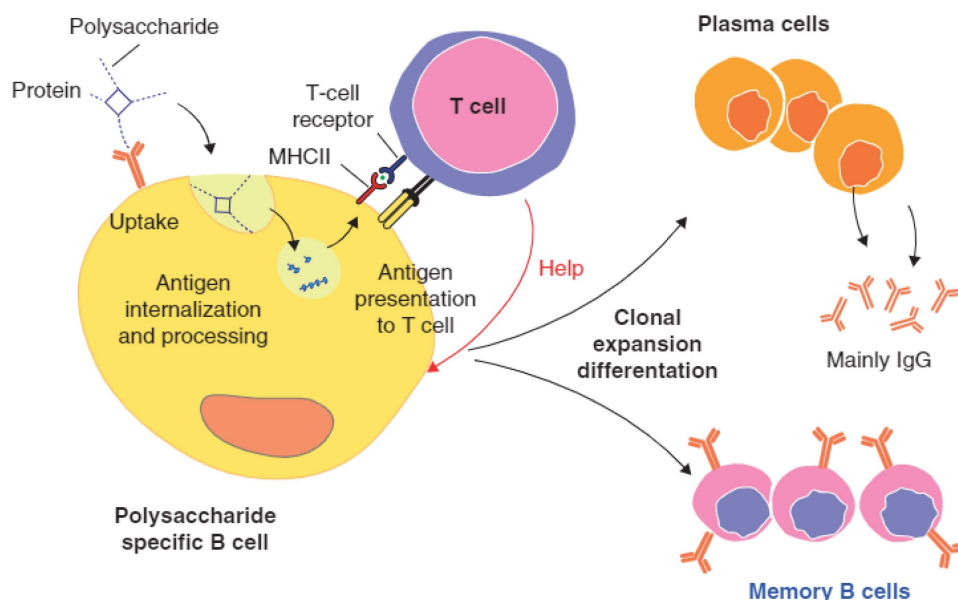


Figure 2. Schematic diagram of mechanism of action for conjugate vaccines.

which, starting from suitable monosaccharides can provide pure and well-defined oligosaccharides for coupling to protein carriers. Recently, a new approach called bioconjugation has received increasing attention. The approach is based on glyco-engineering the N-glycosylation pathway [47,48] in

bacteria such as *Escherichia coli*. The polysaccharide, encoded by the inserted genes, is produced on a polyisoprenoid carrier and then is transferred to an asparagine residue of the carrier protein which has to contain at least one (native or engineered) N-glycosylation site [49,50].

Depending on the conjugation chemistry used, a chemical spacer can be used in order to facilitate the coupling of the protein to the saccharide antigens and, in some cases, conjugation chemistry requires also previous derivatization of the protein carriers. The first conjugate vaccine was developed by the team of John Robbins at the NIH against Hib. Before the introduction of the conjugate vaccine in 1987, Hib was responsible for about 50% of bacterial meningitis worldwide. In a first version, the Hib capsular polysaccharide, a poly-ribosyl-ribitol-phosphate (PRP), was activated with cyanogen bromide and coupled, using the water soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, to diphtheria toxoid (DT) previously derivatized with adipic acid dihydrazide (ADH) used as spacer [9]. Subsequently, it was found more suitable to introduce the ADH spacer into the polysaccharide that was then coupled to the carrier protein via carbodiimide reaction (Figure 3A) [51]. Several conjugation chemistries have been developed by different manufacturers. Connaught and Pasteur Merieux (now Sanofi Pasteur) used the technology developed by John Robbins to couple the Hib polysaccharide to DT and tetanus toxoid (TT), respectively [52]. The original PRP-DT conjugate developed by Connaught Laboratories is no longer in use. Merck developed a different chemistry based on the derivatization of both PRP and the outer membrane protein complex of serogroup B meningococcus as carrier (Figure 3B) [53]. Sugars constituting the repeating units of polysaccharides possess often vicinal hydroxyl groups. These groups readily react with sodium periodate to form aldehydes that can be coupled to the lysine residues of carrier proteins by reductive amination. Pfizer used the periodate chemistry to develop its Hib conjugate vaccine using CRM197, a non-toxic mutant of diphtheria toxin [54,] as carrier (Figure 3C) [12]. A Cuban and Canadian team developed a Hib conjugate vaccine made by synthetic oligosaccharides of about seven repeating units equipped with a terminal maleimido group that were then conjugated to thiolated TT [55].

The periodate chemistry platform has been used by Pfizer and Baxter for their group C meningococcal conjugate vaccines, Baxter removed the O-acetyl groups from the structure of serogroup C meningococcal polysaccharide prior to periodate treatment (Figure 4A) [52]. Novartis Vaccines used a different approach to make Hib and serogroup C meningococcal conjugate vaccines. The purified capsular polysaccharides were size reduced by acid hydrolysis and fractionated to select an intermediate chain length population. The oligosaccharides were then selectively derivatized at their reducing termini introducing first a primary amino group by reductive amination followed by a 6-atom flexible hydrocarbon spacer having a terminal active ester ready for conjugation (Figures 3D and 4B) [13,16,56]. This technology platform was then used by Novartis to develop its quadrivalent A,C,W₁₃₅,Y anti-meningococcal conjugate (Figure 5) [21]. To develop pneumococcal conjugate vaccines, Pfizer has made use of periodate oxidation followed by reductive amination [52], while GlaxoSmithKline (GSK) used the reagent,

1-cyano-4-dimethylamino-pyridinium tetrafluoroborate which activates the hydroxyl groups along the polysaccharide chain [57]. Conjugates vaccines against *Streptococcus* group B (GBS) are also being developed using periodate-controlled oxidation of the sialic acid side chains and subsequent coupling by reductive amination to the protein carriers [52]. The capsular polysaccharide of serogroup B meningococcus is an $\alpha(2-8)$ -polysialic acid and, because of its self nature [58], its use is not recommended for the development of a vaccine against group B meningococcus. Attempts to render it non-self and immunogenic targeted the N-acetyl group of the sialic acid repeating units, which was changed into N-propionyl groups. The N-propionylated polysaccharide was then conjugated to TT. This conjugate was brought till Phase I clinical trials but did not induce bactericidal antibodies against the group B organism [59]. Cox *et al.* have tested a number of chemical strategies in the attempt to develop meningococcal group B candidate vaccines based on conjugates with truncated inner core forms of *N. meningitidis* lipopolysaccharide (LPS) [60]. A variety of other conjugation chemistries can be used to couple carbohydrate antigens to protein carriers [61-65]; a summary of the different functional groups that can be introduced in polysaccharide antigens and proteins for further conjugation is given in Table 2.

2.3 Variables that influence the immunogenicity of conjugate vaccines

Many variables can influence the immunogenicity of conjugate vaccines. Studies to understand the effect that variations on the design of glycoconjugates can have on their immunogenicity have considered the size of the saccharide moiety, the saccharide:protein ratio in the purified conjugate, the conjugation strategy, the nature of the spacer and the protein carrier. A brief discussion on these variables follows below.

2.3.1 The size of the saccharide moiety and saccharide:protein ratio

Seppälä and Mäkelä in one of the first studies on the effect of size and chemistry on the immunogenicity of dextran-protein conjugates found that dextrans of low molecular mass, ranging from 1000 to 4000 Da, conjugated to chicken serum albumin via their reducing end induced strong anti-dextran responses in BALB/c and CBA mice [30], while increasing the dextrans' size resulted in reduced immunogenicity.

Peeters *et al.* [66] showed that a synthetic tetramer of Hib capsular polysaccharide repeating unit, conjugated to a protein carrier, induced in adult mice and non-human primates antibody levels comparable to a commercial Hib conjugate and higher than those induced by a trimer, indicating that for Hib a minimum of eight sugars is needed for a proper immunological response. Benaissa-Trouw *et al.* [67] studying the mouse immunogenicity of synthetic di, tri and tetramer capsular fragments of type 3 *S. pneumoniae* found that only trimer and tetramer conjugates induced homogeneous antibody responses and complete protection on challenge. In these studies, the degree of carbohydrate loading onto the

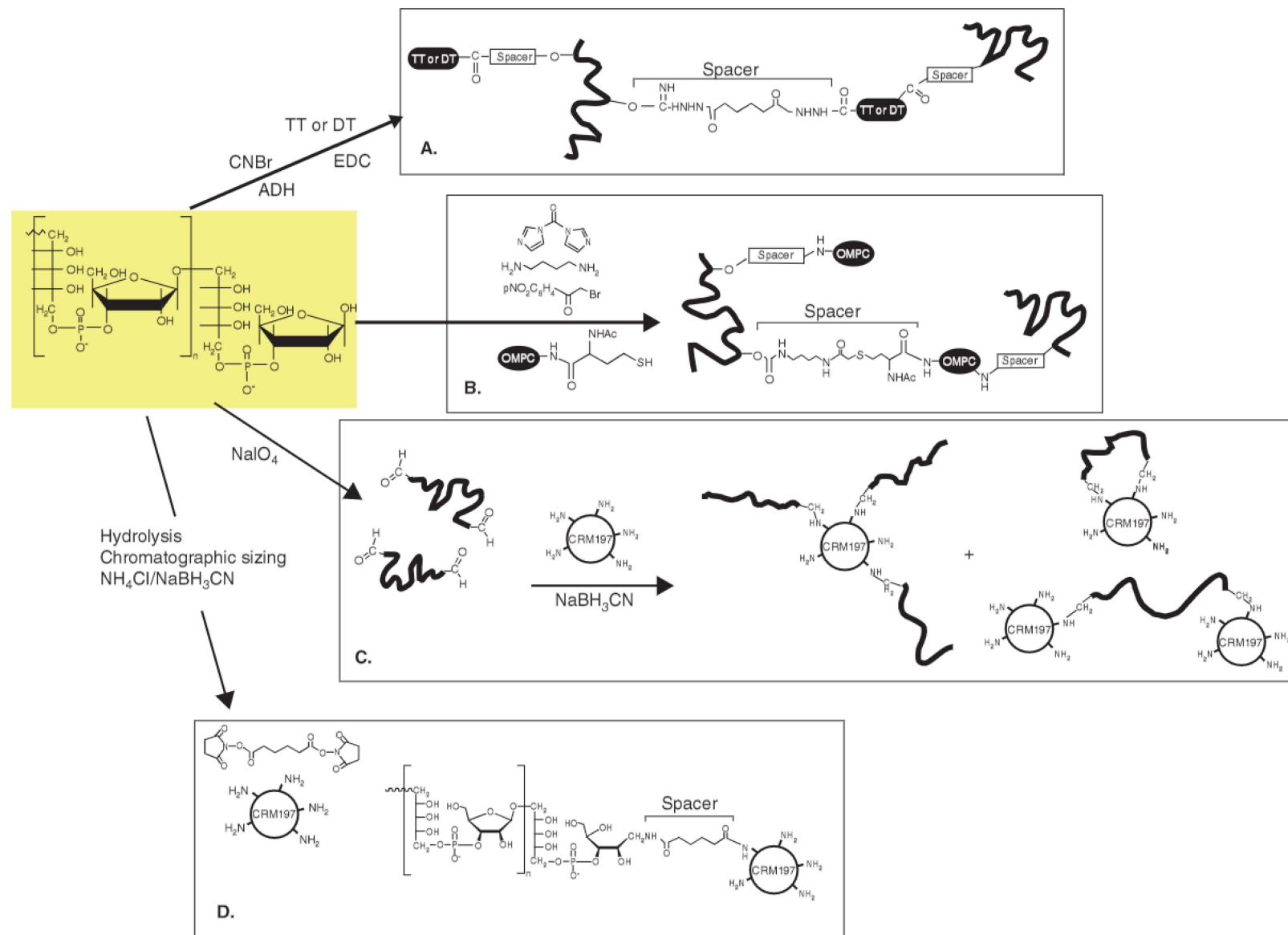


Figure 3. Preparation processes of licensed Hib conjugate vaccines.

