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Expert Review of Vaccines

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ierv20

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**To cite this article:** Charlotte Sorieul, Marta Dolce, Maria Rosaria Romano, Jeroen Codée & Roberto Adamo (2023) Glycoconjugate vaccines against antimicrobial resistant pathogens, Expert Review of Vaccines, 22:1, 1055-1078, DOI: 10.1080/14760584.2023.2274955

**To link to this article:** https://doi.org/10.1080/14760584.2023.2274955



Published online: 08 Nov 2023.

EXPERT REVIEW OF VACCINES
2023, VOL. 22, NO. 1, 1055–1078
https://doi.org/10.1080/14760584.2023.2274955

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REVIEW



# Glycoconjugate vaccines against antimicrobial resistant pathogens

Charlotte Sorieul[a], Marta Dolce[b,c], Maria Rosaria Romano[b], Jeroen Codée[a] and Roberto Adamo [b]

[a]Leiden Institute of Chemistry, Leiden University, Leiden, The Netherlands; [b]GSK, Via Fiorentina 1, Siena, Italy; [c]Department of Biotechnology, Chemistry and Pharmacy, University of Siena, Siena, Italy

**ABSTRACT**

**Introduction:** Antimicrobial resistance (AMR) is responsible for the death of millions worldwide and stands as a major threat to our healthcare systems, which are heavily reliant on antibiotics to fight bacterial infections. The development of vaccines against the main pathogens involved is urgently required as prevention remains essential against the rise of AMR.

**Areas covered:** A systematic research review was conducted on MEDLINE database focusing on the six AMR pathogens defined as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Escherichia coli*), which are considered critical or high priority pathogens by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC). The analysis was intersecated with the terms carbohydrate, glycoconjugate, bioconjugate, glyconanoparticle, and multiple presenting antigen system vaccines.

**Expert opinion:** Glycoconjugate vaccines have been successful in preventing meningitis and pneumoniae, and there are high expectations that they will play a key role in fighting AMR. We herein discuss the recent technological, preclinical, and clinical advances, as well as the challenges associated with the development of carbohydrate-based vaccines against leading AMR bacteria, with focus on the ESKAPE pathogens. The need of innovative clinical and regulatory approaches to tackle these targets is also highlighted.

**ARTICLE HISTORY**
Received 17 July 2023
Accepted 20 October 2023

**KEYWORDS**
Antimicrobial resistance; ESKAPE; carbohydrates; infection; glycoconjugates; vaccines; bioconjugates; multiple presenting antigen system

## 1. Introduction

Bacterial antimicrobial resistance (AMR), a phenomenon resulting in ineffective drugs and persistent infections, is a growing threat and a major global health issue. Associated with around 5 million deaths in 2019, infections by AMR bacteria represent a leading cause of death worldwide, ahead of both human immunodeficiency virus (HIV) and malaria, and is particularly problematic for some of the globe's poorest regions [1]. Antibiotic resistance is a natural process characterized by mechanisms essentially limiting drug uptake, modifying drug targets, or inactivating drugs. Its occurrence has been greatly increased by massive antibiotic (mis)use [2]. High AMR bacterial burdens are prevalently found in sub-Saharan Africa and south Asia, partly due to a lack of or an excessive use of antibiotics respectively, reflecting the need for nationally tailored actions against this global problem [1].

AMR undermines medical care as a whole, by compromising surgeries, organ transplants, as well as treatments for physical trauma and conditions such as cancer, HIV and diabetes [3]. Six of the leading AMR pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacteriacae* including *Escherichia coli*), collectively termed ESKAPE pathogens, along with others, such as *Streptococcus pneumoniae*, have been identified as critical or high priority pathogens by the WHO and the CDC [1,4].

The global AMR challenge is currently tackled with the development of several complementary approaches, amongst which infection prophylaxis through vaccination remains a crucial one [2]. Vaccination can diminish the spread of AMR pathogens by reducing secondary infections and decrease the need for antibiotics, thereby combating resistance expansion [2]. Existing vaccination programmes against *S. pneumoniae*, Hib, *S. typhi*, malaria, rotavirus, seasonal flu, and the recently approved RSV already play an important role in containing antibiotic use and AMR [1,5]. For some of those vaccines, such as the pneumococcal conjugate vaccines (PCV), a continuous update of the vaccine formulation to increase valency is requested to counteract serotype replacement [6,7]. It is also worth noting that the development of veterinary vaccines, particularly against livestock pathogens, represents a desirable approach to reduce antibiotic use in animal and fish farming [8].

Vaccination has the potential to avert antibiotic use impacting on public health and economic burden [9]. Nevertheless, for most of the ESKAPE pathogens [2] vaccination remains an unmet need.

Extracellular polysaccharides (PS) of bacterial pathogens significantly contribute to their virulence mechanisms, while the surface localization and unique structures make these PS ideal candidates for the development of vaccines. Most microbial PS are, however, poorly immunogenic and do not induce a memory response [10]. This challenge has been overcome by

**CONTACT** Roberto Adamo roberto.x.adamo@gsk.com GSK, Via Fiorentina 1, Siena 53100, Italy

**Article highlights**

- Developing vaccines against ESKAPE pathogens is a critical priority, as these pathogens are major contributors to AMR, posing a severe threat to public health.
- Carbohydrate-based vaccines harnessing bacterial glycans could be instrumental in the fight against drug-resistant pathogens.
- A deeper understanding of glycoimmunology is crucial and would help comprehend how glycoconjugates work, paving the way for more effective vaccine development.
- The development of effective bacterial vaccines faces multiple challenges, including the need for new technologies and analytical tools to ensure vaccine quality and deliverability.
- The challenging identification of target populations to assess clinical efficacy and impact on AMR is calling for new clinical design and regulatory approaches.

coupling carbohydrates to a carrier protein to generate glycoconjugate vaccines [11,12]. The first glycoconjugate vaccines were licensed in the 1980s and since then have become one of the safest and most successful vaccine categories to prevent bacterial infection and reduce carriage [12].

In this review, we will present the latest data and developments on carbohydrate-based vaccines against leading AMR bacterial pathogens and discuss strategies to overcome the hurdles impeding their success in the clinics.

## 2. From polysaccharides to glycoconjugate vaccines

Most bacteria are coated with a PS network, termed glycocalyx, which includes i) lipid-linked structures, such as lipopolysaccharides (LPS) for Gram-negative and teichoic acids (TA) for Gram-positive species; ii) surface peptidoglycans for Gram-positive species; and iii) capsular polysaccharides (CPS) for Gram-positive and -negative encapsulated bacteria. These surface PS contribute to biofilm formation and fuel virulence by increasing host attachment and supporting immune evasion [12,13].

Plain PS-based vaccines have shown limited clinical efficacy due to their T cell (or thymus)-independent (TI) antigenic character [12,14]. PS are built up with highly repetitive epitopes, and can activate specific B cells in a polyvalent fashion by crosslinking membrane-bound pentameric immunoglobulin M (IgM) B cell receptor (BCR) clusters [14]. However, they typically induce B cells differentiation into plasma cells, without formation of memory B cells (MBC) [12,15–18], resulting in secretion of low-affinity IgM, and to a lesser extent IgG antibodies (mainly IgG2) [17,19]. The mechanism of B cell activation, based on the crosslinking of multiple BCR, explains why the immunogenicity of these vaccines is size-dependent, with only high molecular weight (MW) antigens being able to induce an effective immune response [12]. PS antigens thus activate the immune system without involving major histocompatibility complex class 2 (MHCII) molecules, and consequently without T cell help [17,19].

Adult individuals who have acquired PS-specific MBC prior to vaccination, likely through exposure to relevant pathogens [11,14] or to cross-reacting PS from commensal bacteria or ingested food, can produce PS-specific antibodies after vaccination [11,20]. However, most of these antibodies are IgM and IgG2, which are poor complement activators and therefore less effective [12,17,19]. Additionally, repeated immunization does not lead to increased antibody titers, but can rather trigger hyporesponsiveness [21–23].

PS-based vaccines are not effective in immunologically naïve populations, such as children younger than 2 years, because of the lack of preexisting MBC and the immaturity of their splenic marginal zone (MZ), which is the primary site for B cell stimulation [17,19,24]. In the elderly, limited production of naïve B cells in the splenic MZ prevents induction of a robust antibody production toward PS vaccines [17,19,25].

Of note, not all PS are TI antigens: zwitterionic polysaccharides (ZPS) such as *S. aureus* CPS type 5 and 8, *S. pneumoniae* Sp1 and *B. fragilis* PSA are able to bind MHCII molecule and trigger T cell help[17].

Eliciting a T cell-dependent (TD) immune response against PS can be generally accomplished by coupling carbohydrates to a carrier protein to generate a glycoconjugate vaccine, which contains both B and T cell epitopes (Figure 1). Glycoconjugate vaccines induce a PS-specific adaptive immune response, a normal B cell affinity maturation in germinal centers, IgM to IgG class-switch recombination, B and T cell memory, and significantly higher protection compared to plain PS vaccines [11,26].

Glycoconjugates are proposed to bind to carbohydrate-specific B cells through BCR or pattern recognition receptors and, upon internalization, the protein component is digested by proteases in the endosomal compartment, forming peptides which interact with MHCII molecules. The resulting complex is then transported to the B cell surface and presented to T cells. Activation of T cells induces B cell maturation, resulting in IgM to IgG class-switching and production of high-affinity carbohydrate-specific antibodies. The generated antigen-specific memory B and T cells can be activated upon exposure to pathogens and consequently provide efficient protection [17,26]. Critical events for mounting an appropriate humoral response, particularly T and B cell interactions in the germinal center, are outside the scope of this review but have been detailed elsewhere [11].

More recently, it has been shown that glycan-peptide fragments can be loaded onto MHCII molecules via the peptide and displayed at the B cell surface resulting in the generation of so-called Tcarb cells (carbohydrate-recognizing CD4+ T helper cells) [27–29]. Carbohydrate-specific T cell-mediated humoral responses have been found for a variety of glycoconjugates (*S. pneumoniae* type 3 CPS, *S. Typhi* Vi, *S. agalactiae* type Ib and *H. influenzae* type b), although it has not been seen for others, such as meningococcal group C CPS conjugate [30] which suggests coexistence of different processing mechanisms depending on the type of PS [31]. Tcarb activation may be an important element of the anti-carbohydrate immune response and should be taken into account in the design of new glycoconjugates.