Hib: *Haemophilus influenzae* type b.

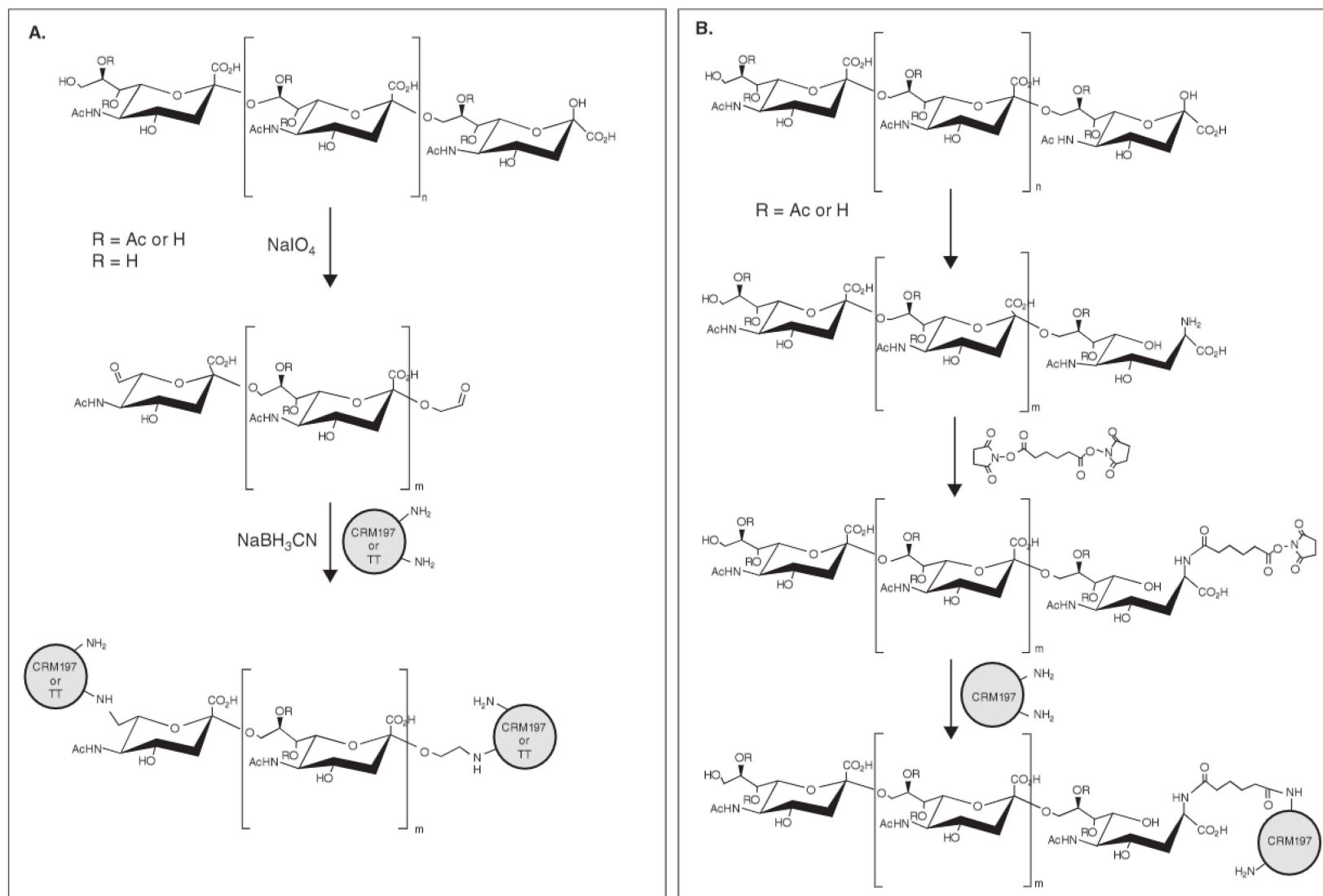


Figure 4. Preparation approaches for licensed meningococcal group C conjugate vaccines of: **A)** Pfizer (R: Ac or H) and Baxter (R: H) and **B)** Novartis Vaccines.

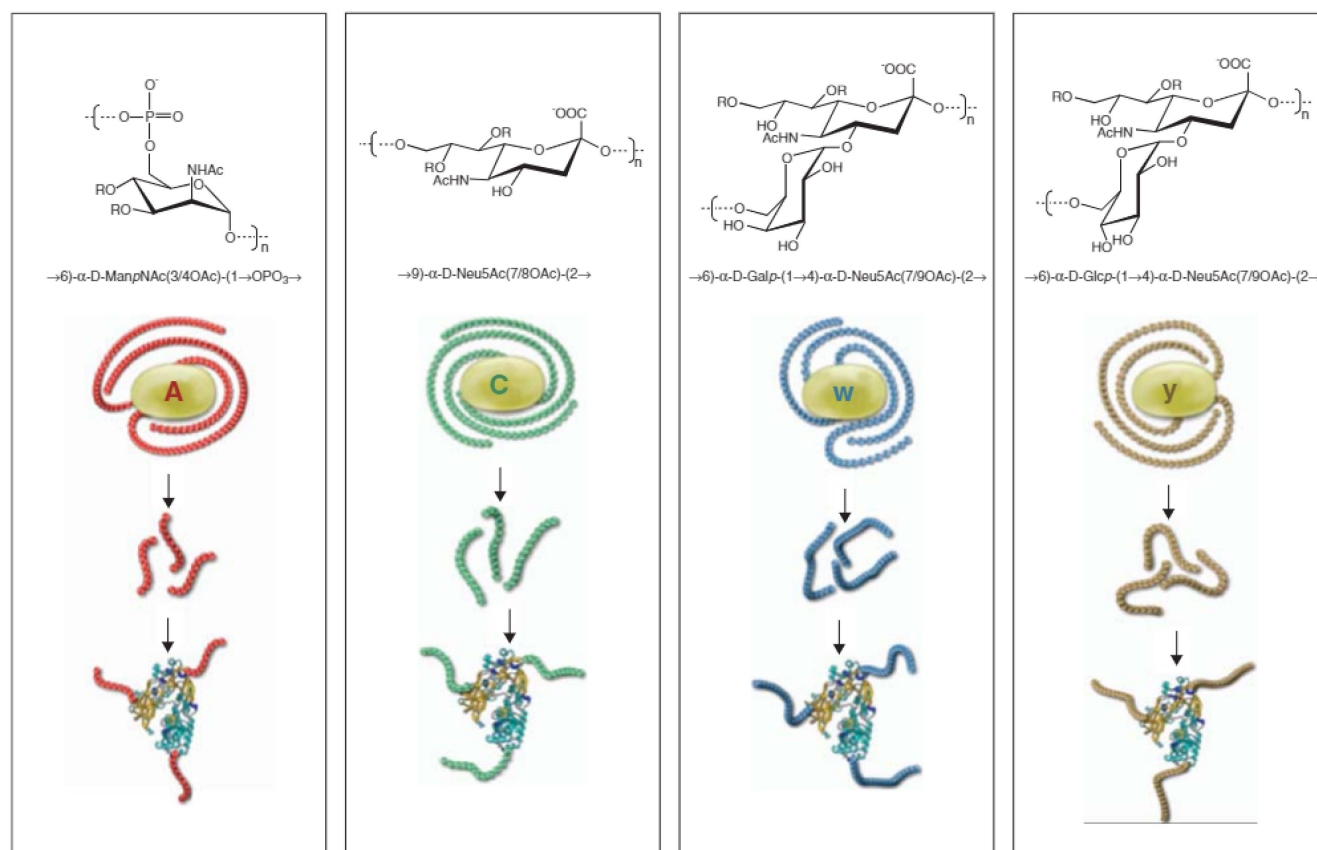


Figure 5. Polysaccharide molecular structures and conjugation technology of licensed meningococcal group A, C, W135, and Y conjugate vaccine produced by Novartis Vaccines.

protein did not influence the immunogenicity of the vaccine. Laferriere *et al.* found little influence of the carbohydrate chain length on the immunogenicity of pneumococcal conjugate vaccines in mice [27]. Anderson *et al.* determined in clinical studies that the immunogenicity in 1-year-old infants of Hib glycoconjugates was superior when the average saccharide polymerization degree (avDP) was 20 as compared to 8 [12]. In a subsequent study, however, they found that conjugates containing Hib oligosaccharides with avDP of 7 were, in 1-year-old infants, equally highly immunogenic to those made with avDP 20, provided that a higher saccharide:protein ratio was used [26]. Pozsgay *et al.* [31] studied the immunogenicity in mice of synthetic *Shigella dysenteriae* type 1 LPS oligosaccharides conjugated to human serum albumin (HSA). The authors found that octa-, dodeca- and hexadecasaccharide fragments induced high levels of LPS binding IgG antibodies in mice after three injections and were superior to a tetrasaccharide conjugate. The influence of the carbohydrate:protein ratio was different for the three conjugates. The octasaccharide-HSA conjugate with the highest density evoked a good immune response, while in the case of dodeca- and hexadecasaccharide conjugates, the median density was optimal. Phalipon *et al.* [68] found that a synthetic sequence containing three repeating units of the O-specific polysaccharide of *Shigella flexneri* 2a LPS is a better functional mimic of the native

polysaccharide as compared to one or two repeating units, as evidenced by the highest immunogenicity of the respective TT conjugates and better protective efficacy of the antibodies induced in mice. This work also determined that the optimal saccharide:protein ratio for the pentadecasaccharide conjugate was 14:1 on a molar basis. These studies suggest that oligosaccharide chain length and hapten loading might be interconnected in determining the immunogenicity of glycoconjugate vaccines.

2.3.2 The conjugation strategy

In the cited study on dextrans-protein conjugates, Seppälä and Mäkelä [30] found also that generating multiple bonds between the saccharide and the protein resulted in reduced immunogenicity. The effect on immunogenicity of molecular size of the conjugate, molecular size of the polysaccharide and degree of polysaccharide:protein crosslinking were studied for GBS type 3-TT conjugates [69]. The authors concluded that immunogenicity and protective efficacy increase with molecular size of the polysaccharide and also with molecular size of the conjugates. An intermediate crosslinking degree was found optimal suggesting that high crosslinking could evoke non-protective epitopes. Specific conjugation through the saccharide end reducing groups was found to induce higher antibodies titers also by Pozsgay *et al.* in the case of *S. dysenteriae* type 1 [31].

Table 2. Examples of functional groups used in chemical conjugation.

Chemistry	Functional groups involved		Product
Carbodiimide-mediated condensation	$\text{R}-\text{C}(=\text{O})-\text{OH}$	$\text{H}_2\text{N}-\text{R}_1$	$\text{R}-\text{C}(=\text{O})-\text{NH}-\text{R}_1$
Active ester	$\text{R}-\text{C}(=\text{O})-\text{O}-\text{N}$ (maleimide ring)	$\text{H}_2\text{N}-\text{R}_1$	$\text{R}-\text{C}(=\text{O})-\text{NH}-\text{R}_1$
Reductive amination	$\text{R}-\text{C}(=\text{O})-\text{H}$	$\text{H}_2\text{N}-\text{R}_1$	$\text{R}-\text{CH}_2-\text{NH}-\text{R}_1$
Thioalkylation [66]	$\text{Br}-\text{CH}_2-\text{C}(=\text{O})-\text{R}_1$		$\text{R}-\text{S}-\text{CH}_2-\text{C}(=\text{O})-\text{R}_1$
Thiol addition [55]	$\text{R}-\text{SH}$	Maleimide ring with $\text{N}-\text{R}_1$	Thioether-linked maleimide ring with $\text{N}-\text{R}_1$
Disulfide formation [62]	$\text{HS}-\text{R}_1$		$\text{R}-\text{S}-\text{S}-\text{R}_1$
Thiol-ene [61,63]	$\text{R}-\text{SH}$	$\text{CH}_2=\text{CH}-\text{R}_1$	$\text{R}-\text{S}-\text{CH}_2-\text{CH}_2-\text{R}_1$
Cyanogen bromide or CDAP activation	$\begin{array}{c} \\ -\text{C}-\text{OH} \\ \\ -\text{C}-\text{OH} \\ \end{array}$	$\text{H}_2\text{N}-\text{R}_1$	$\begin{array}{c} \\ -\text{C}-\text{O}-\text{CH}=\text{N}-\text{R}_1 \\ \\ -\text{C}-\text{OH} \\ \end{array}$
Oxime formation [64]	$\text{R}-\text{C}(=\text{O})-\text{H}$	$\text{H}_2\text{N}-\text{O}-\text{R}_1$	$\text{R}-\text{C}(\text{H})=\text{N}-\text{O}-\text{R}_1$
'Click chemistry' [65]	$\text{R}-\text{N}=\text{N}^+=\text{N}^-$	$\text{HC}\equiv\text{C}-\text{R}_1$	1,4-disubstituted 1,2,3,4,5-pentazoles

CDAP: 1-Cyano-4-dimethylamino-pyridinium tetrafluoroborate.

2.3.3 The nature of the spacer

The spacer is a short linear molecule that is generally linked to the polysaccharide chain or to the protein or to both moieties, depending on the coupling chemistry used. It is used to facilitate the subsequent coupling by alleviating the steric hindrance factors that might render difficult the interaction between two large or relatively large molecules. The spacer is designed with built-in functional groups to allow an efficient and consistent coupling step that is easy to control and scale up. There are evidences in the literature which suggest that rigid, constrained spacers such as cyclohexyl maleimide elicit a significant amount of undesirable antibodies [70,71] with the risk of driving the immune response away from the

targeted epitope on the hapten. The use of a flexible alkyl type maleimido spacer has been reported as a way to overcome the previous observed immunogenicity of cyclic maleimide linkers [68]. The issue might depend on the relative antigenicity of the spacer and the carbohydrate hapten. For example, it has been shown that the serogroup B meningococcus polysaccharide which mimics a self antigen once conjugated to carrier proteins even using the flexible ADH spacer induced an immunological response in mice that was predominantly directed against the junction region between the saccharide and the protein [72]. Cox *et al.* studying meningococcal group B candidate vaccines based on conjugates with truncated inner core forms of *N. meningitidis* LPS found a considerable

response against the spacer region, which they hypothesize is dominated by the hydrazido functionality present in the linker reagent [73]. The creation of immunogenic neo-epitopes as a result of the conjugation chemistry is another event that might drive the antibody response of the conjugate away from targeted epitopes located on the hapten [74]. Interestingly, the triazole moiety present in the linker region of synthetic β -mannan disaccharides conjugated to chicken serum albumin has been reported to be immunosilent in mice and rabbits [75].

In our opinion, the impact of variables such as size, degree of carbohydrate loading onto the protein and O-acetylation of the saccharide chain [76-78] has to be determined on a case by case basis for each carbohydrate antigen. Issues related to optimal chain length of the saccharide moiety might not apply to carbohydrate-based cancer vaccines as the target antigens are short oligosaccharides. We also believe that, whenever possible, the conjugation chemistry should be very specific and involve only poly- or oligosaccharide endgroups, thus avoiding chemical modification along the saccharide chains and consequent formation of crosslinked structures and risk of immunogenicity loss.

2.3.4 The protein carrier

A number of protein carriers have been used so far in preclinical and clinical evaluation of conjugate vaccines [19,79-85]. Proteins such as diphtheria and TTs, which derive from the respective toxins after chemical detoxification with formaldehyde, were initially selected as carrier because of the safety track record accumulated with tetanus and diphtheria vaccination. CRM197, a non-toxic mutant of diphtheria toxin [54] which instead does not need chemical detoxification, has been extensively used as carrier for licensed Hib, pneumococcal, meningococcal conjugate vaccines and for other vaccines being developed. An outer membrane protein complex of serogroup B meningococcus has been used by Merck as carrier for their Hib conjugate vaccine [86]. GSK in their multivalent pneumococcal conjugate vaccine introduced the use of the Hib-related protein D as carrier for most of the polysaccharides included into the vaccine [57,87]. The team of John Robbins made extensive use of the recombinant non-toxic form of *Pseudomonas aeruginosa* exo-toxin as carrier for *Staphylococcus aureus* types 5 and 8 as well as for *Salmonella typhi* Vi conjugates [62,88].

A number of clinical trials have been conducted to compare the immunogenicity of different conjugate vaccines with different carrier proteins [89-93]. We believe that a direct comparison of the effect of different protein carrier is difficult due to the coexistence of other variables such as conjugation chemistry, saccharide chain length, adjuvant, formulation technology, and previous or concomitant vaccination with other antigens.

Immune interferences arising from simultaneous or previous exposure to the carrier or carrier related protein (i.e., multivalent combinations of conjugate vaccines) or to combination or co-administration with other vaccines can

result in a positive or negative effect on the immune response to the conjugated carbohydrate [94-97]. Attempts to explain these effects considered several mechanisms including: i) carrier-specific enhancement of T-cell help characterized by increased anti-hapten antibody responses, ii) carrier-induced epitope suppression where previous or concomitant carrier immunity has a negative effect on anti-hapten responses, iii) bystander effect where multiple antigens not necessarily related are competing for the immunological resources of the host and iv) epitope overload suppression characterized by competition for limited T-helper lymphocytes among different conjugates having the same carrier [94,98-99]. The clinical significance of these observations remains unclear as a number of combinations or concomitant administrations tested in clinical trials were able to elicit antibody levels above the accepted protection threshold [100-104]. The complexity of the interaction among vaccines containing different carrier proteins and the difficulties in predicting their relative effects in the context of combined or concomitantly administered vaccines has been recently reviewed [105].

With the goal of identifying an optimized carrier protein, Falugi *et al.* designed a recombinant protein (N19) containing strings of promiscuous human CD4⁺ T-cell epitopes derived from various pathogens including tetanus, influenza virus, *Plasmodium falciparum* and hepatitis B virus, which proved to be a very good carrier for Hib oligosaccharides [106]. Subsequently, Baraldo *et al.* showed that N19 works nicely as carrier also for meningococcal oligosaccharides [107] and interestingly the anti-carrier antibodies induced by the glycoconjugate vaccine did not recognize TT, an antigen that is commonly found in pediatric combinations. These findings suggest that N19 might be a candidate carrier protein for future or optimized conjugate vaccines. Other carrier proteins such as keyhole limpet hemocyanin (KLH) and the virus-like particle Q β are taken into consideration in developing conjugate vaccines for non-infectious diseases [108,109].

It is the opinion of the authors that preferable features for a protein carrier are: high level of purity and safety, the possibility to be fully characterized by physicochemical methods and the ability to induce a strong T-cell help. A low antibody response to carrier-specific B-cell epitopes might be useful to drive the response against the hapten and to facilitate the combination with other antigens without or minimal immune interference, even though the potential for a dual role of carrier and protective antigen is an attractive perspective.

2.4 Synthetic approaches for glycoconjugate vaccines

Currently, most of the conjugate vaccines are produced from poly- or oligosaccharides extracted and purified from microbial cultures. They can be obtained with very high purity, although heterogeneity and traces of bacterial contaminants (endotoxins, variants containing non-protective epitopes, etc.) cannot be avoided.

The chemical synthesis of oligosaccharides instead provides homogeneous, well defined and characterized molecules with

built-in chemical terminal functionalities suitable for conjugation to carrier proteins. It is also the only choice when dealing with carbohydrate antigens that cannot be easily obtained from biological sources or cannot be obtained at all (fungal antigens, viral oligosaccharides, tumor-associated carbohydrate antigens (TACAs)). The first conjugate vaccine for human use based on a synthetic approach consisting of about seven Hib repeating units coupled to TT through maleimido linker was the one against Hib developed by Bencomo *et al.* [55]. A number of oligosaccharides representing the surface carbohydrates of pathogens such as *S. pneumoniae* serotypes, *Shigella* species, *Candida albicans*, *Cryptococcus neoformans*, Group A *Streptococcus*, *S. aureus*, *Vibrio cholerae* and *P. falciparum* have been synthesized, coupled to carrier protein and tested in animal models [31-33,67,110-119]. All these studies, besides suggesting the potential feasibility of developing synthetic carbohydrate-based conjugate vaccines, provide the possibility of getting additional insights on what are the minimum structural requirements to induce protective antibodies. This information becomes then extremely useful in designing glycoconjugate vaccines for human use. Carbohydrate chemistry and automatic approaches now provide access to many structures that can be synthesized with high purity and reasonable yields for initial research studies [120,121]. There are clear advantages of the synthetic approach in terms of purity and molecular definition of the carbohydrate antigens and resulting conjugates. However, additional efforts concerning the optimization of the synthetic schemes are probably still required for its broad applicability and scalability, especially when dealing with oligosaccharides containing challenging structural motifs and/or made by several repeating units. A combination of chemical and enzymatic approaches could help further and broader implementation of the synthetic approach.

An attractive concept, although challenging, for future conjugate vaccines is based on fully synthetic glycopeptide vaccines where not only the hapten moiety is obtained by chemical synthesis but also the carrier is constituted by synthetic peptides representing CD4⁺ T-cell epitopes [122,123]. The biggest challenge for human application is the MHC class II genetic restriction. Promiscuous T-cell peptides able to interact with different MHC class II alleles have been identified and some of them successfully tested in animal models, setting the basis for further development of fully synthetic conjugate vaccines [106,124].

2.5 Characterization of glycoconjugate vaccines

Glycoconjugate vaccines need to be carefully characterized and controlled to guarantee consistent production and immunogenicity. Precise characterization of conjugate vaccines is a complex procedure that involves tests on starting materials such as purified polysaccharides and carrier proteins, the derivatized intermediates before conjugation, the purified conjugate and the final vaccine formulation. For a proper characterization of glycoconjugate vaccines, a variety of physicochemical methodologies including spectrophotometric methods, HPLC, mass spectrometry and NMR have been developed in order to

achieve a tight control of the manufacturing process [125-129]. An overview of the analytical tests typically applied to conjugate vaccines and their intermediates is shown in Table 3. Important factors to ensure manufacturing consistency and, therefore, production batches with comparable immunogenicity to those used in clinical trials are the size of the saccharide chain, the saccharide:protein ratio, the size of the conjugate and the level of free saccharide [130]. The level of free saccharide is the amount of saccharide that is not covalently linked to the protein carrier. Free saccharide can be present as a process residual after the purification of the glycoconjugate, but can also be formed during vaccine storage as a result of hydrolysis of the glycosidic linkages which hold together the monosaccharide units of the polysaccharides. Excess of free saccharide inevitably results in decrease of glycoconjugate vaccines potency as this is based on the covalent linkage between the saccharide and the protein moiety.