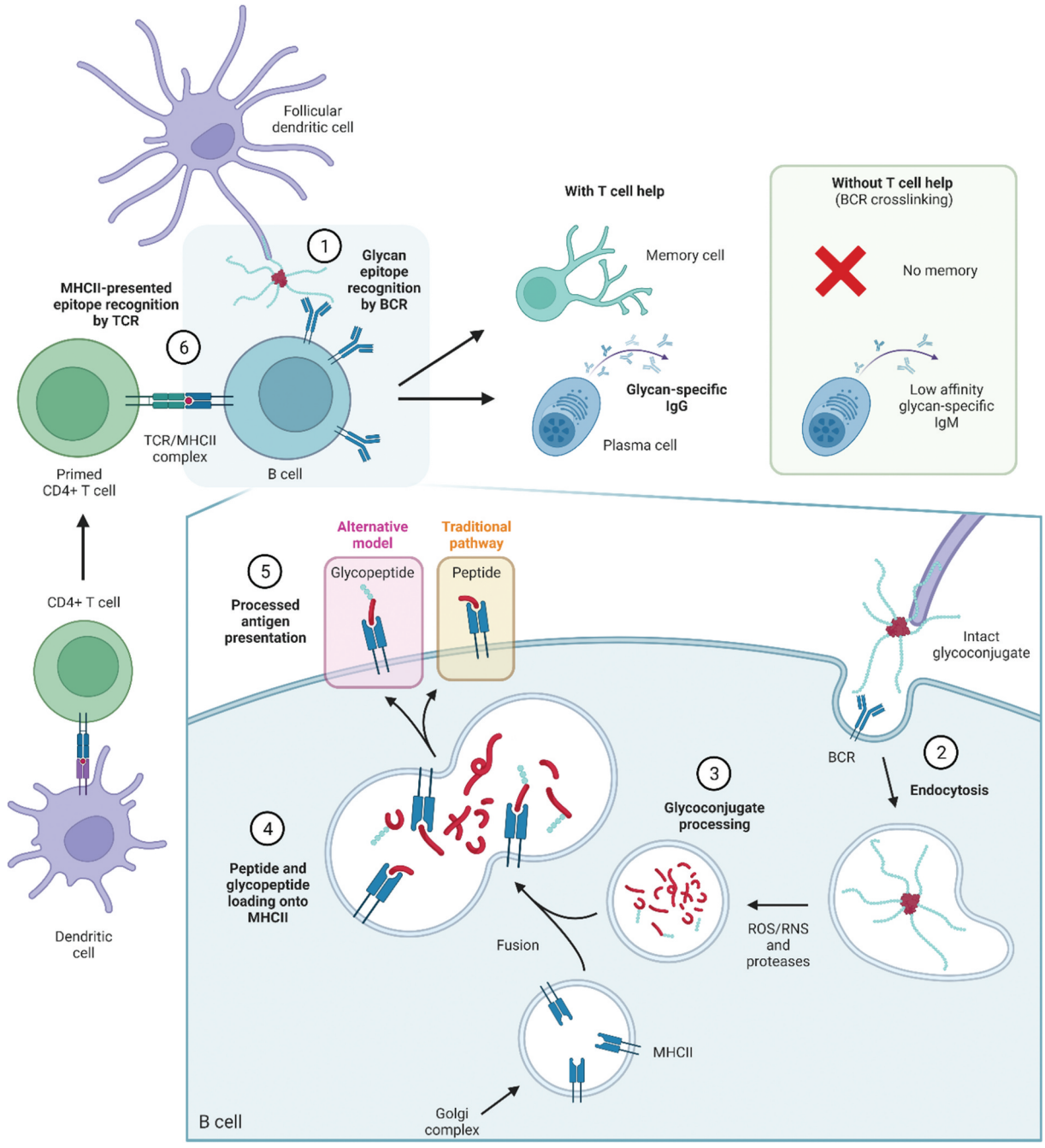


Figure 1. Immunological pathways for processing glycoconjugates. **1)** Glycoconjugates vaccines are taken up by antigen-presenting cells (APC) and presented to B cells, which recognize glycan epitopes through the B cell receptor (BCR). **2)** Upon BCR recognition, glycoconjugates are engulfed into the B cell via endocytosis. **3)** In the cell, glycoconjugates are exposed to acidic conditions and further degraded to fragments by enzymes and reactive oxygen/nitrogen (ROS/RNS) species. **4)** The resulting peptides and glycopeptides are loaded onto peptide-binding MHCII complexes, responsible for trafficking and exposing antigens to the cell surface. **5)** As MHCII molecules can only bind peptides (and ZPS), only processed peptides were thought to be displayed at the surface for T cell recognition, however, studies have shown that T cells can also recognize carbohydrates, suggesting glycopeptides can also bind MHCII. **6)** Primed T helper cells can recognize the presented antigen through their T cell receptor (TCR), and can, upon co-stimulation, initiate B cell maturation and production of memory cells and glycan-specific antibodies. In the absence of peptides, B cells cannot recruit T cell help and do not mount an efficient immune response.

## 3. Carbohydrate-based vaccine technologies

### 3.1. Glycan antigen production

Licensed glycoconjugate vaccines are commonly produced by chemical conjugation of extracted bacterial PS to a carrier protein. PS are generally purified from bacteria and covalently linked randomly along the saccharide chain to a carrier with the formation of high MW, cross-linked and heterogeneous structures (Figure 2a) [32,33]. The production of well-defined and uniform PS populations with a lower MW is sometimes preferred for consistency, as the elicited immune response can then better be correlated with the oligosaccharide's chemical structure [33,34]. Recently, alternative approaches have been developed to produce oligosaccharides through organic synthesis or chemoenzymatic approaches (Figure 2a) [32,33].

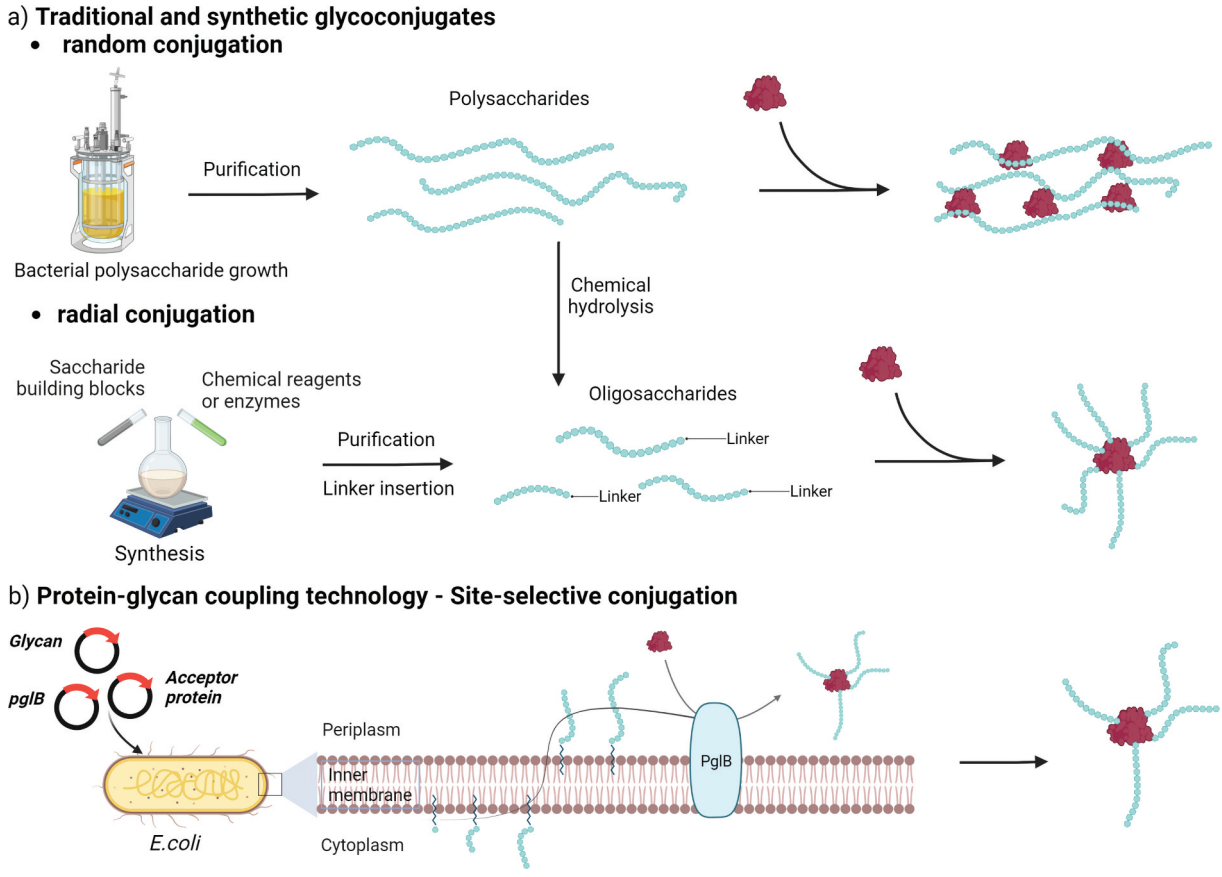


Figure 2. Simplified pathways for production of glycoconjugate vaccines. Oligosaccharides can be obtained from isolated PS through hydrolysis or oxidation. Various conjugation chemistries can be employed, resulting in site-selective or random conjugation on the carrier and/or the saccharide. Classical random conjugation strategies (**a**) usually involve lysine or Cysteine residues (e.g. N-hydroxysuccinimide (NHS) esters or thiol-maleimide reactions) for the protein, and hydroxyl groups for the saccharide (e.g. 1-cyano-4-dimethylaminopyridine (CDAP) activation and conjugation). Oligosaccharides obtained from PS depolymerization or chemoenzymatic methods can be conjugated at the reducing end through reductive amindation or through a linker providing a radial structure. Site-selective conjugation (**b**) can be achieved by glycoengineering (protein-glycan coupling techonology) or through incorporation of uAA.

Specifically, organic synthesis has the advantages of producing well-defined and highly pure structures, which are more easily characterizable and devoid of any potential bacterial contaminants [33,34]. Fully synthetic antigens have reached the clinical development phase, notably with a synthetic *H. influenzae* type b (Hib) vaccine licensed in Cuba [35], a PNAG conjugate that completed Phase I (NCT02853617), and more recently a vaccine against shigellosis that progressed to Phase II [36]. Novel synthetic transformations and deepened insight into glycosylation reaction mechanisms, combined with swifter synthetic protocols, based on one-pot reactions and automated synthesis, are allowing complex bacterial glycans to be more readily available [37]. Achievements in the production of synthetic glycans from PS expressed by AMR pathogens have recently been reviewed [33]. Despite the cost reduction provided by these modern methodologies, the manufacturing of complex multivalent vaccines remains a challenge.

Enzyme-catalyzed oligosaccharide assembly is an environment-friendly alternative to the chemical synthesis of bacterial carbohydrates. which could be attractive for industrialization [34]. Development of enzyme-based approaches has been shown feasible, particularly for meningococcal CPS synthesis [38]. Oligomers with a defined length could be obtained through solid-supported enzyme catalysis with simplified manufacturing and purification [39,40]. The enzymatic approach, however, is limited by the availability of glycosyl donors, due to the variability of bacterial monosaccharides and the potential presence of rare sugars which would need *ad hoc* synthetic protocols for their preparation. No example of chemoenzymatic glycoconjugate vaccine has so far reached the clinical stage.

### 3.2. Approaches for glycoconjugation

For long PS, conjugation to the carrier protein is carried out randomly along the carbohydrate chain, taking advantage of the abundant lysine residues and the high reactivity of activated saccharide residues. Because there is no or little control in the regiochemistry of this conjugation chemistry, the random approach results in cross-linked glycoproteins with variable saccharide loading and antigen positioning (Figure 2a) [34]. In contrast, conjugation of oligosaccharides to the protein can take place directly through the reducing end of the sugar or via a spacer, to reduce steric hindrance, with the formation of more defined and easy to characterize radial structures (Figure 2a) [32]. More selective approaches are required for

proteins that serve a dual role as antigen and glycan carrier. Two main strategies for site-selective protein engineering are applied for glycoconjugation: the incorporation of unnatural amino acids (uAA) and the *in vivo* bioconjugation referred to as protein-glycan coupling technology (PGCT) (Figure 2b) [41].

Recent examples of glycoconjugates relying on uAA include the development of a malarial vaccine, in which a glycosylphosphatidylinositol antigen was incorporated at the C-terminal p-azidomethyl phenylalanine of a Malaria protein Pfs25, that was expressed *E. coli* [42]. A conjugate obtained through a similar approach, based on the polyrhamnose backbone of the universally conserved cell wall carbohydrate from Group A *Streptococcus* (GAS) and the toxin antigen streptolysin O, generated in mice functional antibodies against both virulence factors and provided protection against systemic GAS challenges [43]. A 24–valent pneumococcal vaccine where PS were site-selectively coupled to engineered $CRM_{197}$, bearing multiple uAA for conjugation away from the most relevant T cell epitopes, induced opsonophagocytic antibodies in rabbit, raising IgG levels comparable to PCV13 for the same serotypes [44]. The vaccine completed Phase II clinical studies, while a 31–valent vaccine is under preclinical development.

PGCT is a single-step *in vivo* bioconjugation of the saccharide antigen and the carrier protein, which are both expressed in *E. coli*, thus avoiding the need for separate expression and purification of each component [45]. The technology is based on the N-linked glycosylation system from *Campylobacter jejuni* that can be functionally expressed in *E. coli* [46]. More specifically, PglB, the oligosaccharyltransferase enzyme, couples the PS synthesized on a undecaprenol pyrophosphate lipid anchor within the cytoplasm to the acceptor carrier protein having the acceptor sequon (D/E – X–N – X–S/T) to generate the bioconjugate [32]. Generally, up to 4–5 consensus sequences are inserted within the protein, although full occupancy can be difficult to achieve. PGCT is not limited to the use of PglB, and new enzymes are continuously being discovered to further extend bioconjugation toolbox [47,48].

A variety of bioconjugate vaccines based on O-antigens (*A. baumannii*, *B. pseudomallei*, *E. coli*, *F. tularensis*, *K. pneumoniae, S. dysenteriae,* and *S. flexneri*) and CPS (*S. aureus*, *S. pneumoniae*) have been generated and found immunogenic in animal preclinical models [47,49–51]. Some of these bioconjugates are currently undergoing clinical evaluation: a *Shigella* 2a vaccine [52] was shown to induce anti-lipooligosaccharide antibodies associated with a reduction of disease in a Phase II study [53]; a 9–valent vaccine against extraintestinal pathogenic *E. coli* (ExPEC9V) to prevent invasive infection in older adults with a history of UTI has advanced to Phase III (NCT04899336) [54] while a *S. pneumoniae* (NCT03303976) and a 4–valent *K. pneumoniae* vaccine have entered Phase I studies (NCT04959344) (Table 3) [32]. The limited amount of saccharides that can be incorporated

Table 1. Main advantages and drawbacks for each carbohydrate-based vaccine platform reviewed. We refer the readers to sections 3.1 and 3.2 for carbohydrate production and conjugation details.

| | | Pros | Cons |
|---|---|---|---|
| Chemical conjugation | Traditional conjugates | • Well-established technology with several licensed carbohydrate-based vaccines<br>• Manufacturing scaled up to 20–valent vaccines<br>• Characterization can be simplified by using defined oligosaccharides obtained by PS sizing or chemoenzymatic synthesis | • Frequent use of the same carrier could lead to lower immunogenicity of the PS (CIES effect)<br>• Suboptimal immunogenicity compared to unconjugated PS in the adult population<br>• Challenges in establishing the structure immunogenicity relationship in the case of isolated PS |
| | Site-selective conjugates | • Suitable for the dual role of the protein as carrier and antigen<br>• Manufacturing scaled up to 24–valent vaccines<br>• Compatible with the use of oligosaccharides obtained by PS sizing or chemoenzymatic synthesis | • Limited number of conjugation sites<br>• To be tested for vaccination of infants |
| | Glyconanoparticles | • Several VLP-based vaccines are commercially available<br>• Potential higher immunogenicity due to particle size<br>• A wide range of expression systems can be used to produce VLP<br>• Compatible with the use of oligosaccharides obtained by PS sizing or chemoenzymatic synthesis | • To date, used only at the preclinical level for conjugate vaccines<br>• Technically challenging and costly |
| Glycoengineering | GlycoGMMA | • High immunogenicity benefitting from its size and the presentation of lipidated proteins and LPS<br>• Scalable and cost-efficient<br>• Compatible with chemical conjugation (PS and oligosaccharides) and bioconjugation<br>• Presentation of O-antigens in their natural conformation | • Safety data in children and infants are under consolidation<br>• Complex analytical characterization due to multiple key attributes |
| | Bioconjugates | • Cost-efficient and simplified manufacturing thanks to its one-step production<br>• Manufacturing scaled up to 4–valent vaccines<br>• Suitable for the dual role of the protein as carrier and antigen | • Not suitable for the production of all types of sugars<br>• Full-site occupancy challenging to reach<br>• Limited sugar length (inferior to PS) |
| MAPS | | • Customizable with multiple glycan and protein antigens<br>• Manufacturing scaled up to 24–valent vaccines<br>• Protein attachment can be controlled by the rizhavidin-dependent PS localization | • The polysaccharide serves as anchor for the protein with limited capacity to play on sugar–protein ratio<br>• To be tested for vaccination of infants |

using site-selective technologies might pose some challenges for less immunogenic PS, and clinical studies will be relevant to inform on this.

### 3.3. Nanotechnologies for glycoconjugates

Several new technologies to allow the generation of complex multivalent glycoconjugate vaccines are under development. In the last decade, alternative delivery systems have been receiving attention to improve vaccine efficacy and immunogenicity, and a variety of platforms for the multiple presentation of saccharide antigens on pre-arranged and well-ordered matrices have been developed, among which (self-assembling) nanoparticles (NP), generalized modules for membrane antigens (GMMA), and more recently multiple antigen-presenting system (MAPS) technology [64,65]. Table 1 summarizes the pros and cons associated with each technology.

#### 3.3.1. Glycoconjugated (self-assembling) nanoparticles

Glyconanoparticles can display antigens with high density in a high order-structure arrangement, as naturally presented by a pathogen, enabling multiple binding events between the nanoparticle (NP) and the host cell BCR. The resulting high avidity can induce a more potent immune response than the single soluble recombinant antigens [64].

In addition, particles in the 50–200 nm size range can be actively transported by migratory cells, such as dendritic cells or macrophages, to lymph nodes which are the primary location of B cell maturation [66]. NP carriers could thus enhance antigen uptake by APC and BCR cross-linking as compared to classic protein carriers [67].

Self-assembling protein NP are protein oligomers assembling in three-dimensional structures which recently attracted interest in the field of vaccine development because of their unique properties, such as biocompatibility, enhanced stability and molecular specificity, leading to efficient delivery and antigen display at their surface [68,69]. Virus-like particle (VLP)-based vaccines deriving from self-assembling viral structural proteins are already used in commercial vaccines (Figure 3a) [70]. Examples of VLP-based licensed vaccines include the hepatitis B virus (HBV) vaccine Recombivax HB [71], the human papillomavirus (HPV) vaccines, Gardasil (Merck) and Cervarix (GSK), and the hepatitis E virus vaccine Hecolin [72].

Recently, VLP have been tested as carrier for carbohydrates. An efficacious pneumoccocal glycoconjugate vaccine based on Qβ VLP has been recently shown feasible [73]. VLP from the HBV core antigen have been exploited as carrier for the meningococcal group C CPS, generating a strong immune response in mice [74]. A conjugate vaccine developed by linking purified O-antigen from *V. cholerae* O1 to Qβ VLP using squarate chemistry generated in mice prominent and long-lasting anti O-antigen IgG antibodies that recognized native LPS from *V. cholerae* [75].

New self-assembling NP scaffolds computationally designed with precisely control of morphology and characteristics are emerging as tools to assemble different immunological components (i.e. antigens and adjuvants) in order to amplify their immune responses [68,69,76,77]. Vaccine candidates designed through this approach and exposing glycoprotein antigens from respiratory syncytial virus (RSV), influenza and SARS-CoV-2 have been advanced in clinical trials [69,78–81].

#### 3.3.2. Generalized modules for membrane antigens

Gram-negative bacteria generate low amounts of outer membrane vesicles (OMV) through a blebbing process, which can be stimulated through strain engineering to destabilize the membrane and effectively produce generalized modules for membrane antigens (GMMA) (Figure 3b). GMMA faithfully resemble the bacterial outer membrane and possess self-adjuvanting properties through the display of several pathogen-associated molecular patterns, which are conserved small

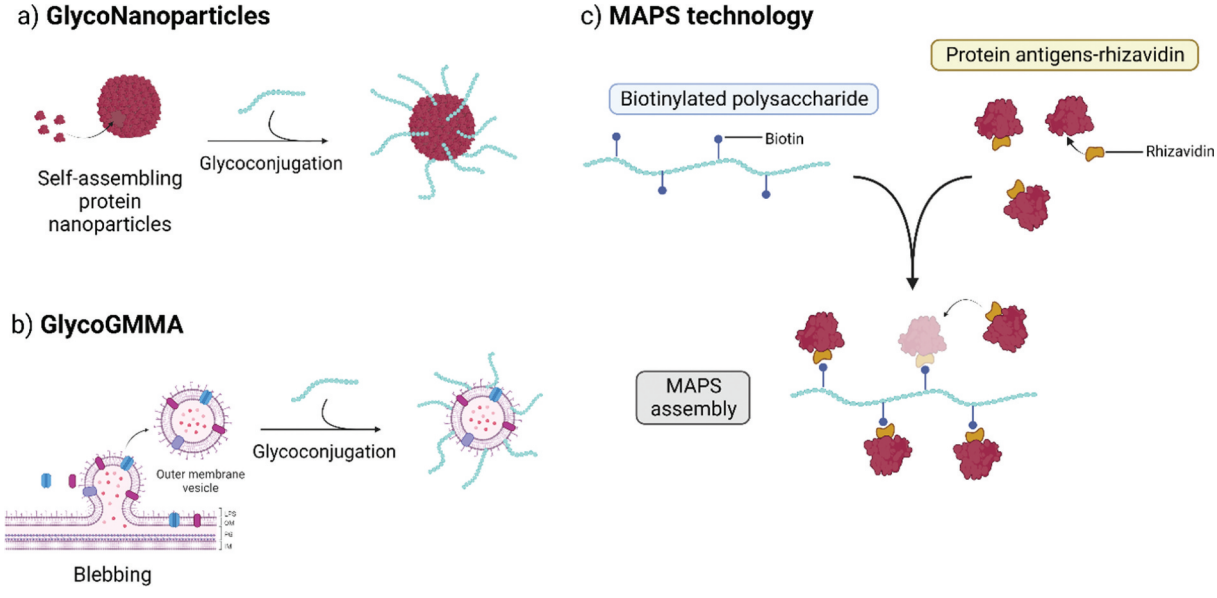


Figure 3. Innovative glycoconjugate technologies.

molecular motifs in bacteria that are recognized by pattern recognition receptors on mammalian cells. For example, GMMA expose surface O-polysaccharide (OPS) chains in their natural environment and conformation, thus promoting uptake by immune cells and inducing strong immune responses. GMMA in which the lipid A portion of its LPS has been modified to modulate endotoxicity have, therefore, been proposed as vaccine candidates [82,83]. The safety of *S. sonnei* GMMA was demonstrated in a Phase I study [34,84,85]. A number of PS from different pathogens (*N. meningitidis* serogroup A, *H. influenzae* type b, GAS, and *S. Typhi* Vi) have been successfully conjugated to GMMA and shown to be immunogenic in animal models [86–88]. The chemical linkage was directed either to proteins or to the LPS expressed on the vesicle surface [87,89,90].

Alternatively, strategies to engineer *E. coli* to express heterologous PS on GMMA and OMV have been developed [91]. GlycoOMV have been generated by exploiting the PS biosynthesis on the native undecaprenyl pyrophosphate carrier in the cytoplasmic side of the inner membrane and subsequent translocation to the periplasm side through the endogenous flippase Wzx, to enable the final transfer on the lipid A by the endogenous O-antigen ligase WaaL [91]. Glycans can also be assembled from the terminal sugars of the truncated lipid A-core expressed on the cytoplasmic side of the inner membrane and then flipped to the periplasm by the Lpt protein complex for final transfer onto the outer membrane of the vesicles [92]. GlycoOMV expressing OPS from various pathogenic bacteria, including the highly virulent *F. tularensis* ssp. *tularensis* type A strain Schu S4 (generating Ft-glycOMV) have been produced through this approach [93]. The glycoOMV induced antibodies protecting mice from a lethal challenge with *F. tularensis* after passive transfer and elicited a protective IgA-mediated mucosal immune response when subcutaneously administered. GlycoOMV decorated with pneumococcal CPS were also generated and induced serum IgG opsonophagocytic titers comparable to the corresponding chemical conjugates in PCV13 [94]. Finally, the hypervesiculating JC8031 strain of *E. coli* was engineered for expression of *S. aureus* PNAG, both in acetylated and deacetylated form (dPNAG) [95]. After mice immunization, the dPNAG-OMV-elicited antibody mediated *in vitro* killing of PNAG-positive *S. aureus* and *F. tularensis holarctica* and protected mice from challenges with the two bacterial species.