Preclinical testing of conjugate vaccines involves immunogenicity assays in animal models. Polysaccharides are not or scarcely immunogenic in mice and other animal models such as rabbit and guinea-pigs, while after conjugation to protein carriers, they become immunogenic and induce specific IgG anti-carbohydrate antibodies. This change in immunological behavior reflects the acquisition of TD character. Potency tests based on animal immunogenicity might not correlate with human efficacy/immunogenicity. For example, some post-licensure batches of the Merck Hib vaccine were less immunogenic in humans even though they were judged to be satisfactory based on release criteria, including a mouse potency test. By reviewing the manufacturing process and adding some physicochemical tests, Merck was able to find a chemical correlate of immunogenicity in humans [129]. In principle, *in vivo* potency tests could be used to assess manufacturing consistency, but for this purpose physicochemical methodologies are much more reliable due to their reproducibility, specificity and sensitivity. Glycoconjugate vaccines, especially those with a well-defined structure, can be appropriately characterized by physicochemical assays. We believe that during the research and preclinical phases of conjugate vaccines development, immunogenicity tests in animal models can be useful to verify that the conjugation process has conferred TD help to the carbohydrate antigens. However, in our opinion, the routine release specifications for this type of vaccines should be based only on appropriate physicochemical tests that can allow a tight control of the manufacturing process and of the key product profile variables [129].

3. Conjugate vaccines against non-infectious diseases

3.1 Anticancer vaccines

Therapeutic vaccines are among the strategies currently considered to combat cancer [131]. Tumor cells are characterized by altered regulation of glycosyl transferases which results in heterogeneity, overexpression and truncation of surface oligosaccharides [37]. The early work of Springer, Lloyd and Hakomori [132-135] contributed to the elucidation of the

Table 3. Example of analytical tests for quality control of glycoconjugate vaccines.

Process intermediates		Quality attributes	Typical tests
Polysaccharide	Structure	Identity	NMR, immunochemical methods
		Molecular size and poly-dispersity	SEC-UV/RI/F-MALLS
	Purity	Chemical composition O-acetyl Proteins, nucleic acids, cetavlon Water and volatile substances Organic solvents residuals Bioburden Endotoxins	HPAEC-PAD, colorimetric assays Colorimetric assay, NMR Colorimetric assays KF, thermogravimetry GC- Flame Ionization Detection Total viable aerobic count of microbial contamination LAL assay
Derivatized poly-/oligosaccharides	Structure	Identity	NMR, immunochemical methods
		Molecular size and poly-dispersity Saccharide content Degree of polymerization (total saccharide:endgroups ratio) O-acetyl Derivatizing groups content (e.g., dihydrazide, bromo acetate, active ester)	SEC-UV/RI/F-MALLS HPAEC-PAD, colorimetric assay Colorimetric assay, HPAEC-PAD, NMR Colorimetric assay, NMR Colorimetric assay, NMR
	Purity	Process-related residuals	Colorimetric assay, GC
Carrier protein	Structure	Identity and molecular size Protein sequence Protein folding Derivatizing groups content (e.g., dihydrazide, bromo acetate)	SDS-Page, WB, SEC-UV/F-MALLS, RPC MS CD, F MS, colorimetric assay
		Nucleic acids <i>Escherichia coli</i> host cell proteins Other proteins as impurities Toxicity, endotoxins Bioburden	Colorimetric assay WB, ELISA SDS-Page, SEC-UV/F, RPC LAL assay Total viable aerobic count of microbial contamination
	Purity		
Bulk conjugate	Structure	Identity Conjugation proof Conjugated and unconjugated saccharide Conjugated and unconjugated protein O-acetyl Molecular size	NMR, immunochemical methods SDS-Page, MS-MS SPE or UF or HIC followed by HPAEC-PAD or colorimetric analyses, CE SEC-UV/RI-fluorescence, CE NMR SEC-UV/RI/F-MALLS, MALDI-MS
		Process-related residuals Endotoxins Bioburden	Colorimetric assay, GC LAL assay Total viable aerobic count of microbial contamination
	Purity		
Formulated vaccine	Structure	Identity Conjugated and unconjugated saccharide Conjugated and unconjugated protein Adjuvant content (e.g., alum) Preservative content (e.g., thimerosal) Excipient and diluents content Residual moisture in lyophilized products	WB, immunochemical methods SPE or UF or HIC followed by HPAEC-PAD or colorimetric analyses, nephelometry, CE SEC-UV/RI/F, CE Colorimetric, titrimetric, ICP-MS-AA Specific assay Specific assay Thermogravimetry
		Endotoxins Pyrogenicity Sterility	LAL assay Pyrogen test B&F
	Purity		

B&F: Bacteriostasis and fungistasis test; CD: Circular dichroism; CE: Capillary electrophoresis; F: Fluorescence; GC: Gas chromatography; HIC: Hydrophobic chromatography; HPAEC-PAD: High performance anionic exchange chromatography-pulsed amperometric detection; ICP-MS-AA: Inductively coupled plasma-mass spectrometry- atomic absorption; KF: Karl fisher; LAL: Limulus amoebocyte lysate; MALDI: Matrix-assisted laser desorption/ionization; MALLS: Multi angle light laser scattering; MS: Mass spectrometry; RI: Refractive index; RPC: Reverse phase chromatography; SEC: Size exclusion chromatography; SPE: Solid phase extraction; UF: Analytical ultrafiltration; UV: Ultraviolet visible; WB: Western blot.

structural and tissue specificities of carbohydrates associated with tumor cells. These antigens have been called TACAs. As opposed to surface carbohydrate of bacterial pathogens, which are generally high molecular mass polysaccharides made by several repeating units covalently linked together, TACAs are small oligosaccharides structurally related to blood group substances and gangliosides. They are associated with glycolipids, as in the case of Globo-H and Fucosyl GM₁ ganglioside, to both glycolipids and glycoproteins as in the case of Lewis antigens, or to glycoproteins only [135] such as Thomsen-Friedenreich (T/TF), Thomsen-Friedenreich new (Tn) and Sialyl-Tn (STn) that are found in the epithelial glycoprotein MUC1 as described by Taylor-Papadimitriou *et al.* and Hanisch [136,137]. A list of TACAs and relative structures associated with tumors is reported in Table 4. Thanks to the recent advances in synthetic carbohydrate chemistry, these antigens can now be provided in the form of well-defined structures with high purity.

TACAs have been proposed for active immunization against tumors and form the basis for one of the current approaches for developing anticancer vaccines [138]. However, TACAs are poor immunogens because, besides being TI antigens, their low level of expression in normal tissues is probably making them self-molecules. Therefore, breaking tolerance is one of the key issues in developing cancer vaccines. Strategies are based on conjugation to molecules which provide T-cell epitopes as proposed in the early work by Kunz *et al.* [139], the use of suitable adjuvants and in some cases chemical modification to escape tolerance [37,38,40,108,139-141]. Several teams are contributing to the development of carbohydrate-based anticancer vaccines. The work of Danishefsky and his team at the Sloan-Kettering Institute evolved from monomeric conjugated vaccines containing a single TACA to a unimolecular model consisting of up to six TACAs on a peptide backbone covalently linked to KLH [38,108,142]. Recent work of Kaiser *et al.* and Hoffman-Roder *et al.* focused on TT as carrier for STn and (T)-MUC1 glycopeptides [143,144], while Sørensen *et al.* used enzymatic glycosylation of synthetic MUC1 to reproduce mucine type O-glycans expressed in tumor cells and subsequent conjugation to KLH [145]. In the case of cancer carbohydrate-based conjugate vaccines, variables such as the degree of loading might play a major role as suggested by Li *et al.* who found that Tn antigen conjugated to bovine serum albumin at high density induced in rabbits a broader spectrum of antibodies than a conjugate at low density of glycosylation. This finding might be of particular importance considering that the Tn antigen can be found in different forms, single and clustered on the surface of glycopeptides [146].

The choice of the carrier protein is particularly critical in designing cancer vaccines. In fact, its role is to make possible, through T-cell epitopes, the interaction between carrier-specific T cells and TACA-specific B cells, which then differentiate into plasma cells and produce antitumor antibodies. However, the carrier protein, especially in the context of

multiple injection schedules, can induce also high titers of carrier-specific antibodies and decrease the response against self antigens such as TACAs.

With the aim of overcoming the issue of poor immunogenicity of TACAs, Boons and co-workers are developing totally synthetic vaccines which incorporate the carbohydrate antigen (B-cell epitope), a selected universal helper T-cell peptide and a synthetic adjuvant [37,147].

While for most infectious diseases humoral immunity is believed to be a key factor to achieve protection, in the case of cancer vaccines, both humoral and cellular immunity are considered important. Humoral responses generate antibodies that facilitate tumor cells killing by complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity with the intervention of NK cells and macrophages. The cellular immunity acts through CD8⁺ cytotoxic T lymphocytes (CTL) that recognize tumor-derived peptide-MHC class I complexes and subsequently cause cytolysis of tumor cells [37,131,148]. Therefore, it seems reasonable that the optimal immune response induced by a cancer vaccine should activate many components of the immune system [131]. Interestingly, there is evidence that MUC-1 glycopeptides can bind to MHC class I molecules and that engineered glycopeptides containing TF and Tn TACAs are able to induce specific anti-TF/Tn CTL responses in mice [149,150]. In order to induce both humoral and cellular immune responses, Renaudet *et al.* developed a multi-epitope construct which incorporates clustered Tn analogs as B-cell epitopes linked in series to CD4⁺ and CD8⁺ T-cell epitopes as well as to a palmitic acid moiety as adjuvant. This construct resulted as immunogenic and protective in a tumor mouse model [151].

Other potential anticancer conjugate vaccines currently under investigation are based on the covalent linking to carrier proteins of: i) peptides representing B-cell epitopes of tumor antigens such as Her-2/neu, which is overexpressed in aggressive forms of breast and prostate cancer, ii) mimotope peptides of the high molecular mass melanoma-associated antigen and iii) mimotope peptides of the EGFR, which is also overexpressed in multiple tumors and especially in epithelial cancer [39,152-154].

When tested in animal models, a number of conjugate vaccines directed against cancer antigens have been shown to elicit specific antibodies that recognize the native structures on cancer tissues and, in some cases, induced protection in tumor mouse models or complement-mediated lysis of cancer cells.

Results of human clinical trials, mostly on terminal ill patients who might have been already immuno-compromised due to previous chemotherapeutic interventions, suggest that experimental anticancer conjugated vaccines are generally immunogenic. However, there is still a long way to go before efficacious vaccines could be claimed. Optimization might include chemical improvement of the constructs and the use of potent adjuvants that can be built in the molecular architecture of the conjugate vaccine or physically mixed as

Table 4. Tumor-associated carbohydrate antigens.

	Structure	Localization
Le ^y	Fuca(1-2)Galβ(1-4)[Fuca(1-3)] GlcNAcβ(1-	Ovary, breast, colon, prostate, NSCLC
sLe ^x	Neu5Acα(2-3)Galβ(1-4)[Fuca(1-3)] GlcNAcβ(1-	
sLe ^a	Neu5Acα(2-3)Galβ(1-3)[Fuca(1-4)] GlcNAcβ(1-	Pancreatic, prostate, colon cancer
KH-1	Fuca(1-2)Galβ(1-4)[Fuca(1-3)] GlcNAcβ(1-3)Galβ(1-4) [Fuca(1-3)]GlcNAcβ(1-	Colon carcinoma cancer
Globo-H	Fuca(1-2)Galβ(1-3)GalNAcβ(1-3) Galα(1-4)Galβ(1-4)Glcβ(1-	Breast, ovary, colon, prostate, lung, small-cell lung cancers
Fucosyl GM ₁	Fuca(1-2)Galβ(1-3)GalNAcβ(1-4) [Neu5Acα(2-3)]Galα(1-4) Galβ(1-	
Tn	GalNAcα-O-Ser/Thr	Breast, ovarian, lung, colon, pancreatic carcinoma cancer
STn	Neu5Acα(2-6)GalNAcα-O-Ser/Thr	
TF	Galα(1-3)GalNAcα-O-Ser/Thr	

Fucosyl GM₁: Fucosylated monosialoganglioside antigen; Globo-H: Also known as MBr-1 antigen; KH-1: Heterodimeric Le^y-Le^x heptasaccharide antigen; Le: Lewis antigen; sLe: Sialyl Lewis antigen; sTn: Sialyl tumor-associated MUC-1 antigen; TF: Thomsen-Friedenreich antigen; Tn: Tumor-associated MUC-1 antigen.

part of the vaccine formulation [37,155]. The current concept is that cancer vaccines might be a promising tool to prevent the growth of small quantities of tumor cells, for example, those residual after a first surgical/chemotherapeutic intervention [156,157]. Nevertheless, investigations on a prophylactic approach might be considered for those categories at hereditary risk to develop cancer during their life [131].