#### 3.3.3. Multiple antigen-presenting system technology

The multiple antigen-presenting system (MAPS) enables the creation of a macromolecular complex by integrating glycan and protein antigens to reproduce the antigenic and immunologic strengths of whole-cell vaccines (Figure 3c). In the MAPS, differently from classic conjugate vaccines, the PS and protein are not covalently linked, but affinity binding is exploited to generate multivalent immune complexes, thus mimicking chemical and physical features of a whole-cell constructs for B and T cell activation [96,97]. The MAPS complex is obtained by mixing a target protein antigen, genetically fused to an avidin moiety (termed rhavi), and a biotinylated PS [96]. The immunogenicity of the formed macromolecular construct depends on the size of the PS scaffold, as the protein becomes exposed along its chain. As the MAPS approach allows for the combination of a variety of protein and carbohydrate components, it appears advantageous in the design of vaccines against challenging pathogens, such as *S. aureus*, a bacterium known to have complex host–pathogen interactions [96,98]. Depending on the size of the glycan-protein complex, the MAPS also allows to achieve Th1/Th17–biased immune responses [96].

MAPS technology appears also to streamline the manufacturing process and this feature has been exploited for the development of 24–valent pneumococcal vaccine that was successful in Phase II clinical studies [99] A multivalent vaccine to fight invasive *K. pneumoniae* and *P. aeruginosa* infections is also in progress (Patent no. US11612647B2) [100].

## 4. Carbohydrates as antigens to target ESKAPE pathogens

Bacterial surface polysaccharides are a very diverse class of biomolecules, that includes capsular polysaccharides (CPS), lipopolysaccharides (LPS), teichoic acids (TA) and exopolysaccharides (Figure 4). These extracellular carbohydrates are usually crucial to the survival of bacteria and heavily contribute to their virulence. Consequently, they represent a rich source of protective antigens. Notably, some structural overlap in glycans between bacterial strains and even species makes it possible to generate broad-spectrum glycoconjugate vaccines, while passive immunotherapy may be a suitable strategy for treating patients unable to mount an effective immune response during or after an infection. The most recent advances and trends in active and passive immunizations against the ESKAPE pathogens presented in Box 1 are described here and summarized in Tables 2 and 3. Although *S. pneumoniae* is not part of the ESKAPE group, it is considered a major AMR driver. For an overview of the state of art of pneumococcal conjugate vaccine we refer the readers to other recent reviews [146,147].

### 4.1. Capsular polysaccharides and lipopolysaccharides

Capsular and bacterial lipopolysaccharides represent the best-known classes of bacterial PS and have consequently been in focus for the development of carbohydrate-based vaccines (Figure 4). Bacterial capsular polysaccharides (CPS), or K-antigens, are long and incredibly diverse PS chains, produced by both Gram-positive and -negative bacteria. Capsules are covalently linked to the cell surface, compose the outermost layer and have variable functions that can enhance bacterial fitness for survival and alter immune responses to pathogens [152]. Lipopolysaccharides (LPS) are endotoxins found in the outer membrane of Gram-negative bacteria. LPS contain three distinct regions: the lipid A, a glycophospholipid moiety anchoring LPS into the bacterial membrane (Figure 4a); a core oligosaccharide, commonly composed of keto-deoxyoctulosonate (KDO) attached to the lipid A; and the O-antigen, or O-specific polysaccharide (OPS), a repetitive outer glycopolymer unique to each bacterial strain

[153]. LPS have long been studied as immunogens and LPS-based conjugates could soon emerge as potent vaccines against Gram-negative bacteria using approaches described in section 4 [154].

#### 4.1.1. *A. baumannii*

There are more than 90 expected serotypes for the CPS of *A. baumannii*. Although CPS are the most explored carbohydrate vaccine antigen because of their surface accessibility [155], only 20 CPS structures have been elucidated to date [156]. Monoclonal antibodies (mAb) against *A. baumannii* K1 CPS bound 13 out of 100 isolates, while showing neutrophil-mediated killing activity *in vitro* and decreasing bacterial loads in various rat organs after infection [157]. Specific antibodies obtained after immunization with the *A. baumannii* SK44 CPS reacted to 62% of the 554 tested clinical isolates, suggesting its potential as protective antigen against heterologous strains [118]. The *A. baumannii* K3 CPS type (Figure 4b) consists of

Table 2. Current key preclinical carbohydrate-based approaches against ESKAPE pathogens. Use of synthetic glycan is meant by the term 'semi-synthetic chemical conjugate'.

| Carbohydrate antigen(s) | Targeted pathogen(s) | Carrier | Technological platform | Reference(s) (year) |
|---|---|---|---|---|
| CP8 | *S. aureus* | $CRM_{197}$ | Semi-synthetic chemical conjugate | [116] (2020) |
| CP5, CP8 | *S. aureus* | $CRM_{197}$ | Chemical conjugate | [117] (2017) |
| SK44 CPS | *A. baumannii* | – | Polysaccharide | [118] (2017) |
| K9 CPS | *A. baumannii* | BSA, OVA, KLH | Chemical conjugate | [119] (2022) |
| CPS2 | *K. pneumoniae* | – | Polysaccharide | [120] (2018) |
| DHG | *E. faecalis* (*E. faecium*) | SagA, PpiC | Chemical conjugate | [121,122] (2019) |
| DHG | *E. faecalis* | BSA | Semi-synthetic chemical conjugate | [123] (2020) |
| K1, K2 CPS | *K. pneumoniae* | EPA | Bioconjugate | [124] (2019) |
| K2 CPS | *K. pneumoniae* | DT | Semi-synthetic chemical conjugate | [125] (2020) |
| LPS | *P. aeruginosa* | PLGA | NP | [126] (2022) |
| LPS | *P. aeruginosa* | AuNP | NP | [127] (2021) |
| O-antigens (O1, O2, O3 and O5) | *K. pneumoniae*, | FlaA, FlaB (*P. aeruginosa*) | Chemical conjugate | [128] (2018) |
| KP O-antigens (O1, O2, O3 and O5) and PA OPS | *K. pneumoniae*, *P. aeruginosa* | MrkA | MAPS | [129] (2019) |
| O25b PS | *E. coli* | $CRM_{197}$ | Semi-synthetic chemical conjugate | [108,130] (2022) |
| O148, O78 PS | *E. coli* | $CRM_{197}$, PD | Bioconjugate | [131] (2023) |
| $Hep_2Kdo_2$ | *N. meningitidis*, *E. coli*, *P. aeruginosa* | DT, BSA | Semi-synthetic chemical conjugate | [132] (2016) |
| A-band PS | *P. aeruginosa* | HSA | Semi-synthetic chemical conjugate | [133] (2022) |
| Alginate | *P. aeruginosa* | HSA | Semi-synthetic chemical conjugate | [134] (2022) |
| Alginate | *P. aeruginosa* | PGLA | NP | [135] (2021) |
| Alginate | *P. aeruginosa* | SLN | NP | [136] (2021) |
| Alginate | *P. aeruginosa* | OMV | OMV | [137] (2021) |
| Psl | *P. aeruginosa* | PHA inclusions | NP | [138] (2021) [139] (2017) |
| EPS (54149) | *A. baumannii* | $CRM_{197}$ | Chemical conjugate | [140] (2018) |
| Pse | *A. baumannii* | $CRM_{197}$, BSA | Semi-synthetic chemical conjugate | [141] (2021) |
| Several surface PS | *E. faecium* | $CRM_{197}$ | Chemical conjugate | [142] (2015) |
| PNAG | *S. aureus*, *E. faecalis*, *E. coli* | TT | Semi-synthetic chemical conjugate | [60] (2019) |
| PNAG | *S. aureus* | OMV | OMV bioconjugate | [95] (2018) |
| PNAG, TA | *S. aureus* | – | Chemical conjugate | [143] (2019) |
| LTA | *S. aureus*, *E. faecalis*, *E. faecium* | BSA | Semi-synthetic chemical conjugate | [144] (2014) |
| WTA | *E. faecium* | KLH | Semi-synthetic chemical conjugate | [145] (2017) |
| In the industrial preclinical pipeline | | | | |
| O-antigens (O1, O2, O2ac, O8 and ST258) | *K. pneumoniae* | $CRM_{197}$ | Semi-synthetic chemical conjugate | Idorsia Pharmaceuticals, Patent no. US20230122752A1 |
| Unknown | *E. coli*, *P. aeruginosa* | – | O-glycosylated proteins | GlyProVac, Patent no. US10647749B2 |
| KP K19 CPS and PA O-antigens (O2, O3, O4, O5, O6, O10 and O11) | *P. aeruginosa*, *K. pneumoniae* | Rhavi-FlaBD2-PcrV, Rhavi-FlaBD2-MrkA | MAPS | Affinivax/GSK, Patent no. US11612647B2 |

Abbreviations: **AuNP**, gold nanoparticle; **BSA**, bovine serum albumin; **COPS**, core and O-polysaccharides; **CP(5/8)**, capsular polysaccharide serotype 5 or 8 (*S. aureus*); **CPS**, capsular polysaccharide (or K antigens); **$CRM_{197}$**, cross-reactive material 197, a detoxified mutant of diphtheria toxin; **DHG**, diheteroglycan; **DT**, diphtheria toxoid; **EPA**, exotoxin protein A (*P. aeruginosa*); **EPS**, extracellular polysaccharide; **HSA**, human serum albumin; **KLH**, keyhole limpet hemocyanin; **KP**, *K. pneumoniae*; **LPS**, lipopolysaccharide; **MAPS**, multiple antigen-presenting system technology; **NP**, nanoparticle; **OMV**, outer membrane vesicle; **OVA**, ovalbumin; **PA**, *P. aeruginosa*; **PD**, protein D from *H. influenzae*; **PGLA**, poly(lactic-co-glycolyc acid); **PHA**, polyhydroxyalkanoates; **PNAG**, poly-N-acetyl glucosamine; **PS**, polysaccharide; **Pse**, pseudaminic acid; **SLN**, solid lipid nanoparticle; **(L/W)TA**, (lipo- or wall) teichoic acid; **TT**, tetanus toxoid; **VLP**, virus-like particle.

Table 3. Recent clinical trials for vaccines and passive immunizations against ESKAPE.

| Carbohydrate antigen (s) | Name | Targeted pathogen(s) | Tech. platform | Phase; status | Reference(s) |
|---|---|---|---|---|---|
| **CPS** | SA4Ag (Pfizer) | *S. aureus* | Chemical conjugate | Phase IIb; terminated (2019) | NCT02388165 [55–57] |
| | StaphVax (Nabi) | *S. aureus* | Chemical conjugate | Phase III; terminated (2005) | NCT00071214 |
| | Staph4V (GSK) | *S. aureus* | Chemical conjugate | Phase I; completed (2017) | NCT01160172 |
| **LPS/OPS** | KlebV4 (LimmaTech/GSK) | *K. pneumoniae* | Bioconjugate | Phase I/II; completed (2022) | NCT04959344 |
| | ExPEC4V (Janssen/J&J) | *E. coli* | Bioconjugate | Phase II; completed (2019) | NCT03500679 [58,59] |
| | ExPEC9V (Janssen/J&J) | *E. coli* | Bioconjugate | Phase III; active | NCT04899336 |
| | ExPEC10V (Janssen/J&J) | *E. coli* | Bioconjugate | Phase I/II; active | NCT03819049 |
| **PNAG** | AV0328 (Alopexx) | *S. aureus, S. pneumoniae* | Chemical conjugate | Phase I/II; completed (2018) | NCT02853617 |
| | F598 (Alopexx) | – | Monoclonal antibody | Phase I; completed | [60] [10] |
| **TA** | Pagibaximab (Biosynexus Inc.) | *S. aureus* | Monoclonal antibody | Phase III; completed (2011) | NCT00646399 [61] |
| **PsI** | MEDI3902 (MedImmune/ AstraZeneca) | *P. aeruginosa* | Monoclonal antibody | Phase II; completed (2019) | NCT02696902 [62] |
| **Alginate** | AR-105 (Aridis Pharmaceuticals) | *P. aeruginosa* | Monoclonal antibody | Phase II; completed (2019) | NCT03027609 [63] |

Abbreviations: **CPS**, capsular polysaccharide (or K antigens); **LPS**, lipopolysaccharide; **OPS**, O-polysaccharide (or O-antigen); **PNAG**, poly-N-acetyl glucosamine; **TA**, teichoic acid.

a branched pentasaccharide, with a core trisaccharide RU found in the CPS of several strains, including SK44, explaining this cross-reactivity. Oligosaccharides resembling that pentasaccharide were synthesized and screened against sera from infected patients to identify potential vaccine candidates [158]. Li *et al*. demonstrated that K3 CPS could be combined with recombinant cholera toxin B subunit into a bioconjugate with significant protective effect [159]. Rudenko *et al*. also recently reported CPS-based glycoconjugates with conserved K9 CPS fragments and several classical carrier proteins (bovine serum albumin (BSA), ovalbumin (OVA) and keyhole limpet hemocyanin (KLH)), which improved carbohydrate immunogenicity and mice survival upon bacterial challenge [119].

Along with the capsules, several O-antigen structures have been identified for *A. baumannii* and methodologies for serotyping are currently under investigation [160]. The O-antigen diversity and lack of understanding of highly expressed structures in clinical isolates remain a challenge for the development of OPS-based vaccines.

### 4.1.2. *E. coli*

*E. coli* produces a remarkable variety of CPS, 80 of which have been isolated [161]. However, studies on the O-antigen distribution in some European countries and in the United States (US) suggest that 10 serotypes would cover the majority of UTI or bacteremia [54,108], which explains the focus on vaccines against these O-antigens in clinical development. ExPEC4V, a bioconjugate containing O-antigens from four *E. coli* serotypes (O1A, O2, O6A, and O25B, conjugated to exotoxin protein A (EPA) from *P. aeruginosa*), was shown to be well tolerated and immunogenic in a Phase II study [58,59]. However, ExPEC4V did not meet the expected endpoint in terms of UTI recurrence rate reduction, although a *post hoc* analysis showed an impact on UTI episodes regardless of serotype [162]. The failure was attributed to the limited vaccine coverage (30–35% of serovars). Therefore, a 10–valent bioconjugate candidate vaccine (ExPEC10V) was tested in Phase I/II and a combination of 9 bioconjugates (ExPEC9V) was selected based on immunogenicity data, to advance in Phase III for prevention of invasive disease in older adults with a history of recurrent UTI.

The main challenge in the development of multivalent vaccines has been the low immunogenicity observed for some O-antigen serotypes. In particular, the glycans of serotypes O25A and O25B were demonstrated to be poor immunogens in healthy volunteers, which is unfortunate as serotype O25B has emerged as the leading cause of invasive multidrug-resistant ExPEC infections [108]. Chorro *et al*. demonstrated that a linkage switch, from radial (as in bioconjugates) to lattice-like, in an elongated O25B PS glycoconjugate to $CRM_{197}$ provided superior immunogenicity in mice. The conjugate was also shown to protect the animals from a lethal challenge with invasive MDR ST131 *E. coli* and to induce a robust functional antibody response in immunized nonhuman primates [108]. In this context, a set of oligosaccharides from O25B serotype differing in RU frameshift and length were chemically synthesized and conjugated to $CRM_{197}$ [130]. Among them, three conjugates of glycan antigens induced antibody titers comparable to a conventional conjugate and enabling identification of relevant PS glycoepitopes.

Cell-free extracts of nonpathogenic *E. coli* were used for PglB mediated production of a bioconjugate vaccine candidate against two of the most prevalent ETEC O serogroups, O148 and O78. The two bioconjugates stimulated in mice induction of IgG antibodies with bactericidal activity against the cognate pathogens. Also, the bioconjugate containing O148 O-PS reduced ETEC colonization in mice by oral administration providing evidence of vaccine-induced mucosal protection [131].

The glycosylation pattern of the protein SslE or YghJ, a metalloprotease known as potential *E. coli* vaccine candidate [163], has been shown to occur in numerous ETEC strains has been shown to be recognized by patients infected with the pathogen suggesting potential use of glycopeptides as

vaccine antigens. Glycopeptide [164]-specific antibodies have been also identified in patients with UTI [165].

#### 4.1.3. *E. faecalis and E. faecium*

After the initial discovery of an LTA-like structure composed of α-Glc-(1,2)-α-Glc-(1,2)-glycerol-3-phosphate, different classes of polysaccharides have been isolated from *E. faecalis* [166,167]. Based on the analysis of the biosynthetic CPS locus, four serotypes A-D have been established. A recent analysis of clinical isolates indicated that most pathogenic strains belong to serotype C, although another study pointed at a broader serotype diversity [168]. Early studies showed that purified CPS depleted the OPK activity of immune rabbit sera, and elicited high titers of antibodies in rabbit mediating OPK of bacteria [166,169]. Diheteroglycan (DHG) (Figure 4b) has been recently characterized as an immunogenic component of CPS-C and -D, and a target to generate antibodies with OPK activity [170]. An anti-DHG opsonic mAb was isolated and demonstrated to have efficacy in passive immunotherapy [121]. DHG-SagA or DHG-PpiC proved to be valid glycoconjugate vaccines against several enterococcal species, with SagA and PpiC (*E. faecium* proteins) having a dual role as antigen and carrier [121,122]. Further studies are needed to inform on the best glycoconjugate design for maximal immunogenicity while maintaining control over the production. In that direction, synthetic oligomers mimicking DHG from *E. faecalis* type 2 conjugated to BSA have recently been evaluated in mice [123]. Interestingly, cell wall-associated antigens of *E. faecium* U0317 were recognized by anti-DHG antibodies [171].

By contrast, *E. faecium* CPS are far less known and though putative CPS biosynthetic genes have been predicted [172,173], primarily teichoic acids have been structurally elucidated [166,174]. Four distinct extracellular PS from *E. faecium* were isolated by Kodali *et al*. and conjugated to $CRM_{197}$ for rabbit immunization. Of these vaccines, $CRM_{197}$-Pf2, a conjugate bearing altruronic acid-containing heteroglycans, proved to be the most effective. Anti-Pf2-$CRM_{197}$ antibodies had strong OPK activity and significantly reduced bacterial loads in mouse liver and kidney tissue. Altruronic acid, a glucuronic acid derivative, is a rare carbohydrate that can be found in the LPS or CPS of other pathogens, and could become part of multivalent formulations [142].

#### 4.1.4. *K. pneumoniae*

Among the most studied virulence factors for *K. pneumoniae* are CPS, or K antigens, 79 serotypes of which have been identified to date, with only 25 representing about 70% of clinical isolates [100,175]. A 24–valent PS K-antigen vaccine (administered in addition to 8 *P. aeruginosa* O-antigens conjugated to EPA as carrier protein) initially moved forward through multiple clinical trials demonstrating safety, immunogenicity and opsonic activity [176–178]. However, the vaccine was never authorized on the market because of the advanced formulation needed for its development [100]. Because the K1 and K2 serotypes (Figure 4b) are responsible for an important part of hypervirulent infections, glycoconjugate vaccines targeting these two serotypes have been explored. In a recent study [124], a glycoengineered *E. coli* strain was used to produce a bioconjugate vaccine expressing K1 and K2 antigens, which afforded protection to mice against a lethal challenge with two *K. pneumoniae* strains. Another promising example in preclinical studies is a synthetic K2 CPS conjugated with diphtheria toxin (DT) that induced the production of specific capsular antibodies in mice [125].

As opposed to CPS, which are highly variable, *K. pneumoniae* O-antigens could represent alternative targets thanks to their limited structural range. Four serotypes (O1, O2, O3 and O5) are responsible for around 80% of clinically relevant *K. pneumoniae* strains [179]. In mid-2021, a tetravalent bioconjugate candidate vaccine (Kleb4V), produced by LimmaTech Biologics AG in collaboration with GSK, completed clinical Phase I/II and proved to be safe and immunogenic in humans. The tetravalent bioconjugate vaccine covers OPS of the most predominant *K. pneumoniae* serotypes (NCT04959344) [32]. Recently, Hegerle *et al*. combined two *P. aeruginosa* flagellins (FlaA, FlaB) to O1, O2, O3, O5 *Klebsiella* O-serotypes to obtain a 4–valent chemical conjugate that induced antibody titers against all antigens in rabbits [128]. Similarly, Cross *et al*. reported functional *in vitro* and *in vivo* activity of antibodies produced by a 12–valent vaccine comprised of four *K. pneumoniae* (O1, O2, O3 and O5) and eight *P. aeruginosa* core and O-polysaccharides (COPS), and interesting protein antigens such as MrkA (*K. pneumoniae* virulence factor), combined through the MAPS platform. The vaccine induced a robust immune response against the carbohydrate antigens of both bacteria as well as against the proteins [129]. Synthetic glycans resembling the PS coating *K. pneumoniae* surface have been also targeted by Idorsia (former Vaxxilon) to develop a multivalent semisynthetic conjugate vaccine (Patent no. US20230122752A1).

#### 4.1.5. *P. aeruginosa*

For *P. aeruginosa*, more than 20 different O-antigen structures can be distinguished, but only 11 of these are expressed in the majority of pathogenic serotypes. LPS-based vaccines against *P. aeruginosa*, initially included 7 (Pseudogen) and subsequently 16 different serotypes, and these have been tested in the clinics since the 1970s, ultimately failing to show efficacy [180–184]. In one study in patients with cystic fibrosis (CF), Pseudogen exacerbated the infection possibly because of the presence of lipid A [185]. Increasing the coverage of these LPS-based vaccines did not provide significant improvements in terms of reduction of bacterial colonization [114,186–188].

A conjugate vaccine, Aerugen, composed of EPA and O-antigens from eight serotypes, was proven safe and immunogenic in diverse populations, including plasma donors, bone marrow transplant and non-colonized CF patients [189–191]. The vaccine was also shown to induce opsonophagocytic antibodies with a three year persistency in a small cohort of non-colonized CF patients [192]. However, in a larger trial in CF patients, Aerugen did not show efficacy compared to the placebo and it was the last carbohydrate-based vaccine against *P. aeruginosa* reported in the clinics [193]. Despite these failures, O-antigens remain promising targets and new approaches have been recently investigated at a preclinical level, including conjugation to poly(lactic-co-glycolic acid) (PGLA)- [126] or gold-based (AuNP) [127] nanoparticles.

**Box1.** Key AMR pathogens and associated challenges for vaccine development.

***Escherichia coli*** (WHO critical priority [4], CDC urgent/serious threat [101])

*E. coli* is a Gram-negative bacterium commonly found in the human gut. Typically, strains are classified into commensal microbiota, enterovirulent or extraintestinal pathogenic *E. coli* (ExPEC) on the basis of their genetic features and clinical outcomes [102]. Strains causing gastroenteritis are in turn subdivided in enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC), enteroinvasive (EIEC), and lastly diffusely adhering *E. coli* (DAEC). ExPEC can cause severe (extra)intestinal infections and diseases, urinary tract infections (UTI), bacteremia and meningitis. ETEC is a major cause of traveler's diarrhea and death from diarrheal disease worldwide, especially in infants and young children [103]. Antibiotic resistance has become a major concern in the treatment of infections, and the production of an effective *E. coli* vaccine is challenging due to i) the heterogeneity of *E. coli* strains, ii) the high probability of side effects on the intestinal flora, iii) the dependence of the disease to the expression of multiple virulence factors [104], especially since an effective vaccine should provide protection against the bacteria at different stages of pathogenesis [105–107]. Most carbohydrate-based vaccines under development are targeting urinary and invasive infections due to the well-known capacity of anti O-antigen antibodies to induce bacterial killing in animal models [108].