3.2 Alzheimer vaccines

Alzheimer disease (AD) is characterized by progressive loss of cognitive abilities and memory due to pathological changes occurring in the brain. These changes consist of the accumulation in the brain of the amyloid- β peptide (A β), the product of a proteolytic cleavage of the amyloid precursor protein; the subsequent formation of aggregates starts neurodegenerative processes [36]. Preventing the A β aggregates formation and increasing their clearance is the goal of the immunotherapeutic approach against AD. Preclinical studies in AD mouse models and in non-human primates have shown that actively induced anti-A β antibodies can reduce/prevent plaque formation in the brain [158]. A first non-conjugated AD vaccine tested in humans consisted of the synthetic A β amino-terminal region 1 – 42 formulated with the adjuvant QS21. The development of this vaccine was stopped in Phase II trials following serious adverse effects in about 6% of vaccinated individuals who developed an aseptic form of meningoencephalitis. These adverse effects were attributed to the induction of anti-A β T_H1 response, possibly due to the use of QS21 adjuvant, and to the presence of both B- and T-cell epitopes in the 1 – 42 sequence [36]. In order to direct the response only toward the A β B-cell epitope, which has been identified within the first 15 aa, new improved generations of conjugated AD vaccine consisting of short amino-terminal A β sequences conjugated to protein carriers such as the virus-like particle Q β and a DT molecule are currently in clinical trials [109,159]. Some of the issues related to the construction of an effective anti-Alzheimer conjugate vaccine are similar to those for cancer vaccines, that is, the haptens against which antibodies should be generated have the characteristic of a self antigen and, therefore, the induction of high and persistent antibody titers might be difficult.

3.3 Conjugate vaccines for drug abuse

The immunotherapeutic approach is being considered as a treatment for drug addiction [160]. The goal of this approach is to form antibody-hapten complexes that would prevent the drug or its active metabolite to cross the blood-brain barrier. This would avoid the interaction with receptors and the occurrence of the psychological effects characteristics of drugs consumption, therefore, reducing or abolishing drug addiction. Drugs of abuse are small molecules and need to be conjugated to a protein carrier that provides CD4⁺ T-cell epitopes. Accordingly, a number of conjugates of nicotine, cocaine and heroin molecules, or their derivatives, with protein carriers are currently being studied [161,162]. One of the key issues in drug addiction vaccine development is the

ability to induce high and persistent titers of high affinity antibodies that could sequester the drug in the blood stream. Consequently, the antibody response of a conjugate vaccine has to be driven towards the hapten while response to the carrier and conjugation linker should be kept as low as possible. Meijler *et al.* showed that conjugates with conformational constrained derivatives of nicotine induce higher titers of high affinity and specific antibodies as compared to the non-constrained molecule [163].

4. Expert opinion

Thanks to glycoconjugate vaccines against *H. influenzae* type b, *S. pneumoniae* and *N. meningitidis* group C, the incidence of diseases caused by these pathogens has been dramatically reduced in those countries where vaccination has been implemented [164-166]. Several technologies have been developed to make glycoconjugate vaccines, giving rise to products with different molecular architecture. Although in all cases safe and effective vaccines have been obtained, we believe that whenever possible the conjugation chemistry should involve preferentially the endgroups of the carbohydrate moieties in such a way to preserve the antigenicity of the saccharide hapten. More defined conjugation chemistry allows also better characterization of conjugate vaccines by means of physicochemical techniques thus facilitating the control of manufacturing consistency.

Variables such as length of the saccharide hapten and loading degree onto the carrier protein have been matter of a number of studies. Based on the results obtained so far, the two variables seem to be interconnected and, after having identified the minimal structure containing protective epitopes, the optimal value for both hapten chain length and loading degree has to be identified for each type of conjugate vaccine.

If a spacer is used in the conjugation procedure, attention should be placed to avoid those that have been shown to induce significant levels of antibodies against the protein-hapten junction region.

A variety of protein carriers have been used so far in conjugate vaccines. The more extensively and successfully used in glycoconjugate vaccines for the prevention of bacterial diseases are TT, CRM₁₉₇ and DT. Different manufacturing and formulation processes involved in the production of glycoconjugate vaccines, as well as the concomitant administration of other vaccines, render difficult a precise comparison of these carriers in terms of their potency in inducing anti-carbohydrate responses. In the case of AD, cancer and drug addiction, the current experimental therapeutic vaccines are making large use of protein carriers such as Q β VLP and KLH. In the case of cancer vaccines, synthetic peptides are increasingly used as T-cell epitope providers, with the aim of avoiding anti-carrier antibody responses which could jeopardize the response against the tumor antigen. In our opinion, preferable features for a protein carrier are a high level of safety and purity, the possibility to be fully characterized by

physicochemical methods and the ability to induce a strong T-cell help in conjunction with a relatively low antibody response to carrier-specific B-cell epitopes. Progress in carbohydrate chemistry gives now access to pure and well-defined oligosaccharides with built-in chemical functionalities for coupling to carrier protein. This would allow the development of extremely well defined conjugate vaccines. The synthetic approach is at the basis of the conjugate vaccines against cancer and we believe that future generations of carbohydrate-protein conjugate vaccines can increasingly make use of fully synthetic oligosaccharides, although additional efforts are still required to make this technology broadly applicable and scalable.

More research efforts are needed to develop fully synthetic conjugate vaccines where also the carrier moiety is made synthetically and represents promiscuous T-cell peptides.

Based on the experience on licensed Hib, pneumococcal and meningococcal vaccines, the characterization of conjugate vaccines is generally focused on the determination of the purity, integrity and molecular architecture of the conjugates. It requires also testing of all the starting materials involved in the conjugation process: carrier protein, hapten (independently if it is of carbohydrate, peptidic or small organic molecule nature) and derivatized intermediates. This type of testing can be achieved with physicochemical methods that can provide a profound characterization especially if the conjugate vaccine has been prepared with methods that result in well-defined products. During the development of conjugate vaccines, immunogenicity studies in animal models are useful to assess the acquisition of TD character by the hapten. However, in our opinion, routine batch release should be essentially based on physicochemical tests.

On the wave of the successful vaccines against pathogenic encapsulated bacteria, the conjugation technology is being extended to vaccines against non-infectious diseases. Although the technological approach is similar, this last category of vaccines presents different issues. A significant issue in cancer vaccines is represented by the self character of TACAs and the heterogeneity of expression during the different phase of tumor growth. Also, in the case of AD the self nature of the

target antigen might render the induction of high and persistent level of IgG antibodies difficult. As to vaccines for drug addictions the requirement is a high and persistent level of circulating antibodies that can block the drug in the blood stream. Despite the challenges posed by these last categories of vaccines and the likely considerable additional development time, we think that the conjugate vaccine approach together with other therapeutic measures have the potential to ameliorate the life conditions and expectation of patients and that continuous research efforts in this field should be granted.

In summary, the rational design of glycoconjugate vaccines has to consider a number of variables including conjugation chemistry, saccharide chain length, carbohydrate loading onto the protein, nature of spacer and type of carrier protein. In order to achieve optimal immunogenicity and better characterization of the conjugates we believe that conjugation chemistry should be based on minimal modification of the saccharide chain and possibly endgroup-specific chemistry. In the case of the other variables, our opinion is that they have to be optimized on a case by case basis. Processes standardization and consistency should be assured by careful physicochemical characterization of the conjugates and control of the key variables discussed in this review.

As far as cancer vaccines based on a hapten-carrier model are concerned, we believe that optimal constructs capable of eliciting a protective humoral and cellular response in humans, in conjunction with optimal adjuvants, have to be further developed.

Acknowledgements

The authors thank S Bertholet and G Del Giudice for discussion and G Corsi for artwork.

Declaration of interest

All of the authors are employees of Novartis Vaccines, Italy. The authors have received no payment in preparation of this manuscript.

Bibliography

Papers of special note have been highlighted as either of interest (●) or of considerable interest (●●) to readers.