***Staphylococcus aureus*** (WHO high priority [4], CDC serious threat [101])

*S. aureus* is a commensal Gram-positive bacterium increasingly resistant to the first-line antibiotic vancomycin [109]. It is the most common microbial cause of nosocomial and community-acquired skin and soft tissue infections (SSTI) [110], and of surgical site infections (SSI) [111]. Around 50% of all staphylococcal infections are estimated to be methicillin-resistant [112]. Given the high infection frequency, contagiousness and the prevalence of resistant strains, SSTI highly contribute to AMR spread [2]. Although a few vaccines are in the pipeline, a successful development will likely depend on a better understanding of pathogen-host interactions.

***Klebsiella pneumoniae***

*K. pneumoniae* is an opportunistic Gram-negative bacterium found in the human mouth and gut. *K. pneumoniae* is associated with hospital-acquired persistent UTI, liver abscess, SSTI, pneumonia and sepsis, particularly affecting the elderly and immunocompromised patients. Infections are also widespread in communal settings. Resistance to cephalosporins and last-line carbapenems through beta-lactamase production generates invasive infections associated with high mortality [101]. *K. pneumoniae* is considered a reservoir and propagator of AMR-related genes [2]. *K. pneumoniae* is also a leading cause of neonatal sepsis in low and middle-income countries which has been associated with spread of multi-resistant lineages [113].

***Acinetobacter baumannii*** (WHO critical priority [4], CDC urgent threat [101])

*A. baumannii* is a commensal Gram-negative bacterium commonly found in hospitals, where it causes pneumonia, SSI, UTI, as well as bloodstream and wound infections. *A. baumannii* is intrinsically resistant to antibiotics and can acquire the ability to produce carbapenemases through gene transfer. Carbapenem-resistant *A. baumannii* ranks as the most critical pathogen in the WHO priority list for research and development of new antibiotics and as a serious threat for the CDC [4,101]. It is however not considered a strong candidate for vaccine development, due to its low incidence and difficulties to identify key target populations, therefore developing alternative strategies such as passive immunization is currently recommended [112].

***Pseudomonas aeruginosa*** (WHO critical priority [4] CDC serious threat [101])

*P. aeruginosa* is an opportunistic Gram-negative bacterium causing life-threatening infections in immunocompromised individuals and patients with cystic fibrosis (CF). It is increasingly recognized as a nosocomial pathogen in the elderly and in combat-related wounds, suggesting an appropriate target population for vaccination [114]. *P. aeruginosa* exhibits most of the known resistance mechanisms to antibiotics, that are both intrinsic chromosomally encoded or originate from genetically imported plasmids. Some strains are resistant to more than three classes of antibiotics and pan-drug resistant strains have been reported [115]. *P. aeruginosa* is also able to form a biofilm in the lungs of infected patients thus impeding the access of antibiotics to bacterial cells. Despite years of academic and industrial research efforts, no commercial vaccine is available, and no potential candidates are currently in clinical trials [2,112].

***Enterococcus faecium*** (CDC serious threat [101])

*E. faecium* is a Gram-positive commensal bacterium commonly found in the gastrointestinal tract and causing nosocomial infections, such as UTI, endocarditis and meningitis. Immunocompromised individuals and patients in intensive care units are particularly at risk. Up to 30% of infections are currently resistant to vancomycin, and additional resistance is on the rise [101]. Due to the low incidence, morbidity and mortality associated with enteroccocal infections, vaccine development is currently not recommended and other approaches are explored [112]. Infections from *E. faecium* and *faecalis* are often undistinguishable.

*P. aeruginosa* also produces a surface PS antigen termed A-band polysaccharide (A-PS) (Figure 4b), that can be attached to the LPS, which is then referred to as A-band LPS, in contrast to the LPS O-antigens, which are sometimes termed B-band LPS. Notably, A-band LPS is found to be conserved in clinical isolates, while serotype-specific B-band LPS disappears over time. A-PS is composed of a rhamnan RU, and several other non-rhamnose components that remain to be confirmed. Preliminary studies, however, determined the rhamnan chain to be non-immunogenic. Recently, Cairns *et al*. established that the A-PS is capped with a 3-O-methyl-D-rhamnose pentasaccharide linked through a methyl mannose unit to a neutral mannose trisaccharide. Further study on an

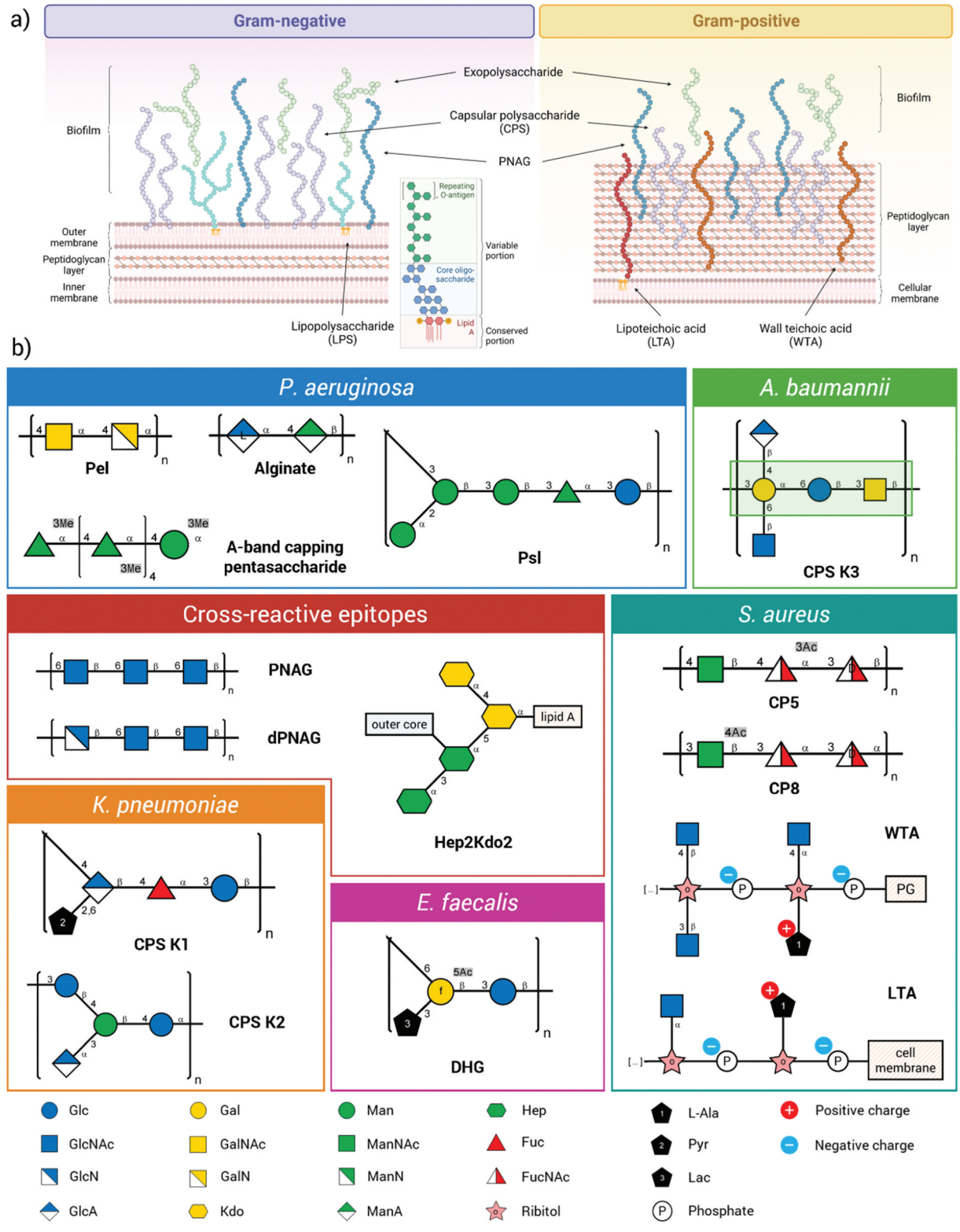


Figure 4. **a**) Overview of the main surface carbohydrate classes and their surface localization on Gram-negative and -positive bacteria. **b)** Selected examples of polysaccharide RU and relevant oligosaccharides from ESKAPE pathogens. LPS and O-antigens structures are not shown for clarity. For WTA and LTA enterococcal structures see reference [148]. Cross-reactive trisaccharide highlighted for *A. baumannii* CPS K3. Pel is a partially deacetylated polymer of α-1,4–GlcNAc made up predominantly of the dimeric repeat here shown [149]. Alginate is composed of ManA, that can bear O-acetyls at the C2′ and/or C3′ positions, and interspersed with L-GlcA, resulting in random structures [150]. Common *S. aureus* WTA is shown with all three possible linkages for GlcNac residues, catalyzed by glycosyltransferases TarS (β-1,4), TarM (α-1,4) and TarP (β-1,3) [151]. dPNAG can be fully or partially deacetylated. All carbohydrates are represented in pyranose form, except when noted f for furanose. All carbohydrates are in D configuration (save Fuc naturally occurring in L configuration), except when stated otherwise (D/L within the symbol). At physiological pH, GlcN, GalN, ManN and Ala residues carry positive charges, while GlcA, ManA and phosphate residues carry negative charges.

Abbreviations: **Ac**, O-acetyl group; **CP(5/8)**, capsular polysaccharide serotype 5 or 8 (*S. aureus*); **CPS**, capsular polysaccharide; **DHG**, diheteroglycan; **Fuc**, L-fucose; **FucNAc**, N-acetyl-L-fucosamine; **Gal**, D-galactose; **GalN**, D-galactosamine; **GalNAc**, N-acetyl-D-galactosamine; **Glc**, D-glucose; **GlcA**, D-glucuronic acid; **GlcN**, D-glucosamine; **GlcNAc**, N-acetyl-D-glucosamine; **Hep**, D-glycero-D-manno-heptose; **Kdo**, 3-deoxy-D-manno-octulosonic acid; **Lac**, lactic acid residue; **L-Ala**, L-alanine residue; **LPS**, lipopolysaccharide; **Man**, D-mannose; **ManA**, D-mannuronic acid; **ManN**, D-mannosamine; **ManNAc**, N-acetyl-D-mannosamine; **Me**, O-methyl group; **PG**, peptidoglycan; **(d)PNAG**, (deacetylated) poly-N-acetyl glucosamine; **Pyr**, pyruvate ketal modification; **(L/W)TA**, (lipo- or wall) teichoic acid.

antibody that was broadly cross-reactive to LPS hinted at the potential of A-PS as immunogen for vaccine or therapeutic development against *P. aeruginosa* [194]. The same group reported the synthesis of the terminal methylated oligosaccharide and confirmed the immunogenicity of the A-PS tip structure through conjugation to human serum albumin (HSA) [133].

#### 4.1.6. S. aureus

CPS 5 and 8 (CP5 and CP8, respectively) (Figure 4b) are the most prevalent capsular serotypes in clinical isolates of *S. aureus* and are associated with approximately 80% of infections [195]. CP5 and CP8 conjugates have been evaluated as vaccine candidates since the 1990s, most notably in Nabi's StaphVax (CP5/CP8 glycoconjugates to EPA), and more recently in Pfizer's SA4Ag (containing CP5/8 glycoconjugates to $CRM_{197}$, with two additional protein antigens) as well as in GSK's trivalent vaccines (composed of CP5/8–tetanus toxoid (TT) conjugates) [55,196]. However, no vaccine has been licensed so far.

While the unadjuvanted CP5 and CP8 EPA conjugates initially showed efficacy [197,198], no benefit compared to placebo was shown in further clinical studies involving end-stage renal disease patients as target population [199]. In the GSK vaccine, the two CP5–and 8–TT conjugates were mixed with the detoxified α-toxin (Hla H35L) and clumping factor A (ClfA) and tested with and without adjuvant AS03B in a Phase I study in healthy volunteers. The vaccine was safe and induced a strong humoral response [200]. CP5–and CP8–$CRM_{197}$ conjugates combined with ClfA and manganese transporter C, without adjuvant, were well tolerated and immunogenic in a Phase I study conducted by Pfizer [201,202]. Challenges associated with the development of these vaccines include the lability of the linker used for conjugation, specifically considering the disulfide chemistry conjugation exploited by Nabi's approach [196,203]. More generally, the lack of established correlates of protection with studies in humans and the complexity of staphylococcal pathogenesis have hampered the success of vaccination [204,205].

Preclinical research is still ongoing on CP5 and CP8 [117], through emerging technologies including bioconjugate vaccines, MAPS and semi-synthetic glycoconjugates [116] which could improve the manufacturing and stability issues associated with classical conjugates.

#### 4.1.7. Conserved LPS epitopes

An LPS mimic of a conserved core tetrasaccharide, Hep2Kdo2 (Figure 4b), was synthesized and evaluated as protective antigen against multiple Gram-negative pathogens. Sera raised against the Hep2Kdo2 glycoconjugate were cross-reactive to carbohydrate extracts from various bacteria, including *P. aeruginosa*. Interestingly, this vaccine proved efficacious against *N. meningitidis* and *E. coli* when administered with an inhibitor of the CPS transporter to allow access to the inner LPS core. Besides solving the lack of antigen exposure, this 'vaccination + block' approach could bring a level of control and minimize off-target damage to other commensal bacteria, as either element is not sufficient for bacterial killing [132]. However, this method and other similar approaches could prove too complicated for clinical trials.

Epitope mapping of a mAb targeting the LPS inner core revealed binding to a heavily phosphorylated heptose, characteristic of Hep2Kdo2 in *P. aeruginosa* strains. The mAb was suggested to be serotype-independent but exclusive to *P. aeruginosa* over other Gram-negative bacteria [206]. The strain specificity of conserved antigens could prove useful in limiting negative effects on the normal flora, at the cost of reduced antibody binding breadth against other Gram-negative pathogens.

### 4.2. Teichoic acids

Teichoic acids (TA) are important adhesive macromolecules from Gram-positive bacteria. They are phosphodiester-based glycopolymers, built up from a ribitol phosphate or glycerolphosphate backbone and include the membrane-anchored lipoteichoic acid (LTA) and the peptidoglycan-bound wall teichoic acid (WTA) (Figure 4b). Both polymers are associated with biofilm formation and immune evasion [151]. Because TA antigens are highly expressed, easily accessible, and with limited structural variation, passive immunization against TA could be a particularly suitable strategy against *S. aureus* [151], especially for immunocompromised patients for whom active immunization could prove too taxing. Pagibaximab, a chimeric monoclonal antibody against LTA, was developed by Biosynexus Inc. to prevent sepsis in neonates, unfortunately to no avail (NCT00646399) [61].

Besides passive immunotherapy, TA remain interesting targets for vaccine development [207], and various efforts have been made toward their synthesis and immunological evaluation [148,208–210]. Importantly, cross-reactivity of LTA was reported, pointing out LTA as a potential single vaccine antigen to target multiple Gram-positive pathogens, including *S. aureus*, *E. faecalis* and *E. faecium* [144,211].

Despite their inherent low immunogenicity as standalone antigens, it was reported that mouse immunization with purified PNAG and/or TA PS could have an inhibitory effect on biofilm formation in *S. aureus* [143]. Along with DHG, LTA is responsible for the strain diversity of *E. faecalis*. LTA was shown to be a viable vaccine candidate for CPS-A and -B serotypes, which lack DHG that masks LTA in CPS-C and -D strains (see previous section) and prevent opsonization by anti-LTA antibodies [170]. The genetically similar pathogen *E. faecium* also expresses TA, several glycoforms of which have already been successfully synthesized [148,210]. Pf3 is a glucosylated LTA and one of the four *E. faecium* surface PS identified by Kodali and colleagues. Nonlipidated Pf3 was conjugated to $CRM_{197}$ and used for rabbit immunization, where the raised antibodies showed significantly enhanced OPK activity compared to those raised with unconjugated and lipidated Pf3 [142]. Zhou *et al*. conjugated a synthetic *E. faecium* U0317 WTA monomer to KLH for mouse immunization, eliciting high IgG titers against the conjugate. However the raised sera were not tested against naturally occurring WTA and their functionality was not investigated [145]. Finally, an LTA-mimicking

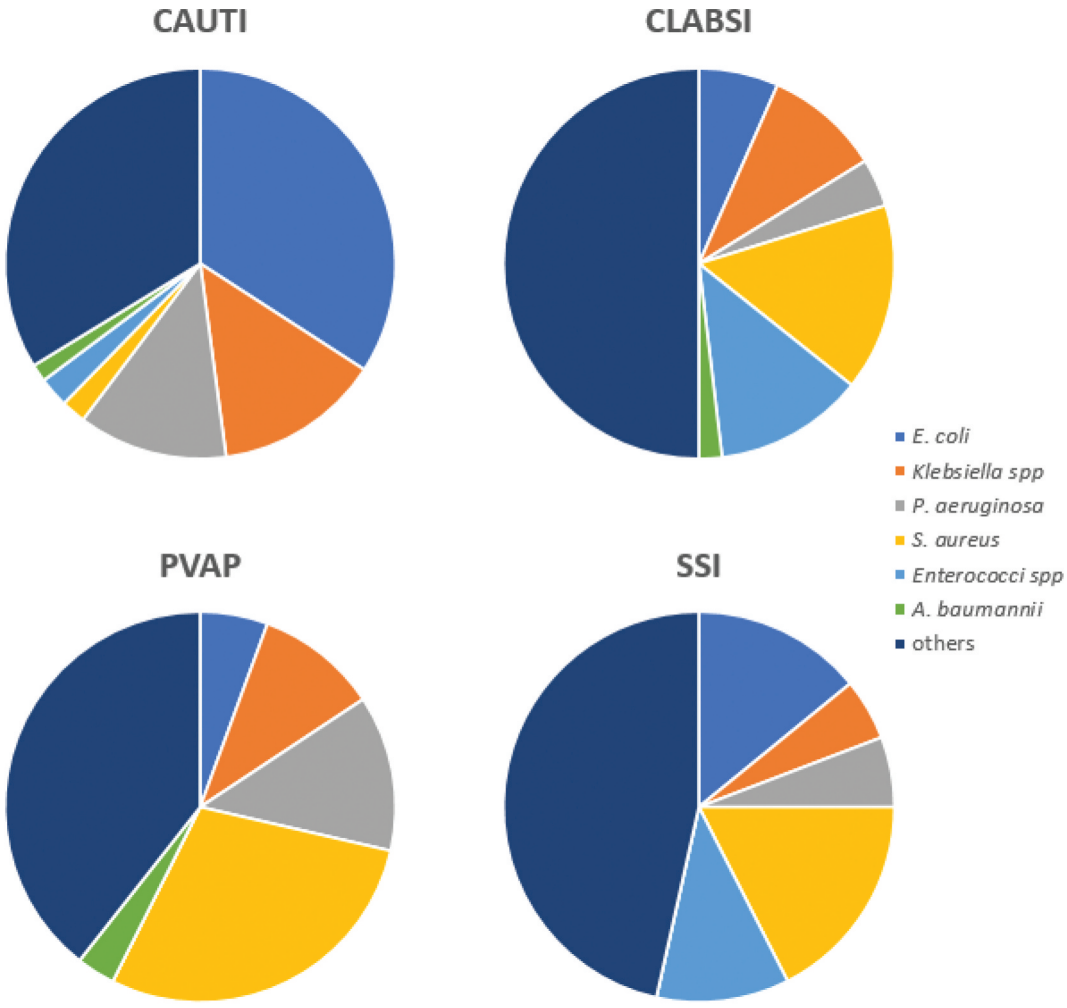


Figure 5. Incidence of AMR pathogens in adult healthcare-associated infections (US data 2015–2017). Among the ESKAPE pathogens, *E. coli*, *S. aureus*, *klebsiella* spp. and *P. aeruginosa* emerge as the ones with higher incidence across different syndromes. Vaccines against these pathogens remains a priority. Figure adapted from reference [232]. Abbreviations: **CAUTI**, catheter-associated urinary tract infections; **CLABSI**, central line-associated bloodstream infections; **PVAP**, possible ventilator-associated pneumoniae; **SSI**, surgical site infections.

peptide was recently reported to protect mice from *S. aureus* infection, which could represent another clever use of carbohydrate antigens [212].

### 4.3. Pseudomonas extracellular or exopolysaccharides

Bacterial extracellular polymeric substances (EPS) are biofilm components and include extracellular or exopolysaccharides. These PS represent a major proportion of the EPS matrix for both Gram-positive and -negative bacteria and are involved in most biofilm-related functions [213]. *P. aeruginosa* can produce three different exopolysaccharides, alginate (or mucoid exopolysaccharide, MEP), Psl and Pel, each of which is associated with different biofilm development stages (Figure 4b) [213].

Alginate is a promising immunogen that could be used to produce opsonizing antibodies [193], but it failed in Phase I due to its poor immunogenicity as a stand-alone antigen. However, these vaccine preparations contained no protein component nor adjuvant which could have amplified the immune response. Preclinical research and immunological evaluation of alginate is still ongoing [134–137], with several NP approaches in development. PGLA [135], solid lipid nanoparticles (SLN) [136] and OMV [137] conjugates decorated with *P. aeruginosa*-extracted alginate are being developed. Recently, Zhang *et al*. synthesized alginate fragments up to the 24–mer and assessed their immunogenicity in mice through HSA conjugates [134]. Interestingly, the data seems to indicate that an alginate tetrasaccharide can serve as an optimal antigen to elicit a sufficient immune response. Passive immunization against alginate is currently also being explored. Aridis Pharmaceutical's AR-105 (Aerucin), a human IgG1 mAb targeting *P. aeruginosa* alginate, with a broad recognition of several clinical isolates, demonstrated safety in a Phase I clinical trial in 16 healthy volunteers [63] and recently completed a Phase II study in ventilator-associated pneumonia patients (NCT03027609), though no report has yet been published.

Pel and Psl are the two other exopolysaccharides produced by *P. aeruginosa* [214,215]. Presence of Psl has been ascertained in 76% of analyzed clinical isolates, and its expression has been shown to favor persistent infection. Psl is involved in the formation and maintenance of biofilms and recently has been shown to protect *P. aeruginosa* from host defenses within the CF lung by inhibiting opsonization and phagocyte killing, prior to the conversion to the mucoid phenotype [216]. Anti-Psl mAb have

demonstrated to be protective in various animal models with an OPK ability against several bacterial strains [217]. One of these mAb, MEDI3902, binding in a bivalent mode to Psl and PcrV, a protein involved in host cell cytotoxicity, was initially shown safe in a dose escalation Phase I study in healthy subjects [218]. It failed however to reduce *P. aeruginosa* nosocomial pneumonia incidence in *Pseudomonas*-colonized mechanically ventilated subjects [62]. Recently, three mAb targeting three distinct Psl epitopes exhibited stratified binding in mature *in vitro* biofilms and bound Psl within the context of a chronic biofilm infection. The mAb also inhibited biofilm formation to a varying extent but all favored biomass reduction in the presence of neutrophils [219].

Polyhydroxyalkanoates (PHA) are natural polymeric intracellular inclusions often produced by bacteria to store energy and materials. These NP can be engineered to express vaccine antigens at their surface, a feature recently harnessed by Rehm's group for a particulate vaccine against *P. aeruginosa* [138,139]. While only protein antigens were expressed at the surface of PHA particles, the authors observed that by deliberately co-purifying PHA with other host cell proteins and Psl, they could broaden the immune response induced by the PHA vaccine. Indeed, a significant anti-Psl response was shown in the sera from PHA-immunized mice [138].