- Avery OT, Goebel WF. Chemo-immunological studies on conjugated carbohydrate-proteins : ii. immunological specificity of synthetic sugar-protein antigens. *J Exp Med* 1929;50(4):533-50
- Finland M, Sutliff WD. Specific antibody response of human subjects to intracutaneous injection of pneumococcus products. *J Exp Med* 1932;55(6):853-65
- Macleod CM, Hodges RG, Heidelberger M, Bernhard WG. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. *J Exp Med* 1945;6(6):445-65
- Peltola H, Makela H, Kayhty H, et al. Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age. *N Engl J Med* 1977;297(13):686-91
- Makela PH, Peltola H, Kayhty H, et al. Polysaccharide vaccines of group A *Neisseria meningitidis* and *Haemophilus influenzae* type b: a field trial in Finland. *J Infect Dis* 1977;136(Suppl):S43-50
- Artenstein MS, Gold R, Zimmerly JG, et al. Prevention of meningococcal disease by group C polysaccharide vaccine. *N Engl J Med* 1970;282(8):417-20
- Gold R, Artenstein MS. Meningococcal infections. 2. Field trial of group C meningococcal polysaccharide vaccine in 1969 - 70. *Bull World Health Organ* 1971;45(3):279-82
- Peltola H, Kayhty H, Sivenon A, Makela H. *Haemophilus influenzae* type b capsular polysaccharide vaccine in children: a double-blind field study of 100,000 vaccinees 3 months to 5 years of age in Finland. *Pediatrics* 1977;60(5):730-7
- Schneerson R, Barrera O, Sutton A, Robbins JB. Preparation, characterization, and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. *J Exp Med* 1980;152(2):361-76
- Eskola J, Peltola H, Takala AK, et al. Efficacy of *Haemophilus influenzae* type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy. *N Engl J Med* 1987;317(12):717-22
- Black SB, Shinefield HR, Fireman B, et al. Efficacy in infancy of oligosaccharide conjugate *Haemophilus influenzae* type b (HbOC) vaccine in a United States population of 61,080 children. The Northern California Kaiser Permanente Vaccine Study Center Pediatrics Group. *Pediatr Infect Dis J* 1991;10(2):97-104
- Anderson PW, Pichichero ME, Insel RA, et al. Vaccines consisting of periodate-cleaved oligosaccharides from the capsule of *Haemophilus influenzae* type b coupled to a protein carrier: structural and temporal requirements for priming in the human infant. *J Immunol* 1986;137(4):1181-6
- Costantino P, Viti S, Podda A, et al. Development and phase 1 clinical testing of a conjugate vaccine against meningococcus A and C. *Vaccine* 1992;10(10):691-8
- Eby R. Pneumococcal conjugate vaccines. *Pharm Biotechnol* 1995;6:695-718
- Ravenscroft N, Averani G, Bartoloni A, et al. Size determination of bacterial capsular oligosaccharides used to prepare conjugate vaccines. *Vaccine* 1999;17(22):2802-16
- Costantino P, Norelli F, Giannozzi A, et al. Size fractionation of bacterial capsular polysaccharides for their use in conjugate vaccines. *Vaccine* 1999;17(9-10):1251-63
- Lakshman R, Finn A. Meningococcal serogroup C conjugate vaccine. *Expert Opin Biol Ther* 2002;2(1):87-96
- Paoletti LC, Kasper DL. Glycoconjugate vaccines to prevent group B streptococcal infections. *Expert Opin Biol Ther* 2003;3(6):975-84
- Snape MD, Perrett KP, Ford KJ, et al. Immunogenicity of a tetravalent meningococcal glycoconjugate vaccine in infants: a randomized controlled trial. *JAMA* 2008;299(2):173-84
- Bardotti A, Averani G, Berti F, et al. Physicochemical characterisation of glycoconjugate vaccines for prevention of meningococcal diseases. *Vaccine* 2008;26(18):2284-96
- Broker M, Dull PM, Rappuoli R, Costantino P. Chemistry of a new investigational quadrivalent meningococcal conjugate vaccine that is immunogenic at all ages. *Vaccine* 2009;27(41):5574-80
- Black S, Klein NP, Shah J, et al. Immunogenicity and tolerability of a quadrivalent meningococcal glycoconjugate vaccine in children 2-10 years of age. *Vaccine* 2010;28(3):657-63
- Taylor CM, Roberts IS. Capsular polysaccharides and their role in virulence. *Contrib Microbiol* 2005;12:55-66
- Ravenscroft N. Saccharide based vaccines. *AIM Mag* 2001;55(1):40-6
- Dintzis RZ. Rational design of conjugate vaccines. *Pediatr Res* 1992;32(4):376-85
- Anderson PW, Pichichero ME, Stein EC, et al. Effect of oligosaccharide chain length, exposed terminal group, and hapten loading on the antibody response of human adults and infants to vaccines consisting of *Haemophilus influenzae* type b capsular antigen unterminally coupled to the diphtheria protein CRM197. *J Immunol* 1989;142(7):2464-8
- Laferriere CA, Sood RK, de Muys JM, et al. The synthesis of *Streptococcus pneumoniae* polysaccharide-tetanus toxoid conjugates and the effect of chain length on immunogenicity. *Vaccine* 1997;15(2):179-86
- Laferriere CA, Sood RK, de Muys JM, et al. *Streptococcus pneumoniae* type 14 polysaccharide-conjugate vaccines: length stabilization of opsonophagocytic conformational polysaccharide epitopes. *Infect Immun* 1998;66(6):2441-6
- Michon F, Huang CH, Farley EK, et al. Structure activity studies on group C meningococcal polysaccharide-protein conjugate vaccines: effect of O-acetylation on the nature of the protective epitope. *Dev Biol (Basel)* 2000;103:151-60
- Seppala I, Makela O. Antigenicity of dextran-protein conjugates in mice. Effect of molecular weight of the carbohydrate and comparison of two modes of coupling. *J Immunol* 1989;143(4):1259-64
- Pozsgay V, Chu C, Pannell L, et al. Protein conjugates of synthetic

- saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from *Shigella dysenteriae* type 1. *Proc Natl Acad Sci USA* 1999;96(9):5194-7
- **An important paper which studies the effect on immunogenicity of the variables saccharide chain length and loading degree onto the carrier protein for a conjugate vaccine.**
32. Bromuro C, Romano M, Chiani P, et al. Beta-glucan-CRM197 conjugates as candidates antifungal vaccines. *Vaccine* 2010;28(14):2615-23
33. Schofield L, Hewitt MC, Evans K, et al. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. *Nature* 2002;418(6899):785-9
34. Astronomo RD, Burton DR. Carbohydrate vaccines: developing sweet solutions to sticky situations? *Nat Rev Drug Discov* 2010;9(4):308-24
- **An excellent review which describes the evolution of the glycoconjugate approach from the bacterial pathogens to new targets such as fungi, viruses and cancer.**
35. Chackerian B. Virus-like particle based vaccines for Alzheimer disease. *Hum Vaccin* 2010;6(11):926-30
36. Lemere CA, Masliah E. Can Alzheimer disease be prevented by amyloid-beta immunotherapy? *Nat Rev Neurol* 2010;6(2):108-19
- **This review nicely describes the potential of the immunotherapeutic approach against Alzheimer disease.**
37. Buskas T, Thompson P, Boons GJ. Immunotherapy for cancer: synthetic carbohydrate-based vaccines. *Chem Commun (Camb)* 2009;36(36):5335-49
- **An important review which describes different recent approaches and challenges associated with the development of synthetic carbohydrate based cancer vaccines.**
38. Zhu J, Warren JD, Danishefsky SJ. Synthetic carbohydrate-based anticancer vaccines: the Memorial Sloan-Kettering experience. *Expert Rev Vaccines* 2009;8(10):1399-413
- **An excellent overview of all the steps taken by this pioneer research team towards the structural optimization of synthetic cancer vaccines.**
39. Wagner S, Jasinska J, Breiteneder H, et al. Delayed tumor onset and reduced tumor growth progression after immunization with a Her-2/neu multi-peptide vaccine and IL-12 in c-neu transgenic mice. *Breast Cancer Res Treat* 2007;106(1):29-38
40. Krug LM, Ragupathi G, Ng KK, et al. Vaccination of small cell lung cancer patients with polysialic acid or N-propionylated polysialic acid conjugated to keyhole limpet hemocyanin. *Clin Cancer Res* 2004;10(3):916-23
41. Do TH, Chen Y, Nguyen VT, Phisitkul S. Vaccines in the management of hypertension. *Expert Opin Biol Ther* 2010;10(7):1077-87
42. Lai Z, Schreiber JR. Antigen processing of glycoconjugate vaccines; the polysaccharide portion of the pneumococcal CRM(197) conjugate vaccine co-localizes with MHC II on the antigen processing cell surface. *Vaccine* 2009;27(24):3137-44
43. Avci FY, Kasper DL. How bacterial carbohydrates influence the adaptive immune system. *Annu Rev Immunol* 2010;28:107-30
44. Pollard AJ, Perrett KP, Beverley PC. Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. *Nat Rev Immunol* 2009;9(3):213-20
- **An excellent review which describes the immune response to polysaccharides and glycoconjugates as well as challenges associated with maintaining antibody persistence.**
45. Ada G, Isaacs D. Carbohydrate-protein conjugate vaccines. *Clin Microbiol Infect* 2003;9(2):79-85
46. Kelly DF, Pollard AJ, Moxon ER. Immunological memory: the role of B cells in long-term protection against invasive bacterial pathogens. *JAMA* 2005;294(23):3019-23
47. Szymanski CM, Yao R, Ewing CP, et al. Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. *Mol Microbiol* 1999;32(5):1022-30
48. Kelly J, Jarrell H, Millar L, et al. Biosynthesis of the N-linked glycan in *Campylobacter jejuni* and addition onto protein through block transfer. *J Bacteriol* 2006;188(7):2427-34
49. Feldman MF, Wacker M, Hernandez M, et al. Engineering N-linked protein glycosylation with diverse O antigen lipopolysaccharide structures in *Escherichia coli*. *Proc Natl Acad Sci USA* 2005;102(8):3016-21
50. Ihssen J, Kowarik M, Dilettoso S, et al. Production of glycoprotein vaccines in *Escherichia coli*. *Microb Cell Fact* 2010;9:61
51. Chu C, Schneerson R, Robbins JB, Rastogi SC. Further studies on the immunogenicity of *Haemophilus influenzae* type b and pneumococcal type 6A polysaccharide-protein conjugates. *Infect Immun* 1983;40(1):245-56
52. Zou W, Jennings HJ. Preparation of glycoconjugate vaccines. In *Carbohydrate-Based Vaccines and Immunotherapies 2009*, Eds Zhongwu Guo and Geert-Jan Boons. John Wiley & Sons, Inc; Hoboken, New Jersey; 2009. p. 55-88
- **A book chapter which describes the preparation methods of glycoconjugate vaccines used by different manufacturers.**
53. Marburg S, Jorn D, Tolman RL, et al. Bimolecular chemistry of macromolecules: synthesis of bacterial polysaccharide conjugates with *Neisseria meningitidis* membrane protein. *J Am Chem Soc* 1986;108(17):5282-7
54. Giannini G, Rappuoli R, Ratti G. The amino-acid sequence of two non-toxic mutants of diphtheria toxin: CRM45 and CRM197. *Nucleic Acids Res* 1984;12(10):4063-9
55. Verez-Bencomo V, Fernandez-Santana V, Hardy E, et al. A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type b. *Science* 2004;305(5683):522-5
56. Matjila MJ, Phohu TC, Banzhoff A, et al. Safety and immunogenicity of two *Haemophilus influenzae* type b conjugate vaccines. *S Afr Med J* 2004;94(1):43-6
57. Prymula R, Schuerman L. 10-valent pneumococcal nontypeable *Haemophilus influenzae* PD conjugate vaccine: Synflorix. *Expert Rev Vaccines* 2009;8(11):1479-500
58. Finne J, Bitter-Suermann D, Goridis C, Finne U. An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural

- and extraneural tissues. *J Immunol* 1987;138(12):4402-7
59. Bruge J, Bouveret-Le Cam N, Danve B, et al. Clinical evaluation of a group B meningococcal N-propionylated polysaccharide conjugate vaccine in adult, male volunteers. *Vaccine* 2004;22(9-10):1087-96
 60. Cox AD, St Michael F, Neelamegan D, et al. Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: chemical strategies to prepare glycoconjugates with good carbohydrate loading. *Glycoconj J* 2010;27(4):401-17
 61. Wittrock S, Becker T, Kunz H. Synthetic vaccines of tumor-associated glycopeptide antigens by immune-compatible thioether linkage to bovine serum albumin. *Angew Chem Int Ed Engl* 2007;46(27):5226-30
 62. Fattom A, Schneerson R, Watson DC, et al. Laboratory and clinical evaluation of conjugate vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides bound to *Pseudomonas aeruginosa* recombinant exoprotein A. *Infect Immun* 1993;61(3):1023-32
 63. Floyd N, Vijayakrishnan B, Koeppe JR, Davis BG. Thiol glycosylation of olefinic proteins: S-linked glycoconjugate synthesis. *Angew Chem Int Ed Engl* 2009;48(42):7798-802
 64. Robbins JB, Kubler-Kielb J, Vinogradov E, et al. Synthesis, characterization, and immunogenicity in mice of *Shigella sonnei* O-specific oligosaccharide-core-protein conjugates. *Proc Natl Acad Sci USA* 2009;106(19):7974-8
 65. Wan Q, Chen J, Chen G, Danishefsky SJ. A potentially valuable advance in the synthesis of carbohydrate-based anticancer vaccines through extended cycloaddition chemistry. *J Org Chem* 2006;71(21):8244-9
 66. Peeters CC, Evenberg D, Hoogerhout P, et al. Synthetic trimer and tetramer of 3-beta-D-ribose-(1-1)-D-ribitol-5-phosphate conjugated to protein induce antibody responses to *Haemophilus influenzae* type b capsular polysaccharide in mice and monkeys. *Infect Immun* 1992;60(5):1826-33
 67. Benaissa-Trouw B, Lefeber DJ, Kamerling JP, et al. Synthetic polysaccharide type 3-related di-, tri-, and tetrasaccharide-CRM(197) conjugates induce protection against *Streptococcus pneumoniae* type 3 in mice. *Infect Immun* 2001;69(7):4698-701
 68. Phalipon A, Tanguy M, Grandjean C, et al. A synthetic carbohydrate-protein conjugate vaccine candidate against *Shigella flexneri* 2a infection. *J Immunol* 2009;182(4):2241-7
 - **A good example of optimization of glycoconjugate vaccine parameters such as saccharide chain length, carbohydrate:protein ratio and spacer.**
 69. Wessels MR, Paoletti LC, Guttormsen HK, et al. Structural properties of group B streptococcal type III polysaccharide conjugate vaccines that influence immunogenicity and efficacy. *Infect Immun* 1998;66(5):2186-92
 70. Peeters JM, Hazendonk TG, Beuvery EC, Tesser GI. Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates. *J Immunol Methods* 1989;120(1):133-43
 71. Buskas T, Li Y, Boons GJ. The immunogenicity of the tumor-associated antigen Lewis(y) may be suppressed by a bifunctional cross-linker required for coupling to a carrier protein. *Chemistry (Easton)* 2004;10(14):3517-24
 72. Bartoloni A, Norelli F, Ceccarini C, et al. Immunogenicity of meningococcal B polysaccharide conjugated to tetanus toxoid or CRM197 via adipic acid dihydrazide. *Vaccine* 1995;13(5):463-70
 73. Cox AD, St Michael F, Neelamegan D, et al. Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: immunology of glycoconjugates with high carbohydrate loading. *Glycoconj J* 2010;27(7-9):643-8
 74. Lipinski T, Luu T, Kitov PI, et al. A structurally diversified linker enhances the immune response to a small carbohydrate hapten. *Glycoconj J* 2011;28:149-64
 75. Poolman J, Frasch C, Nurkka A, et al. Impact of the conjugation method on the immunogenicity of *Streptococcus pneumoniae* 19F polysaccharide in conjugate vaccines. *Clin Vaccine Immunol* 2010;18(2):327-36
 76. Berry DS, Lynn F, Lee CH, et al. Effect of O acetylation of *Neisseria meningitidis* serogroup A capsular polysaccharide on development of functional immune responses. *Infect Immun* 2002;70(7):3707-13
 77. Gudlavalleti SK, Lee CH, Norris SE, et al. Comparison of *Neisseria meningitidis* serogroup W135 polysaccharide-tetanus toxoid conjugate vaccines made by periodate activation of O-acetylated, non-O-acetylated and chemically de-O-acetylated polysaccharide. *Vaccine* 2007;25(46):7972-80
 78. Fusco PC, Farley EK, Huang CH, et al. Protective meningococcal capsular polysaccharide epitopes and the role of O acetylation. *Clin Vaccine Immunol* 2007;14(5):577-84
 79. Pace D. Quadrivalent meningococcal ACYW-135 glycoconjugate vaccine for broader protection from infancy. *Expert Rev Vaccines* 2009;8(5):529-42
 80. Ruggeberg JU, Pollard AJ. Meningococcal vaccines. *Paediatr Drugs* 2004;6(4):251-66
 81. Rennels M, King J Jr, Ryall R, et al. Dosage escalation, safety and immunogenicity study of four dosages of a tetravalent meningococcal polysaccharide diphtheria toxoid conjugate vaccine in infants. *Pediatr Infect Dis J* 2004;23(5):429-35
 82. Hwang KW. *Haemophilus influenzae* type b (Hib) vaccine and its carrier proteins. *Arch Pharm Res* 2010;33(6):793-5
 83. Ostergaard L, Lebacqz E, Poolman J, et al. Immunogenicity, reactogenicity and persistence of meningococcal A, C, W-135 and Y-tetanus toxoid candidate conjugate (MenACWY-TT) vaccine formulations in adolescents aged 15-25 years. *Vaccine* 2009;27(1):161-8
 84. Kabanova A, Margarit I, Berti F, et al. Evaluation of a Group A *Streptococcus* synthetic oligosaccharide as vaccine candidate. *Vaccine* 2010;29(1):104-14
 85. Micoli F, Rondini S, Pisoni I, et al. Vi-CRM 197 as a new conjugate vaccine against *Salmonella* Typhi. *Vaccine* 2011;29(4):712-20

86. Donnelly JJ, Deck RR, Liu MA. Immunogenicity of a Haemophilus influenzae polysaccharide-Neisseria meningitidis outer membrane protein complex conjugate vaccine. *J Immunol* 1990;145(9):3071-9
87. Forsgren A, Riesbeck K, Janson H. Protein D of Haemophilus influenzae: a protective nontypeable H. influenzae antigen and a carrier for pneumococcal conjugate vaccines. *Clin Infect Dis* 2008;46(5):726-31
88. Szu SC, Stone AL, Robbins JD, et al. Vi capsular polysaccharide-protein conjugates for prevention of typhoid fever. Preparation, characterization, and immunogenicity in laboratory animals. *J Exp Med* 1987;166(5):1510-24
89. Decker MD, Edwards KM, Bradley R, Palmer P. Comparative trial in infants of four conjugate Haemophilus influenzae type b vaccines. *J Pediatr* 1992;120(2 Pt 1):184-9
90. Granoff DM, Anderson EL, Osterholm MT, et al. Differences in the immunogenicity of three Haemophilus influenzae type b conjugate vaccines in infants. *J Pediatr* 1992;121(2):187-94
91. Halperin SA, Gupta A, Jeanfreau R, et al. Comparison of the safety and immunogenicity of an investigational and a licensed quadrivalent meningococcal conjugate vaccine in children 2-10 years of age. *Vaccine* 2010;28(50):7865-72
92. Southern J, Borrow R, Andrews N, et al. Immunogenicity of a reduced schedule of meningococcal group C conjugate vaccine given concomitantly with the Prevenar and Pediacel vaccines in healthy infants in the United Kingdom. *Clin Vaccine Immunol* 2009;16(2):194-9
93. Jackson LA, Baxter R, Reisinger K, et al. Phase III comparison of an investigational quadrivalent meningococcal conjugate vaccine with the licensed meningococcal ACWY conjugate vaccine in adolescents. *Clin Infect Dis* 2009;49(1):e1-10
94. Dagan R, Poolman J, Siegrist CA. Glycoconjugate vaccines and immune interference: a review. *Vaccine* 2010;28(34):5513-23
95. Pollabauer EM, Petermann R, Ehrlich HJ. The influence of carrier protein on the immunogenicity of simultaneously administered conjugate vaccines in infants. *Vaccine* 2009;27(11):1674-9
96. Usonis V, Bakasenas V, Lockhart S, et al. A clinical trial examining the effect of increased total CRM(197) carrier protein dose on the antibody response to Haemophilus influenzae type b CRM(197) conjugate vaccine. *Vaccine* 2008;26(35):4602-7
97. Shinefield HR. Overview of the development and current use of CRM(197) conjugate vaccines for pediatric use. *Vaccine* 2010;28(27):4335-9
98. Bar-Zeev N, Buttery JP. Combination conjugate vaccines. *Expert Opin Drug Saf* 2006;5(3):351-60
99. Fattom A, Cho YH, Chu C, et al. Epitopic overload at the site of injection may result in suppression of the immune response to combined capsular polysaccharide conjugate vaccines. *Vaccine* 1999;17(2):126-33
100. Knuf M, Szenborn L, Moro M, et al. Immunogenicity of routinely used childhood vaccines when coadministered with the 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV). *Pediatr Infect Dis J* 2009;28(4 Suppl):S97-108
101. Esposito S, Tansey S, Thompson A, et al. Safety and immunogenicity of a 13-valent pneumococcal conjugate vaccine compared to those of a 7-valent pneumococcal conjugate vaccine given as a three-dose series with routine vaccines in healthy infants and toddlers. *Clin Vaccine Immunol* 2010;17(6):1017-26
102. Dinleyici EC, Yargic ZA. Current knowledge regarding the investigational 13-valent pneumococcal conjugate vaccine. *Expert Rev Vaccines* 2009;8(8):977-86
103. Dinleyici EC, Yargic ZA. Pneumococcal conjugated vaccine: PHiD-CV. *Expert Rev Anti Infect Ther* 2009;7(9):1063-74
104. Gasparini R, Conversano M, Bona G, et al. Randomized trial on the safety, tolerability, and immunogenicity of MenACWY-CRM, an investigational quadrivalent meningococcal glycoconjugate vaccine, administered concomitantly with a combined tetanus, reduced diphtheria, and acellular pertussis vaccine in adolescents and young adults. *Clin Vaccine Immunol* 2010;17(4):537-44
105. Knuf M, Kowalzik F, Kieninger D. Comparative effects of carrier proteins on vaccine-induced immune response. *Vaccine* 2011;29:4881-90
106. Falugi F, Petracca R, Mariani M, et al. Rationally designed strings of promiscuous CD4(+) T cell epitopes provide help to Haemophilus influenzae type b oligosaccharide: a model for new conjugate vaccines. *Eur J Immunol* 2001;31(12):3816-24
107. Baraldo K, Mori E, Bartoloni A, et al. Combined conjugate vaccines: enhanced immunogenicity with the N19 polyepitope as a carrier protein. *Infect Immun* 2005;73(9):5835-41
108. Danishefsky SJ, Allen JR. From the laboratory to the clinic: a retrospective on fully synthetic carbohydrate-based anticancer vaccines. *Angew Chem Int Ed Engl* 2000;39(5):836-63
109. Bengt GW, Minthon L, Floesser A, et al. Results of the first-in-man study with the active Abeta Immunotherapy CAD106 in Alzheimer patients. *Alzheimer's & dementia: the journal of the Alzheimer's Association* 2009;5(4):P113-14
110. Snippe H, Jansen WT, Kamerling JP, Lefeber DJ. Towards a synthetic pneumococcal vaccine: synthetic oligosaccharides as tools for improving the specificity of enzyme-linked immunosorbent assays. *Clin Diagn Lab Immunol* 2000;7(2):325
111. Jansen WT, Hogenboom S, Thijssen MJ, et al. Synthetic 6B di-, tri-, and tetrasaccharide-protein conjugates contain pneumococcal type 6A and 6B common and 6B-specific epitopes that elicit protective antibodies in mice. *Infect Immun* 2001;69(2):787-93
112. Safari D, Dekker HA, Joosten JA, et al. Identification of the smallest structure capable of evoking opsonophagocytic antibodies against Streptococcus pneumoniae type 14. *Infect Immun* 2008;76(10):4615-23
113. Kubler-Kielb J, Vinogradov E, Mocca C, et al. Immunochemical studies of Shigella flexneri 2a and 6, and Shigella dysenteriae type 1 O-specific polysaccharide-core fragments and their