### 4.4. PNAG

Poly-N-acetyl glucosamine (PNAG), also known as the polysaccharide intercellular adhesin (PIA), capsular polysaccharide/adhesin (PS/A) or slime-associated antigen (SAA), is a β-(1–6)-linked surface polymer, sometimes categorized as a CPS or as an exopolysaccharide (Figure 4). PNAG plays an essential role in the biofilm production and immune evasion mechanisms of different bacterial species and has been identified as a virulence factor in most ESKAPE pathogens. This prevalence makes for a particularly interesting protective antigen with a broad vaccination potential [213,220]. The deacetylated form of PNAG has been shown to induce protective antibodies against *S. aureus* [221,222], as opposed to the acetylated form that generates poorly opsonic unprotective antibodies [223]. A synthetic dPNAG composed of nine monosaccharides, conjugated to staphylococcal Hla H35L-induced antibodies capable of reducing staphylococcal skin abscesses, pneumonia, and nasal colonization in mice [224]. A synthetic PNAG pentamer TT-conjugate vaccine candidate (AV0328) was tested to offer protection against *S. pneumoniae*, methicillin-resistant *S. aureus* and *N. meningitidis*, and progressed in Phase I and II (NCT02853617) [225]. The comparability of antibodies raised against synthetic nonamer and pentamer-GlcN-TT conjugates and X-ray studies of a highly opsonic monoclonal antibody (F598) backed the hypothesis that a pentasaccharide is the shortest repeat for effective immunization against PNAG. The data also suggested that a single acetyl group is required for binding, explaining how protective antibodies are able to bind both highly and poorly acetylated PNAG forms [226]. Alopexx reported the *in vitro* OPK activity of AV0328–derived antibodies against a wide range of pathogens, including *E. coli*, *A. baumannii,* and *K. pneumoniae*. Notably, rabbit antibodies induced against the synthetic oligosaccharide conjugated to TT induced complement-mediated killing of *A. baumannii* S1, a high PNAG-producing strain, but not its PNAG-negative mutant [227]. Immunization significantly reduced post infection levels of *A. baumannii* in the lungs and blood, demonstrating that the PNAG conjugate could prevent pneumonia and pathogen caused bacteremia. Recently, different modalities to present PNAG have been explored. GlycoOMV, where PNAG was expressed in OMV, were recently described by Stevenson *et al*. to increase OPK activity against *S. aureus* in comparison to the pentameric GlcN-TT conjugate, while avoiding the chemical conjugation step [95].

Active and passive immunization against PNAG has been described by Hülsdünker *et al*., who observed a decrease in neutrophil activation and in the severity of graft-versus-host disease in mice [60]. Anti-PNAG mAb F598 has completed Phase I clinical studies, and combination with other anti-PNAG mAb, such as TG10 may have synergistic effects.

### 4.5. Prokaryotic nonulosonic acids

Nonulosonic acids (NulO) are a superfamily of carbohydrates which includes the well-known sialic acids and several other structures unique to prokaryotes that decorate cell surface glycans (e.g. CPS and LPS) and glycoproteins (e.g. flagellin, pili proteins). Much like hypersialylated cancer cells, prokaryotic NulO help pathogens evade the host's immune defenses, but also have roles in bacterial motility and biofilm formation [228]. Targeting selected repeats rather than whole PS can be more effective [229], and these signature glycans can therefore become privileged antigens.

Lee *et al*. recently isolated pseudaminic acid (Pse)-containing oligosaccharides from the exopolysaccharide of *A. baumannii* through phage-assisted digestion. *A. baumannii* strain 54,149 exopolysaccharide was essentially depolymerized to two RU and conjugated to $CRM_{197}$. Rabbit antibodies raised against the glycoconjugate displayed *in vitro* bactericidal activity and identified Pse as a strong immunogen and main epitope [140]. Subsequently, Wei *et al*. synthesized Pse and evaluated the immunogenicity of synthetic Pse-based conjugates with different glycosylation degrees. All Pse-vaccines provided protection against a lethal challenge with *A. baumannii*, with the conjugate bearing 8 Pse units affording 100% survival (as compared to the 80% of survival afforded by the lower and higher glycosylated $CRM_{197}$, carrying 4 or 14 Pse units respectively) [141]. Acinetaminic acid is another prokaryotic NulO, isolated from *A. baumannii* K13 and K73 CPS, which differ in their forms [230].

Enterococcal glycans can also be decorated with NulO. Of the four PS isolated from *E. faecium* by Kodali *et al*., Pf4, a legionaminic acid-containing heteroglycan, conjugated to $CRM_{197}$ generated opsonic antibodies and reduced bacterial loads in mouse tissue. While the immunological impact of legionaminic acid in Pf4 is unknown, a similar branched pentamer has been shown to form the core repeat of O-antigen from *C. tuicensis* [142]. Legionaminic acid can also be found in the capsules of *E. coli* and *P. aeruginosa* [228].

These studies highlight how targeting single immunodominant carbohydrate moieties or designed repeats could be

effective and lead to more rationally designed multivalent cross-reactive formulations where well-defined synthetic oligosaccharides would play a central role. Potential cross-reactivity with mammalian sialic acids should however be taken into consideration.

## 5. Conclusion

Glycoconjugate vaccines are widely recognized tools for preventing bacterial infections. This class of vaccines has demonstrated the ability to generate opsonic or bactericidal antibodies in humans, effectively thwarting invasive infections such as pneumoniae and impacting bacterial carriage. Consequently, carbohydrate-based vaccines could play a crucial role in combating infections caused by the so-called ESKAPE pathogens, which are primary drivers of AMR. In addition to traditional methods, emerging technologies such as chemically synthesized carbohydrates, bioconjugation, glyconanoparticles/GMMA and MAPS are enabling the development of purpose-designed novel glycoconjugate vaccines.

## 6. Expert opinion

The global AMR burden seems primarily associated with the ESKAPE pathogens. As such, identifying syndromes where future vaccines can be clinically tested will be key to successfully develop a preventive strategy against AMR. Lower respiratory and thorax infections, bloodstream infections, and intra-abdominal infections have been estimated to account for almost 80% of deaths attributable to AMR pathogens in 2019, with lower respiratory infections alone accounting for more than 400,000 attributable deaths and 1.5 million associated deaths [1]. These infections are often associated with large consumptions of antibiotics driving the emergence of resistant strains. Nosocomial infections have been a significant source of AMR. Central line-associated bloodstream infections (CLABSI), ventilatory-associated infection, catheter-associated UTI (CAUTI) and surgical site infections (SSI) represent the most relevant syndromes [231], with four pathogens accounting as primary causative agents (*S. aureus*, *E. coli*, *P. aeruginosa,* and *K. pneumoniae*) (Figure 5) [232]. This suggests that developing tools to prevent infections caused by these targets will not only help alleviate these diseases but also fight the development of AMR.

rRemarkably, AMR is not exclusively linked to nosocomial infections. UTI accounts for 150,000 episodes globally per year and is responsible for 11% of antibiotic prescriptions. The consequence of community-acquired infections should not be underestimated: for example, *E. coli* bacteremia [233] seems to be primarily linked to UTI in both nosocomial and community settings. Therefore, impacting community-acquired infections could also reduce the burden of bacteremia.

It is worth underscoring that *E coli* and *K. pneumoniae* are also major causes of neonatal sepsis and a vaccine has been advocated by the WHO [234–236].

Carbohydrate-based vaccines have the potential to play a relevant role to counteract AMR. However, in order to design effective glycoconjugate vaccines against AMR pathogens some challenges will need to be addressed.

Considering that hospital-acquired infections associated with AMR are often polymicrobial, complex multivalent formulations will be needed to ensure disease prevention and reduce the AMR burden. In some of these infections (e.g. CAUTI or CF), therapy is not very effective because of the formation of biofilms in which pathogens are hidden and poorly accessible to therapeutics as well as antibodies. Targeting biofilm-associated glycans (e.g. PNAG, Psl) and proteins along with disrupting biofilm agents could be key to succeeding in the development of novel therapeutic approaches.

Also, currently approved glycoconjugate vaccines are all made from CPS, while it remains to be seen whether OPS can be successful. For example it has been pointed out that the capsule might obstruct O-antigen specific antibody binding in *E. coli* [237] and *K. pneumoniae* [238]. In some cases, as in bronchiectasis patients, high titers of IgG2 specific for the O-antigen resulted in impaired *Pseudomonas* serum-mediated killing [239], which could explain the inconsistent results of LPS-based approaches thus far explored for *P. aeruginosa* vaccines. Considering that patients with *P. aeruginosa*-associated bloodstream infections presented non-protective antibodies against Psl, boosting the immune response against this PS might be challenging [240]. For other biofilm-associated PS like PNAG, which are ubiquitous among different species, safety concerns need to be addressed. The difficult path for a *S. aureus* vaccines well exemplifies challenges associated with AMR vaccine development as various glycoconjugate formulations have been tested in clinical trials with no success. While the chemical features of some conjugates tested in early clinical trials could not ensure adequate stability to the vaccine [203], challenges are also associated with the existence of multiple evasion mechanisms. Consequently, current vaccine formulations under development are based on either non-capsular carbohydrate antigens (e.g. teichoic acids) or on protein antigens combined to glycoconjugates. Also, training the immune system for the production of specific antibody subclasses could be relevant for an appropriate immune response against *S. aureus* [241].

Glycoconjugate vaccines typically elicit suboptimal immune response in individuals with underlying comorbidities, immunocompromised and aging populations, who are often exposed to antibiotic use [11,12]. Current vaccination schedules include several booster immunizations [242], without reaching the glycoconjugates' full protection potential [11] and fail to quickly mount a strong immune response [243]. In today's AMR context, identifying novel glycoconjugate designs combined with potent adjuvants to enhance immunogenicity is pivotal. For instance, potent single-dose vaccines are highly desirable to counteract nosocomial infections and expand access where supply issues could occur.

A more profound understanding of immunological mechanisms behind glycoconjugate vaccines would be beneficial in this regard [244]. Carrier-induced epitope suppression (CIES) is one of such immune phenomena which has not yet been fully unraveled and is directly relevant to the choice of carrier for safe and efficient conjugate vaccines. CIES involves the dampening of the immune response against PS epitopes by prior or simultaneous immunization with the same protein carrier as used in the glycoconjugate, and has been observed in several conjugate combination vaccines [244,245]. CIES has been proposed to be caused by the clonal expansion of B cells specific for carrier protein epitopes following intramolecular antigenic competition. Nonetheless, clinical data assessment [246] has determined that CIES is not the only underlying interference mechanism and is not observed for all regimens. As additional antigens are added to routine vaccinations, further research is required to confirm the impact of CIES and understand the patterns involved in either the enhancement or hindrance of the immune response [244,245].

All these challenges call for technologies enabling access to complex multi-antigen and even multi-pathogen nanosized vaccines. In this regard, the development of innovative platforms such as MAPS, NP and GMMA, as well as advances in glycoengineering is likely critical. These emerging technologies are currently under evaluation at the clinical level and the coming years will be an exciting time to learn which ones will be validated by licensure of new vaccines.

## Declaration of interests

C Sorieul is a PhD student at Leiden University and a former employee of GSK in the context of the industrial EU program Horizon 2020 PAVax, grant no 861194. M Dolce is a PhD student and participated in a post graduate studentship program at GSK in collaboration with the University of Siena. M Rosaria Romano and R Adamo are employees of the GSK group of companies. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or material discussed in the manuscript apart from those disclosed.

## Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

## Acknowledgments

Figures 1 to were created with BioRender.com. Carbohydrate structures from Figure 4 were generated with the CSDB/SNFG structure editor.

## Funding

This manuscript was funded by EU program Horizon 2020 through grant no 861194 (PAVax). This work was sponsored by GlaxoSmithKline Biologicals SA.

## Author contributions

All authors have substantially contributed to the conception and design of the review article and interpreting the relevant literature, and have been involved in writing the review article or revised it for intellectual content.

## ORCID

Roberto Adamo http://orcid.org/0000-0001-5228-6088

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**Papers of special note have been highlighted as: • of interest, •• of considerable interest.**

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