- protein conjugates as vaccine candidates. *Carbohydr Res* 2010;345(11):1600-8
114. Pozsgay V. Recent developments in synthetic oligosaccharide-based bacterial vaccines. *Curr Top Med Chem* 2008;8(2):126-40
 - **This reference highlights how synthetic advances made possible chemical assembly of a number of carbohydrate antigens and how studies on the respective conjugates can help in understanding the requirements for optimal immunogenicity.**
 115. Wu X, Lipinski T, Paszkiewicz E, Bundle DR. Synthesis and immunochemical characterization of S-linked glycoconjugate vaccines against *Candida albicans*. *Chemistry (Easton)* 2008;14(21):6474-82
 116. Slattegard R, Teodorovic P, Kinfe HH, et al. Synthesis of structures corresponding to the capsular polysaccharide of *Neisseria meningitidis* group A. *Org Biomol Chem* 2005;3(20):3782-7
 117. Nakouzi A, Zhang T, Oscarson S, Casadevall A. The common *Cryptococcus neoformans* glucuronoxylomannan M2 motif elicits non-protective antibodies. *Vaccine* 2009;27(27):3513-18
 118. Gening ML, Maira-Litran T, Kropec A, et al. Synthetic {beta}-(1-> 6)-linked N-acetylated and nonacetylated oligoglucosamines used to produce conjugate vaccines for bacterial pathogens. *Infect Immun* 2010;78(2):764-72
 119. Kabanova A, Margarit I, Berti F, et al. Evaluation of a Group A *Streptococcus* synthetic oligosaccharide as vaccine candidate. *Vaccine* 2010;29(1):104-14
 120. Seeberger PH. Automated oligosaccharide synthesis. *Chem Soc Rev* 2008;37:19-28
 - **An important review describing the status of the art of automated oligosaccharide synthesis, key issues and examples of complex oligosaccharides that can be prepared with this technology.**
 121. Jaipuri FA, Pohl NL. Toward solution-phase automated iterative synthesis: fluororous-tag assisted solution-phase synthesis of linear and branched mannose oligomers. *Org Biomol Chem* 2008;6(15):2686-91
 122. Xin H, Dziadek S, Bundle DR, Cutler JE. Synthetic glycopeptide vaccines combining beta-mannan and peptide epitopes induce protection against candidiasis. *Proc Natl Acad Sci USA* 2008;105(36):13526-31
 - **An example of a completely synthetic glycoconjugate vaccine that can induce a protective immunity against a fungal disease.**
 123. Chong P, Chan N, Kandil A, et al. A strategy for rational design of fully synthetic glycopeptide conjugate vaccines. *Infect Immun* 1997;65(12):4918-25
 124. Alexander J, del Guercio MF, Maewal A, et al. Linear PADRE T helper epitope and carbohydrate B cell epitope conjugates induce specific high titer IgG antibody responses. *J Immunol* 2000;164(3):1625-33
 125. Ravenscroft N, D'Ascenzi S, Proietti D, et al. Physicochemical characterisation of the oligosaccharide component of vaccines. *Dev Biol (Basel)* 2000;103:35-47
 126. Ravenscroft N, Wheeler JX, Jones C. Bioanalysis of meningococcal vaccines. *Bioanalysis* 2010;2(2):343-61
 - **An important review describing the physicochemical methodologies applicable to characterization and control of glycoconjugate vaccines.**
 127. Jones C. NMR assays for carbohydrate-based vaccines. *J Pharm Biomed Anal* 2005;38(5):840-50
 - **An important review which describes how NMR can be applied to the characterization and quality control of carbohydrate-based vaccines and process intermediates.**
 128. Suker J, Feavers IM, Corbel MJ, et al. Control and lot release of meningococcal group C conjugate vaccines. *Expert Rev Vaccines* 2004;3(5):533-40
 129. Egan W, Frasch CE, Anthony BF. Lot-release criteria, postlicensure quality control, and the *Haemophilus influenzae* type b conjugate vaccines. *JAMA* 1995;273(11):888-9
 130. Jones C. Vaccines based on the cell surface carbohydrates of pathogenic bacteria. *An Acad Bras Cienc* 2005;77(2):293-324
 - **An excellent review on glycoconjugate vaccines which discusses the issues associated with their preparation, characterization and the important variables for their immunogenicity.**
 131. Finn OJ. Cancer vaccines: between the idea and the reality. *Nat Rev Immunol* 2003;3(8):630-41
 - **An excellent review describing the immune response to tumors, their ability to evade the immune system and the challenges facing the development of cancer vaccines.**
 132. Springer GF. T and Tn, general carcinoma autoantigens. *Science* 1984;224(4654):1198-206
 133. Sakamoto J, Furukawa K, Cordon-Cardo C, et al. Expression of Lewis, Lewisb, X, and Y blood group antigens in human colonic tumors and normal tissue and in human tumor-derived cell lines. *Cancer Res* 1986;46(3):1553-61
 134. Lloyd KO. The chemistry and immunochemistry of blood group A, B, H, and Lewis antigens: past, present and future. *Glycoconj J* 2000;17(7-9):531-41
 135. Hakomori S. Antigen structure and genetic basis of histo-blood groups A, B and O: their changes associated with human cancer. *Biochim Biophys Acta* 1999;1473(1):247-66
 136. Taylor-Papadimitriou J, Burchell J, Miles DW, Dalziel M. MUC1 and cancer. *Biochim Biophys Acta* 1999;1455(2-3):301-13
 - **An important review which describes the epithelial mucine MUC1, its glycosylation changes in cancer and discusses implications in immunotherapy.**
 137. Hanisch FG. O-glycosylation of the mucin type. *Biol Chem* 2001;382(2):143-9
 138. Hakomori S. Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. *Adv Exp Med Biol* 2001;491:369-402
 - **An excellent review which describes the various tumor associated antigens, their structure and localization in different cancer tissues.**
 139. Kunz H, Birnbach S. Synthesis of O-Glycopeptides of the Tumor-Associated TN- and T-antigen type and their binding to bovine serum albumin. *Angew Chem Int Ed Engl* 1986;25(4):360-2
 140. Zhu J, Wan Q, Lee D, et al. From synthesis to biologics: preclinical data on a chemistry derived anticancer

- vaccine. *J Am Chem Soc* 2009;131(26):9298-303
141. Krug LM, Ragupathi G, Hood C, et al. Vaccination of patients with small-cell lung cancer with synthetic fucosyl GM-1 conjugated to keyhole limpet hemocyanin. *Clin Cancer Res* 2004;10(18 Pt 1):6094-100
142. Kudryashov V, Glunz PW, Williams LJ, et al. Toward optimized carbohydrate-based anticancer vaccines: epitope clustering, carrier structure, and adjuvant all influence antibody responses to Lewis(y) conjugates in mice. *Proc Natl Acad Sci USA* 2001;98(6):3264-9
143. Kaiser A, Gaidzik N, Westerlind U, et al. A synthetic vaccine consisting of a tumor-associated sialyl-T(N)-MUC1 tandem-repeat glycopeptide and tetanus toxoid: induction of a strong and highly selective immune response. *Angew Chem Int Ed Engl* 2009;48(41):7551-5
144. Hoffmann-Roder A, Kaiser A, Wagner S, et al. Synthetic antitumor vaccines from tetanus toxoid conjugates of MUC1 glycopeptides with the Thomsen-Friedenreich antigen and a fluorine-substituted analogue. *Angew Chem Int Ed Engl* 2010;49(45):8498-503
145. Sorensen AL, Reis CA, Tarp MA, et al. Chemoenzymatically synthesized multimeric Tn/STn MUC1 glycopeptides elicit cancer-specific anti-MUC1 antibody responses and override tolerance. *Glycobiology* 2006;16(2):96-107
146. Li Q, Rodriguez LG, Farnsworth DF, Gildersleeve JC. Effects of hapten density on the induced antibody repertoire. *ChemBioChem* 2010;11(12):1686-91
147. Ingale S, Wolfert MA, Gaekwad J, et al. Robust immune responses elicited by a fully synthetic three-component vaccine. *Nat Chem Biol* 2007;3(10):663-7
- **An interesting example of a fully synthetic approach to cancer vaccines which combines in the same construct a glycopeptide antigen, a T-cell epitope and a synthetic adjuvant.**
148. Finn OJ. Cancer immunology. *N Engl J Med* 2008;358(25):2704-15
149. Apostolopoulos V, Yuriev E, Ramsland PA, et al. A glycopeptide in complex with MHC class I uses the GalNAc residue as an anchor. *Proc Natl Acad Sci USA* 2003;100(25):15029-34
150. Xu Y, Gendler SJ, Franco A. Designer glycopeptides for cytotoxic T cell-based elimination of carcinomas. *J Exp Med* 2004;199(5):707-16
151. Renaudet O, BenMohamed L, Dasgupta G, et al. Towards a self-adjuvanting multivalent B and T cell epitope containing synthetic glycolipopeptide cancer vaccine. *ChemMedChem* 2008;3(5):737-41
- **An interesting fully synthetic approach to cancer vaccine which includes multivalence of B-cell epitope, humoral and cellular response as well as synthetic adjuvant.**
152. Wiedermann U, Wiltshcke C, Jasinska J, et al. A virosomal formulated Her-2/neu multi-peptide vaccine induces Her-2/neu-specific immune responses in patients with metastatic breast cancer: a phase I study. *Breast Cancer Res Treat* 2010;119(3):673-83
153. Riemer AB, Kurz H, Klinger M, et al. Vaccination with cetuximab mimotopes and biological properties of induced anti-epidermal growth factor receptor antibodies. *J Natl Cancer Inst* 2005;97(22):1663-70
154. Wagner S, Krepler C, Allwardt D, et al. Reduction of human melanoma tumor growth in severe combined immunodeficient mice by passive transfer of antibodies induced by a high molecular weight melanoma-associated antigen mimotope vaccine. *Clin Cancer Res* 2008;14(24):8178-83
155. Helling F, Zhang S, Shang A, et al. GM2-KLH conjugate vaccine: increased immunogenicity in melanoma patients after administration with immunological adjuvant QS-21. *Cancer Res* 1995;55(13):2783-8
156. Buskas T, Thompson P, Boons G-J. Semisynthetic and fully synthetic carbohydrate-based cancer vaccines. In *Carbohydrate-Based Vaccines and Immunotherapies*. Eds Zhongwu Guo and Geert-Jan Boons. John Wiley & Sons, Inc. Hoboken, New Jersey; 2009. p. 263-311
- **A book chapter describing the different models of carbohydrate-based cancer vaccines.**
157. Wandall HH, Tarp MA. Therapeutic cancer vaccines: clinical trials and applications. In *Carbohydrate-Based Vaccines and Immunotherapies*, Eds Zhongwu Guo and Geert-Jan Boons. John Wiley & Sons, Inc. Hoboken, New Jersey; 2009. p. 333-66
- **A book chapter describing the main clinical trials performed with carbohydrate-based cancer candidate vaccines.**
158. Lemere CA, Maier M, Jiang L, et al. Amyloid-beta immunotherapy for the prevention and treatment of Alzheimer disease: lessons from mice, monkeys, and humans. *Rejuvenation Res* 2006;9(1):77-84
159. Tabira T. Immunization therapy for Alzheimer disease: a comprehensive review of active immunization strategies. *Tohoku J Exp Med* 2010;220(2):95-106
160. Moreno AY, Janda KD. Immunopharmacotherapy: vaccination strategies as a treatment for drug abuse and dependence. *Pharmacol Biochem Behav* 2009;92(2):199-205
161. Kinsey BM, Jackson DC, Orson FM. Anti-drug vaccines to treat substance abuse. *Immunol Cell Biol* 2009;87(4):309-14
162. Cerny EH, Cerny T. Vaccines against nicotine. *Hum Vaccin* 2009;5(4):200-5
163. Meijler MM, Matsushita M, Altobelli LJ III, et al. A new strategy for improved nicotine vaccines using conformationally constrained haptens. *J Am Chem Soc* 2003;125(24):7164-5
164. Adams WG, Deaver KA, Cochi SL, et al. Decline of childhood Haemophilus influenzae type b (Hib) disease in the Hib vaccine era. *JAMA* 1993;269(2):221-6
165. Ruggeberg J, Heath PT. Safety and efficacy of meningococcal group C conjugate vaccines. *Expert Opin Drug Saf* 2003;2(1):7-19
166. Black S, Shinefield H, Baxter R, et al. Impact of the use of heptavalent pneumococcal conjugate vaccine on disease epidemiology in children and adults. *Vaccine* 2006;24(Suppl 2):S279-80

Affiliation

Paolo Costantino, Rino Rappuoli[†] & Francesco Berti
[†]Author for correspondence
 Novartis Vaccines,
 Via Fiorentina 1,
 Siena 53100, Italy
 E-mail: rino.rappuoli@novartis.com